Hematopoiesis

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SUMMARY

Enormous numbers of adult blood cells are constantly regenerated throughout life from hematopoietic stem cells through a series of progenitor stages. Accessibility, robust functional assays, well-established prospective isolation, and successful clinical application made hematopoiesis the classical mammalian stem cell system. Most of the basic concepts of stem cell biology have been defined in this system. At the same time, many long-standing disputes in hematopoiesis research illustrate our still limited understanding. Here we discuss the embryonic development and lifelong maintenance of the hematopoietic system, its cellular components, and some of the hypotheses about the molecular mechanisms involved in controlling hematopoietic cell fates.

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1 INTRODUCTION

The blood system contains more than 10 different blood cell types (lineages) with various functions: Leukocytes represent many specialized cell types involved in innate and acquired immunity. Erythrocytes provide O₂ and CO₂ transport, whereas megakaryocytes generate platelets for blood clotting and wound healing. All blood cell types arise from hematopoietic stem cells (HSCs) that reside mainly in the bone marrow (BM), a major site of adult hematopoiesis. Blood is one of the most regenerative and plastic tissues, and millions of “old” blood cells are replenished with new ones each second during life. In emergency situations such as anemia or infections, blood cell counts rapidly increase. The cell number then declines back to normal after recovery. The lifetimes of various mature blood cell types range from hours to years.

The hematopoietic system is a prime example of successful applied regenerative medicine. For more than 30 years, stem cell transplantation has become a routine treatment for blood disorders and malignant diseases. After the eradication of the patient’s own hematopoietic system, the transplanted donor hematopoietic stem and progenitor cells (HSPCs) provide lifelong reconstitution of the blood system of the patient. The experimental evidence that HSCs naturally migrate back and forth from the BM periodically, as well as the identification of agents that increase HSC mobilization (e.g., granulocyte colony-stimulating factor [G-CSF]), have opened new avenues for stem cell transplantation. However, although stem cell transplants work successfully in the clinic, further improvement of the method is needed to minimize engraftment failure and post-transplant infections. The ex vivo expansion of HSCs would be beneficial for grafts with limiting numbers of HSCs (umbilical cord blood) and for gene therapy approaches for monogenetic inherited blood disorders. However, despite decades of research, the robust expansion (or even maintenance) of HSCs ex vivo is not yet routinely achieved.

HSCs are the most-studied adult stem cells. Five decades ago researchers passed the stage of providing purely descriptive data to start quantitative HSC research (Till and McCulloch 1961; Becker et al. 1963; Siminovitch et al. 1963). Several properties of hematopoietic cells are favorable for stem cell research. First, they are not tightly interconnected in a tissue. Therefore, cells can be physically separated without too much stress. The isolation from peripheral blood is minimally invasive, and millions of cells can easily be harvested. Many blood cell types are naturally capable of extravasating into tightly packed tissues and sustaining enormous shear forces. Therefore, they tolerate isolation by flow cytometry well. This enabled the early

correlation of surface marker expression patterns with functional tests examining self-renewal capacity, clonogenicity, and lineage potential, leading to successful prospective enrichment of distinct HSPC populations (Morrison and Weissman 1994; Osawa et al. 1996; Kondo et al. 1997; Akashi et al. 2000; Kiel et al. 2005; Inlay et al. 2009). Last, HSPCs grow into colonies from single cells under appropriate culture conditions, allowing assays at the clonal level.

Only functional tests in vivo can retrospectively determine the true identity of HSCs by demonstrating their unique abilities of long-term or even lifelong self-renewal and multilineage differentiation. Robust HSPC transplants between congenic mouse strains, in which the donor cells differ from the recipient cells by only a single surface marker, allow quantitative functional HSC readouts. Once injected intravenously, even single transplanted HSCs can find their way to the appropriate location in the BM to initiate lifelong blood regeneration (Osawa et al. 1996). Serial transplantation shows that HSC self-renewal can last longer than the normal lifetime of the organism in the mouse model (Morrison and Weissman 1994). The HSC frequency within a mixture of undefined cells can be quantified by transplanting limiting dilutions of cells (Szilvassy et al. 1990).

The murine hematopoietic system is by far the best understood of all species. The generation of genetically modified mouse strains for gain- and loss-of-function studies and for lineage and cell tracing enables the study of hematopoiesis in vivo. Conditional and inducible systems allow the timed and tissue-specific manipulation of gene expression under homeostatic conditions. For this approach, mouse strains expressing Cre recombinase only in specific hematopoietic cell types have proven extremely valuable (Kuhn et al. 1995; de Boer et al. 2003). Murine transplantation models for murine and human HSPCs have been established for decades. The homing and engraftment of murine cells transplanted into congenic mouse strains is very efficient, although not absolute. Sophisticated humanized mouse strains with an incompetent adaptive immune system have been developed to prevent xenograft rejection for reconstitution studies with human BM cells (McDermott et al. 2010). Existing species incompatibilities in receptor–ligand or adhesion molecule binding can be partly compensated for by intrafemoral cell injection (Notta et al. 2011) or by transgenic recipient mice expressing human growth factors (Rongvaux et al. 2011). Less commonly used but extremely valuable model organisms for hematopoiesis research are *Danio rerio* (zebrafish) (Jing and Zon 2011), and increasingly also *Drosophila melanogaster* (fruit fly) (Martinez-Agosto et al. 2007). The genetic manipulation of these organisms is faster and cheaper than in the murine model, and genetically modified

offspring can be generated within days and weeks. Moreover, their transparent embryos, which develop extremely fast and ex utero, allow relatively easy live imaging.

2 EMBRYONIC HEMATOPOIESIS

The analysis of how blood cells are first produced during embryonic development is not only of great scientific interest, but it can also offer important insights into the cellular and molecular mechanisms that lead to the specification of clinically important HSCs. This could lead to their improved expansion or even de novo generation in vitro from other cells like embryonic stem cells or induced pluripotent stem cells by improved differentiation protocols or from any other cell type by direct reprogramming.

The earliest stages of embryonic development actually happen in the absence of any blood cells. Only after the embryo gets too big to be supplied with oxygen and other essential factors by diffusion are blood cells first generated (Fig. 1). The sites of both de novo HSPC generation and maintenance/expansion are constantly changing in the embryo (Fig. 1). Anatomically, the first hematopoietic cells are generated in the extraembryonic yolk sac before the first heartbeat and later in the allantois and placenta. Finally, HSCs are then generated de novo in the intraembryonic aorta-gonad-mesonephros (AGM) region and the placenta. Interestingly, although all adult and many of the later embryonic blood cells are produced from HSCs (see below), there is a transient population of so-called primitive blood cells in the early embryo. These cells emerge in the extraembryonic yolk sac and placenta and possibly also in intraembryonic sites before the first HSCs. They do not propagate into the adult and are soon replaced by their definitive counterparts in the embryo. They have specific effector functions in the embryo and differ from their adult counterparts. As an example, primitive erythrocytes, in contrast to adult “definitive” erythrocytes, mostly retain

their nuclei and express fetal forms of hemoglobin. Other primitive blood cells like macrophages and megakaryocytes exist, but this field of hematopoiesis remains poorly understood and many questions await investigation (Palis et al. 1999; Chen and Zon 2009). In addition, definitive erythroid/myeloid progenitors have been described before HSC formation (Bertrand et al. 2010b). These very recent discoveries of previously unrecognized embryonic cell types and mechanisms of their generation (Chen et al. 2011) well illustrate how little we still know.

The first cells with functional properties of adult HSCs (i.e., regeneration of the hematopoietic system upon transplantation into adult recipient mice; see below) are generated in the intraembryonic AGM region (Muller et al. 1994; Medvinsky and Dzierzak 1996; Ivanovs et al. 2011) and the placenta (Gekas et al. 2005; Ottersbach and Dzierzak 2005). Interestingly, their location quickly changes throughout development. After being generated in the AGM, they soon migrate to the placenta and fetal liver, which are major sites of their expansion. Soon thereafter they migrate to the spleen, and around birth to the BM. BM, and to a reduced extent the spleen, then remain the main sites of HSC-derived hematopoiesis after birth.

The development of the blood system in the embryo is a very complex and dynamic process, and in contrast to most other tissues, different cell types constantly appear in different intra- and extraembryonic tissues (Fig. 1). The transient generation of different embryonic cell types before establishment of HSCs, which then exclusively drive later hematopoiesis, complicates the analysis of how exactly this tissue is generated. This has led to many decades-old disputes about the cellular sources of their de novo generation, differentiation hierarchy, and molecular control (Medvinsky et al. 2011). One of those disputes concerned the cellular source of the first HSCs. Because of the facts that hematopoietic cells (both primitive and definitive) are always found initially in proximity to endothelial cells and

Figure 1. Embryonic hematopoietic development. Anatomical sites and timing of hematopoietic cell generation, maintenance, and expansion during embryonic development. Hematopoietic cells are generated de novo in both extra- (yolk sac, allantois, placenta) and intraembryonic (AGM region) tissues. Cells are migrating to other sites for expansion and ultimately long-term maintenance throughout adult life. dpc, days after conception; wpc, weeks after conception.

that these cell types share many molecular markers, it had been hypothesized for decades that the first hematopoietic cells are actually generated from endothelium (reviewed by Dieterlen-Lievre et al. 2006; Medvinsky et al. 2011). These hemogenic endothelial cells would be integrated into vessel walls but would be able, under the influence of unknown molecular signals, to generate hematopoietic cells. However, proving the existence of hemogenic endothelial cells was surprisingly difficult. For decades, static analyses of cells either before or after the endothelial-to-hematopoietic transition yielded data that could also be explained by the alternative conclusion that hematopoietic cells were generated elsewhere and then migrated toward endothelial cells. Final proof of direct generation of hematopoietic cells from hemogenic endothelium came from continuous single-cell observations of endothelial-to-hematopoietic transitions by time-lapse microscopy (Eilken et al. 2009). Subsequent studies could then also detect this direct transition in vivo in the zebrafish embryo (Bertrand et al. 2010a; Kissa and Herbomel 2010). It is worth mentioning that these studies do not rule out the possible existence of other sources of hemogenic cell types in the embryo. Importantly, comparable timing and location of hemogenic activity in human embryos makes it likely that the same cells, molecules, and mechanisms are also involved in human embryonic hematopoiesis (Ivanovs et al. 2011).

The proof of the existence of hemogenic endothelium now allows the improved analysis of the molecular mechanisms involved in the generation of the first blood cells, including the therapeutically important HSCs. This molecular mechanism is only partially understood. It involves complex signaling from different cell types surrounding developing hemogenic endothelium at different sites and times in the fast-developing and -changing embryo. Mesodermal cells expressing Flk1 and ETV2 were recently identified as the common precursors for endothelial and hematopoietic cells (Lee et al. 2008; Kataoka et al. 2011a). The signals inducing hemogenic fate in these cells and their progeny are the subject of ongoing research. Molecules like membrane-bound Notch ligands or soluble hedgehog, bone morphogenetic protein, or vascular endothelial growth factor, but also shear stress and nitric oxide signaling, have been involved in the induction of the hemogenic program in endothelial cells—possibly even in mesodermal precursors before they have reached endothelial identity (Medvinsky et al. 2011). Within the hemogenic endothelial cell, a number of transcription factors (TFs) seem to be crucial for the induction of the hemogenic program, with Runx1 and CBF β apparently being critical core factors (Chen et al. 2011).

Importantly, fetal HSCs differ from adult HSCs in their regeneration behavior. In contrast to mostly quiescent

adult HSCs, they are actively cycling and regenerate hematopoiesis faster and more robustly upon transplantation. The switch from this fetal to adult HSC behavior occurs in a very short time window a few weeks after birth of a mouse (Bowie et al. 2007). This sharp switch at a defined time is useful for the identification of the involved molecular mechanisms (Kim et al. 2007; He et al. 2011), which could be of great interest for the induction of a clinically preferred fetal phenotype in HSCs.

3 ADULT HEMATOPOIESIS

3.1 The Hematopoietic Differentiation Hierarchy

In adult mammals, HSCs are at the apex of a hierarchy of numerous progenitor cell stages with increasingly restricted lineage potentials that give rise to all blood cell lineages (Fig. 2). Of all blood cells, only HSCs fulfill the criteria for somatic stem cells, namely, long-term (and possibly lifelong) self-renewal and differentiation potential. In the murine system they robustly self-renew (produce more HSCs) while also reconstituting all blood cell lineages for >24 weeks in congenic transplant recipients (Benveniste et al. 2010), and are further able to repopulate secondary recipients. These functional assays have also revealed multipotent progenitor (MPP) populations with intermediate- or short-term repopulating capacity (formerly called IT- and ST-HSCs) with a finite self-renewal potential (Osawa et al. 1996; Benveniste et al. 2010). The stepwise identification of multiple surface markers in the last 25 years enabled the prospective isolation of defined stem and progenitor cell populations by flow cytometry-based cell sorting. Immature multipotent cells express CD117 (c-Kit) and stem cell antigen (Sca)-1 and are low in mature cell marker expression (lineage markers) (Spangrude et al. 1988; Okada et al. 1992; Uchida and Weissman 1992; Morrison and Weissman 1994). About one in 30 cells within this so-called KSL (c-Kit⁺/Sca-1⁺/Lineage⁻) population (0.06% in BM) is an HSC (0.002% in BM), and there is no HSC activity outside the KSL fraction. These findings laid the ground for the identification of additional surface markers for improved prospective HSC identification (Table 1). Currently, the highest purity of at least 50% HSCs in the sorted fraction is obtained with CD150⁺/CD48⁻/CD34^{low} KSL cells (Kiel et al. 2005). Moreover, CD150 expression levels might indicate distinct subpopulations of HSCs with predisposed myeloid- or lymphoid-biased reconstitution patterns (see also Sec. 3.2.1) (Morita et al. 2010). CD49b expression could be attributed to intermediate-term repopulating MPPs, a population that shares many functional properties of myeloid-biased HSCs (Benveniste et al. 2010). In addition to surface markers, distinct functional

Figure 2. Adult hematopoietic differentiation hierarchy. Long-term self-renewing HSCs are at the apex of a hierarchy of multiple progenitor cell stages giving rise to all blood cell lineages. Distinct HSPC stages have been described by correlating surface marker expression and functional properties for prospective isolation, both in the murine and human systems. Murine hematopoiesis is currently defined in more detail and therefore displayed here. Corresponding human HSPC populations with their markers are indicated. HSCs differentiate into all blood cell lineages via long described (bold arrows) and potentially also or alternatively more recently described differentiation routes (thin arrows). It is important to point out that this model is only a simplified representation of current knowledge and will continue to change. Individual murine HSCs show an intrinsic lineage-biased repopulating ability, generating either more myeloid or lymphoid cells or a balanced mixture. These HSC subgroups can be enriched by detecting various levels of CD150 expression or Hoechst efflux. HSC, hematopoietic stem cell; MPP, multipotent progenitor; LT-, long-term repopulating; IT-, intermediate-term repopulating; ST-, short-term repopulating; LMPP, lymphoid-primed MPP; ELP, early lymphoid progenitor; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GMP, granulocyte–macrophage progenitor; MEP, megakaryocyte–erythrocyte progenitor; CDP, common dendritic progenitor; MDP, monocyte–dendritic cell progenitor; NK, natural killer cell.

properties of HSCs have been used for prospective isolation, like the increased efflux of the dye Hoechst 33342 (so-called side population, or SP) (Goodell et al. 1996) and low staining for Rhodamine123 dye (Bertoncello et al. 1985). A further discrimination of SP^{lower} and SP^{higher} HSCs also dissected HSCs with myeloid- and lymphoid-biased repopulation capacity, respectively (Challen et al. 2010). It is important to point out that although transplantation experiments functionally identify HSC properties, these are stress conditions for the HSCs and for the organism. They therefore may not reflect functional properties of homeostatic HSCs. Furthermore, functional HSC

reconstitution behavior depends on the chosen transplantation model. This is particularly problematic for xenograft settings with human HSCs repopulating murine recipients. Although this is an extremely important methodology for analyzing human HSCs, it is important to remember that HSC properties and behavior in such a different environment could differ massively from those under conditions in the human niches (McDermott et al. 2010).

HSCs differentiate into a cascade of progenitor cell stages with declining multilineage potential before unilineage commitment occurs (Kondo et al. 1997; Akashi et al. 2000; Adolfsson et al. 2005; Wilson et al. 2008; Rieger et al.

Table 1. Prospective HSC isolation

HSPC population	Transplanted cells	Assay/model	HSCs (%)	References
Murine				
Thy-1 ^{low} /Sca-1 ⁺ /Lin ⁻	30	Competitive	1.5–3	Spangrude et al. 1988
KSL	Limiting dilutions	Competitive	3–7	Okada et al. 1992; Bryder et al. 2006
Thy-1 ^{low} KSL	5–10	Competitive	10–20	Morrison and Weissman 1994
CD34 ⁻ KSL	1	Competitive	21	Osawa et al. 1996
SP Rho ^{low} /CD45 ^{mid} /Lin ⁻	1	Sublethal W-41	42/33	Uchida et al. 2003; Dykstra et al. 2006
SP CD201 ⁺	10	Competitive	>10	Balazs et al. 2006
CD150 ⁺ /CD48 ⁻ /CD41 ⁻ KSL	1	Competitive	47	Kiel et al. 2005
CD150 ⁺ /CD48 ⁻ /CD49b ^{low} KSL	1	Sublethal W-41	29	Benveniste et al. 2010
CD150 ⁺ /CD48 ⁻ /CD201 ⁺ /CD45 ⁺	1	competitive Sublethal W-41	43	Kent et al. 2009
Human				
CD34 ⁺ /CD38 ⁻ /Lin ⁻	Limiting dilutions	NOD/SCID	<1	Bhatia et al. 1997
CD34 ⁺ /CD38 ⁻ /CD90 ⁺ /CD45RA ⁻ /Lin ⁻	Limiting dilutions	Sublethal NSG	5	Majeti et al. 2007; Notta et al. 2011
Rho ^{low} /CD49f ⁺ /CD34 ⁺ /CD38 ⁻ /CD90 ⁺ /CD45RA ⁻ /Lin ⁻	1	Sublethal NSG	15	Notta et al. 2011

HSPC, hematopoietic stem or progenitor cell; KSL, CD117⁺, Sca-1⁺, and Lin⁻ cells; SP, side population; Rho, Rhodamine123; W-41, mouse line with homozygous *Kit*^{W-41}/*Kit*^{W-41} mutation; NOD/SCID, nonobese diabetic/severe combined immune deficiency mouse line; NSG, NOD/SCID/IL2 γ -chain knockout mouse line; competitive, transplantation in lethally irradiated recipients with “competitor-recipient” HSPCs.

2009b). Current models of the hematopoietic hierarchy describe a successive loss of individual lineage potentials (Adolfsson et al. 2005; Arinobu et al. 2007; Mansson et al. 2007; Pronk et al. 2007). MPPs first lose their erythroid/megakaryocytic potential and develop into lymphoid-primed MPPs (LMPPs) and early lymphoid progenitors (ELPs) (Adolfsson et al. 2005). Then they lose their myeloid potential and become common lymphoid progenitors (CLPs) (Kondo et al. 1997; Inlay et al. 2009; Schlenner et al. 2010), and finally differentiate into lymphoid cells (Fig. 2). However, the successive restriction to solely lymphoid fate should not imply a picture of default lymphoid cell fate. The opposite might be the case, namely, that active repression of the myeloid cell fate is required to induce lymphoid development. Interestingly, B- and T-cell progenitors retain, at least in vitro, some myeloid potential (Kawamoto et al. 2010). In contrast, lineage-tracing experiments revealed only a minor contribution of myeloid cells from interleukin-7 (IL-7) receptor-positive CLPs in murine homeostatic hematopoiesis (Schlenner et al. 2010). It is important to point out that this model of the hematopoietic differentiation hierarchy clearly only reflects current knowledge and will continue to change over time. In particular, the early progenitor stages are still ill defined, and numerous novel maturation stages and differentiation pathways will certainly be described in coming years.

According to current data, the human hematopoietic differentiation hierarchy closely resembles the murine one (Majeti et al. 2007; Doulatov et al. 2010). However, mostly as a result of more difficult experimentation, and despite their clinical importance, purification methods for human HSPCs are far less advanced than for murine HSPCs (Table 1). Human HSCs express the surface molecule CD34, in contrast to their murine counterparts (Osawa et al. 1996; Dick 2008). However, although many reports describe CD34⁺/CD38⁻/Lineage⁻ cells as human HSCs, it is very important to point out that this population comprises <1% functional HSCs (as determined by xenograft transplantation assays). Only recently have improved methods for the prospective enrichment of human HSCs been described that now allow enrichment to purities of ~15% (Notta et al. 2011) (Table 1). Prospective isolation of human hematopoietic progenitors has also recently been improved, allowing the functional and molecular characterization of these important cell types with much improved resolution (Manz et al. 2002; Majeti et al. 2007; Doulatov et al. 2010). However, the percentage of prospectively isolated human HSCs may be underestimated by xenotransplantations because the murine environment is less permissive to human HSCs, and advanced humanized mouse models have improved engraftment efficiency (Rongvaux et al. 2011).

3.2 Molecular Control of Hematopoietic Fate Choices

To continuously regenerate the hematopoietic system, the right number of specific cell types must permanently be generated at the right time and place. To achieve this, correct fate decisions constantly have to be chosen in HSPCs: quiescence versus proliferation, self-renewal versus differentiation, lineage choice, survival versus death, and sessility versus migration (Fig. 3A). Exact timing and sequential order of all choices in each cell underpin normal hematopoiesis, both in steady-state and injury situations. Disruptions of normal cell fate decisions underlie hematological disorders. A comprehensive analysis of their molecular control and the exact interplay of these fate decisions are therefore crucial for a full understanding of normal and malignant hematopoiesis and the development of novel therapies.

The molecular circuitries underlying HSPC fate choice must enable cells to respond to changing external stimuli but also allow specific cellular states to be stable independent of their environment. As an example, plasticity must enable the choice of HSPCs between multiple lineages but ensure stability of lineage commitment after the choice has been made. The expression, activity, or changing function of fate-control molecules therefore must be well timed, cell type specific, and controllable by other factors. For a comprehensive understanding, these parameters have to be quantified in a cell-stage-specific manner, at the single-cell level, and ideally over time. Experimental analysis of molecular cell-fate control requires demanding technical prerequisites: (1) the prospective isolation of distinct cell

stages in sufficient numbers and purity, (2) continuous quantitative measurements of functional molecular activity during differentiation, and (3) the observation of multiple factors in complex genetic programs. Many of these requirements have been achieved individually, but their integrated analysis is still limited by current technologies.

Hematopoiesis is usually analyzed in heterogeneous populations and at individual time points of the process of interest. However, these snapshot analyses only allow limited conclusions about the involved sequence of individual molecular and cellular events (Fig. 3B). Experimental data can then usually be explained by a multitude of hypotheses, leading to ambiguous conclusions. Only when the behavior and fate of each individual cell and its progeny are continuously known can a comprehensive understanding of developmental steps and their molecular control be drawn (Fig. 3B). Ideally, therefore, cellular and molecular HSPC behavior is continuously quantified at the single-cell level and throughout the process of interest (Schroeder 2008). One approach to fulfill these requirements is long-term video microscopy of HSPCs and computer-aided single-cell tracking (Eilken et al. 2009; Rieger et al. 2009a). However, despite its potential, this field is still in its infancy, and because of the required high level of interdisciplinary technical know-how, its use is still limited to few expert laboratories (Schroeder 2011).

3.2.1 A Stem Cell's Decision: Self-Renewal versus Differentiation

The HSC's choice between self-renewal and differentiation must be tightly regulated to enable both the generation of

Figure 3. Continuous single-cell observations are required for a comprehensive understanding of dynamic cellular behavior. (A) Individual cell-fate decisions are the reason for the behavior of the complete hematopoietic system in health and disease, and must therefore be quantified. They are influenced by factors like cytokines, extracellular matrix (ECM), or membrane-bound signaling molecules in many different niches. (B) Only continuous single-cell observation allows unambiguous conclusions about cell-fate choices in complex cell systems. Discontinuous input/output analyses—even when done at the single-cell level—cannot distinguish between different combinations of cell-fate decisions leading to the same output of a cellular system, and therefore lead to ambiguous conclusions.

differentiated cells and the accurate maintenance of the right HSC number. The BM provides the environment for sustained HSC function, and HSCs rapidly lose their self-renewal capacity once isolated from their *in vivo* niches. Extrinsic signals from membrane-bound, soluble, or extracellular matrix-associated ligands from the niche are necessary for appropriate HSC behavior. Two major HSC niches are currently proposed to exist in BM: the endosteal osteoblastic niche (Calvi et al. 2003; Zhang et al. 2003; Lo Celso et al. 2009; Xie et al. 2009) and the perivascular endothelial niche (Kiel et al. 2005; Ding et al. 2012). It is unclear if both niches, although spatially separated, fulfill a similar function; if both niches provide distinct properties, for example, for dormant versus cycling HSCs; or if the niche comprises both osteoblasts and endothelial cells working synergistically on HSC function. Also, BM comprises a heterogeneous mixture of various cell types including blood cells, mesenchymal cells, osteoblasts, osteoclasts, endothelial cells, reticular cells, fat cells, and many other less-defined types, which will also influence hematopoietic fates. A multitude of signaling pathways have been shown to be activated in HSCs by the niche (e.g., the cytokine receptors c-Kit and Mpl, Wnt, Notch, Sonic hedgehog, and integrin signaling). However, their precise involvement in HSC maintenance remains surprisingly controversial. For most of them, contradictory conclusions have been drawn in separate studies. Our incomplete understanding of the molecular control of HSC self-renewal is well illustrated by the fact that therapeutically relevant robust maintenance or even expansion of these clinically important cells *ex vivo* remains elusive without genetic manipulation. Nevertheless, several recent studies with murine and human HSCs describe very promising approaches for the *ex vivo* expansion of HSCs without genetic manipulation (Zhang et al. 2008; Boitano et al. 2010). A comprehensive discussion of all potential molecular HSC self-renewal modulators goes beyond the scope of this chapter, and can be found elsewhere (Ehninger and Trumpp 2011; Mercier et al. 2011).

At the single-cell level, even HSCs with retrospectively proven functionality (>1% long-term contribution to mature myeloid and lymphoid cells in peripheral blood) are heterogeneous in their reconstitution efficacy and their lineage contribution pattern. They range from 1% to almost 100% total contribution, with varying ratios of myeloid and lymphoid lineages (Muller-Sieburg et al. 2002, 2004; Sieburg et al. 2006; Dykstra et al. 2007; Kent et al. 2009). Importantly, these patterns are conserved through serial transplantations, indicating the existence of stable inheritable stem cell intrinsic programs (Muller-Sieburg et al. 2002, 2004; Sieburg et al. 2006; Dykstra et al. 2007; Kent et al. 2009). Therefore, not only are self-

renewal and differentiation controlled by the environment, but intrinsic regulators of HSC functionality exist (Table 2).

Remarkably, ectopic expression of HoxB4 and in particular of Hox fusion proteins to NUP98 (NUP98–HoxB4 and NUP98–HoxA10 homeodomain) lead to a massive net expansion of HSCs *in vitro*, while still maintaining their normal multilineage differentiation *in vivo* (Antonchuk et al. 2002). However, despite years of research, the exact

Table 2. Examples of HSC intrinsic molecules implicated in control of self-renewal

Molecule	Effect on self-renewal (SR)	References
Transcription factors		
HoxB4	Expression enhances SR	Antonchuk et al. 2002
Gfi1	Loss reduces SR upon HSC exhaustion	Hock et al. 2004; Zeng et al. 2004
Evi1	Loss reduces SR	Kataoka et al. 2011b
c-Myc	Loss leads to HSC accumulation with differentiation defect	Laurenti et al. 2008
Signaling modulators		
Pten	Loss reduces SR upon HSC exhaustion	Yilmaz et al. 2006
Lnk	Loss increases SR	Ema et al. 2005; Seita et al. 2007
STAT5A/B	Loss reduces SR	Wang et al. 2009
Epigenetic modifiers		
Dnmt1	Loss reduces SR by reduced DNA methylation	Broske et al. 2009
Dnmt3a/b	Loss alters SR and/or differentiation	Tadokoro et al. 2007; Challen et al. 2011
Bmi-1 (PRC1)	Loss reduces SR, expression increases SR	Lessard and Sauvageau 2003; Park et al. 2003; Iwama et al. 2004
Suz12 (PRC2)	Loss increases SR	Majewski et al. 2010
Ezh2 (PRC2)	Expression enhances SR, loss reduces SR (mainly in fetal liver)	Herrera-Merchan et al. 2012
Cell cycle regulators		
p57 ^{KIP2}	Loss reduces SR upon HSC exhaustion	Matsumoto et al. 2011; Zou et al. 2011
p18 ^{Ink4c}	Loss increases SR	Yuan et al. 2004
microRNAs		
miR125a	Loss reduces SR and increases apoptosis	Guo et al. 2010
miR125b	Expression enhances SR by blocking apoptosis	O'Connell et al. 2010; Ooi et al. 2010
RNA-binding proteins		
Msi2	Loss reduces SR	Hope et al. 2010; Ito et al. 2010; Kharas et al. 2010

PRC, polycomb repressive complex.

molecular mechanism and the relevant target genes inducing HSC expansion remain poorly defined.

Although all the discussed and numerous additional molecules could be identified to be involved in regulating HSC self-renewal, their exact interplay remains to be unraveled. More sensitive large-scale methods will likely be a key to drawing a more complete picture of the stem cell self-renewal network and to identifying a potential core mechanism of self-renewal control. It is interesting that deletion of many factors that are essential for fetal HSC generation and self-renewal (e.g., Runx1, Tal1, Notch1 and -2, RBP-J, β -catenin, and HoxB4) only have minimal consequences upon deletion in adult HSCs, whereas overexpression often results in enhanced HSC maintenance, self-renewal, and leukemia (Mikkola et al. 2003; Ichikawa et al. 2004).

3.2.2 Hematopoietic Lineage Choice and Stability

Differentiation of multipotent cells into different lineages must be well controlled to enable the timely production of the right number and type of mature cells. Despite intensive research over decades, we are only just beginning to understand how cells manage to establish and maintain a lineage-committed stage at the molecular level. The molecular mechanisms of lineage stability are better defined than those of lineage choice. Here we discuss some exemplary mechanisms of intrinsic and extrinsic control of lineage choice.

The differentiation of a multipotent cell to a specific lineage involves a global change of gene expression. Lineage choice and commitment are accompanied by the induction and maintenance of lineage-affiliated genetic programs. These include not only the expression of lineage-specific genes but also the repression of those specific for other lineages. Stable gene expression requires the presence and activity of a set of distinct TFs, which are integrated in complex networks with other TFs, modulating cofactors, chromatin modifiers, microRNAs, and other regulatory RNAs (Davidson 2010).

Most current knowledge about TF function in hematopoiesis has been gained in static gene-by-gene analyses. Genetically modified mouse models dissect TF function in distinct cell stages during hematopoiesis. These analyses unravel central players of genetic networks but ignore less prominent components that are vital for the orchestration of the whole program and the dynamic regulation of these networks. In the future we will also need to systemically understand the dynamic changes of expression or activity of the whole ensemble of lineage regulators. Deep parallel sequencing methods allowing quantitative whole-genome information about TF binding to DNA, chromatin status, and resulting transcriptional activity will be of great use

for this endeavor. Most of these studies have elucidated far more binding sites and DNA motifs than originally predicted, often at sites far from expected promoter regions. These potential *cis*- or *trans*-acting sites would have been undetected by conventional methods. However, the technical requirement for high cell numbers interferes with the necessity to analyze distinct primary cell populations at many different time points during differentiation. These usually are very infrequent, and enrichments still yield insufficient numbers and purity. Technical improvement is eagerly awaited to allow these comprehensive analyses from small cell numbers and ideally from single cells (Islam et al. 2011).

3.2.2.1 Stability of Lineage Commitment. In contrast to other tissues, plasticity between lineages, with frequent physiological “transdifferentiation” of cells of one lineage into another, has not been observed in the hematopoietic system. Although maintenance of lineage choice is critical for normal hematopoiesis, its targeted manipulation could also be used to induce dedifferentiation or differentiation in another lineage for therapeutic purposes.

The expulsion of the nucleus during late stages of erythroid maturation is—although implemented for other reasons—probably the most drastic way of preventing the activation of genetic programs of another lineage. In most other cell types, lineage commitment is not entirely an irreversible state. In contrast, it must be actively maintained by sustained commitment factor expression or network stability in committed cells and their progeny. Positive autoregulation of a lineage-specific factor while inhibiting opposing factors leads to stable situations with one factor expressed and the other repressed (Kerenyi and Orkin 2010). One excellent example is the switch from high levels of GATA2 to high levels of GATA1, which precedes erythropoiesis from HSCs and is called the “GATA switch” (Fig. 4A). GATA2 is mainly expressed in early progenitors and induces GATA1 expression, which in turn activates its own expression and represses GATA2. The switch is mediated by the displacement of GATA2 from its own upstream enhancer by increasing levels of the interacting TF pair GATA1 and Friend of GATA1 (FOG1) (Grass et al. 2003). Moreover, the displacement of GATA2 by GATA1–FOG1 at the *c-Kit* locus results in rearranged chromatin looping and down-regulated *c-Kit* expression, demonstrating the ability of TFs to directly alter long-range chromatin interactions (Jing et al. 2008). These switches can be implemented rapidly, taking only one specific S phase for their implementation, thus driving cells to the next stage of maturation in very short time (Pop et al. 2010).

Lineage commitment in B-cell development is orchestrated in a regulatory network of key TFs with feed-forward

Figure 4. Network motifs for induction and maintenance of lineage commitment. Simplified examples of molecular mechanisms and networks of stable commitment induction and propagation in erythroid (A), B-lymphoid (B), T-lymphoid (C), and myeloid (D) cells. Direct or indirect activation (arrows) and repression (barred lines) of individual factor expression are indicated. Transcription factors are depicted in white, surface receptors in gray. Dashed lines represent indirect interactions.

regulatory cascades (Fig. 4B). PU.1 and E2A are critical for lymphoid cell-fate determination and induce specific B-lineage commitment factors like early B-cell factor 1 (EBF1) and Pax5. EBF1 is a primary determinant of B-cell fate, and its expression is controlled by high expression of IKAROS and E2A in conjunction with PU.1 and by extrinsic signals of the IL-7 receptor. EBF1-deficient cells arrest in early B-cell development (before the immunoglobulin heavy-chain rearrangement) with a failure to initiate the early B-lineage program of gene expression. E2A might promote the generation of LMPPs by antagonizing megakaryocyte/erythroid-lineage programs and priming the transcription of the B-cell-specific factors EBF1 and Pax5 (Dias et al. 2008). B-cell differentiation in the absence of E2A can be rescued with EBF1 and Pax5 (Seet et al. 2004; Kwon et al. 2008). Progenitors with genetically ablated E2A or EBF1 retain their multilineage potential (with the exception of the erythroid lineage) (Dias et al. 2008; Semerad et al. 2009). EBF1 can antagonize the expression of myeloid determining factors such as PU.1, C/EBPα, and Id2 and induce synergistically with E2A the expression of Pax5 as the main determinant factor of B-cell commitment. The stable B-cell fate is locked in by a feedback loop of Pax5 and IKAROS to sustain EBF1 expression. All factors function in a concerted manner in pro-B cells to ensure the repression of myeloid genes and thus ensure stability of B-cell commitment (Pridans et al. 2008; Decker et al. 2009). Pax5 deletion leads not only to a B-cell differentiation block at the pre-/pro-B-cell stage, but it also enables B cells to dedifferentiate into an LMPP-like stage and to allow entry into normal alternative differentiation paths into various myeloid and lymphoid lineages in vivo and in vitro (Nutt et al. 1999;

Rolink et al. 1999; Mikkola et al. 2002; Delogu et al. 2006; Cobaleda et al. 2007). Pax5 is directly involved in epigenetic alterations of B-lineage-specific genes (Schebesta et al. 2007; Gao et al. 2009; McManus et al. 2011). Interestingly, ectopic expression of EBF1 in Pax5-deficient hematopoietic progenitors restricts their alternate lineage potential in vivo (Nutt et al. 1999; Rolink et al. 1999; Mikkola et al. 2002; Delogu et al. 2006; Pongubala et al. 2008). This repression of other lineage-specific molecular programs by Pax5 and partners is an excellent example of how a lineage choice can be kept stable. This mechanism also requires sustained expression and activity of these factors and might therefore be less stable against perturbations than other mechanisms, for example, involving chromatin modifications. Subtle changes and misregulation of individual TFs will directly impact normal differentiation and can lead to leukemia (Rosenbauer et al. 2006).

Epigenetic activation or silencing of lineage-restricting programs by chromatin and DNA modifications may ultimately determine lineage commitment. As one example, the *c-fms* locus (coding for the macrophage CSF [M-CSF] receptor) becomes epigenetically silenced during B-cell differentiation in a gradual process, inhibiting remaining myeloid potential (Tagoh et al. 2004). However, sustained Pax5 expression is still required for maintaining *c-fms* locus silencing, because mature B cells rapidly up-regulate M-CSF receptor expression upon Pax5 deletion. These findings well illustrate that TFs function by altering chromatin states and directly recruiting epigenetic modifying complexes (McManus et al. 2011).

In contrast to the intrinsically stabilized B-cell commitment network, T-lineage fate choice remains provisional for

an extended period of differentiation with high dependency on extrinsic stimulation from the thymic environment (Fig. 4C). T-cell fate initiation and determination requires persistent extrinsic Notch receptor signaling, which is stimulated by Delta-like 1 and 4 expressing cortical endothelial cells in the thymus (Radtke et al. 1999). Notch signaling turns LMPPs into early T-cell progenitors by inducing a pro-T-cell developmental program with direct and indirect activation of GATA3, Tcf7, and other T-cell-restricting genes, which, once initiated, positively autoregulate their own expression. The activation of a T-lineage program by Notch signaling is, however, not sufficient for T-cell fate determination alone. Additionally, Notch suppresses B-cell-lineage-determining EBF1 and E2A expression and diminishes remaining myeloid potential by down-regulation of PU.1 and C/EBP α (Ordentlich et al. 1998; Smith et al. 2005; Laiosa et al. 2006b). Id2 expression is blocked by Notch signaling to circumvent NK cell development (Ikawa et al. 2001). Conversely, the TF leukemia/lymphoma-related factor (LRF) inhibits Notch signaling in the BM, thus preventing T-cell development. Consequently, the conditional deletion of LRF in HSCs results in the generation of T-lineage progeny in the BM (Maeda et al. 2007).

3.2.2.2 Extrinsic Regulation of Lineage Choice—Instructive versus Selective Function of Cytokines.

The mechanisms discussed above can explain how a lineage choice, once it is made and implemented, can be maintained. Before this state, molecular changes in uncommitted cells must lead to the actual lineage choice and the induction of these maintenance programs. One way of inducing lineage choice is by signals from outside the cells, for example, by hematopoietic cytokines. Cytokines are very important regulators of hematopoiesis. They affect multiple cell fates of every hematopoietic cell type, including survival, proliferation, maturation, and functional activation (Rieger and Schroeder 2009). In addition to these functions, the instruction of hematopoietic lineage choice by cytokines has been postulated for decades (Metcalf 1998, 2010). However, it proved to be surprisingly difficult to show beyond doubt that cytokines do not merely allow the survival and proliferation of precursors that have already intrinsically—and independently of the presence of a cytokine—committed to one lineage (permissive/selective model) (Fig. 5) (Enver et al. 1998). The pleiotropic, redundant, and cell-type-dependent effects of cytokines are one reason for the difficulty in precisely defining their effects on cell fates (Rieger and Schroeder 2009). Loss-of-function studies have thus provided limited insight. Most studies suggesting lineage instruction by cytokines have therefore relied on ectopic molecular overexpression and/or transformed cell lines. As an example, the ectopic expression of

Figure 5. Possible influence of extracellular signals on production of lineage-committed cells. Extrinsic signals by, for example, hematopoietic cytokines may instruct the lineage choice of multipotent cells. Alternatively, extrinsic signals could merely enable survival, proliferation, and maturation signals of cells that have already committed to one lineage independently of the extrinsic signal. Importantly, static analyses at the start and end of an experiment would lead to identical data in both situations, and continuous single-cell observations are required for definitive conclusions about cytokine function.

IL-2 receptor converted CLPs from the lymphoid to the myeloid lineage, probably through ectopic up-regulation of granulocyte–macrophage CSF (GM-CSF) receptor upon IL-2 stimulation (Kondo et al. 2000; Iwasaki-Arai et al. 2003). However, the effects of ectopically expressed molecules may not be representing physiologically relevant programs of lineage choice. Only recently has lineage instruction of unmanipulated primary hematopoietic progenitors by cytokines to which they physiologically respond been proven (Rieger et al. 2009a). Culture of granulocyte–macrophage progenitors (GMPs) in only G-CSF or M-CSF will eventually lead to production of only granulocytic or monocytic cells, respectively. In the selective model, GMPs would produce progeny of both lineages even if only one cytokine was present. The absence of one lineage-specific cytokine would then lead to the death of cells that have committed to this “wrong” lineage, leading to a monolineage output (Fig. 5). However, the continuous observation of individual GMPs and all of their progeny throughout the differentiation process demonstrated the lack of cell death events during the cytokine-dependent monolineage differentiation of GMPs (Fig. 5). A purely selective effect could therefore be excluded, demonstrating GMP lineage instruction by these cytokines (Rieger et al. 2009a). However, the involved molecular mechanisms are poorly understood. A signal strength-dependent model of dendritic cell development has recently been proposed, in which dendritic cell development signals are integrated from cytokine receptor expression, downstream signal transduction, and the presence of the cytokines M-CSF, Flt3 ligand, and GM-CSF in specific niches (Schmid et al. 2010). This kind of model could have broader relevance in understanding the role of cytokines in instructing lineage development.

Despite this accumulating evidence for lineage instruction by cell-extrinsic signals in some of the more restricted progenitors, their impact on earlier branching points of the hematopoietic hierarchy still remains poorly understood. Cytokines contribute in a major way to the survival and proliferation of different hematopoietic cell types, and not all lineage-affiliated cytokines have a lineage-instructive ability. Even if specific lineages can be instructed by cytokines, this does not preclude the existence of additional cell-intrinsic lineage choice mechanisms that could lead to lineage choice in the absence of cell-extrinsic instructive signals. The capacity of individual extrinsic signals to instruct lineage choice, as well as its integration with other cytokine signals and with other intracellular molecular states like TF expression, must be further analyzed for each individual cytokine, in specific cell types, under chemically defined conditions, and ideally continuously at the single-cell level.

3.2.2.3 Intrinsic Regulation of Lineage Choice by TFs—Decision Makers or Only Executors? It is clear that individual TFs can instruct lineage choice and are even able to reprogram committed cells, leading to crossing of lineage borders. For example, ectopic expression of GATA1 in multipotent cells or cells committed to other than the megakaryocyte–erythrocyte lineages (e.g., CLPs) enforces the development into erythroid and megakaryocytic cells (Heyworth et al. 2002). C/EBP α and PU.1 are essential for the generation of granulocyte–macrophage progenitors. C/EBP α expression in committed lymphoid cells (B and T cells) instructs the development of macrophages (Xie et al. 2004; Laiosa et al. 2006a,b). Furthermore, committed T cells transdifferentiate into myeloid dendritic cells upon ectopic PU.1 expression (Laiosa et al. 2006b). TFs also actively repress lineage programs. To this end, the myeloid potential as well as PU.1- or C/EBP α -dependent myeloid reprogramming of thymic precursors can be blocked by active Notch signaling (Franco et al. 2006; Laiosa et al. 2006b; Rothenberg 2007), suggesting an instructive extracellular non-cytokine-mediated induction of T-cell identity. Findings like these clearly demonstrate the ability of many lineage-specific TFs to drive cells into one lineage. Upon their up-regulation or activation, they will start the transcription of myriads of direct and indirect target genes and chromatin modifications, which lead to the changing phenotypes of lineage-committing and maturing cells.

However, the ability of TFs to drive a cell into one lineage does not yet explain how the initial decision for this lineage was made—it only explains how this decision is then executed. What led to the up-regulation or activation of, for example, the executing TF remains unclear. One

possibility is the instruction of lineage choice by cell-extrinsic signals. In this case, TFs are most likely the final executors, implementing the lineage decision, but are not involved in the actual decision making. Another intriguing possible mechanism is the stochastic output of fluctuating cell-intrinsic networks of TFs. In this case, TFs would not only execute a previously made decision, they would also be the relevant components of the molecular machinery generating the lineage choice. This hypothesis stems from the initially surprising findings that multiple “lineage-specific” TFs are coexpressed in multipotent cells before lineage commitment (“lineage priming”; see, e.g., Miyamoto et al. 2002). These factors are expressed only exclusively in different mature lineages, and they can drive cells into this lineage upon overexpression. Initially, therefore, it was assumed that multipotent cells have the potential to differentiate into multiple lineages because they lack the expression of these lineage-specific TFs. However, with the availability of improved prospective purification of multipotent cells and more sensitive molecular methods for the detection of TF expression, it then became clear that the opposite could be true. Multipotency might in fact be characterized not by the absence of lineage-specific properties but by the presence of properties of multiple lineages, in particular the coexpression of multiple “lineage-specific” TFs. The additional finding that these TFs often inhibit each other by binding mutually or to cofactors of opposing TFs led to the idea that these factors may neutralize each other in uncommitted cells, thus retaining multipotency. Random fluctuations in their expression could then lead to one TF gaining dominance; positive autoregulation and repression of other TFs would lead to a lineage-committed state in which only one lineage-specific TF is expressed (Enver et al. 1998; Cantor and Orkin 2001; Graf and Enver 2009). The production of all lineages would be ensured by the overall wiring of the TF network leading to stable stochastic lineage output. Although not predictable for an individual cell, different cells within a population would fluctuate into decisions for different lineages with specific probabilities. Adaptation of the blood system to stress or injury would then be regulated by the selection of existing lineage-committed progenitors for survival and proliferation (Suda et al. 1984).

Studies on transcriptional pathways that control binary lineage choices in hematopoiesis revealed some similarities in the gene regulatory network circuits of different lineages. Pairs of TFs that mutually antagonize each other's activity and expression are often involved. One example is the antagonistic interplay between the lineage-determining factors PU.1 and GATA1 as a molecular mechanism of lineage choice between myeloid and megakaryocytic–erythroid fate, respectively (Fig. 4D). PU.1 and GATA1 physically

bind each other and cross-antagonize their activity (Zhang et al. 1999, 2000; Stopka et al. 2005; Liew et al. 2006). PU.1 directly inhibits GATA1 DNA-binding capacity, while GATA1 inhibits the transactivation potential of PU.1. Both PU.1 and GATA1 are autoregulatory for their own expression (Nishimura et al. 2000; Okuno et al. 2005; Laio-sa et al. 2006a), thereby providing stability to their levels, once expressed. The genetic ablation of these factors underpins their central role in implementing lineage choice. Loss of GATA1 demonstrates its absolute necessity for megakaryocyte/erythrocyte development, whereas PU.1 deficiency leads to a lack of granulocytes, macrophages, and B cells. Furthermore, both factors have instructive lineage commitment ability by implementing lineage-affiliated gene programs (Iwasaki et al. 2003, 2006). However, recent studies suggest a more complicated dynamic implementation. In fish hematopoiesis, the interplay of PU.1 and GATA1 differs in various cell stages during hematopoiesis and is influenced by other factors, such as the transcription intermediate factor 1g (tif1g), a RING domain E3 ubiquitin ligase (Monteiro et al. 2011). Moreover, PU.1 showed positive autoregulation in all analyzed cell stages, but GATA1 only in some of them. C/EBP α and FOG1 have recently been implicated in the lineage choice between myeloid and megakaryocyte/erythrocyte lineages by exhibiting transcriptional cross-regulation, potentially being better candidates for lineage choice making than PU.1 and GATA1 (Mancini et al. 2011).

Again, all of these models suffer from the fact that they are based on expression analyses with very low resolution. Precise timing of changing PU.1 and GATA1 protein levels before and during lineage choice in the individual cell would be critical for this mechanism of lineage choice. However, current expression analyses measure only population averages and/or RNA levels and/or single time points or maturation stages. This static data allows many alternative explanations and is therefore not sufficient to prove the mechanism of lineage choice. Ideally, novel technology will enable the simultaneous quantification of protein levels of multiple TFs, at the single-cell level, continuously with high temporal resolution, and throughout lineage choice of living HSCs.

4 CONCLUSION/OUTLOOK

In conclusion, hematopoiesis is an excellent model for studying the molecular mechanisms of cell-fate control. Being the classical mammalian stem cell model, with high clinical relevance, robust quantitative functional assays, and well-established culture and prospective isolation techniques, it allows us to address the underlying mechanisms of cell-fate control. Most of the basic concepts in

stem cell research have been defined in the hematopoietic system, and many novel technical approaches are first developed or applied in hematopoiesis research. Because of the routine use of hematopoietic cells for clinical therapy, the chances for a quick transfer of novel basic insights to patient benefit in the clinic are higher than for most other tissues. Nevertheless, despite decades of successful research, many questions posed very long ago are still awaiting an answer, and long-standing disputes illustrate the need for ever improving technological approaches. These continue to be exciting times in hematopoiesis research.

ACKNOWLEDGMENTS

M.A.R. is thankful for the support of the LOEWE Center for Cell and Gene Therapy Frankfurt (HMWK III L 4-518/17.004 [2010]) and institutional funds of the Georg-Speyer-Haus. The Georg-Speyer-Haus is funded jointly by the German Federal Ministry of Health (BMG) and the Ministry of Higher Education, Research and the Arts of the state of Hessen (HMWK).

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