Characterization of human myeloid progenitors and their differentiation

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Characterization of human myeloid progenitors and their differentiation

Doctoral Thesis

by

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Till Mamma och Pappa

Thus grew the tale of Wonderland:
Thus slowly, one by one,
Its quaint events were hammered out –
And now the tale is done,
And home we steer, a merry crew
Beneath the setting sun.

Lewis Carroll

ORIGINAL PAPERS

The thesis is based on the following papers, which are referred to in the text by their respective Roman numerals (I-III).

I. Edvardsson L, Dykes J, Olsson ML and Olofsson T. Clonogenicity, gene expression and phenotype during neutrophil versus erythroid differentiation of cytokine-stimulated $CD34^+$ human marrow cells *in vitro*. *Br J Haematol*. 2004; 127: 451-463.

II. Edvardsson L, Dykes J and Olofsson T. Isolation and characterization of human myeloid progenitor populations. – TpoR as discriminator between common myeloid and megakaryocyte/erythroid progenitors. *Exp Hematol*. *In press*. 2006.

III. Edvardsson L and Olofsson T. Phenotypic and functional heterogeneity within the human common myeloid progenitor (CMP) – population. *Manuscript*.

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SELECTED ABBREVIATIONS

AML(-1)	acute myeloid leukemia(-1)
β_c	common β-subunit
C/EBP	CCAAT/enhancer-binding protein
CFDA,SE	carboxyfluorescein diacetate, succinimidyl ester
CFU	colony-forming unit
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
Epo(R)	erythropoietin (receptor)
FACS	fluorescence-activated cell sorting
FL	flt3-ligand
G-CSF(R)	granulocyte colony-stimulating factor (receptor)
GF(R)	growth factor (receptor)
G/M	granulocyte and monocyte
GM-CSF(R)	granulocyte/macrophage colony-stimulating factor (receptor)
GMB	granulocyte/monocyte/B cell
GMP	granulocyte/monocyte progenitor
GMPL	granulocyte/monocyte/lymphocyte progenitor
GMT	granulocyte/monocyte/T cell
GPA/C	glycophorin A/C
GPIIb/IIIa	glycoprotein IIb/IIIa
HSC	hematopoietic stem cell
IFN	interferon
IL	interleukin
Lin	lineage marker
LTC-IC	long-term culture-initiating cell
M-CSF(R)	macrophage colony-stimulating factor (receptor)
Meg/E	megakaryocyte and erythrocyte
MEP	megakaryocyte/erythrocyte progenitor
NF-E2	nuclear factor erythroid-derived 2

NK	natural killer
PR3	proteinase 3
RT-PCR	reverse transcription-polymerase chain reaction
SCF	stem cell factor
SCL	stem cell leukemia
SDF-1	stromal cell-derived factor-1
TGF-β	transforming growth factor-β
TNF	tumor necrosis factor
Tpo(R)	thrombopoietin (receptor)

INTRODUCTION

Hematopoiesis, or the formation of blood cells, is a continuous process where the production of new cells is tightly regulated to maintain normal cell counts during steady-state, and to meet the demands put on the system during stress. The hematopoietic cells are crucial for our survival, with diverse functions such as oxygen transport, blood coagulation and innate and adaptive immune defense, and when parts or all of the hematopoiesis are disrupted in diseases such as leukemia and myelodysplastic syndromes the outcome is severe and often deadly. Knowledge of the different stages of development in normal hematopoiesis and the mechanisms of hematopoietic regulation is also the key to a better understanding of how these mechanisms go awry in hematological diseases, and consequently to the development of more effective treatments. However, despite extensive studies of the hematopoiesis and its regulation, involving a network of transcription factors and microenvironmental factors, we still have a somewhat limited understanding of this process.

The aim of this thesis was to characterize the earliest hematopoietic myeloid progenitors and events associated with commitment of these cells to specific myeloid lineages, particularly the neutrophil and erythroid lineages, through studies of normal adult human bone marrow cells. The background focuses on what is known so far of human hematopoiesis and its regulation, although on occasion data and models derived from studies in other species (primarily mice) are included, necessitated by an insufficient knowledge of the human hematopoietic system in some areas.

BACKGROUND

Hematopoiesis

The site of hematopoiesis changes throughout development, with embryonic hematopoiesis being localized to the yolk sac, the para-aortic splanchnopleura (PAS) and aorta-gonad mesonephros (AGM) regions and later also the fetal liver, while adult hematopoiesis occurs primarily in the bone marrow [1,2]. In hematopoiesis, blood cells are derived from the hematopoietic stem cells (HSCs) in a continuous and dynamic process. Stem cells are self-renewing and multipotent i.e. they can sustain hematopoiesis throughout life and they can differentiate to all hematopoietic lineages. Part of the stem cell population enters the cell-cycle either to self-renew, or to proliferate, differentiate and give rise to progenitor cells with gradually more restricted lineage potential and no self-renewing capacity. Eventually these progenitors commit to a certain lineage and terminally differentiate to mature blood cells with distinct functions and limited lifespan. As the cells differentiate the cell surface changes, some membrane components are downregulated and others are upregulated, rendering surface markers useful and often used tools for identifying cells at different stages of hematopoiesis.

Although the actual mechanisms are still largely uncharacterized the survival, proliferation and differentiation of hematopoietic cells appear to ultimately depend on the expression of different transcription factors. Also extrinsic signals from hematopoietic cytokines, cell-cell interactions with adjacent stromal cells and other factors in the microenvironment are believed to have an important role, even though their specific functions in hematopoiesis are more debated [3-7].

Hematopoietic cells

Hematopoietic stem cells

HSCs constitute a small number of cells, which by their extremely high proliferation potential, coupled with their ability to self-renew, support a constant production of new cells without being depleted, and which by their capacity to differentiate to all

hematopoietic lineages, i.e. B, T and natural killer (NK) cells (lymphocytes), erythrocytes, megakaryocytes/platelets, basophils, mast cells, eosinophils, neutrophils, monocytes/macrophages and dendritic cells, all with very different phenotypes, provide the system with a diversity of functions [8]. It was long thought that most HSCs existed in a dormant, non-dividing state, but now it seems that this apparent quiescence is only relative and that HSCs actually do cycle although very slowly [8,9]. Furthermore, since the size of this population is more or less constant despite the continuous production of hematopoietic cells, approximately half of the divisions HSCs go through must be self-renewing. The mechanisms regulating the choice between differentiation and self-renewal (or the third choice apoptosis) are not well understood, but recently regulators such as the wingless (Wnt) ligand/signaling molecules, the transmembrane molecules Notch and Sonic hedgehog, the transcription factor HoxB4 and transcriptional repressor Bmi-1 have been suggested to promote self-renewal, whereas *in vitro* exposure to cytokines such as stem cell factor (SCF), interleukin (IL)-11, IL-6, thrombopoietin (Tpo), granulocyte/macrophage colony-stimulating factor (GM-CSF), granulocyte-CSF (G-CSF) and macrophage-CSF (M-CSF) appears to primarily result in differentiation, albeit that some combinations (e.g. IL-11, flt3-ligand (FL) and SCF or SCF, Tpo and IL-11/IL-3) may achieve limited HSC expansion [2,8-10].

In mice HSCs are functionally identified by their ability to give rise to a sustained reconstitution of all hematopoietic lineages in the bone marrow of lethally irradiated recipients, often in serial transplantation experiments where cells from a transplanted individual give rise to all lineages in yet another recipient. Even single cells from stem cell-enriched populations have been shown to successfully reconstitute hematopoiesis [6,10,11]. In humans it has been more difficult to assay HSC-activity, since the corresponding *in vivo* repopulating assays obviously cannot be performed in human subjects. Instead, two types of assays have been used. There are *in vitro* assays such as long-term culture-initiating cell (LTC-IC) assays, where candidate cells are cultured for about 5-12 weeks on adherent human stromal cells from bone marrow, mimicking the bone marrow microenvironment [11,12]. This is followed by a secondary colony-

forming assay in semisolid cultures or murine stromal cell cultures, where any remaining primitive cells will give rise to myeloid and lymphoid colonies (primarily of the NK and B cell lineages, as T cells despite recent advances [11,13] are generally difficult to culture in *in vitro* assays,), indicating the presence of self-renewing capacity and multipotency. Secondly, *in vivo* xenotransplantation models have been used, which test the ability of human cells to repopulate sheep fetuses or sublethaly irradiated immunodeficient mice such as nonobese diabetic/severe combined immunodeficiency (NOD-SCID) mice [8,11,12].

HSCs do not have any specific morphological appearance among other mononuclear cells. Instead other characteristics are used to isolate candidate stem cells, for example the expression of surface antigens, intracellular enzyme markers (aldehyde dyhydrogenase), dye efflux properties (rhodamine-123 and/or Hoechst 33342), sensitivity to cycle-active cytotoxic agents (5-fluorouracil) and cell-cycling properties [8]. Among these methods fluorescence-activated cell sorting (FACS) based on surface marker expression has emerged as one of the major tools for identification and isolation of HSCs [2,8]. So far a surface marker that exactly pinpoints the HSC-population has not been found and may not exist at all, but specific combinations of antigens can be used to isolate populations considerably enriched for these cells. Generally, murine HSCs are defined as Sca-1^{+}c-kit^{+}lineage marker $(Lin)^{-/lo}$, where Lin represents a collection of surface antigens primarily expressed on lineage-committed cells [2,6,8]. Within this population it is primarily the Thy-$1.1^{lo}flt3^{-}$ cells that give rise to long-term (LT) multilineage reconstitution in irradiated mice, but also differential expression of CD34 have been used to subdivide the Sca-1^{+}c-$kit^{+}Lin^{-/lo}$ population, where $CD34^{-/lo}$ cells provide a more LT reconstitution of the myeloid and lymphoid lineages than $CD34^{+}$ cells, indicating a higher self-renewing capacity in the former population [2,6,8]. Notably, it was recently suggested that the simple use of three surface receptors belonging to the SLAM family; CD150, CD48 and CD244, can replace or improve the previously used methods of HSC isolation by defining HSCs as $CD150^{+}CD48^{-}CD244^{-}$, multipotent progenitors as $CD150^{-}CD48^{-}CD244^{+}$ and lineage-restricted progenitors as $CD150^{-}CD48^{+}CD244^{+}$ [14,15].

However, even though the mouse is a frequently used model system for human hematopoiesis, there appear to be discrepancies between the hematopoietic immunophenotypes of these species. In humans the CD34 antigen (a sialomucin with a possible non-essential role in adhesion to bone marrow stroma and in homing) is the most widely used positive marker for HSCs and progenitor cells [1,16]. The $CD34^+$ population constitutes about 0.5-5% of all hematopoietic cells in fetal liver, cord blood and bone marrow, and contains almost all cells with *in vitro* colony-forming potential [2,16]. However, the $CD34^+$ population is very heterogeneous and HSCs and pluripotent progenitor cells represent only a small fraction (1-10%) of this population [2,12]. More specifically the stem cells seem to be included in the $Lin^-CD34^+CD38^-$ population, whereas most lineage-restricted progenitors localize to the $CD34^+CD38^+$ population [2,12], and $Lin^-CD34^+CD38^-$ expression is the most commonly used definition of human HSCs [2], even though this population is also quite heterogeneous. Further attempts to narrow down the HSCs have been made by introducing markers such as Thy-1, HLA-DR, c-kit, vascular endothelial growth factor receptor 2 (VEGFR2) and flt3 [17-23], several of which are used to define murine HSCs, but which present a different expression on human HSCs e.g. $flt3^+$ rather than $flt3^-$ and $c\text{-}kit^{-/lo}$ rather than $c\text{-}kit^{-+}$ [19-22]. On the other hand a small fraction of human Lin^-CD34^- cells have, in concurrence with findings in mouse, been found to contain *in vivo* repopulating activity [2,8], although the physiological relevance of expression versus no expression of CD34 on primitive cells is not known [24,25]. Possibly, yet another surface marker, CD133, can be used to positively identify both $CD34^+$ and $CD34^-$ human HSCs [26].

Hematopoietic progenitor cells and the hematopoietic lineage map

Progenitor cells are generally considered to be multipotent, oligopotent or unipotent cells without any significant ability to self-renew [10]. However, not all adhere to this model. For example Quesenberry *et al.* [27-29] have proposed that the same cells act as both stem cells and multipotent progenitors and that the function of the cell is dependant upon the cell-cycle phase. Hence, the HSCs and early progenitors would exist as a reversible continuum rather than a hierarchy of distinct cell types. The model is based on observations of reversible changes in repopulation-potential, surface

adhesion molecules and gene expression profiles, as well as an inverse correlation between progenitor numbers and repopulating potential, as HSCs progress through cell-cycle initiated by growth factor (GF)-stimulation [27-29]. Alternatively, these variations with cell-cycle can be interpreted as a variable function within a HSC compartment separate from the progenitor cells [8,30], and even though there are other models also advocating some sort of continuum rather than hierarchy of hematopoietic development or even a completely stochastic lineage commitment with no preferred lineage-combinations [31-33], the most generally accepted model postulates that development from HSCs to terminally differentiated cells involves a progressive loss of self-renewing potential and lineage potential and that this occurs in a hierarchal and linear process where the ability to become specific branches or lineages is essentially irreversibly lost [2]. Nevertheless, there are several hypotheses for how this gradual restriction of cellular fate occurs, primarily based on different *in vitro* clonogenic assays used to quantify multi- and unipotent progenitors. All models so far seem to agree on an initial separation of megakaryocyte and erythrocyte (Meg/E) development from lymphoid development, but disagree on whether the granulocyte and monocyte (G/M) branch co-localize with the former or latter or both (reviewed by Katsura [34] and Traver *et al.* [35]) (Fig. 1). The classical model, based on findings from immunophenotyping, *in vitro* culture assays, experimental bone marrow transplantation and clinical experience [36], proposes that HSCs or rather primitive multipotent progenitors first commit either to the lymphoid or myeloid branch through a common lymphoid progenitor (CLP) or a common myeloid progenitor (CMP), which have potential for all lineages of their respective branches (Fig. 1A). The CMP is then posited to further commit to the granulocyte/monocyte progenitor (GMP) or the megakaryocyte/erythrocyte progenitor (MEP) [2,37]. However, the co-existence of an alternative pathway to lymphoid and G/M commitment has been suggested, with an initial loss of Meg/E potential and the formation of a granulocyte/monocyte/lymphocyte progenitor (GMLP) (Fig. 1B). In a third model the CMP does not exist at all, the singular first branch point being between the Meg/E pathway and a GMLP (Fig. 1C). Moreover, even though there is little debate on the

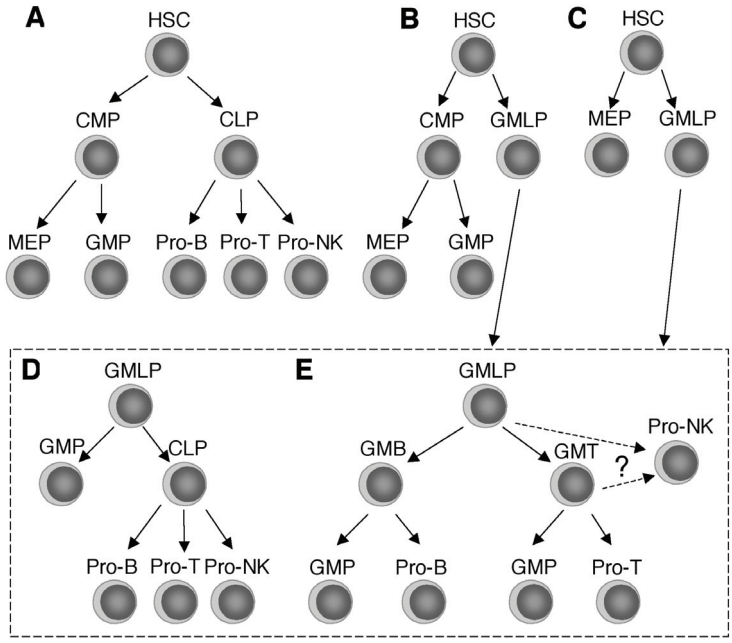

Figure 1. Models of the hematopoietic lineage map. There are several models for how lineage-committed progenitors develop from HSCs. These models postulate that the HSCs give rise to **(A)** a CMP and a CLP, **(B)** a CMP and a GMLP or **(C)** a MEP and a GMLP. The CMP and CLP then further commit to more restricted myeloid (MEP and GMP) and lymphoid progenitors (pro-B, -T and –NK), respectively, while the GMLP is proposed to give rise either to **(D)** a GMP and a CLP or **(E)** GMB- and GMT-progenitors. (Pro-B, T and NK, progenitor-B, T and NK cell)

co-differentiation of the Meg/E lineages or the G/M lineages, there are different opinions on the continued fate of the proposed GMPL, where some suggest a separation of a GMP from a CLP (Fig. 1D), whereas others believe that the progenitor gives rise to a granulocyte/monocyte/T cell (GMT)- or granulocyte/monocyte/B cell (GMB)-progenitor (Fig. 1E) [34,35]. Several studies in mice indicate the existence of a common G/M and lymphoid pathway, and even though most of these studies demonstrate a relationship only between the monocyte and B cell lineages and only in fetal hematopoiesis, which appear to differ from the adult system with a wider developmental potential and less restricted lineage potential [35,38], there are also data including granulocytes and T cells, some with adult cell sources [34,35,39]. In accordance, findings of a murine GMPL containing granulocyte, monocyte, B and T cell potential separated from cells with Meg/E potential by their surface expression of flt3 was

recently presented [40], but there is little evidence contradicting at least a co-existence of a CMP. Especially since the classical and most widely accepted model found strong support when a murine CLP ($Lin^-Sca\text{-}1^{lo}c\text{-}kit^{lo}Thy\text{-}1.1^-IL\text{-}7R\alpha^+$) [41] and CMP ($Lin^-Sca\text{-}1^-c\text{-}kit^+IL\text{-}7R\alpha^-CD34^+Fc\gamma RII/III^{lo}$), further differentiating into GMPs ($Lin^-Sca\text{-}1^-c\text{-}kit^+IL\text{-}7R\alpha^-CD34^+Fc\gamma RII/III^{hi}$) and MEPs ($Lin^-Sca\text{-}1^-c\text{-}kit^+IL\text{-}7R\alpha^-CD34^-Fc\gamma RII/III^{lo}$) [42], were characterized. Regarding the CLP versus GMB/GMT models, the latter have been indicated in studies on fetal liver cells [34], which in general display more retention of G/M potential in lymphoid development than adult cells [35,38]. However, in adult hematopoiesis there appear to be a separation between G/M cells and a CLP [41,43], although some suggest that a polarization (but not restriction) towards either T cell or B cell development occurs prior to complete loss of G/M potential [44-46]. Altogether, the collected evidence imply that there may be alternative pathways for development of the hematopoietic lineages in mouse, but so far the relative importance of these pathways has not been determined [39]. The accumulation of myeloid colony-forming activity in the $IL\text{-}7R\alpha^-Lin^-Sca\text{-}1^-c\text{-}kit^+$ CMPs, GMPs and MEPs and the often limited G/M proliferation capacity found in potential GMLPs, could suggest that the former three populations represent the major myeloid differentiation pathway [35].

As regards human hematopoiesis, there do exist mixed G/M/B-type and rarer G/M/T-type human leukemia [47], but despite a number of findings indicating the possible existence of a common G/M and lymphocyte differentiation pathway in mouse, there are only a few reports of such findings in humans. Moreover, these studies either directly display a co-existing erythroid differentiation or do not exclude it [48,49], and consequently do not provide reliable support for a considerable human G/M/lymphoid differentiation separate from Meg/E differentiation. Overall it is difficult to translate isolation of murine populations into isolation of their human counterparts and whether this just represents a difference in the immunophenotypes of essentially alike pathways or more fundamental differences between the two systems is not known. However, by using other sets of surface markers candidate populations for a human CLP were identified as $Lin^-CD34^+CD38^+CD10^+$ cells in bone marrow (containing common progenitors for B, NK and dendritic cells, T lymphocyte progenitors and very little

G/M potential) and $CD34^+CD38^-CD7^+$ cells in cord blood (with common B, NK and dendritic progenitors, but virtually no myeloid potential) [50,51]. CXCR4, the stromal cell-derived factor-1 (SDF-1) receptor, has also been proposed to distinguish lymphoid progenitors, but in that study lymphoid bipotentiality was not investigated at the single-cell level [52]. There have also been several attempts to phenotypically identify and isolate the human CMP, GMP and MEP, where markers such as CD45RA, CD71, CCR1, CD64, M-CSF receptor (M-CSFR), flt3 and IL-3Rα [53-65] were used for separation of the G/M and erythroid branches from multipotent cells, but a convincing definition including all three progenitors was not presented. Then a few years ago, promising candidates for the human myeloid progenitors were isolated from bone marrow as well as cord blood, by using a combination of some of the above mentioned antigens and defining the CMP, GMP and MEP as $Lin^-CD34^+CD38^+IL\text{-}3R\alpha^{lo}CD45RA^-$, $Lin^-CD34^+CD38^+IL\text{-}3R\alpha^{lo}CD45RA^+$ and $Lin^-CD34^+CD38^+IL\text{-}3R\alpha^-CD45RA^-$, respectively [66]. CD45RA, an isoform of CD45, acts as a negative regulator of GF receptor (GFR)-signaling [67] and IL-3Rα is a subunit of the receptor for an important GF in early myeloid hematopoiesis (see below) [2,35]. Notably, by including lymphoid-expressed antigens such as CD7, CD10 and CD19 among the removed lineage markers this definition essentially prevented co-isolation of lymphoid progenitors, as demonstrated by the lack of *in vitro* NK or B cell potential, albeit that the CMPs (like their murine counterparts [42,68,69]) generated a small number of $CD19^+$ B cells *in vivo*. Furthermore, a significant fraction of single CMPs gave rise to both G/M and Meg/E cells, despite the removal of most multipotent LTC-IC activity through exclusion of $CD38^-$ cells, and the population generated both GMPs and MEPs in *in vitro* culture, identifying the two latter as progeny of the CMPs [66]. The isolation of the above discussed progenitor populations seems to support a linear model where differentiating human HSCs irreversibly choose either the lymphoid or myeloid pathway [2] and though the existence of a common lymphomyeloid pathway [48] or redirected differentiation between these branches [49] cannot be excluded, there is little data indicating that this would contribute in any major way to the physiological myeloid differentiation in human hematopoiesis. Therefore, this work is based on the

classical model with a CMP and CLP, and this model will be depicted and referred to from now on (Fig. 2).

According to this model the CLP subsequently gives rise to B, T and NK cells whereas in the myeloid branch, the focus of this work, the CMP-derived GMPs and MEPs further differentiate to neutrophils/monocytes/macrophages and megakaryocytes/ erythrocytes, respectively. Mature neutrophils, one of three granulocytes, have a multilobed nucleus and numerous granules with microbicidal activity, and constitute a central part of our first-line defense against infecting agents and tumor cells [70], whereas circulating monocytes, also they equipped with granules, migrate into tissue where they transform into phagocytic macrophages with different properties and functions depending on the tissue, but generally involving cell-cell interactions, antigen presentation, killing of pathogens etc [71]. Developing erythroid precursors extrude their nuclei and form mature biconcave erythrocytes, covered with numerous blood group antigens and with transport of oxygen and carbon dioxide to and from tissues, respectively, as their main function [72]. In megakaryocytes an uncoupling of cell division and genome replication (endomitosis) gives rise to polyploid megakaryocytes, from which exvagination and subsequent fragmentation of long proplatelet-processes result in the formation of platelets, with a crucial role in thrombus formation and the repair of vascular damage [73,74].

Although the developmental pathways of the above mentioned lineages is extensively studied, much less is known about the origin and pathways of the remaining two granulocytes, eosinophils and basophils, which have been suggested to share early progenitor stages with a number of different myeloid lineages, including each other (as a colony-forming unit-eosinophil/basophil, CFU-Eo/Baso) [75]. However, recent studies in mouse indicate that eosinophil progenitors and separate bipotent basophil/mast cell progenitors develop from GMPs, with possible alternative differentiation pathways directly from the CMP (or even earlier cells [76]) [77,78], but it should be noted that the human counterparts of these progenitor populations have yet to be defined. The origin of dendritic cells, bone marrow-derived leukocytes with high antigen presenting

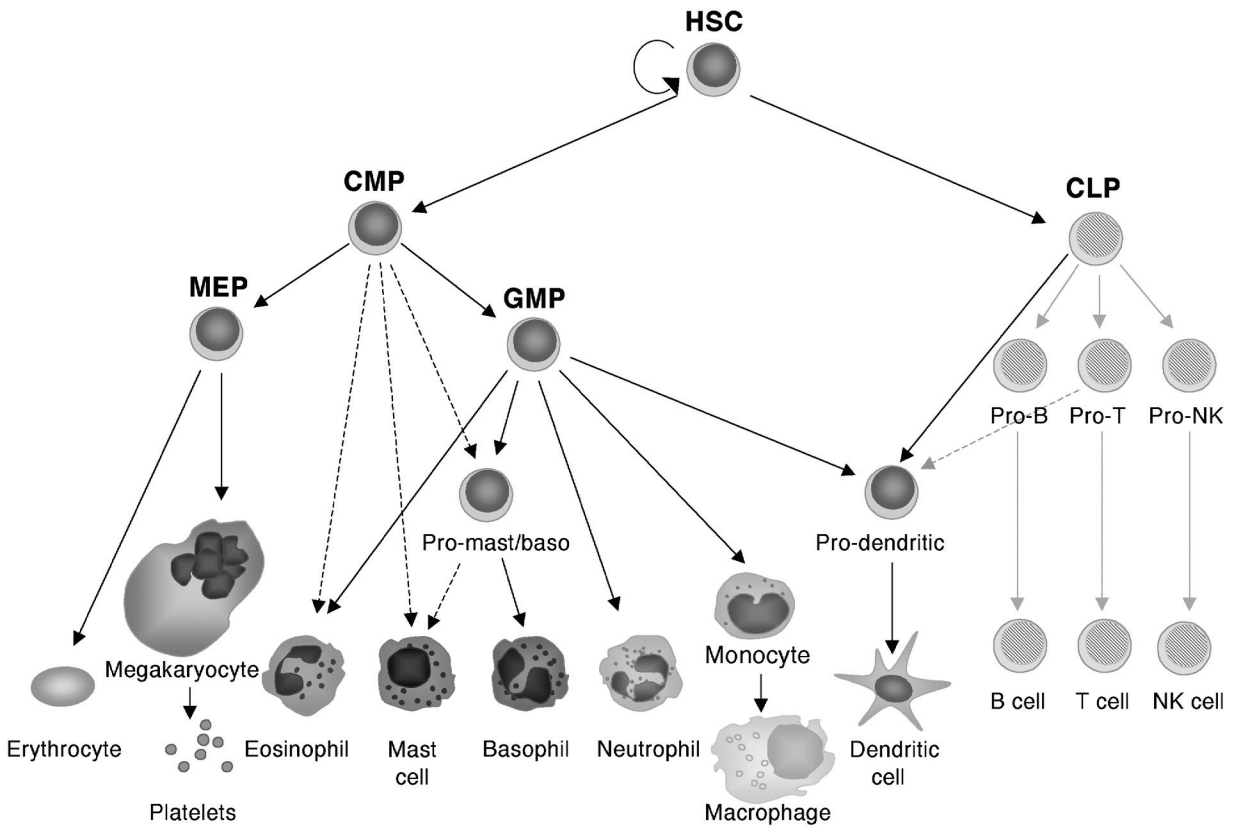

Figure 2. The hematopoietic lineage map. Schematic picture of hematopoietic development from HSCs via CMPs or CLPs and subsequent, more restricted progenitor stages to mature cells of the different hematopoietic lineages. (Pro-B, T or NK, progenitor-B, T, or NK cell; pro-mast/baso, progenitor-mast cell/basophil; pro-dendritic, progenitor-dendritic cell)

capacity, has also been debated, but in mice they are now believed to differentiate from both myeloid and lymphoid precursors [68], primarily associating with the G/M and T cell pathways, respectively (although association with B cells have also been observed [43]) and human dendritic cells appear to develop in a similar manner [35].

Plasticity

In later years the possible existence of plasticity or fate conversion of cells in the hematopoietic compartment has been widely discussed. It has been suggested that cells apparently committed to this compartment can assume non-hematopoietic fates or that cells can switch lineage-affiliation within the compartment [2,79]. Some believe that the apparent transdifferentiation between non-hematopoietic and hematopoietic cells is actually due to circulating HSCs entering other tissues, the presence of tissue-specific non-hematopoietic stem cells in bone marrow or cell-fusion between hematopoietic and non-hematopoietic cells. However there are reports of purified HSCs giving rise to other tissue cells (liver and epithelial cells) and transdifferentiation in this direction is further supported by the finding that HSCs express multiple non-hematopoietic genes as well as hematopoietic genes, thus implying a multitissue developmental potential in these cells, even though the normal *in vivo* frequency (if it occurs at all) is not known [2,35,79,80]. Conversion of lineage fate within the hematopoietic compartment has been achieved in studies with cell lines and genetically modified cells (transcription factor knock-out or introduction, GFR introduction etc.), but so far little evidence for an occurrence of such events in normal *in vivo* settings have been presented. If transdifferentiation does take place it is presumably with very low incidence, and dependant on how closely related the two lineages are and how much of their transcriptional machinery they share [2,35,36,79].

Hematopoietic regulation

To be able to maintain the normal steady-state production of the different hematopoietic cell types and respond to specific demands during physiological stress such as infection, hypoxia or hemorrhage, the hematopoiesis is under stringent control. Proliferation, commitment, differentiation and survival of hematopoietic cells are

regulated by a complex network of interactions with extrinsic factors such as surrounding cells, extracellular matrix components and soluble or bound cytokines (GFs or growth inhibitors), and ultimately by cell-intrinsic changes in the expression of transcription factors and other crucial molecules, but the exact mechanisms that govern the hematopoietic development are far from completely elucidated [70,81].

Opinions on how the different regulating factors contribute to the regulation of hematopoiesis differ. The role of transcription factors in directing hematopoiesis and commitment is generally accepted today, but the role of cytokines and other extrinsic factors is more debated. According to the stochastic model random cell-intrinsic events establish a dominating expression of transcription factors associated with one hematopoietic lineage or branch, a pattern which is subsequently enforced by positive autoregulation and cross-antagonizing effects on opposing lineage factors (see below) and results in commitment to the favored differentiation pathway. Transcription factors in turn initiate expression of lineage-affiliated genes e.g. GFRs. GFs then act on the cell in a permissive rather than instructive way, being required for the continued survival, proliferation and complete maturation of already committed cells, but not for inducing commitment [6,82,83]. In contrast, the instructive (extrinsic) model postulates that signals from lineage-affiliated hematopoietic GFs and other external influences, such as cell-cell or cell-matrix interactions, instruct uncommitted cells to differentiate to the respective lineages by initiating changes in the transcription factor expression [35,81,84]. Both models have experimental support. For example the fact that all myeloid lineages are produced to some extent despite null mutations of myeloid GFs in mice, whereas null mutations or overexpression of transcription factors often have major effects on differentiation, seems to corroborate the stochastic model [81,83,85]. On the other hand, the instructive model is substantiated by findings that exogenous introduction of some GFRs can redirect differentiation and that GF-signaling can induce increased expression of lineage-affiliated transcription factors, thus favoring differentiation along a particular lineage [6,81,84]. Perhaps the most likely model is a combination of both i.e. that some cell compartments are open for the instructive effects of extrinsic factor, whereas others (particularly primitive cells) are not, or that

both types of regulation can act on the same cells depending on the demands put on the system [81,83].

Transcription factors

Even though there are still many unanswered questions regarding commitment and differentiation in the hematopoietic compartment there is little doubt that the process is regulated by the expression and function of a wide range of transcription factors. Different transcription factors are expressed in different maturation stages and lineages. However, accumulating evidence indicate that few factors are truly lineage-specific, instead it seems that different levels and combinations of expression characterize the different lineages. In fact, several lineage-associated transcription factors (as well as GFs and lineage-linked genes such as globins and neutrophil granule proteins) are promiscuously co-expressed at low levels already in early progenitor cells and even in HSCs [66,81,86-88]. This has by many been interpreted as a sort of pre-activation or priming of these genes, the result of an open chromatin-structure required to keep potentially needed genes transcriptionally accessible [35,80,83]. Notably, these patterns of co-expression support previously described CMP and CLP definitions in mouse, as a large fraction of single CMPs co-express G/M- and Meg/E-associated genes, but not lymphoid genes, while CLPs co-express T , B and NK cell-associated genes, but not myeloid genes and multipotent progenitors express both myeloid and lymphoid genes [80,88]. It appears that in commitment to specific lineages, transcription factors, co-activators and co-repressors interact to initiate and consolidate expression of the genes necessary for differentiation of the favored lineage or lineages, while concomitantly repressing expression and function of gene-products linked to unrelated lineages [6,36,81,89]. These events then lead to further production of transcription factors, GFRs and other proteins conferring lineage-specific properties, eventually resulting in terminally differentiated cells. Several transcription factors with pivotal roles in hematopoiesis have been identified, although primarily through studies in mice and cell lines [90-92]. Here, the focus is on the early and myeloid development.

Transcription factors such as stem cell leukemia (SCL), LMO2, acute myeloid leukemia (AML)-1, GATA-2 and c-Myb are believed to be important in early hematopoiesis, often before any lineage commitment occurs [5,6,90,91]. In addition, AML-1 and c-Myb are expressed in early G/M development [91,93], whereas SCL, LMO2 and GATA-2 are expressed in early Meg/E development [90,94]. It is also at these stages of development that the expression of more obviously lineage-associated transcription factors becomes important. PU.1 and CCAAT/enhancer-binding proteins (C/EBPs) appear to be the major regulators of G/M differentiation and the associated gene expression [36,95], in co-operation with c-Myb, AML-1 and retinoic acid receptor (RAR)α and later on in granulocyte and/or monocyte lineage committed cells, Sp1 and Egr-1 [6,93,95,96]. Similarly, GATA-1 has emerged as a key factor for erythroid and megakaryocytic development together with friend of GATA (FOG)-1, SCL/LMO2, GATA-2 and at later stages erythroid krüppel-like factor (EKLF) and nuclear factor erythroid-derived 2 (NF-E2) [6,90,92]. (see Fig. 3) In addition, members of the homeobox family of transcription factors and many other factors, participate in the regulation of hematopoiesis [5,6,12].

SCL

The basic helix-loop-helix (bHLH) transcription factor SCL, which binds DNA heterodimerized with splicing products of the E2A gene and often in complex with LMO2, appears to have a crucial role very early in hematopoiesis possibly in the actual specification of the HSC from mesoderm, as indicated by the complete absence of normal hematopoietic development in SCL null mice and cells [6,90,97,98]. It has also been suggested that the factor is involved in processes such as proliferation, progression through cell-cycle, self-renewal and possibly inhibition of apoptosis, and dysregulation of the gene is strongly associated with leukemogenesis [97,98]. The identification of c-kit, the receptor of the early-acting SCF (see below), as a target gene of a SCL transcription-complex further supports an early role in hematopoietic development [99,100].

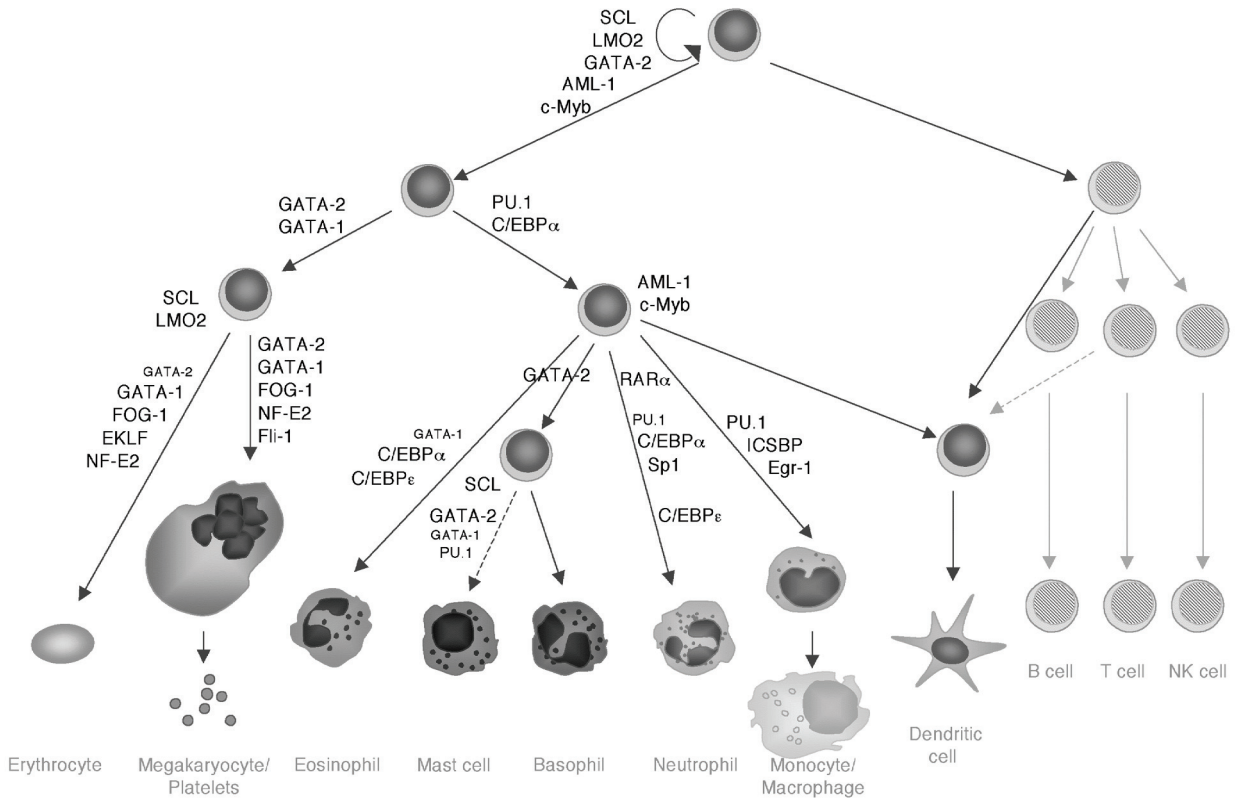

Figure 3. Transcriptional regulation of early and myeloid hematopoiesis. Selected transcription factors with important roles in early and myeloid development are depicted at their suggested sites of action. Low-level expression is indicated with smaller font.

In addition, studies have also indicated a strong association between SCL and megakaryocytic and erythroid differentiation [97,98,101,102]. SCL gene expression increases in early erythroid differentiation and the resulting protein forms an erythroid transcription-activating complex with GATA-1 among others, with LMO2 acting as a bridge [97,103]. This complex has potential binding sites in the genes of erythroid-associated transcription factors GATA-1 and EKLF and is essential for activation of the glycophorin A (GPA) gene, an erythroid-specific blood group antigen [98,104]. In accordance with these pro-erythroid effects, SCL has also been suggested to repress differentiation along the G/M branch [90,97], even though it may subsequently have a role in mast cell differentiation [97,98]. These temporal- and lineage-specific effects of SCL could be the result of a varying composition of the SCL-containing complexes needed for gene transactivation, e.g. with GATA-2 being the preferred partner in regulation of early targets such as c-kit, whereas GPA-regulating complexes primarily include GATA-1 [98,104].

GATA transcription factors

The GATA transcription factor family has six family members with two highly conserved zinc finger domains and all bind a core GATA consensus-motif in gene regulatory elements [90,105]. Two family members, GATA-2 and 1 are key factors in early and myeloid hematopoiesis (while the third hematopoiesis-expressed GATA factor, GATA-3, has its primary role in lymphopoiesis [35]).

GATA-2 is expressed in HSCs and progenitors [105] and knock-outs of the GATA-2 gene display severe and general defects in hematopoiesis, suggesting an early pivotal role in this process [106], although the detection of some remaining hematopoietic cells indicates that GATA-2 acts downstream of SCL [107]. Rather than taking part in the initiation of hematopoiesis, GATA-2 seems to have a crucial role in maintenance and proliferation of immature hematopoietic progenitors [90,105], but looses its importance prior to terminal differentiation [108].

Even though GATA-2 most likely has its main functions in early hematopoiesis, it may also have a role in cells committed to the Meg/E pathway, possibly in the subsequent choice between the two lineages, with high GATA-2 expression favoring the megakaryocytic lineage [109]. Its importance for Meg/E differentiation is supported by the apparent transactivating role at the onset of GATA-1 gene expression [105] and the inhibiting effect on the G/M transcription factor PU.1 [110]. At the same time a subsequent decrease in GATA-2 expression (probably through GATA-1-mediated repression) is required for terminal erythroid differentiation [103,111-114]. In addition, an involvement in eosinophil, basophil and mast cell differentiation have been suggested. What the role of GATA-2 is in these pathways has yet to be determined, but it appears to differ between the lineages and to be most crucial in the development of mast cells [115,116].

GATA-1, the founding member of the GATA family, displays high levels of expression in erythroid cells [103,113] and studies in mice and cell lines have indicated a major role in erythroid proliferation and differentiation, and possibly in commitment to this lineage, as well as a role in the closely linked megakaryocytic differentiation [92,105,117]. Furthermore, germline and somatic mutations of the GATA-1 gene, or the GATA binding site of a number of target genes, have in humans been found to result in hematological disorders involving these two lineages [105,111,118]. The GATA binding site is found in regulatory elements of many erythroid- and megakaryocyte-associated genes such as globins, EKLF, FOG-1, erythropoietin receptor (EpoR), glycoprotein IIb (GPIIb), the 45kda subunit of NF-E2 (p45) (see below) and Tpo receptor (TpoR) [90,119,120]. Moreover, GATA-1 positively autoregulates its own expression [105] and affects the function of the G/M-associated transcription factor PU.1, by binding to it and displacing co-factors such as c-Jun and thus reducing the expression of PU.1 target genes [6,110]. Through these mechanisms GATA-1 can stabilize the erythroid lineage choice at the expense of the G/M lineages.

Additionally, GATA-1 is expressed in eosinophil and mast cell differentiation [115,116,121,122] and its importance is demonstrated by defects in differentiation of these

lineages, as well as the erythroid and megakaryocytic lineages, in GATA-1 knock-out mice [118]. The specific effects in the separate lineages are probably achieved by different levels of GATA-1, combined with additional transcription factors [118,123]. Accordingly, besides GATA-1, FOG-1 and the erythroid-specific EKLF seem to be required for the expression of a number of erythroid genes (e.g. α- and β-globin) [90,92]. FOG-1 is also expressed together with NF-E2 and Ets proteins (e.g. Fli-1) in megakaryocytic differentiation [119,124,125], while it on the contrary appears to inhibit eosinophil differentiation, which instead requires lower levels of GATA-1 combined with C/EBPα [116,126]. Mast cell development involves simultaneous expression of GATA factors (1 and 2) and the G/M-associated factor PU.1, indicating great complexity in the interactions between these otherwise mutually inhibitory factors [115,127-129].

NF-E2

NF-E2 is a heterodimeric basic-leucine zipper transcription factor consisting of a ubiquitously expressed 18 kDa subunit (small Maf family proteins, Maf G and Maf K) and a 45 kDa subunit (p45) with a more limited expression. In the absence of p45 the Maf proteins bind the NF-E2 site as a homodimer, acting as a repressor rather than activator of transcription [130]. Expression of the p45 subunit has generally been considered to be restricted to hematopoietic progenitors and cells committed to the erythroid, megakaryocytic and mast cell lineages [103,113,131], but expression in peripheral granulocytes have been reported as well [130]. NF-E2 appears to have important functions in the later stages of erythropoiesis by regulating α- and β−globin gene expression and controlling the gene expression of two enzymes involved in heme-synthesis [131]. However, disruption of the gene encoding the p45 subunit has only a mild effect on erythropoiesis in mice, suggesting that there are alternative molecules or mechanisms that can perform these functions [119,131]. On the other hand the knock-out mice display severe thrombocytopenia with numerous immature, dysplastic megakaryocytes in the bone marrow, reflecting a crucial role of NF-E2 (and p45) in terminal megakaryocyte differentiation and proplatelet formation. The same phenotype is obtained if both genes of the alternative small-Maf proteins are disrupted [119,132]. The

few identified target genes have diverse function e.g. β1-tubulin (with an important cytoskeletal function in platelet formation), the signal protein Rab27b, thromboxane synthase and caspase-12 [119,124,131,132].

PU.1

PU.1 is a member of the Ets transcription factor family and its expression seems to be restricted to hematopoietic tissues [36,107]. Like many other genes it is expressed at low levels already in the HSCs, but even though recent findings actually imply a role already at this stage (e.g. in self-renewal) [133], its function in myeloid and lymphoid differentiation, revealed by the selective expression in G/M, B lymphoid and mast cell development [86,107,127,134] and the defective G/M and lymphoid development seen in mice with a disrupted PU.1 gene [36], is more well-established. Further evidence for the importance of this factor in G/M differentiation is introduced by the apparent association between perturbed levels of PU.1 expression (rather than complete absence) and development of AML [36,135]. As in the case of GATA-1 the expression levels appear to be critical for the different functions of the transcription factor. G/M development seems associated with a higher level of expression than B cell development [136,137] and if maintained this expression results in the formation of monocytes, whereas differentiation towards neutrophils requires a subsequent decline [138,139]. PU.1 is involved in the regulation of numerous genes characteristic of G/M development such as CD11b, CD64, the receptors of M-CSF, GM-CSF and G-CSF, granule proteins (e.g. proteinase 3 (PR3), myeloperoxidase, neutrophil elastase) [95,107], as well as PU.1 itself, and the differentiation block in granulopoiesis and monopoiesis seen in PU.1 knock-out mice is also associated with loss of expression of these target genes [36]. PU.1 is believed to be important for the initial commitment to the G/M lineages and although its presence may not be absolutely necessary for this process, it seems to be required for the production of normal mature neutrophils and monocytes [36]. In neutrophils PU.1 is combined with C/EBPα (see below), whereas interferon consensus sequence binding protein (ICSBP) and Egr-1 appear to be important in monocytes [6]. Equally important, PU.1 interacts with and blocks the DNA-binding domain of the erythroid transcription factor GATA-1 [140], which coupled with positive

autoregulation, makes it possible for only small initial changes to result in significant reinforcement of the G/M lineages. Yet, there are reports suggesting a role for low levels of PU.1 in regulating proliferation of erythroid progenitors [141,142]. Most likely the ratio of GATA-1/PU.1 proteins at a critical time point in the CMP, determines the lineage fate of the cell [6].

C/EBPs

The C/EBP family has six members; α, β, δ, ε, γ and CHOP, which bind to DNA as homo- and heterodimers and are expressed in multiple hematopoietic and non-hematopoietic cell types [36,95]. So far C/EBPα and ε have the most well defined roles in G/M development, even though the β- and δ-forms are also clearly expressed in the neutrophil, monocyte and eosinophil lineages [36,95].

The importance of C/EBPα in G/M development is supported by the apparent pathogenic effects of mutations and dysregulation of the factor, found in human AML [6,36,143]. C/EBPα is expressed early on in G/M differentiation [86,134], with general G/M genes such as M-CSFR, GM-CSF receptor (GM-CSFR), G-CSF receptor (G-CSFR) and C/EBPα itself as targets, and it has been suggested that this factor is involved in the commitment to the GMP through induction of or in co-operation with PU.1 [95,144]. In addition, C/EBPε and neutrophil granule protein genes are also targets of C/EBPα, a fact which together with the markedly increased expression in neutrophil development and the concurrent decrease in monocytic development suggest a role in commitment to neutrophil differentiation [36,95]. In further support, the C/EBPα null mice display a specific differentiation block of neutrophils and eosinophils [93,145]. As mentioned above the level of PU.1 expression have also been indicated as an important regulating factor in the bifurcation of the neutrophil and monocyte lineages and in fact it is probably the ratio between C/EBPα and PU.1 that determines whether the cells will mature into neutrophils (high C/EBPα/low PU.1) or monocytes (low C/EBPα/high PU.1) [138,139]. Accordingly, the two transcription factors exhibit a complex interplay, where C/EBPα can activate the promoter of the PU.1 gene [144], the expression of which is needed in late neutrophil differentiation, but also has the ability

to bind PU.1 and block its function by displacing c-Jun from PU.1 [146], thus preventing the autoregulating PU.1 from reaching the high levels necessary for monocyte development and consequently favoring neutrophil maturation. However, it should be noted that the block in neutrophil differentiation seen in C/EBPα null mice can be overcome, indicating the existence of alternative, possibly GF-mediated, pathways for neutrophil differentiation [36,144]. GFs are most likely important in the C/EBPα-mediated pathway as well and it has been proposed that signaling through the C/EBPα target gene G-CSFR induce C/EBPα expression, thus creating a co-operative autocrine loop between C/EBPα and G-CSFR activities [93].

In addition to its role in commitment, C/EBPα is also a key factor in terminal neutrophil differentiation being one of the factors inducing the necessary cell-cycle arrest [6,95]. At this later stage of development, another C/EBP factor functions as well. C/EBPε is almost exclusively expressed in hematopoietic cells and predominantly in late neutrophil development (and in T –lymphocytes) [147-149], where it is induced by C/EBPα among others and in turn induces expression of genes such as G-CSFR and the granule protein lactoferrin [95,147]. In accordance with this late appearance, the knock-out mice do develop neutrophilic cells, but these cells display characteristics of an immature stage, lacking later developed (secondary and tertiary) granules. In addition, also eosinophilic differentiation is perturbed [147,150]. Furthermore, mutations within the C/EBPε gene have been found in humans with neutrophil-specific granule deficiency, a disorder characterized by the lack of secondary and tertiary neutrophil granules (and eosinophil-specific granules) [95,151]. Together these data indicate an important role for C/EBPε in terminal differentiation of granulocyte (neutrophil in particular) precursor cells, even though the less severe phenotype of humans with C/EBPε-mutations compared to knock-out mice, may indicate a more crucial role in murine than human hematopoiesis [36,151]. Furthermore, it has been suggested that G-CSF, one of the key cytokines in neutrophil differentiation, can induce C/EBPε expression [152], implying the existence of a positive feedback loop via the C/EBPε-induced G-CSFR, like the one proposed for the earlier expressed family member C/EBPα and G-CSF/G-CSFR.

Hematopoietic cytokines and their receptors

Cytokines are a group of proteins or glycoproteins that are crucial for survival, proliferation, differentiation and mature function of hematopoietic cells. The large number of cytokines that act on the hematopoietic process from HSCs to mature cells, are either produced in other organs, arriving at the site of hematopoiesis with the circulation (endocrine action) or generated in the local environment, acting on neighboring cells (paracrine action) or even the producing cell itself (autocrine action) and the production often occurs in response to different stimuli [70,153]. The effects of these factors are influenced by their concentration, the presence of other cytokines and whether they are presented to their target cell in soluble form or bound to a cell membrane or extracellular matrix in the microenvironment [8]. Cells of different stages of development and of different lineages respond to signals from different GFs i.e. Tpo, FL, SCF and IL-6 act on stem cells and multipotent progenitors, whereas IL-3 and GM-CSF have important effects on myeloid oligo- and early bi- and unipotent progenitors, and G-CSF, M-CSF, Tpo, Epo and IL-5 are associated with specific lineages [4,154]. In addition, there are also a number of inhibitors of hematopoietic growth and development such as tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β and interferons (IFNs) [70]. (see Fig. 4) All of these cytokines interact by antagonizing each other or by synergizing (having a greater combined effect than the sum of their individual effects) and they display both functional pleitropy, by exhibiting numerous functions on different target cells and redundancy, i.e. overlapping effects on specific cells [70]. Hence, during steady-state this network of growth-promoting factors and growth-inhibitory factors maintains balance with a normal cell production, but in stress conditions these factors can also cause major shifts in the production to meet the new demands [155]. The redundancy and widespread existence of alternative pathways in this network can probably at least partly explain why knock-out mice of GFs or their receptors rarely display the dramatic or specific phenotypes seen with the abrogation of many transcription factor genes [70,83,156].

Cytokines exert their effect by binding to specific transmembrane receptors on target cells, thereby initiating signaling cascades that ultimately alter gene expression. These

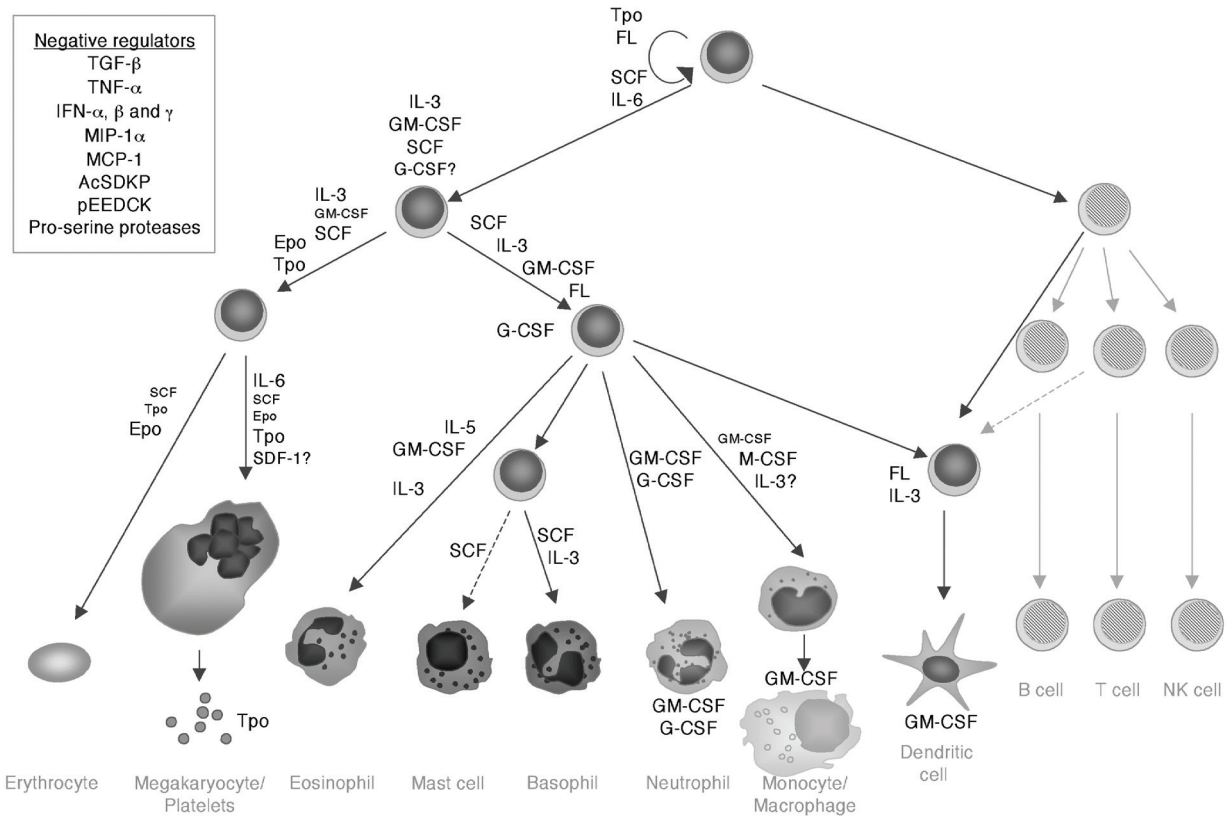

Figure 4. Cytokines regulating early and myeloid hematopoiesis. Selected GFs with important roles in early and myeloid development are depicted at their suggested sites of action. Font size indicates relative stimulating effect. Negative regulators with widespread and varying effects in the hematopoietic system are listed to the left (box).

cytokine receptors are divided into families (Fig. 5). The GFRs flt3, c-kit and M-CSFR belong to a subfamily of the immunoglobulin superfamily, which has an extracellular domain with five immunoglobulin-like domains and a cytoplasmic domain containing a tyrosine kinase motif (split in two by a kinase insert sequence), and upon ligand-binding these receptors homodimerize, autophosphorylate and initiate multiple downstream signaling pathways [155,157,158]. Most receptors of hematopoietic GFs, however, belong to the type I cytokine receptor family and have conserved motifs in the extracellular domain; a sequence of four positionally conserved cystein residues (CCCC) and a tryptophan-serine-(nonconserved amino acid)-tryptophan-serine (WSXWS) sequence. This family is further subdivided according to the subunit composition, where one group, including receptors such as EpoR, G-CSFR and TpoR, forms homodimers upon ligand-binding, whereas others form heterodimers or higher order complexes upon ligand-binding. For example, members of the GM-CSFR subfamily have one ligand-specific α-subunit and share a common signal-transducing β-subunit (β_c), whereas in the IL-6 receptor subfamily the receptor is composed of the common signal-transducing gp130 and one or two cytokine-specific subunits [153,159,160]. Members of the type II cytokine receptor family, with ligands such as IFN-α, β and γ, contain a CCCC sequence just like type I receptors, but not a WSXWS motif, although like the latter family these receptors are composed of more than one subunit, where

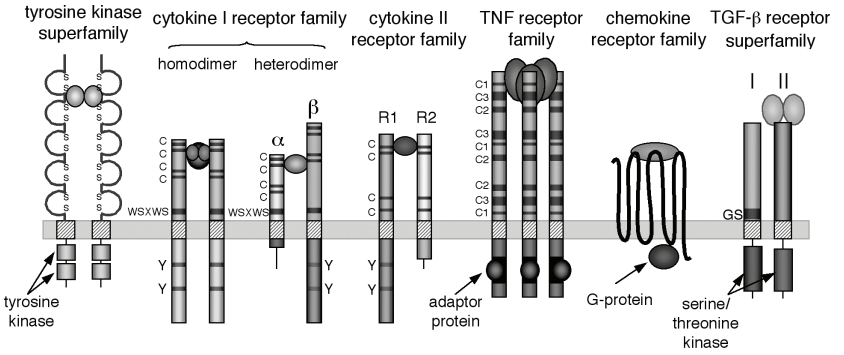

Figure 5. Cytokine receptors are divided into families. Schematic picture of the cytokine receptor families. (C, cystein ; WSXWS, tryptophan-serine-(nonconserved amino acid)-tryptophan-serine; Y, tyrosine; GS, glycine-serine; α, α-subunit; β, β-subunit; R1 and 2, receptor subunit 1 and 2; I and II, type I and II receptors)

one subunit is shared among several cytokines [153,161,162]. Also in common for these two families is the induction of tyrosine phosphorylation of the receptors and activation of subsequent signaling events without an intrinsic tyrosine kinase activity, but rather through closely associated cytoplasmic tyrosine kinases such as Janus kinases (JAKs). JAKs phosphorylate the receptor, thereby creating docking site for other signaling-molecules e.g. STATs, which after activation by phosphorylation forward a signal resulting in gene transcription alterations [153,161,162]. Notably, the JAK/STAT pathway is not the only signaling pathway activated by these receptors [160]. In yet another family, the TNF receptor family, most receptors are transmembrane proteins with extracellular domains containing one to four cystein-rich domains [163,164]. The TNFs e.g. TNF-α and TNF-β, all of which exists as trimers, generally bind three receptor molecules to initiate a signal, although it is possible that multiple such complexes are needed to activate the signaling pathway [163,165], and the signal is then propagated by cytoplasmic adaptor proteins associated with the cytoplasmic domain [164]. The structure of the chemokine receptors differ quite a lot from the other families, with seven-transmembrane G-protein-coupled receptor-chains, which activate large GDP/GTP-binding proteins upon ligand binding and thereby downstream signaling pathways [166,167]. Finally, transmembrane type I and type II serine/threonine kinase receptors of the TGF-β receptor superfamily bind a dimer of TGF-β (or other members of the TGF-β superfamily), on binding forming a heterodimer, which transphosphorylates and propagates the signal to downstream molecules [168,169].

Which cells that respond to a particular cytokine is determined by the expression of the receptor specific for that factor. Hence, it is the expression pattern of the receptor rather than of the cytokine itself that give indications to what function a particular ligand-receptor pair has, with immature cells expressing receptors like c-kit, flt3, TpoR, IL-3R and GM-CSFR, whereas EpoR dominates on erythroid cells, G-CSFR on neutrophil cells and so on. Consequently, when the receptors for some early-acting GFs like Tpo, IL-3 and GM-CSF are retained in specific lineages, it usually indicates a sustained function in those particular lineages [4,170]. Besides identifying target cell types for certain GFs, the expression patterns of GFRs have on numerous occasions

(described above) been used in the separation of different hematopoietic populations both through type and level of expression, even though the specific receptors used for these separations have varied [22,40,41,54-57,65,66] .

FL/flt3

FL belongs to a small GF family also including SCF and M-CSF [157]. It plays an important role in early hematopoiesis [6] and displays potent synergistic effects in combinations with other GFs such as Tpo, SCF, IL-3, GM-CSF, G-CSF and M-CSF, but has little activity on its own [155,157,171]. FL acts on primitive hematopoietic cells, inducing survival and proliferation and differentiation and also seems to act on cells committed to the G/M branch, the lymphoid branch and dendritic precursors, but not the Meg/E lineages [171,172]. The importance of this factor in hematopoietic development is emphasized both by the high frequency of activating mutations found in its receptor (flt3) in all AML types (higher than any other genetic abnormality) and the poor prognosis they confer, and by the reduction of early hematopoietic cells in general and lymphoid cells in particular seen in FL knock-out mice [155,157]. FL mRNA is expressed in most tissues, but the protein is chiefly detected in T cells and bone marrow stromal fibroblast [155,157]. Although there are several isoforms of the factor, it is primarily produced as a biologically active transmembrane protein, which can also be proteolytically cleaved generating a likewise biologically active soluble form [155,171]. The GF is normally constitutively expressed and suitable serum levels are maintained through storage and regulated release of preformed FL from these intracellular stores [155,157].

Flt3 (CD135) shares structural features with c-kit, M-CSFR and platelet-derived GF (PDGF) α/β receptors, all members of a immunoglobulin receptor subfamily [155,157]. Ligand-binding to the receptor appears to induce the formation of a tetrameric complex, consisting of a receptor-homodimer and a ligand-homodimer, which results in phosphorylation and initiation of downstream signaling cascades that regulate apoptosis, proliferation and differentiation [157,171]. In mice, flt3 has been suggested to be expressed on a GMPL separated from cells with Meg/E-potential [40], but in humans this

has not been shown, instead the receptor appears to be expressed on *in vivo* repopulating HSCs with potential for all lymphoid and myeloid lineages, including the Meg/E branch [21,22]. The expression is maintained and even slightly upregulated initially in the G/M pathway followed by a gradual downregulation, whereas the expression is quickly shut down in erythroid and megakaryocytic differentiation [56,57,172-175]. In addition, the receptor appears on early stages of lymphoid development, on most dendritic precursor cells and in some non-hematopoietic tissues [157,171,176].

SCF/c-kit

Like FL, SCF is crucial in early hematopoiesis [6], where it acts mainly in synergy with other GFs (e.g. IL-3, GM-CSF, G-CSF and Epo) [171,177]. However, in humans it most likely primarily acts a little later in the process than FL [22], inducing proliferation, survival and perhaps even migration of multipotent and committed myeloid progenitors [22,64,171,177]. In addition, SCF synergizes with factors such as IL-3 and Epo to induce proliferation and differentiation of erythroid progenitors and it has synergistic pro-proliferative effects on megakaryocytic progenitors and a profound effect on mast cell production [56,171,177]. Furthermore, SCF stimulates adhesion of hematopoietic cells to stromal elements in the bone marrow microenvironment [171,177]. In line with its important function in hematopoiesis, partial or complete absence of activity in the SCF/c-kit system in mice results in severe defects in hematopoiesis (and other systems) and death, respectively. Although notably, a human counterpart of the c-kit mutations only result in some of these features [171,177]. SCF is constitutively produced primarily by endothelial cells and fibroblasts in bone marrow, and surprisingly the production does not appear to be materially affected by cell counts or inflammation, or the subject of any other control [155,177]. The factor is produced as two major biologically active isoforms, one soluble and one membrane-bound, which have different signaling properties [171,177,178]. The membrane-bound form confers a prolonged activation compared to the transient activation produced by the soluble form, possibly caused by the membrane-association preventing receptor-mediated internalization. These differences also result in different downstream signaling and there are some

indications that the two isoforms differ in their effects on G/M and erythroid progenitors [171,177,178].

Also for c-kit (CD117), another member of the immunoglobulin subfamily, ligand-mediated receptor dimerization results in activation of a multitude of downstream signaling pathways [158,178]. HSCs express only low or no levels of c-kit [19,20], but the receptor is present on the surface of nearly all myeloid progenitors and clonogenic potential is almost completely localized to the c-kit$^+$ cells [64,171,177], concurring with the reported effects of its ligand. Eventually c-kit is downregulated in both G/M and Meg/E development, but the process is slower in the Meg/E lineages [4,64,171] where c-kit can interact directly with and enhance EpoR-signaling [179,180]. In addition, c-kit is present on more differentiated cells of the basophil lineage, mature mast cells and activated platelets, as well as on early lymphocyte progenitors [155,171,177] .

Tpo/TpoR

Whereas most GFs can be fairly easily divided into groups of primarily early-acting or late lineage-affiliated factors, Tpo defies such categorization. This factor supports survival, self-renewal and expansion of HSCs without influencing their lineage fate, as well as continuously stimulating megakaryocyte development, from the earliest progenitors and throughout differentiation, and though it does not affect actual platelet formation it is involved in platelet function [73,74,154]. Besides having stimulating effects on its own, Tpo also works in synergy with other GFs such as SCF, IL-3, IL-11 and Epo on HSCs, megakaryocytic cells and to some extent erythroid cells [181-189]. The dual effect of Tpo is confirmed by the phenotypes of Tpo/TpoR null mice and congenital absence of TpoR in humans, all of which present with low levels of HSCs and progenitor cells of all lineages, as well as severely reduced platelet counts [73,74,190]. However, the residual presence of some platelets indicates that even though Tpo is the major regulating factor in megakaryopoiesis there are alternative stimulating factors, possibly the chemokine SDF-1 [191]. Tpo is constitutively produced in liver, kidney and bone marrow, and receptor-mediated internalization and degradation has a key role in maintaining an appropriate level of platelet production by removing the GF from

circulation. When platelet counts are high so are receptor numbers and consequently a large part of the produced ligand is internalized, whereas low platelet counts leave more Tpo free to stimulate megakaryopoiesis and thus an autoregulatory loop is created [73,74]. Moreover, in severe thrombocytopenia bone marrow stromal cells display an increased production of Tpo, induced by a yet uncharacterized factor(s) and in inflammation the steady state levels can be abrogated by IL-6 mediating an increased Tpo expression in the liver, resulting in elevated platelet counts [73].

The TpoR (CD110), a member of the type I cytokine receptor family, binds a ligand molecule as a homodimer resulting in conformational changes that allow activation of adjacent intracellular tyrosine kinases, phosphorylation, recruitment of signaling molecules and subsequent signal transduction [73]. There are indications that different regions of the cytoplasmic part of the receptor are involved in proliferation, differentiation, stress response and so on [74]. Although there is some expression of the receptor on non-hematopoietic cells (e.g. endothelial cells), it primarily appears on hematopoietic cells, but there are conflicting data on where in this system it is expressed. Some report an expression solely on HSCs [192] and the megakaryocytic lineage, from precursors to platelets [73,74,193]. Others have suggested that TpoR is additionally expressed on bipotent Meg/E and unipotent erythroid progenitors [185], or even has a more general expression on all $CD34^+$ progenitors [194]. Consistent with its function in megakaryopoiesis, GATA transcription factors seem to be involved in the regulation of the TpoR gene [73,74]. There are also several alternative splice forms of TpoR with potential regulatory effects on Tpo/TpoR-signaling, but whether they actually have a biological function is not yet known [73,74].

IL-3/IL-3R

The multipotent IL-3 has a broader target specificity than any other hematopoietic GF and through stimulation of primitive as well as lineage-committed progenitors it plays an important part in the generation of monocyte/macrophages, neutrophils, eosinophils, basophils, mast cells, megakaryocytes and erythrocytes [70,156,195]. IL-3 induces proliferation, survival and differentiation (often in synergy with other GFs) of

these progenitors and also seems to act on later stages of eosinophils, basophils and possibly monocytes [70]. The GF is primarily produced in activated T lymphocytes, but can be released from activated mast cells as well, and perhaps other cells such as NK cells, eosinophils and stromal cells. Unlike the above described GFs IL-3 is not constitutively expressed, instead the production is regulated at the transcriptional level by a number of different transcription factors and by post-transcriptional control of transcript stability [70].

The IL-3R belongs to the same subfamily as the GM-CSFR, also including the receptor of the eosinophil-stimulating IL-5, and is as mentioned above composed of an α- and a β-chain [70,195,196]. The α-chain (CD123) binds specifically to IL-3 with low affinity, but complex formation with the β_c (CD131) either as a heterodimer ($\alpha_1\beta_1$) or a tetramer ($\alpha_2\beta_2$), is necessary for high-affinity binding and signal transduction. [70] The β_c is shared by the three receptors of the subfamily, thus resulting in competition among different α-subunits for the limiting β_c (when the former are expressed on the same cells), in this competition GM-CSF displays the greatest affinity, followed by IL-3 and then IL-5. The common subunit is also the major signal transducer (with different domains mediating different effects e.g. proliferation, survival or differentiation), thus explaining the functional redundancy found for the GFs of this subfamily. [70] However, both chains are needed to obtain the conformational changes necessary for signal initiation and even though the cytoplasmic domain of the α-chain does not signal without the β_c, it appears to be required for signaling to occur, possibly conferring some GF-specific component to the signal. Additionally, differential expression of cytoplasmic signaling-molecules and transcription factors in the different target cells may also contribute to GF-specific effects [70,195]. Like other type I receptors the subunits lack kinase activity and instead forward the signal via cytoplasmic tyrosine kinases [70]. As implied by the multi-lineage effects of IL-3, the specific α-chain is expressed on a variety of hematopoietic cells, including multi-potent and lineage-committed progenitors, granulocytes, monocytes/macrophages, B lymphocytes and a subtype of dendritic cells, but probably not on erythrocytes and megakaryocytes [4,65,197,198]. The receptor is also expressed on non-hematopoietic cells [70].

The regulatory elements of the IL-3Rα gene contain binding sites for hematopoietic transcription factors such as PU.1, C/EBPs and GATA-1, but their respective roles in regulating the gene expression is not quite known [70]. The β_c, needed for signaling through all the receptors of the subfamily, appears to be expressed at low levels in immature cells, followed by increasing levels of expression in early differentiation of all myeloid lineages [199]. However, while the expression subsequently increases further in the G/M lineages, it is downregulated in the erythroid and megakaryocytic cells [199,200], a pattern that fits the reported effects of the GFs in question [70,195,196]. Also in line with this, the promoter of the β_c gene contains binding sites for several hematopoietic transcription factors, both early-acting and more lineage-associated [70] .

GM-CSF/GM-CSFR

GM-CSF acts on immature cells just as the closely related IL-3 (although primarily on slightly more mature cells) and it also stimulates cells of specific lineages [70,195,200,201]. The GF promotes proliferation, survival and differentiation of cells of the monocyte/macrophage, granulocyte, erythrocyte and megakaryocyte lineages by acting on their progenitors, in the two latter lineages by synergizing with other GFs [70,195]. Moreover, it has been suggested that GM-CSF can influence commitment choices at several stages of development i.e. promoting CMP over CLP, GMP over MEP and neutrophil over monocyte fate [6,70,81,84]. Interestingly, different concentrations of the factor appear to mediate different responses, with induction of survival requiring lower concentrations than proliferation. Furthermore, in a parallel to the concentration-dependant effects of hematopoietic transcription factors different cell types respond at different concentrations, with G/M lineages demanding much lower concentrations than Meg/E and multipotent progenitors, and it also seems that it is the level of GM-CSF (alone or in combination with M-CSF) that determines its effect in the neutrophil versus monocyte/macrophage commitment [70,84]. In addition to these effects GM-CSF modulates functions of mature hematopoietic cells such as neutrophils, macrophages, dendritic cells and lymphocytes, and even of some non-hematopoietic cells, making it an important factor in inflammation [70,201]. In accordance with this role an array of cell types can produce GM-CSF including macrophages, neutrophils, eosinophils, T and B

lymphocytes, mast cells and a number of non-hematopoietic cells such as stromal cells, fibroblasts and endothelial cells, primarily in response to specific activating signals that induce increased gene expression or mRNA-stability [70,201].

The GM-CSFR consists of a specific α-subunit (CD116) and the β_c-subunit, which is shared with IL-3R and IL-5R [70,195]. The signaling through an αβ-complex and the effects of a shared subunit is discussed above. GM-CSFRα is mainly expressed on progenitors and mature cells of the monocyte/macrophage, neutrophil and eosinophil lineages, but also appears on more immature ($CD34^+$) cells [4,70,200], all in accordance with the reported effects of the ligand. In addition some expression can be found on cells of the Meg/E pathway and a number of non-hematopoietic cell types [4,70,195]. The GM-CSFRα promoter contains putative binding sites for several transcription factors and a PU.1 site seems to be important for expression of the gene [70].

G-CSF/G-CSFR

Although a role in early hematopoiesis (in synergy with IL-3) have been indicated for G-CSF [6,7,70,159], this GF is primarily associated with the G/M pathway and it is considered to be the principal factor acting on the neutrophil lineage, stimulating proliferation, survival, maturation and functional activation of these cells, while M-CSF has a corresponding or even more specific role in monopoiesis [70,145,159]. G-CSF and G-CSFR deficient mice display reductions in neutrophil development, but still retain some production possibly through redundant signals from other cytokine receptors. However, failed attempts at compensating for this reduction by anti-apoptotic measures [93], together with the apparent ability of G-CSFR-signaling to favor neutrophil over monocyte differentiation through induction of C/EBPα and ε-expression [138,152] , indicate that G-CSF has an instructive role in neutrophil development. The GF is produced by a number of different cells, but primarily by monocytes, macrophages and endothelial cells [70,202]. Under stress conditions stimulation of these cells, mediated for example by bacterial lipopolysacharide or cytokines, results in an augmented G-CSF production (by transcriptional and post-

transcriptional mechanisms) and a subsequent specific increase in neutrophil numbers and function, emphasizing the importance of G-CSF in this pathway [70,159].

Through binding of a G-CSF homodimer to the specific G-CSFR (CD114), a tetrameric complexes with two GFs and two receptor molecules is formed, which results in the activation of cytoplasmic tyrosine kinases and signaling events that eventually give rise to the biological effects of G-CSF. It also appears that different receptor domains are involved in different effects of the GF, like differentiation or proliferation, by activating different signal molecules [70,93,159]. The receptor is predominantly expressed on progenitors and terminally differentiated cells of the neutrophil lineage, displaying an increasing expression with differentiation [4,70,86,159] and (as discussed in the transcription factor chapter) the crucial G/M transcription factors PU.1 and C/EBPα appear to be involved in the regulation of the gene [159,203]. Notably, some expression is also found on early progenitors, on monocytes and mature platelets, as well as on non-hematopoietic cells [70,159].

Epo/EpoR

Epo and EpoR show many structural similarities with the Tpo-system[73,74], but unlike the latter, Epo seems to be a more traditional lineage-affiliated GF. Epo is the principal stimulating factor of erythrocyte production, with erythroid progenitor cells as its main target cells. The fact that Epo and EpoR knock-out mice produce erythroid committed cells, but die as embryos due to apoptosis and failed maturation of these erythroid cells [204], indicates that the factor is at least crucial for survival, proliferation and differentiation of erythroid cells, even though it may not be absolutely required for commitment to the erythroid lineage [90,205,206]. Epo has effects on its own, but often seems to act in synergy with other GFs such as SCF, GM-CSF , IL-3 and insulin growth factor-1 (IGF-1) to induce proliferation and differentiation [206,207]. Additionally, it has been reported that Epo has a stimulating effect on the closely related megakaryocytic lineage in synergy with Tpo, mirroring the effects of Tpo on erythropoiesis [181,185,208]. Production of Epo is primarily regulated by oxygen tension, where tissue hypoxia in the kidneys activates hypoxia-inducible factors (HIFs), which

in turn induce Epo gene expression [90,205], but small amounts of Epo are produced in other tissues as well [207,209].

EpoR exists as an inactive homodimer in the absence of Epo, but binding of a single ligand molecule induces the conformational changes necessary for signal initiation by associated tyrosine kinases [205,206]. Interestingly, c-kit seems to interact directly with EpoR, enhancing its proliferation- and differentiation-inducing effects most likely through phosphorylation of EpoR [179,180]. Cells expressing the receptor primarily belong to the erythroid lineage, with the earliest committed progenitors expressing relatively low levels, followed by a peak in subsequent stages, a gradual decline and finally complete absence on the most mature cells [86,114,205,206]. Furthermore, the erythroid-associated transcription factor GATA-1 appears to play an important part in the regulation of the gene [210]. It has also been suggested, although not directly shown, that the receptor is expressed on megakaryocytic cells [208] and there are even reports of expression on neutrophils and several non-hematopoietic cells even though it is not known whether this reflects a physiological role of Epo in these cells [4,205,211].

Negative regulators

Negative regulators of HSCs and progenitor cells or the growth-inhibitory factors, most likely have an important role in maintaining balance in the hematopoietic cell production [155], even though the biological significance of growth-inhibitory cytokines in steady-state hematopoiesis is not fully determined [171]. These regulators generally act by inhibiting progression through cell-cycle and by inducing apoptosis [212].

TGF-β is one of the most important growth-inhibitory factors [168]. It is a pleitropic factor with effects in many non-hematopoietic tissues and with varying positive or negative effects on proliferation, differentiation and survival of hematopoietic cells depending on the developmental stage of the target cell. Its major effect on stem cells and primitive progenitors is to reversibly inhibit their entry into cell-cycle and at least some of this effect may be mediated through downregulation of receptors of growth-stimulatory factors, e.g. c-kit, flt3 and TpoR [168,213]. Notably, it has less inhibitory or

even stimulating effects on more mature progenitors. The importance of TGF-β in controlling early progenitor proliferation is further implied by the possible involvement of mutations disrupting the TGF-β-pathway in human leukemia development [168]. As described above the five TGF-β isoforms, which differ somewhat in function, all bind members of the type I and II TGF-β-receptor families to initiate intracellular signaling [168].

Another factor with many functions including regulation of hematopoiesis is TNF-α, which can act both as a positive and negative regulator in this system. The factor has a negative effect on self-renewal of HSCs, although possibly through induction of differentiation rather than apoptosis or direct inhibition of cell-cycle [214], and also functions both as a growth-inhibitor and -stimulator on early and more mature myeloid progenitors, where the effect (inhibitory or stimulatory) seems to depend on the concentration, the presence of other cytokines and the branch-affiliation of the target cell [215-217]. TNF-α acts through two different receptors, TNF receptor p55 and p75, which mediate somewhat different effects and the factor appears to have multiple mechanisms of action, since both direct and indirect (on neighboring cells) effects, reversible and irreversible effects, as well as modulation of GFR surface expression and induction of apoptosis, have been found [177,216,218].

IFN-α, β and γ have been reported to have equally inhibitory effects on human CFU-granulocyte/erythroid/macrophage/megakaryocyte (CFU-GEMM) and burst-forming unit-erythroid (BFU-E) colony formation, whereas they display differential suppressive effects on CFU-granulocyte/macrophage (CFU-GM) growth, with IFN-γ and to some extent IFN-α being potent inhibitors also in this branch, but not IFN-β [219]. Furthermore, IFN-γ as well as TNF-α, enhances the suppressive action of TGF-β on proliferation of immature murine progenitors, illustrating that negative regulators cooperate just as stimulating factors do [220].

A family of small cytokines, the chemokines, includes some growth-inhibitors such as the macrophage inflammatory protein (MIP)-1α [166,167], which suppresses proliferation

of multipotent and early erythroid and G/M progenitors, probably mediated by a direct effect on these cells through members of the chemokine receptor family (see above) [166,212,221,222]. This effect is counteracted by another MIP, MIP-1β, thus indicating a place for these factors in the balancing network [221]. Yet another chemokine, the monocyte chemoattractant protein (MCP)-1, can also inhibit S-phase entry of human primitive progenitors, but not of more mature granulopoietic progenitors [212].

In addition to these cytokines, a number of peptides have also been shown to act as negative regulators. The tetrapeptide Acetyl-N-Ser-Asp-Lys-Pro (AcSDKP) displays a direct and reversible effect on primitive multipotent cells (LTC-ICs) and progenitor cells, by blocking their cell-cycle [223,224] and in monomeric form the granulocyte-derived pentapeptide pyroGlu-Glu-Asp-Cys-Lys (pEEDCK) also inhibits proliferation of hematopoietic progenitor cells possibly in synergy with other negative regulators such as TNF-α and IFN-γ, whereas a dimeric form instead stimulates growth of committed myeloid progenitors [225]. Furthermore, some members of the serine proteinase superfamily, more specifically PR3, azurocidin and the granzymes, can as proforms (but not as enzymatically active mature forms) induce a reversible S-phase arrest of G/M progenitors, presumably through a specific conformational presentation of their N-terminal tetrapeptides (Ile-Val-Gly-Gly or Ile-Ile-Gly-Gly) [226-228]. Although the exact mechanism by which these proproteinase inhibitors act is not known, they show different effects compared to e.g. AcSDKP, MIP-α and TGF-β, implying that they work by a separate pathway [227].

Microenvironment

Hematopoietic cytokines are not the only extrinsic factors of importance in hematopoietic development. Rather, they constitute one important component of the microenvironment, in which the hematopoietic cells reside and which plays a critical role in determining cell fate, influencing survival, proliferation and differentiation. The microenvironment comprises stromal cells (fibroblasts, endothelial cells, reticular cells, adipocytes and osteoblasts), the surface expressed, extracellular matrix-associated and soluble cytokines (such as IL-6, SCF, GM-CSF, FL, SDF-1 and MIP-1)

and other proteins the cells produce, as well as the surrounding extracellular matrix itself, which ensures that a suitable local concentration of these regulating molecules is maintained [1,6,229]. However, the overall molecular interactions in the microenvironment are less well defined. Most research in this area concerns the HSC microenvironment or niche. Notably, so far our knowledge of the hematopoietic microenvironment is primarily based on studies in mouse models and even though results from *in vitro* assays with human cells have appeared to support these findings the relevance for human *in vivo* hematopoiesis have been uncertain [229-233]. However, a recent report of *in vivo* studies (in mouse) with human hematopoietic and stromal cells, may provide the means for functional and spatial studies of the human hematopoietic microenvironment [234]. The initial results implied many similarities between the murine and human systems.

Adult HSCs reside primarily in the bone marrow, even though some circulate in the body [8]. It is believed that the specific conditions in specialized anatomical compartments, so called niches, regulate stem cell fate by balancing quiescence, self-renewal and differentiation through physical interactions between HSCs and cells, extracellular matrix components and other factors in the environment, even though cell-intrinsic regulators such as transcription factors ultimately control the outcome in the hematopoietic cells [9,230]. The cells surrounding the hematopoietic cells in bone marrow e.g. endothelial cells and osteoblasts, create these specific microenvironments. In mouse, anatomical studies have indicated that hematopoietic cells localize in a differentiation gradient, with immature cells near the bone in association with the osteoblasts lining the endosteal surface, and more differentiated cells progressively closer to the central marrow region and the endothelial lining of the sinus [9,229,230]. However, it should be noted that recently the use of SLAM receptors to visualize HSCs, suggested that although some stem cell are associated with the endosteum (and presumably osteoblasts), a considerable fraction are localized to the sinusoidal endothelium, which also expresses HSC regulating factors [14]. Thus, implying that more than one niche can support these crucial cells. Either way, osteoblasts appear to have a crucial role in modulating HSCs in bone marrow and when an increased

production of osteoblasts is induced the number of stem cells, but not progenitor cells, also increases [9,230]. Osteoblasts have been shown to physically interact with stem cells, with complexes between N-cadherin and β-catenin adhering HSCs to osteoblasts, interactions between Notch-1 receptors on hematopoietic cells and Notch ligands (Jagged-1) on osteoblasts potentially mediating the differentiation-inhibitory and expansion-inducing signals resulting in self-renewal, perhaps through induction of sustained GATA-2 expression, while interactions of the HSC-expressed Tie2 with angiopoietin-1 on osteoblasts appear to mediate adhesion and relative quiescence [9,229,230,235]. Osteopontin-expression on osteoblasts on the other hand, appears to negatively regulate the HSC-pool, thus providing a mechanism by which the endosteal cells can maintain steady-state. Osteoblasts also have the ability to produce numerous cytokines e.g. G-CSF, GM-CSF, IL-6, TNF- α and TGF-β [229] and members of the bone morphogenic protein (BMP)/TGF-β family appear to regulate HSC numbers by regulating osteoblast proliferation and thus the niche size [233]. Nevertheless, most HSCs do not bind directly to osteoblasts. Most likely the cells are instead anchored to the extracellular matrix in their niche by adhesion molecules, membrane expressed c-kit binding to SCF anchored in the matrix or present on cell surfaces (an interaction which may also change the avidity of other adhesion molecules through inside-out signaling[177]) and the matrix component hyaluronic acid produced by the HSCs themselves etc., whereas the chemokine SDF-1 appears to have a crucial role in directing HSCs to the stem cell niche [230]. Importantly, the niches are probably maintained by a two-way communication where factors produced by HSCs affect the function and organization of stromal cells, and not just the other way around [231,236].

Differentiating cells are believed to reside in the so-called vascular niche of marrow sinuses, where mature cells (and to some extent stem and progenitor cells) pass into circulation, for further direction to other tissues or continued circulation. The microvascular endothelium also generates the microenvironment needed for hematopoietic development, by providing cell-cell interactions and cytokines, either secreted by the endothelial cell themselves or produced by other cells but anchored in the matrix generated by these cells, and it appears that a niche supporting specific

hematopoietic cells is characterized not so much by the stroma cell-type as by the GFs or chemokines secreted by these cells [230]. Relatively little is known about the required microenvironment for different myeloid lineage development. However, megakaryocyte development and platelet production appear to depend on an intact vascular niche, where adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) and very late antigen-4 (VLA-4) and the chemokines SDF-1 (with its receptor CXCR4 expressed on megakaryocyte progenitors) and fibroblast growth factor-4 (FGF-4) are important for localizing and adhering the cells to their optimal environment [230]. The formation of erythrocytes occurs in erythroblastic islands, where a central macrophage extends its cytoplasmic processes around developing erythroid cells (erythroblasts) and interactions between erythroblasts, macrophages, the surrounding stroma cells and extracellular matrix proteins support erythroid survival, proliferation and differentiation [237]. Most likely adhesive interactions between macrophages and erythroblasts, via VCAM-1 (on macrophages)/VLA-4 (on erythroblasts) and perhaps Jagged-1/Notch, co-operate with Epo-signaling to support erythropoiesis, and the macrophages themselves also secrete cytokines which may have modulating functions in erythropoiesis [237,238]. In addition, megakaryocytes recycle and release iron after erythrophagocytosis, thereby providing developing erythroid cells with iron for new heme-synthesis [239].

THE PRESENT INVESTIGATION

Aims

Although advances have been made towards understanding the commitment of early progenitor stages in myeloid development, much remain to be done before the process is completely elucidated. The general objective of my work was to characterize human hematopoietic myeloid progenitors, with focus on early development and commitment to the neutrophil and erythroid lineages.

Specific aims:

I. To characterize clonogenicity, immunophenotypes and gene expression profiles for *in vitro* neutrophil and erythroid differentiation and to identify useful markers for neutrophil and erythroid commitment.

II. To find new surface markers for an improved definition and isolation of the human CMP, GMP and MEP and to characterize the isolated progenitors with regard to surface antigen and gene expression.

III. To further characterize the composition of the defined CMP-population and increase our understanding of the commitment process in the myeloid lineages by using this population.

Experimental considerations

Primary bone marrow cells – an *in vitro* model of normal myeloid development

In this work we used cells from normal human bone marrow to characterize specific myeloid progenitor populations, differentiation-associated changes and regulation. Normal human cells are preferable to immortalized cell lines, which contain a number of modifying mutations, and to animal models due to the existence of considerable species-to-species variations. Furthermore, even though there are other sources of normal hematopoietic cells such as mobilized peripheral blood and cord blood, cells from different hematopoietic sources differ in their surface immunophenotypes and

function [8,12,240] and may not always differentiate along identical pathways [35]. Hence, unmanipulated adult bone marrow cells derived from the primary site of hematopoiesis, constitute the most relevant model of cells and regulation events in human normal adult hematopoiesis, which is the focus of this thesis.

Isolation and characterization of hematopoietic populations by their surface antigen expression

Magnetic cell sorting (MACS)

Mononuclear cells isolated from bone marrow were subjected to MACS for isolation of $CD19^-$ cells (negative selection) and subsequently $CD34^+$ cells (positive selection). In MACS magnetic beads coupled to antibodies directed towards the antigen in question are used to retain the antigen-expressing cells in a magnetic field, while antigen-negative cells pass through unimpeded (negative selection), whereupon the positive cells are eluted (positive selection). $CD34^+$ cells (about 1-5% of the mononuclear cells in human bone marrow) constitute the majority of cells with multi-lineage engraftment potential and colony-forming potential and CD34 is the most commonly used marker for isolation of human HSCs and progenitor cells [1,2,16], whereas CD19 is expressed on the majority of cells differentiating along the B lymphoid lineage [12,38], the major lymphoid component of $CD34^+$ cells. Hence, the remaining $CD19^-CD34^+$ cells are considerably enriched for progenitor cells, but contain little lymphoid potential (paper II).

Flow cytometric analysis and cell sorting

However, even though $CD34^+$ cells are enriched for hematopoietic progenitors, they are still a heterogeneous collection of cells (containing everything from HSCs to relatively mature lineage committed cells) [2,12,16]. These cells can be further separated using fluorochrome-conjugated antibodies and FACS. Notably, this method has some limitations. Flow cytometry has a rather high detection limit and may not detect potentially crucial low-level expression of an antigen e.g. in mapping the distribution of functionally important antigens such as GFRs. Also, when surface markers are used to identify populations and differentiation stages, the purity is very important,

particularly if small populations are sorted, since contaminations with a relatively small number of cells can have dramatic effects. Sorting and re-sorting to achieve a better purity can often overcome this difficulty, but when an antigen is expressed as a continuum rather than on separate populations complete purity may not be obtainable. However, for analysis of the simultaneous expression of specific markers on specific cells and for separation of defined number of cells of characterized subpopulations with high purity, FACS is so far unchallenged. We used flow cytometry to analyze changes or differences in the expression of surface antigens (paper I, II and III) and to isolate populations of interest, such as candidate progenitor populations, for further characterization (paper I, II and III).

In vitro cultures of isolated hematopoietic cells

Colony assays

Colony assay is the standard method used to assess the ability of different hematopoietic cells to divide and differentiate to the myeloid lineages and is generally performed in semi-solid *in vitro* cultures where the clonal progeny of one cell, a CFU, form a discrete colony of mature cells, stimulated by the presence of selected GFs [11]. For general characterization of the developmental potential of a population, a small number of cells were sorted to wells containing methylcellulose and GFs selected to support both erythroid and G/M growth (paper I, II, and III), while single-cell cultures were used to establish the presence of multipotent cells (paper II and III). Furthermore, cultures with combinations and concentrations of GFs selected to favor specific lineages were used in single-cell assays aimed at obtaining information about the susceptibility of individual cells to GF-mediated regulation of lineage fate (paper III). However, the serum-containing methylcellulose-based cultures are not ideal for detection of megakaryocytic potential, since megakaryocyte-differentiation is very sensitive towards inhibitory signal from negative regulators present in the undefined serum, and since CFU-megakaryocytes (CFU-Megs) are best identified through staining of the colonies, which is a cumbersome process in methylcellulose, involving individual removal and staining of each colony [241]. Therefore, bulk cultures in the collagen-based and serum-free system MegaCult-C, were fixed and stained for

expression of the human megakaryocytic marker GPIIb/IIIa, in order to map the presence of megakaryocyte potential in isolated prospective progenitor populations (paper II). The possibility of CFU-Meg and CFU-megakaryocyte/erythroid (CFU-MegE) enumeration (the latter requiring the addition of Epo) makes this assay an important complement to methylcellulose, but not a replacement as it does not support the G/M branch optimally and is difficult to use for single-cell assays.

Notably, a general limitation of the semi-solid colony assays is that they do not support lymphoid development or differentiation of more primitive LTC-ICs or HSCs. Detection of these cells would require *in vitro* lymphoid-supporting cultures, LTC systems, or for true evaluation of HSCs, *in vivo* reconstitution in xenotransplantation models [11,12].

Liquid cultures

Differentiation-inducing liquid cultures were used for two purposes in this work. In paper I the GF-combinations were chosen to selectively support survival and differentiation of the neutrophil or erythroid lineages, in order to allow characterization of changes associated with differentiation of that particular lineage e.g. in gene expression. However, due to the relatively heterogeneous starting population ($CD34^+$ cells), cells at different stages of development and even cells committed to other lineages were present at a given time in culture. Therefore, we sorted for sequential, differentiation-associated changes in surface marker expression (A antigen/CD117 in erythropoiesis and CD15/CD33 in granulopoiesis), to obtain more homogenous collections of cells for subsequent assays (paper I). In paper III the purpose of the culture systems was instead to promote differentiation along one lineage, while simultaneously allowing survival of cells already programmed for differentiation along other lineage(s). Hence, combinations of GFs shown to support survival and proliferation of immature progenitors [242,243], together with different concentrations of lineage-affiliated GFs were chosen.

Real time RT-PCR

Real time reverse transcription-polymerase chain reaction (RT-PCR) was used for analysis of gene expression by detecting the levels of mRNA produced from a specific gene (paper I and II). Cells were sorted directly into PCR-tubes and lysed, whereupon cDNA was produced from the released total RNA with RT. The TaqMan-based real-time RT-PCR (used here) utilizes a dye-labeled probe, which anneals specifically to a segment of the primer-defined sequence. On polymerization a fluorescent signal is emitted from a reporter-dye on the probe and as amplification continues a proportionally stronger fluorescence is detected [Applied Biosystems Essentials of Real Time PCR, PN 105622]. Hence, amplification can be visualized in real time and not just as a final product, allowing for comparison of samples at a time of true exponential amplification, and consequently an exact relative quantification of the original amount of target RNA is possible. Additionally, the probes are designed to span exon-exon boundaries so that no product will be generated from genomic DNA and target amounts are normalized to an endogenous control to make sure that the observed differences are not the result of sample processing and cDNA-synthesis, rather than actual expression differences. (For details on the data analysis see separate papers and [Applied Biosystems User Bulletin #2: Relative Quantitation of Gene Expression, PN 4303859].) The endogenous control should ideally have a constant expression in all target cells, but this proved difficult to find for the diverse and well-defined populations that were analyzed in this work and even though 18S ribosomal RNA was eventually chosen as the most suitable control, the existing variations may introduce some error. Also, RT-PCR only measures the mRNA levels, which due to post-transcriptional regulations, may not always correspond to the actual presence of functional proteins.

Monitoring and characterizing differentiation

A vital part of this work was to identify the appearance of known differentiation markers to confirm the progression of differentiation, as well as to characterize differentiation-associated changes and to correlate them to developmental potential, where colony assays were used for evaluation of the differentiation potential of

specific sorted populations (paper I, II, and III). Flow cytometric analysis of the expression of antigens with known lineage-affiliation, such as CD45RA [58,62], CD15 [244,245], CD66 [246] (G/M associated), CD71 and GPA (erythroid associated) [247,248], on cells in differentiation-inducing cultures was performed to evaluate differentiation-progression and lineage-presence (paper I and III), whereas the expression patterns for other markers, such as blood group antigens A (of the AB0 system), glycophorin C (GPC) and Kell, were analyzed to obtain a complete map over differentiation-associated changes (paper I). Importantly, surface antigen expression was also used to define and characterize specific developmental stages in freshly isolated cells (paper II and III).

Gene expression analysis of transcription factors and GFRs implicated in early (GATA-2 and SCL) [6,90], Meg/E (GATA-1, NF-E2 and EpoR) [6,90,131] and G/M (PU.1, C/EBPα, C/EBPε and G-CSFR) [36,93,95] development and molecules appearing in mature cells, such as blood group antigens GPC, GPA and Kell, the ABO transferase [247-250], the hemoglobin component β-globin [103,114], the megakaryocyte/platelet expressed GPIIb/IIIa [251] and neutrophil granule components PR3 and lactoferrin [252,253], was used to create a map over differentiation-associated changes in different myeloid branches (paper I) and to verify lineage-affiliation (paper II), whereas the gene expression of a number of myeloid-regulating GFRs contributed to the functional characterization of specific populations (paper II).

Tracking cell division with CFDA,SE

Carboxyfluorescein diacetate, succinimidyl ester (CFDA,SE) can be used to directly relate changes in developmental potential and surface antigen expression of a cell population in culture to the number of times the cells have divided [254]. The non-fluorescent CFDA,SE diffuses into cells where it is transformed into a fluorescent molecule, which forms conjugates with intracellular proteins. As cells divide these dye-protein conjugates are distributed equally between daughter cells, resulting in sequential halving of the fluorescent signal, detected with flow cytometry as a series of peaks, which can be analyzed for surface antigen expression or sorted to colony

assays. It should be noted however, that even though one of the great advantages of this method is the labeling of viable cells, the labeling process still has a somewhat toxic effect on the cells.

Results and general discussion

Differentiation profiles of cytokine-induced neutrophil and erythroid development of human marrow cells

During differentiation hematopoietic cells go through extensive changes and their surface antigen and gene expression profiles are gradually altered as they reach new and more restricted stages of development, eventually resulting in morphologically recognizable cells and mature function. Some of these changes involve the complex network of regulating factors, including transcription factors and GFs, which control and balance survival, proliferation and differentiation from HSCs to mature cells. Therefore, an initial and necessary step in characterizing early myeloid development is to identify markers linked to differentiation and the gene expression patterns of factors regulating proliferation and differentiation. For this purpose, two *in vitro* culture systems were set up, which by use of different cytokine-combinations induced isolated $CD34^+$ cells from human bone marrow to differentiate along the neutrophil or erythroid lineages (paper I). This differentiation was verified by morphological studies and the surface expression of antigens previously established to be G/M-affiliated; CD11b, CD13, CD15 and CD66 [53,244-246] and erythroid-affiliated; $CD71^{hi}$ and GPA [64,247,250]. However, these studies also showed that differentiation of the heterogeneous $CD34^+$ cells [2,16] resulted in the simultaneous presence of cells at different stages of development and of some cells committed to other lineages. Therefore, when two surface marker combinations, CD15/CD33 and A antigen/CD117, were found to visualize early to late neutrophil and erythroid differentiation, respectively, we used this fact to obtain more homogeneous collections of cells. CD33 and CD117 were by no means lineage-specifically expressed, but they were preferentially maintained in the respective culture systems and as cells differentiated further they first attained expression of the lineage-affiliated CD15 or A antigen with increasing intensity, followed by loss of CD33 and CD117. Moreover, clonogenic assays showed that

CD15 and A antigen were expressed exclusively on cells committed to the G/M and erythroid lineages, respectively. Hence, these arcs of differentiation could be used to separate subpopulations, representing different development stages along the two lineages, for further analysis.

So far, most of our knowledge of expression patterns and functions of hematopoietic regulating factors is based on studies in mice and cell lines, the functional studies often involving gene disruption or over-expression [6,12]. However, the conclusions that can be drawn about human hematopoiesis from these model systems are somewhat limited and to confirm hypotheses derived from them, experimental studies need to be extended to normal human cells. By sorting defined subpopulations of erythroid and neutrophil differentiating cells to clonogenic assay and gene expression analysis with real time RT-PCR, the gene expression profiles of a relatively large number of genes could be quantified and directly related to changes in clonogenicity in general and lineage potential in particular, also revealing differences between neutrophil and erythroid development (paper I). The expression of several lineage-affiliated genes (e.g. GATA-1, PU.1, C/EBPα, ABO transferase and PR3) in the early populations seemed to support the idea of priming of genes prior to a pronounced role for the gene product [66,86,88], while the appearance of GATA-2 and SCL in these cells coincided with their supposed roles in immature and uncommitted cells [97,98,105]. The G/M regulating transcription factor PU.1 [107] displayed a high expression during early neutrophil differentiation, whereas the directly opposing transcription factors GATA-1 and –2 [110] were eventually completely downregulated, as was SCL. Also C/EBPα, the other major neutrophil-determining transcription factor [36,95], retained a significant expression throughout culture, although as PU.1 it was slightly downmodulated after G/M commitment. G-CSFR is a reported target gene of these G/M transcription factors [203] and also showed a marked G/M-associated expression, while the expression profile of the supposedly neutrophil-specific transcription factor C/EBPε appeared to confirm such a specificity, with a late expression almost exclusively localized to the $CD15^+$ populations and slightly preceding that of lactoferrin, a late granule protein and C/EBPε target protein [147]. PR3 (another granule protein [252,253]) proved its use as a G/M

marker-gene by being distinctly upregulated specifically in neutrophil differentiation. In erythroid differentiation on the other hand GATA-2 was maintained longer before complete downmodulation, in support of a role in early erythropoiesis and a required absence for terminal differentiation [111,112], whereas SCL actually increased, indicating a function also in quite mature erythroid cells for this factor [97,98]. The major erythroid transcription factor GATA-1 was greatly upregulated with erythroid commitment, simultaneously with EpoR and a little prior to a vast increase in β-globin, both of which are suggested target genes of GATA-1 [90,210]. However, EpoR also retained low levels of expression in $CD15^+$ cells, possibly indicating a role for this receptor in neutrophil differentiation as well. In accordance with the lineage restriction of the A antigen, ABO transferase, necessary for surface expression of antigen A and B [255], increased with erythroid commitment. Whereas NF-E2 deviated quite significantly from its expected profile by displaying a clear expression in neutrophil committed cells in addition to the erythroid differentiation, despite its proposed role as an erythroid- and megakaryocyte-specific transcription factor [119,131], thus implying the existence of an as of yet uncharacterized role in neutrophil differentiation.

Altogether, these expression profiles form a basis for conclusions about previously suggested or potential new roles for the investigated genes, but they also provide information about what genes or antigens to use as lineage markers, and allow interpretation of the expression pattern, in regard to lineage-commitment and affiliation, of future isolated cells.

TpoR is a key antigen in the isolation of human myeloid progenitor populations

Isolation of relatively pure populations of cells representing the major hematopoietic branch points is crucial for a better understanding of human hematopoiesis and would greatly facilitate studies of lineage commitment. For example, the ability to correlate changes in expression of specific transcription factors and GFRs with particular stages in differentiation will increase our knowledge of the role of these factors in lineage commitment [12]. There are several models suggesting different branch points in early hematopoiesis [34], but in the human system most evidence support the classical model

with an initial split between the lymphoid and myeloid branches (as discussed in the background). Recently Manz *et al.* [66] presented a method for isolation of a human CMP, GMP and MEP, defining them as $Lin^{-}CD34^{+}CD38^{+}$ cells differing in their expression of IL-3Rα and CD45RA i.e. as IL-$3R\alpha^{lo}CD45RA^{-}$, IL-$3R\alpha^{lo}CD45RA^{+}$ and IL-$3R\alpha^{-}CD45RA^{-}$, respectively. Lineage-commitment in a hematopoietic progenitor population can only be substantiated if these cells give rise exclusively to colonies of a single lineage, even when in the presence of GFs that should have permitted cells of other lineages to survive and proliferate, had they been generated in the colonies [84]. However, we found that even though sorting of $CD45RA^{+}$ cells efficiently separated a pure population of G/M committed cells, it was very difficult to discriminate between IL-$3R\alpha^{lo}$ and IL-$3R\alpha^{-}$ cells, and consequently the suggested MEP-population frequently contained also G/M colony-forming cells. A new surface marker was needed to obtain a more clear-cut separation of Meg/E committed cells and when TpoR was identified as a candidate for such a branch-specific antigen, it was incorporated in the previous definition (paper II). Both the original CMP and the IL-$3R\alpha^{lo/-}CD45RA^{-}TpoR^{-}$ cells (suggested new CMP) contained progenitors of all myeloid lineages (new CMP, Fig. 6A) and the G/M restricted cells included in the GMP-population (Fig. 6B) were the same irrespective of the version used, but whereas

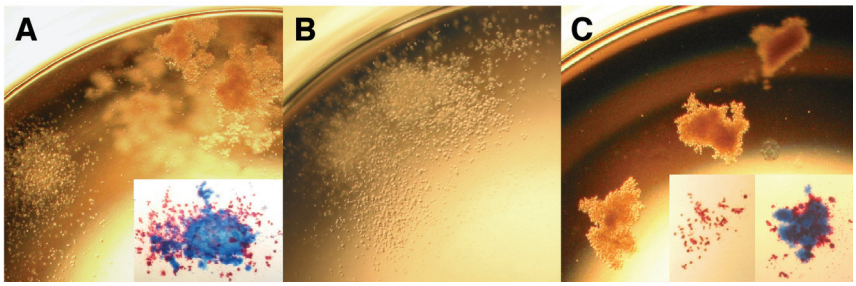

Figure 6. Colony morphology in cultures of isolated progenitor populations. Morphology of day-14 colonies derived from sorted progenitors cultured in methylcellulose and MegaCult™-C (inverted light microscope x 25). In MegaCult™-C (inserts) megakaryocytic cells are visualized by red immunostaining (anti-GPIIb/IIIa antibody), while non-megakaryocytic colonies are counterstained with Evans Blue. **(A)** IL-$3R\alpha^{lo/-}CD45RA^{-}TpoR^{-}$ cells give rise to both CFU-GM (left), large BFU-E (right) and CFU-MegE (insert), **(B)** IL-$3R\alpha^{lo}CD45RA^{+}TpoR^{-}$ cells form CFU-GM and **(C)** IL-$3R\alpha^{lo/-}CD45RA^{-}TpoR^{+}$ cells form BFU-E, CFU-Meg (left insert) and CFU-MegE (right insert, x100). (CFU-MegE, colony-forming unit-megakaryocyte/erythroid; BFU-E, burst-forming unit-erythroid; CFU-Meg, CFU-megakaryocyte; CFU-GM, CFU-granulocyte/macrophage.)

the original MEP displayed a significant fraction of G/M differentiating cells, the IL-3R$\alpha^{lo/-}$CD45RA^{-}TpoR^{+} cells (suggested new MEP) differentiated solely along the Meg/E lineages (Fig. 6C). Notably, bipotent Meg/E progenitors and other more immature megakaryocytic and erythroid colony-forming cells, appeared to localize to the TpoR^{lo}-population, whereas more mature progenitor cells and non-colony-forming precursor cells were concentrated in the TpoR^{hi}-population. Hence, the use of TpoR as a defining marker provides the means for a pure, reproducible separation of Meg/E committed cells and allows further subdivision of these cells according to their maturity. This new definition was corroborated by gene expression analysis of transcription factors and lineage-associated molecules in the isolated populations (Fig. 7), where changes from CMPs to GMPs and from CMPs to MEPs (paper II) coincided with changes previously observed with induced neutrophil and erythroid differentiation, respectively (paper I). Furthermore, when sorted to liquid culture CMPs gave rise to both CD45RA^{+}TpoR^{-} cells and CD45RA^{-}TpoR^{+} cells, confirming that CMPs are precursors of the defined GMPs and MEPs (paper III). The expression

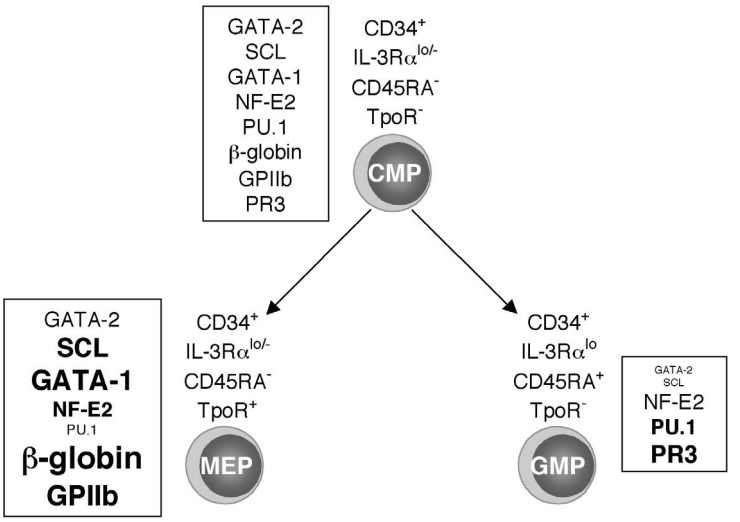

Figure 7. Gene expression profiles of isolated progenitor populations. The gene expression profiles of the proposed CMP-, GMP- and MEP-populations, separated by their expression of CD34, IL-3Rα, CD45RA and TpoR, support this progenitor definition. Relative differences in expression in CMPs compared to MEPs or GMPs are represented as increased or decreased font size or complete absence of the respective transcription factors and other lineage-associated genes (boxes).

of TpoR on MEPs is also functionally supported by the synergistic effects that have been suggested for Epo and Tpo in both megakaryopoeisis and erythropoiesis [185-189,256]. Notably, Tpo has additionally been reported to stimulate survival and proliferation of very early hematopoietic cells [73,154] and TpoR expression have been detected on these cells [192,257], whereas we found no expression on $CD38^{-/lo}$ cells (paper II). This discrepancy is probably caused by different antigen–recognition of the two antibody-clones that were used (3G4 [192,257] or BAH-1 (paper II)). These antibodies may differ in their ability to bind and visualize TpoR expressed in low levels on early cells, but considering the barely overlapping detection profiles and the inconsistencies in relative intensity of expression on different populations (Ninos *et al.* [257] and paper II) it is perhaps more likely that the antibodies recognize different isoforms of TpoR, which are expressed at different stages of development [258-260]. In either case, the TpoR-recognizing BAH-1 antibody specifically identifies Meg/E committed cells, rendering TpoR a key component in the definition of human myeloid progenitors.

Regulating factors of isolated myeloid progenitors – gene expression does not always predict function

TpoR constitutes yet another addition to the many GFRs previously used for isolation of murine and human hematopoietic progenitor populations (e.g. c-kit, flt3, IL-3Rα, IL-7Rα and M-CSFR) [39-41,54,65,66]. Consequently, additional analysis of the expression of other GFRs was preformed, both for the possibility of further improvement of the progenitor definition and in the hope of obtaining clues about the regulation of these progenitors (paper II). We found a widespread expression of c-kit on the three populations in agreement with previous reports of expression [4,64,261,262] and stimulatory effects of SCF on myeloid progenitors [171,177], and the upregulation of flt3 on GMPs and downregulation on MEPs were in line with earlier findings of flt3 expression [56,57] and FL function [171]. Also the β_c of the GM-CSFR, IL-3R and IL-5R, the GM-CSFRα and the lineage-associated G-CSFR displayed expression profiles that coincided with earlier surface expression studies [4,199,263] and suggested functions [70]. However, whereas Manz *et al.* managed to obtain a clearly separable Meg/E restricted IL-3Rα$^-$ population [66], we (paper II) and others did not [65]. Even so, since previous studies have shown that

the receptor subunit is downmodulated with further differentiation along the megakaryocyte or erythrocyte lineage [4,197,200], the detected expression of IL-3Rα on our MEPs (TpoR$^+$) was probably caused by inadequate resolution rather than a significant presence of the receptor subunit on these cells.

Altogether, the obtained expression patterns supported earlier findings of expression and function of these GFRs, but none of them resulted in a clear-cut subdivision of the CMP, GMP or MEP populations. On the other hand, some interesting discoveries were made when the myeloid progenitor populations were analyzed for their gene expression. The mRNA production from most GFRs genes (i.e. c-kit, flt3, GM-CSFRα and G-CSFR) essentially matched the surface expression and the major downmodulation of IL-3Rα in MEPs, even though not corresponding with the observed surface expression profile, probably matched an actual downmodulation also on the surface (see above). However, for both TpoR and β_c there were considerable discrepancies between surface and gene expression patterns (Fig. 8), which cannot be explained by selective detection of specific splice forms, since all known splice forms [199,258,260] are detected by the gene expression assays we used (paper II,

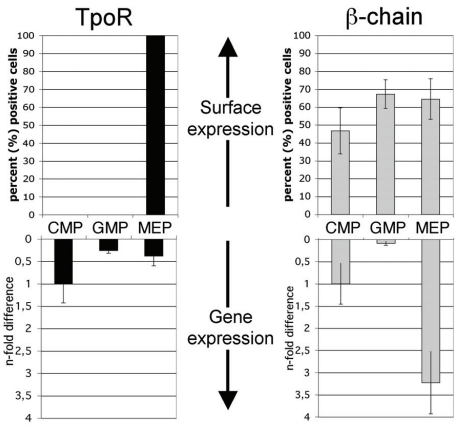

Figure 8. Gene expression does not match surface expression of TpoR and β_c. Surface expression (percent positive cells) versus gene expression (n-fold difference relative to the CMP-population) of TpoR and β_c, for the proposed CMP-, GMP- and MEP-populations. The graphs show the mean value and standard deviation of three bone marrows and target gene expression is normalized to 18S ribosomal RNA.

www.appliedbiosystems.com). Therefore it can be concluded that for some GFRs post-transcriptional mechanisms have a considerable role in the determination of surface expression and that gene expression profiling may not always be sufficient for establishing whether a population expresses a certain receptor and can respond to its ligand.

Composition and regulation of the CMP-population

The fact that most surface markers used for isolation of hematopoietic progenitors are either GFRs (see above) or have a known or suggested function in regulation of GF-signaling (CD45RA [67]) or cell adhesion (CD34, CD7 [16,51]) supports a crucial role for external microenvironment in the differentiation to hematopoietic lineages. However, it does not answer the question of whether these surface markers appear on the surface after commitment, mediating permissive signals that maintain differentiation programs already activated by stochastic events, or whether they are expressed also some time prior to commitment, thereby allowing extrinsic instructive signals to activate previously silent programs. In other words, whether regulating factors in the environment can affect the developmental fate of a single cell (instructive model) or not (stochastic model).

The isolation of putative myeloid progenitor populations (paper II) provided us with the means for further study of commitment in this branch. In permissive cultures the GMPs and MEPs gave rise exclusively to colonies of their respective branch (paper II) and they could not be rerouted to the opposing lineage fates in lineage-promoting liquid cultures (data not shown), indicating that these cells are irreversibly committed to their respective lineages. On the other hand a CMP, which can differentiate along either myeloid branch, would be a very interesting target for commitment studies. However, the true composition of our CMP-population was not fully determined. Single cells expressing both G/M and erythroid potential in culture, i.e. known multipotent myeloid progenitors, constituted a quite small fraction of the CMP-population (paper II). Then again, this fact does not necessarily signify a lack of multipotent cells in the population, since previous studies have shown that prospective

CMPs and populations enriched in stem cells give rise to approximately the same proportions of the different CFUs, with CFU-GEMMs constituting only a relatively small part of the clonogenic cells [42,66], indicating that all multipotent cells may not express their full potential in colony assays. Notably, when single murine and human CMPs were sorted to liquid culture prior to colony assays they displayed a much larger fraction of multipotent cells [42,66] and an additional presence of multipotent cells was suggested also in our CMP-population by the fact that exclusion of Epo from the colony assays resulted in a shift in developmental fate, demonstrated by an increased fraction of G/M colonies and concomitant decrease in erythroid colonies (paper II). On the other hand, $flt3^-$ and $flt3^+$ subpopulations of CMPs showed a significant accumulation of erythroid and G/M differentiating cells, respectively (paper II), implying that the CMP-population is not homogeneous.

To further elucidate the composition of the CMP-population, these cells were sorted to liquid cultures containing low concentrations of the early-acting GFs FL, SCF, IL-3 and IL-6 [70,171,242,243] and different concentrations of the lineage-promoting G-CSF and Epo [70,205], a system designed to promote differentiation along selected lineages while still allowing survival of cells committed to other lineages (paper III). Through flow cytometric immunophenotyping after four and ten days, using surface markers such as CD15, CD33, CD45RA, $CD71^{hi}$, CD117, TpoR and GPA the expression patterns of which were previously established to be lineage-associated (paper I and II), it was found that the inclusion of either G-CSF or Epo resulted in a domination of neutrophil and erythroid differentiation, respectively. In addition, when single cells were sorted to methylcellulose containing the different GF-combinations the absence of Epo again induced a large fraction of the cells that formed erythroid colonies in unselective cultures to differentiate along the G/M pathway instead, and also in cultures with Epo and without G-CSF some cells appeared to shift branch. Hence, it would seem that the presence of different GFs does affect the lineage fate of a fraction of the CMPs, supporting both the existence of an instructive role for GFs in commitment and the presence of more multipotent cells in the CMP-population than the ones actually manifesting themselves as CFU-GEMM. On the other hand, there were also a

significant number of cells that continued to differentiate along the unpromoted lineage both in liquid bulk cultures and single-cell colony assays, indicating that the population contained cells that could not be rerouted to a new developmental fate by extrinsic signals. This was seen again in cell division studies with CFDA,SE-labeled cells in liquid culture, where a fraction of the CMPs always differentiated along the unpromoted lineage (detected by colony assays and their expression of CD15, CD45RA or TpoR). Interestingly, the non-lineage-promoting GFs seemed to stimulate erythroid proliferation more efficiently than G/M proliferation, since regardless of which other GFs were present, and of the size of the erythroid-differentiating population, these cells were always concentrated in the later CFDA,SE-peaks, which contain cells that have passed a greater number of cell divisions. This observation also implies that fewer cells in the original population were pre-destined for the erythroid than for the G/M pathway, seeing as the erythroid cells did not dominate the cultures more than G/M cells did despite the higher proliferation rate and an apparently similar viability (paper III).

Hence, it can be concluded that the proposed CMP-population (IL-3R$\alpha^{lo/-}$CD45RA^{-} TpoR^{-}) is a heterogeneous collection of cells composed of a significant fraction of multipotent myeloid cells susceptible to GF regulation, together with cells preferentially differentiating along the erythroid or the G/M branch. These lineage-biased cells could be irreversibly committed cells functionally belonging to the previously defined MEP- and GMP-populations (Fig. 9A). However, they could also represent a stage of development that unlike the observed multipotent cells is impervious to extrinsic regulation and has an increased propensity for, but not complete restriction to, a particular lineage fate (Fig. 9B), possibly due to different expression levels of crucial transcription factors. The existence of such polarized or pre-committed progenitor cells have previously been hypothesized in murine lymphoid and myeloid development [44-46,264].

It would of course be desirable to identify surface markers that separate the multipotent cells from the polarized or committed cells, but this may prove difficult.

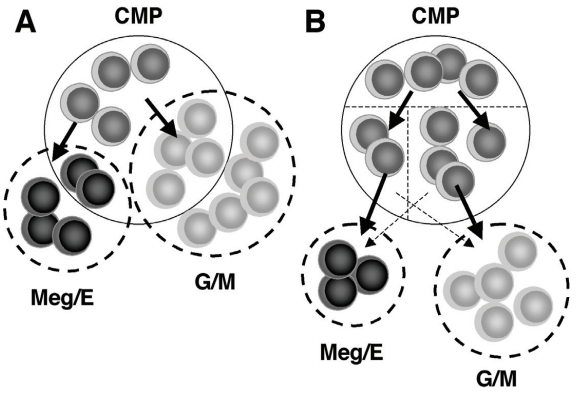

Figure 9. Composition of the heterogeneous CMP-population. The CMPs are composed of multipotent cells susceptible to extrinsic regulation of lineage choice, as well as lineage-biased cells impervious to extrinsic signals. The latter can either be **(A)** irreversibly committed to the Meg/E or G/M branches, constituting immature and undefined fractions of the MEP- and GMP-compartments or **(B)** polarized cells that are not yet fully committed to a specific branch, but that are functionally distinct from the unbiased multipotent CMPs.

So far we have found several antigens that subdivide the CMPs into populations apparently enriched in cells with different developmental potential e.g. CD133, CD33 and the previously used flt3 (paper II and III), again supporting the heterogeneity of this compartment, but none of them can be used to purely separate functionally different populations. In truth, the fact that previous studies with liquid culture and multilineage gene expression profiling of single human and murine CMPs also implied the presence of cells only giving rise to a single branch [42,66,88], indicates that there is some inherent difficulty in separating more lineage-biased cells from multipotent cells. It is quite possible that these developmentally close populations are only separated by intrinsic changes that are not yet manifested on the surface.

Conclusions and future perspectives

Knowledge of developmental pathways and the regulation of survival, proliferation, lineage choice and differentiation in normal human hematopoiesis is essential for understanding the adverse events in hematological diseases such as leukemia and for designing new and more effective treatments. So far most of our knowledge of the hematopoiesis is based on experiments carried out in animal models and cell lines.

However, for complete elucidation of normal human hematopoiesis it is necessary with studies performed directly on this system and isolation of populations representing specific developmental stages, to be used both in expression analysis and functional studies with potential regulating factors, is a crucial step. Therefore, the aim of this thesis was to characterize the early stages of human myeloid development and to study how this development is regulated.

First, we established a map over differentiation-associated changes both on the surface and in gene expression in normal human cells, correlating these changes to clonogenicity and G/M and erythroid commitment and differentiation. This map identifies lineage-specific surface antigens (e.g. CD15 and A antigen) and gene expression (e.g. PR3 and β-globin) with potential use as lineage markers and describes both expected and a few unexpected expression patterns of suggested crucial regulators of myeloid development, thus representing a useful tool for our continued investigations of these pathways (paper I).

In these investigations we found that the addition of the GFR TpoR to the surface markers (IL-3Rα and CD45RA) used in a previously presented definition of the human CMP, GMP and MEP [66] could significantly improve the separation of MEPs ($TpoR^+$) from CMPs and also allow separation of Meg/E cells of different maturation stages based on their level of TpoR expression (paper II). Further characterization of these defined progenitors showed a surface expression of GFRs that coincides with the supposed regulating functions of the corresponding GFs in myeloid hematopoiesis, but which cannot always be predicted by their gene expression (paper II).

More specific studies of the proposed CMP, aimed at furthering our understanding of myeloid commitment, revealed that the CMPs do not constitute a homogenous population. Instead they are composed of a fraction of multipotent cells that are susceptible to extrinsic regulation of lineage fate, as well as lineage-polarized or committed cells, which differentiate along their respective lineage regardless of the presence or absence of different GFs. So far no surface markers have been found that

can separate these subcompartments from each other and it is possible that they only differ in their expression of transcription factors and other intrinsic molecules (paper III).

Access to the different subpopulations within the CMP-population could contribute greatly to our understanding of events associated with commitment and their regulation through elucidation of their relative position in the hematopoietic development, comparison of their expression of crucial transcription factors and their regulation by extrinsic factors. Therefore an important step in future investigations would be to define less heterogeneous subpopulations. Even if selective surface markers may not exist, there could be antigens that at least narrow down the multipotent, GF-inducible CMP-population or perhaps there is some other way to separate them. In addition, single-cell RT-PCR analysis of gene expression profiles could help identify the respective size and functional characteristics (such as partial or complete commitment of the GF-unresponsive cells) of subpopulations even within an unseparated CMP-population [88].

SAMMANFATTNING PÅ SVENSKA

I blodet finns röda och vita blodkroppar eller blodceller, som är nödvändiga för vår överlevnad. Blodcellerna utvecklas i benmärgen från gemensamma ursprungsceller, så kallade stamceller och en konstant nyproduktion pågår genom hela livet. Under produktionen av mogna celler tillväxer och delar sig cellerna och får allt mer specialiserade egenskaper. Denna specialisering kan ske längs olika utvecklingslinjer, tills cellerna når det mogna cellstadiet för en speciell celltyp och transporteras ut i kroppen via blodet. De färdiga blodcellerna kan delas upp i två grupper; de myeloida cellerna och de lymfoida cellerna. Myeloida blodceller innefattar syretransporterande röda blodkroppar, blodplättar med en viktig funktion i blodkoagulering och sårläkning, samt vita blodkroppar, såsom neutrofiler och monocyter, som ger ett snabbt skydd mot infektioner. De likaledes vita lymfoida cellerna verkar istället senare i vårt infektionsförsvar och gör oss immuna mot nya infektioner. För att det alltid skall finnas rätt nivåer av de olika celltyperna regleras produktionen mycket noga. Till exempel är förekomsten av olika transkriptionsfaktorer inne i cellerna central för utmognaden och för cellernas egenskaper. Transkriptionsfaktorerna är en grupp proteiner som styr vilka gener som är aktiva och ger upphov till ny proteinproduktion. Även cellens omgivning påverkar utvecklingsprocessen, bland annat via förekomsten av olika tillväxtfaktorer som binder specifika molekyler på cellens yta, receptorer, vilka sedan vidarebefordrar signaler in i cellen. Dessa signaler påverkar bland annat förekomsten och funktionen av transkriptionsfaktorerna och olika tillväxtfaktorer stödjer utvecklingen av olika celltyper. När blodcellsproduktionen inte fungerar normalt, resulterar det i allvarliga och ofta dödliga sjukdomar såsom leukemi (blodcancer).

Tyvärr är dock vår förståelse av hur blodcellernas utveckling går till och hur den kontrolleras något begränsad. I detta arbete fokuserade jag på att öka förståelsen av den normala utvecklingen av de myeloida cellerna och jag använde mig av celler utvunna från human (mänsklig) benmärg. Under pågående specialisering av blodcellerna ändras deras uttryck av proteiner på ytan (t.ex. tillväxtfaktorreceptorer)

och inuti cellerna (t.ex. transkriptionsfaktorer) kontinuerligt och därför kan karakterisering av detta uttryck användas både för ökad förståelse av utvecklingen och för att identifiera specifika utvecklingsstadier för fortsatta studier. Därför började jag mina försök med att rena fram omogna blodceller från benmärgen och odla dem tillsammans med specifika tillväxtfaktorer för att framkalla utmognad till antingen röda eller neutrofila celler. Sedan användes dessa celler för att kartlägga mognads-associerade förändringar i uttrycket av transkriptionsfaktorer, tillväxtfaktorreceptorer och andra markörproteiner utanpå eller inuti cellerna. Denna ”karta” kan användas för att identifiera cellinjetillhörighet och mognadsstadie för specifika grupper av celler.

Därefter fann jag att ytuttrycket av en speciell tillväxtfaktorreceptor, trombopoietinreceptorn, kan användas tillsammans med tidigare identifierade ytmarkörer för att separera tre olika utvecklingsstadier i den myeloida utmognaden. Det vill säga en grupp celler som kan utvecklas till alla myeloida celltyper (kallad CMP) och två grupper med celler som begränsat sin utvecklingspotential till några av dessa typer. De två senare stadierna mognade antingen till röda celler och blodplättar (MEP) eller neutrofiler och monocyter (GMP).

Vid vidare studier av CMP-cellerna fann jag dock att de innefattar flera olika celltyper. I en grupp CMP-celler kan varje cell utvecklas till alla myeloida celltyper och deras val påverkas av vilka tillväxtfaktorer de omges av. Andra celler ger framför allt upphov till antingen röda celler eller neutrofiler och kan inte förmås att ändra utvecklingsväg med yttre faktorer. Det skulle vara önskvärt att separera dessa olika grupper från varandra, men det är inte säkert att de skiljer sig åt tillräckligt mycket, när det gäller uttryck av ytmarkörer, för att göra detta möjligt.

Mitt arbete har ökat våra kunskaper om de första stegen i myeloid blodutveckling och underlättar avsevärt för fortsatta studier av denna process och dess reglering. Bättre kunskaper om den normala processen, både när det gäller utvecklingsvägar för olika celltyper och deras reglering, kan även förbättra vår förståelse av vad som går fel i olika blodsjukdomar och hur de skulle kunna behandlas.

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