Characterization of signal transduction pathways regulating myelopoiesis

Christian R. Geest

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Characterisatie van signaaltransductie routes betrokken
in de regulatie van myeloide differentiatie

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Christian Robert Geest
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Promotoren:	Prof. dr. P.J. Coffer
	Prof. dr. E. Vellenga
Co-promotor:	Dr. M. Buitenhuis

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Is de kwaal van het vragen een kwaal
van de tijd en ben ik maar een van de
tallozen, bevangen door de zucht de
fouten van het leven te doorgronden.
Arthur van Schendel

CONTENTS

Chapter 1

General Introduction

Hematopoiesis

Hematopoietic stem cells (HSC) are defined as cells which can both undergo self-renewal and differentiate into all types of blood cells throughout an individual's life. Mature hematopoietic cells are categorized into two distinct lineages: the lymphoid, which includes T, B and natural killer (NK) cells, and the myeloid lineage consisting of various subclasses of granulocytes, including neutrophils, eosinophils and basophils, monocytes, macrophages, erythrocytes, megakaryocytes and mast cells. Dendritic cells (DC) can originate from either the myeloid or the lymphoid pathways (1)(2). These two classes are believed to use separate differentiation pathways (3). HSCs were first identified phenotypically as the Lin^{-} $Sca\text{-}1^{+}$ $c\text{-}Kit^{+}$ (LSK) population residing in the murine bone marrow (4)(5)(6). However, there are many differences between the human and mice hematopoietic cell markers for the commonly-accepted type of hematopoietic stem cells. The *Weissman model* proposes that long-term human HSCs are defined phenotypically as Lin^{-} $IL\text{-}7R\alpha^{-}$ $Sca\text{-}1^{+}$ $c\text{-}Kit^{+}$ $Flt3^{-}$ $Thy1^{low}$ $CD34^{-}$, which reside within a low-frequency bone-marrow subpopulation and have the ability for life-long self-renewal and multilineage differentiation potential (7)(8). Long-term HSCs give rise to short-term HSCs (Lin^{-} $IL\text{-}7R\alpha^{-}$ $Sca\text{-}1^{+}$ $c\text{-}Kit^{+}$ $Flt3^{low}$ $Thy1^{low}$ $CD34^{+}$), which retain the potential for multi-lineage differentiation, but have decreased self-renewal capacity (9). Subsequently, lineage-restricted progenitors at various developmental stages have been identified downstream of HSCs based on expression of additional lineage-related markers. The Weissman model (Figure 1) further discriminates a common lymphoid progenitor (CLP) that can generate all lymphoid lineages and its counterpart, the common myeloid progenitor (CMP) that gives rise to all myeloid cells, supporting the concept that lymphoid and myeloid lineages both develop independently downstream of HSCs (10)(11). However, recent studies have provided a more detailed developmental map downstream of HSCs. Importantly, the model that MEPs and GMPs are both progeny of CMP has been adapted by a study of Adolfsson *et al*, who proposed that MEPs directly originate from HSCs, whereas all myeloid and lymphoid lineages are the progeny of a lymphoid-primed multipotent progenitor (LMPP) (12).

Transcriptional regulation of myeloid differentiation

The differentiation of HSCs into lineage-restricted hematopoietic progenitor cells is associated with both loss of self-renewal potential and stage-dependent acquisition of a specific lineage characteristics, which are tightly regulated by genetic programs. Stepwise differentiation of hematopoietic progenitors is directed by a complex network of cooperative and counteracting genes, which are ultimately controlled by transcription factors and together determine cell fate (10)(13). The formation of myeloid cells is regulated by a relatively small number of transcription factors (Figure 2). Among them are PU.1 (14), CCAAT/enhancer binding proteins, C/EBPα, C/EBPβ and C/EBPε (15)(16)(17), growth-factor independent 1 (GFI1) (18) and interferon-regulatory factor 8 (IRF8) (19), and, at the HSC level, runt-related transcription factor 1 (RUNX1) (20) and stem-cell leukemia factor (SCL) (21), as well as other factors, including JUNB, signal transducers and activators of transcription (STAT) 5, Ikaros and Myc (22)(23)(24)(25). These transcription factors are involved in regulating the expression of many myeloid genes, including those encoding granulocyte colony-stimulating factor (G-CSF) and granulocyte/macrophage colony-stimulating factor (GM-CSF) receptors

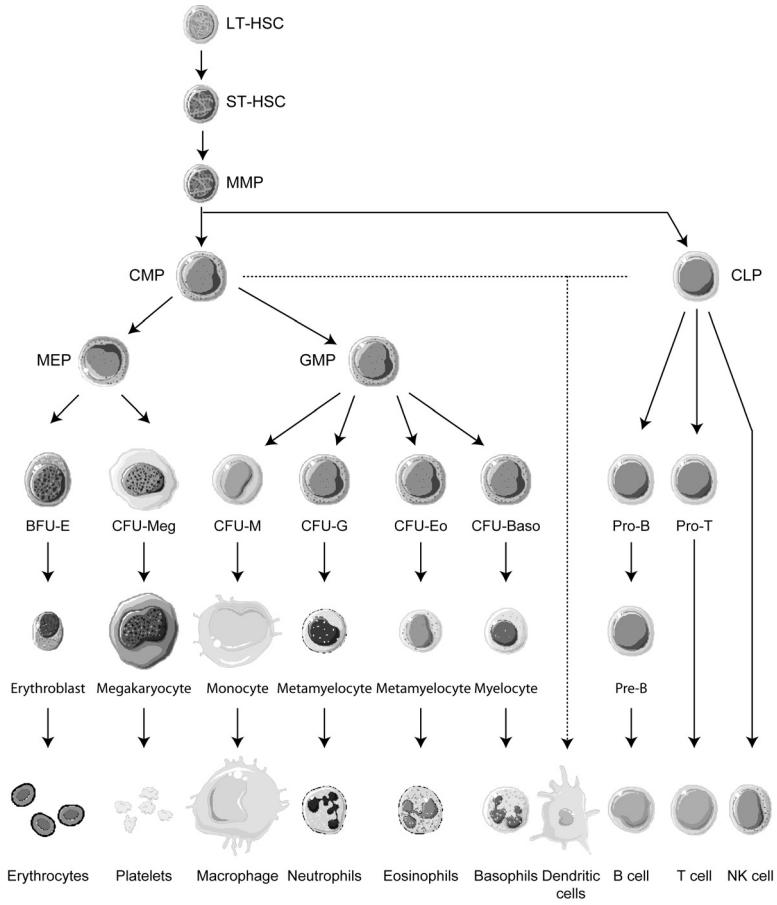

Figure 1. Hematopoietic lineage diversification. Longterm (LT) hematopoietic stem cells (HSC) have the ability for life-long self-renewal and multilineage differentiation potential. Long-term HSCs give rise to short-term (ST) HSCs, which retain the ability for multi-lineage differentiation, but have decreased self-renewal potential. According to the model, ST-HSCs produce multipotential progenitors (MPPs), which have completely lost self-renewal potential, but are able to generate all hematopoietic cell lineages. Common lymphoid progenitors (CLP) are thought to give rise to T and B cells, whereas the common myeloid progenitors (CMP) give rise to granulocyte/macrophage progenitors (GMP), megakaryocyte/erythroid progenitors (MEPs), and mast-cell and basophil progenitors.
Abbreviations: LT-HSC, long-term hematopoietic stem cell; ST-HSC, short-term hematopoietic stemcell; MPP, multipotential progenitor; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte/erythroid progenitor; GMP, granulocyte/macrophage progenitor.

and granule components such as lactoferrin and neutrophil gelatinase (26). Furthermore, transcription of genes that are normally expressed in other lineages are often actively suppressed during differentiation of HSCs into lineage-restricted myeloid progenitor cells (27).

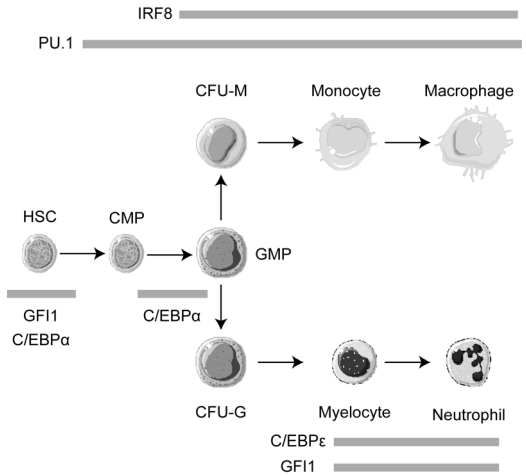

Figure 2. A stepwise requirement for transcription factors during myeloid differentiation. The differentiation of stem cells into the two main myeloid lineages, the monocytic and the neutrophilic lineages, is regulated by a complex network of transcription factors. Growth-factor independent 1 (GFI1) and CCAAT/enhancer binding protein α (C/EBPα) function in self-renewal of existing HSCs, whereas C/EBPα plays an indispensable role in facilitating the transition of CMP into GMP. GFI1 and C/EBPε are crucial for late-stage neutrophil production, whereas macrophage development depends on PU.1 and interferon-regulatory factor 8 (IRF8).

Neutrophil production

During myeloid development, the CMP precursor differentiates into a granulocyte/macrophage progenitor (CFU-GM) from which both neutrophils and monocytes are derived (Figure 1). Neutrophil maturation from the CFU-GM stage requires stimulation with G-CSF. Based on morphology of the cells and appearance of specific granules, different morphological stages can be distinguished (28)(29). Myeloblasts develop from promyelocyte towards myelocytes and metamyelocytes, whereas during terminal maturation, cells with band-shaped and segmented nuclei are formed (Figure 3). During microbial infections, neutrophils migrate from the peripheral blood towards the site of inflammation by chemo-attractants released by the surrounding tissues (30). After adhesion to the endothelial lining of the blood vessel wall, neutrophils diapedese through the endothelial layer, a process which is mediated by integrins. Once at the inflammatory locus, infectious microrganisms can be eliminated through phagocytosis in conjunction with release of toxic oxygen metabolites and cytotoxic proteins (31). The importance of correct neutrophil function is underscored by studies showing that individuals with decreased numbers of neutrophils have an increased mortality rate due to recurrent bacterial infections (32). For example, a high incidence of bacterial infection-related deaths is observed in patients with myelodysplastic syndromes, which suffer from neutropenia and aberrant neutrophil functioning due differentiation defects in the hematopoietic stem cell compartment (33).

Scope of the thesis

So far, studies have demonstrated that hematopoiesis requires coordinated expression of many genes that may directly or indirectly govern HSC and progenitor cell maintenance, lineage commitment, differentiation and mature blood cell function. Although it is evident that correct regulation of proliferation, survival and differentiation is critical for normal homeostasis within the bone marrow, the precise molecular mechanisms involved in the regulation of these processes are still incompletely understood. The aim of the work described in the thesis was to characterize the cytokine-mediated intracellular signal transduction pathways

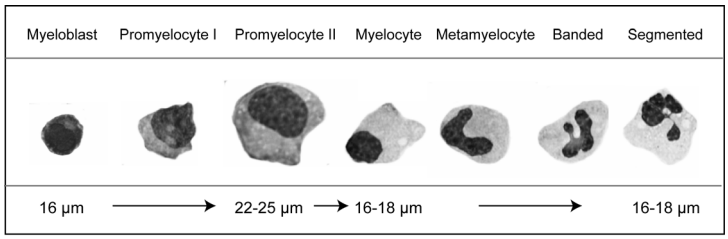

Figure 3. Neutrophil differentiation. Hematopoietic progenitor cells differentiating towards neutrophils undergo distinct morphological stages: myeloblast, promyelocyte I, promyelocyte II, myelocyte, metamyelocyte, banded and segmented mature neutrophils. These stages are characterized by changes in the morphology and development of granules.

regulating HSC and myeloid progenitor function. Furthermore, no studies have been performed to elucidate the molecular mechanism underlying the differentiation defects in MDS patients. In order to investigate the mechanisms underlying myeloid differentiation in human $CD34^+$ cells, an ex-*vivo* hematopoiesis culture system as well as an *in vivo* mouse transplantation model was utilized. Retroviral experiments allowed to ectopically express genes of interest and to study their role in regulation of hematopoiesis. In **Chapter 3**, we show a role for MKK3-p38MAPK signalling in the regulation of lineage choices during myelopoiesis through modulation of C/EBPα activity. In **Chapter 4**, we further studied MAPK function in the regulation of myeloid differentiation and identified a novel role for MEK-ERK signalling in regulating the balance between proliferation and apoptosis of neutrophil progenitors. In **Chapter 5**, we investigated the role of mTOR in regulation of myelopoiesis, and demonstrate that mTOR activity is essential for expansion of $CD34^+$ hematopoietic progenitor cells during myelopoiesis. In **Chapter 6**, we performed a candidate-directed screen for molecules improving defective hematopoiesis in MDS $CD34^+$ progenitor cells and show that ectopic expression of C/EBPα resulted in improved neutrophil maturation of MDS progenitors. This suggests that targeting C/EBPα may be of benefit in the design of novel therapies for low-risk MDS. Finally, in **Chapter 7**, we analyse the effect of histone deacetylase (HDAC) inhibition on the ability of HSC to undergo myelopoiesis and further discuss the application of these drugs in the treatment of hematological malignancies. Aberrant regulation of hematopoiesis can lead to bone marrow failure or development of hematological malignancies. By identifying the molecular mechanisms underlying the function of protein kinases in hematopoietic progenitor cells it will be possible to design novel therapies targeting these kinase activities for treatment of bone marrow disorders.

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Chapter 2

MAPK signalling pathways in the regulation of hematopoiesis

Christian R Geest[1] and Paul J Coffer[1,2]

[1] Molecular Immunology Lab, Department of Immunology, University Medical Center, Utrecht, The Netherlands, [2] Department of Pediatric Immunology, University Medical Center, Utrecht, The Netherlands

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ABSTRACT

The mitogen-activated protein kinases (MAPKs) are a family of serine/threonine kinases that play an essential role in connecting cell-surface receptors to changes in transcriptional programs. MAPKs are part of a three-component kinase module consisting of a MAPK, an upstream MAPK kinase and a MAPK kinase kinase that couples the signals from cell surface cytokine receptors to trigger downstream pathways. Three major groups of MAPKs have been characterized in mammals, including extracellular signal-regulated protein kinases (ERKs), c-Jun NH2-terminal kinases (JNKs), and p38MAPKs. Over the last decade, extensive work has established that these proteins play critical roles in the regulation of a wide variety of cellular processes including cell growth, migration, proliferation, differentiation and survival. It has been demonstrated that ERK, JNK and p38MAPK activity can be regulated in response to a plethora of hematopoietic cytokines and growth factors that play critical roles in hematopoiesis. In this review we summarize the current understanding of MAPK function in the regulation of hematopoiesis. In addition, the consequences of aberrant MAPK activation in the the pathogenesis of various hematological malignancies will be discussed.

The mitogen-activated protein kinases (MAPKs) are a family of serine/threonine kinases that play an essential role in connecting cell-surface receptors to changes in transcriptional programs (Figure 1) (1). They are ubiquitously expressed and are involved in the regulation of a wide variety of critical cellular functions, including proliferation, differentiation, migration and apoptosis (2)(3)(4)(5)(6)(7). In humans, there are at least 11 members of the MAPK superfamily, which can be divided into six distinct subgroups based on sequence similarity: (1) the extracellular signal-regulated kinases (ERK1 and ERK2), (2) c-Jun NH2-terminal kinases (JNK1, JNK2, JNK3), (3) p38 MAPKs (p38α/β/γ/δ), (4) ERK5, (5) ERK3s and (6) ERK7s (4)(8). Each group of MAPKs is activated by distinct kinase cascade in which a MAPK kinase kinase (MAP3K or MEKK) phosphorylates and activates a downstream dual-specificity MAPK kinase (MAP2K or MEK), that in turn stimulates MAPK activity through dual phosphorylation on threonine and tyrosine residues within a conserved tri-peptide motif (Thr-X-Tyr) (9). Phosphorylation of these threonine and tyrosine residues on MAPKs results in a conformational change that increases substrate accessibility and enhances catalysis. Activation of ERK is through phosphorylation by MEK1/2 in response to various cytokines and growth factors, and primarily mediates mitogenic and anti-apoptotic signals, whereas members of the JNK and p38 family of MAP kinases were originally identified as mediators of cellular stress and are activated by MKK4/MKK7 and MKK3/MKK6 respectively (1). This review will focus primarily on recent progress in understanding the function of the ERK, JNK and p38 MAPK pathways in regulating hematopoiesis (10).

Specificity in MAPK activation and function

MAPKs are activated by a plethora of extracellular stimuli, often with a single stimulus activating multiple subgroups, implying however that there is tight and specific control of MAPK activation and function in response to cellular agonists. MAPKs bind their upstream MAP2Ks and downstream targets through specific surface interactions that are achieved through docking motifs located outside of the catalytic domain (11-13). These binding sites or D-motifs are critical in determining substrate specificity of MAPKs. D-motifs are frequently located N-terminal to the typical MAPK conserved phosphorylation sites which are composed of a serine or threonine residue followed by a proline, and the amino acids surrounding these sites further increase the specificity of recognition. In addition, binding of MAPK signalling complexes to scaffold proteins determines the location and duration of MAPK activation, thus regulating specific signalling outcomes (Table 1)(14). For instance, the JIP1 protein functions as a scaffold that organizes JNK1/2, MKK7 and the MAP3K MLK1 into a specific MAPK signalling module. Similar to JIP1, additional scaffold proteins such as JIP2, JIP3 and Axin promote assembly of specific signalling complexes involved in the JNK activation (15). MP1, another scaffold protein specifically interacts with both ERK1 and MEK1, thereby facilitating ERK1 activation (16)(17). This illustrates how a distinct scaffold protein such as MP1, involved activation of ERK1 but not ERK2, can confer signalling specificity through recruiting the different components of a specific pathway simultaneously. Recently, kinase suppressor of Ras-1 (KSR-1) was also identified as a scaffold regulating the ERK MAPK pathway (18). KSR-1 can interact with Raf-1, MEK1/2 and ERK1/2 and some fractions of MEK1/2 were found to remain associated with KSR in quiescent cells. Upon activation by Ras, KSR translocates with MEK1/2 to the plasma membrane, bringing MEK1/2 in close proximity to

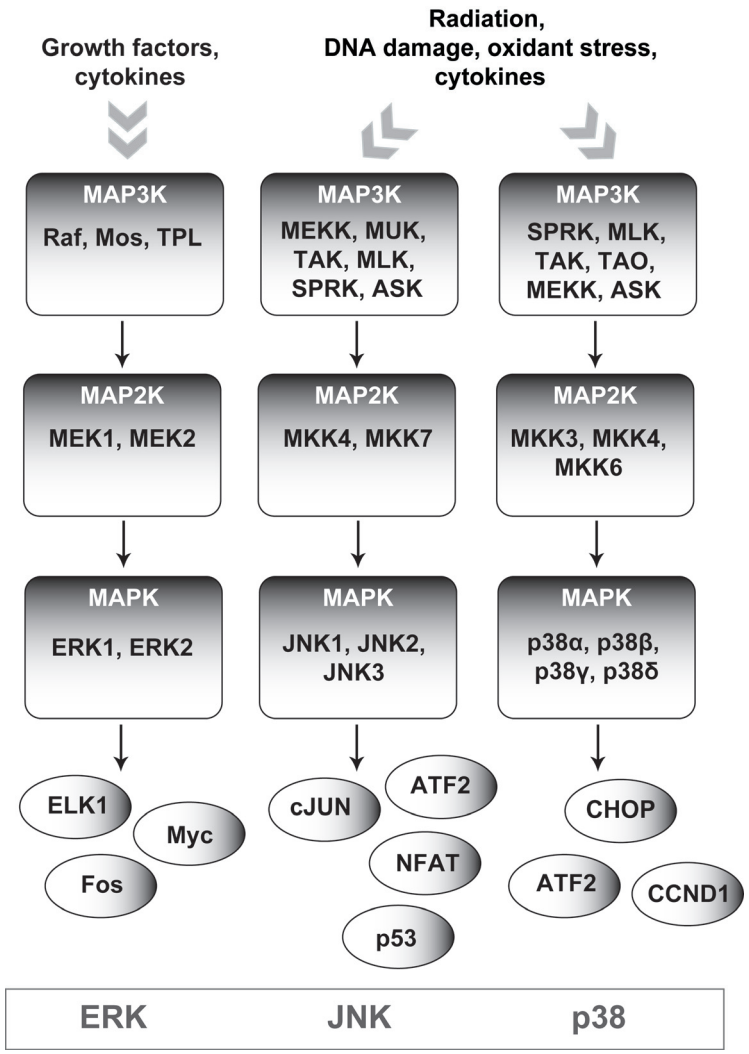

Figure 1. Schematic representation of the three main MAPK signalling cascades: ERK, JNK, p38 family members. MAPKs are part of a three-component kinase module consisting of a MAPK, an upstream MAPK kinase and a MAPK kinase kinase that couples the signals from cell surface cytokine receptors to trigger downstream pathways. Three major groups of MAPKs have been characterized in mammals, including extracellular signal-regulated protein kinases (ERKs), c-Jun NH2-terminal kinases (JNKs), and p38MAPKs. Each group of MAPKs is activated by a distinct kinase cascade in which a MAPK kinase kinase (MAP3K or MEKK) phosphorylates and activates a downstream dual-specificity MAPK kinase (MAP2K or MEK), that in turn stimulates MAPK activity through dual phosphorylation on threonine and tyrosine residues within a conserved tri-peptide motif (Thr-X-Tyr). Upon activation, MAPK itself can phosphorylate specific target substrates on serine and threonine residues.

its activator Raf-1 and its downstream effector ERK1/2, thereby facilitating formation of a Raf/MEK/ERK complex and activation of ERK1/2 (14)(19). So far, only few scaffolds have been described for members of the p38 MAPK pathway. However, some of the JNK scaffolding proteins appear to scaffold components of the p38 MAPK pathway as well. For example, it has been demonstrated that JIP2 can facilitate p38 signalling by binding to p38α and p38δ (20)(21). Furthermore, osmosensing scaffold for MEKK3 (OSM), a specific scaffold for the p38MAPK pathway was found to bind to MEKK3, MEK3 and p38 and facilitate p38 activation in response to osmotic stress (22).

Lastly, the regulated dephosphorylation of MAPKs by phosphoprotein phosphatases also plays an important role in determining the magnitude and duration of kinase activation and thereby the outcome of signalling (15)(23). Phosphoprotein phosphatases involved in the regulation of MAPK signalling can be classified in three groups based on preference for dephosphorylating phospho-tyrosine, phospho-serine/threonine or dual-specificity phosphatases (DSP) that can dephosphorylate both types of residues. DSPs that selectively inactivate MAPKs are known as MAPK phosphatases (MKPs) (23). MKPs have a variety of subcellular locations and can either recognize a single class of MAPK or can regulate more than one MAPK pathway. For example, the cytoplasmic DSP MKP-3 selectively inactivates ERK, whereas nuclear MKP-1 is able to dephosphorylate ERK, JNK and p38. Together these mechanisms cooperate to generate specificity in MAPK activation and function.

The ERK pathway: controlling the balance between expansion, survival and differentiation of hematopoietic progenitors.

ERK1 and ERK2, also known as p44MAPK and p42MAPK respectively, were identified as growth factor-stimulated protein kinases phosphorylating microtubule-associated protein-2 (MAP-2) and myelin basic protein (MBP) (24). They have more than 80% sequence similarity and can be activated by a wide variety of stimuli, including growth factors, serum, ligands for heterotrimeric G protein-coupled receptors, cytokines, osmotic and other cell stresses (25-27). Upon activation, ERK1/2 phosphorylate and regulate the activity of both cytoplasmic molecules and nuclear proteins, which in turn can control gene expression (Table 2) (28). For example, ERK1/2 have been found to phosphorylate (1) p90 ribosomal S6 kinase (RSK), mitogen and stress activated kinase (MSK) and MAPK interacting kinase (MNK), (2) proteins involved in migration, cell attachment including paxillin and calpain, and (3) transcription factors such as Elk-1, c-Fos, and c-Myc (15).

The ability of the ERK MAPK signalling module to promote cellular proliferation and survival has now been well established (29)(30). However, *in vivo* analysis of ERK1/2 function through generation of null mutant mouse strains by gene targeting revealed that ERK signalling is also critically involved in the regulation of other important cellular processes (Table 3). ERK1-/- mice are viable, fertile, and of normal size, indicating that ERK2 may compensate for its loss (31). However, although ERK1 is apparently dispensable during embryonic development, deletion of ERK2 was found to be embryonic lethal due to defects in trophoblast formation, mesoderm differentiation and placental function, demonstrating distinct biological functions for these related kinases (32-34).

In the hematopoietic system, the ERK pathway has been demonstrated to be essential for thymocyte differentiation since mice lacking ERK1 manifest impaired proliferation and

Scaffold	Pathway	MAPK-Scaffold interactions	References
MORG1	ERK	Raf-1, B-Raf, MEK, ERK	Vomastek et al (2004) [137]
Paxillin	ERK	MEK, ERK	Ishibe et al (2003) [138]
MP1	ERK	MEK1, ERK1	Schaeffer et al (1998) [16]
MEKK1	ERK	Raf-1, MEK1, ERK2	Karandikar et al (2000) [139, 140] Xu et al (1997) [140]
β-arrestin-1	ERK	Raf-1, MEK1, ERK2	De`Fea et al (2000) [141]
β-arrestin-2	JNK	Raf-1, MEK1, ERK2	Luttrell et al (2001) [142]
	JNK	ASK1, MKK4, JNK3	McDonald et al (2000) [143]
JIP1	JNK	MLK2/3, DLK, MKK7, JNK1/2	Dickens et al (1997) [144]
JIP2	JNK	MLK2/3, DLK, MKK7, JNK1/2	Yasuda et al (1999) [145] Verhey et al (2001) [146]
	p38	MLK3, MKK3, p38	Buchsbaum et al (2002) [19] Robidoux et al (2005) [147]
JIP3	JNK	MEKK1, MLK3, MKK7, JNK	Kelkar et al (2000) [148] Matsuguchi et al (2003) [149]
JIP4	p38	ASK1, MKK3/6, p38	Kelkar et al (2005) [150]
OSM	p38	MEKK3, MKK3	Uhlik et al (2003) [21]

Table 1. Scaffold proteins regulating MAPK signalling. Scaffold proteins play an important role in modulating the signal strength and duration through interaction with their respective MAPK modules. These proteins can further specify MAPK signalling by interacting with upstream signalling components such as cell surface receptors.

maturation of thymocytes, despite expression of ERK2 (31). Furthermore, ex-*vivo* studies focussing on ERK1/2 function employing differentiation-competent cell lines revealed the importance of the ERK signalling module in regulating myeloid, erythroid as well as megakaryocyte differentiation (35-38). Experiments using pharmacologic inhibitors have demonstrated that the MEK-ERK pathway is also essential for PMA-induced differentiation of K562 (39)(40), TF-1 (41) and U937 cells (42). Furthermore, ERK-mediated phosphorylation of the C/EBPα transcription factor on serine residue 21 was found to negatively regulate the activity of C/EBPα and its ability to induce neutrophil differentiation (43). While the dephosphorylated form of C/EBPα was able to induce neutrophil differentiation, ectopic expression of the phosphomimetic C/EBPα mutant inhibited neutrophil differentiation, suggesting that ERK signalling may indeed play a significant role in the regulation of myelopoiesis. Similarly, the requirement for MEK-ERK activation has been demonstrated during cytokine-induced differentiation using human $CD34^+$ hematopoietic progenitors (Geest *et al*, submitted). Modulating the duration and extent of ERK activation was shown to determine the balance between expansion and survival of hematopoietic progenitors during neutrophil differentiation. The underlying molecular mechanism was found to involve regulation of expression of a variety of cell-cycle modulating proteins, including c-myc, c-fos, p21CIP1, cyclin D1 and cyclin D3. Furthermore, conditional activation of MEK1 also resulted in increased expression of mRNA transcripts encoding a large number of hematopoietic

MAPK Pathway	Example of Substrate	Description	References
ERK	ELK1	Member of ETS oncogene family	Yang et al (1998) 151
	RUNX1/AML1	Runt-related transcription factor 1	Tanaka et al (1996) 152
	E47	Transcription factor E47	Nie et al (2003) 153
	Fos	FBJ osteosarcoma oncogene	Murphy et al (2002) 154
	Myc	Melocytomatosis oncogene	Alvarez et al (1991) 155
	Fra1	Fos-like antigen 1	Young et al. (2002) 156
	STAT1/3	Signal transducer and activator of transcription 1/3	Chung et al (1997) 157
	Lck	Lymphocyte-specific protein tyrosine kinase	Winkler et al (1993) 158
	RSK	Ribosomal protein S6 kinase	Sturgill et al (1988) 159
	MSK1/2	Mitogen and stress-activated kinase 1/2	Deak et al (1998) 160
	MNK1/2	MAP kinase-interacting protein kinase 1/2	Fukunaga et al (1997) 161
	Paxillin	Paxillin	Ku and Meier (2000) 162
	Calpain	Calpain	Glading et al (2004) 163
	EGFR	Epidermal growth factor receptor	Northwood et al (1991) 164
	Bad	Bcl2-antagonist of cell death	Scheid et al (1999) 165
	p21CIP1	Cyclin-dependent kinase inhibitor 1A	Das et al (2000) 166
	p27KIP1	Cyclin-dependent kinase inhibitor 1B	Kawada et al (1997) 167
	Rb	Retinoblastoma protein	Garnovskaya et al (2004) 168
JNK	c-Jun	Jun oncogene	Pulverer et al (1991) 169
	ATF2	Activating transcription factor 2	Gupta et al (1995) 170
	ELK1	Member of ETS oncogene family	Whitmarsh et al (1995) 171
	Pax2	Paired box 2	Cai et al (2002) 172
	p53	p53 tumor suppressor	Buschmann et al (2001) 173
	Myc	Myelocytomatosis oncogene	Alarcon-Vargas et al (2004) 174
	STAT3	Signal transducer and activator of transcription 3	Zhang et al (2001) 175
	FOXO4	Forkhead transcription factor O 4	Essers et al (2004) 176
	NFAT4	Nuclear factor of activated T-cells4	Chow et al (1997) 177
	MAP	Microtubule-associated proteins	Chang et al (2003) 178
	Bax	Bcl2-associated X protein	Kim et al (2006) 115
	Bim	Bcl2 interacting mediator of cell death	Putcha et al (2003) 179
p38	ATF2	Ativating transcription factor 2	Jiang et al (1996) 180
	MEF2	Myocyte enhancing factor 2	Yang et al (1999) 151
	STAT4	Signal transducer and activator of transcription 4	Visconti et al (2000) 181
	ELK1	Member of ETS oncogene family	Yang et al (1998) 182
	Fos	FBJ osteosarcoma oncogene	Tanos et al (2005) 183
	CHOP	C/EBP homologous protein	Wang et al (1996) 184
	C/EBPβ	CCAAT/enhancer binding protein β	Engelman et al (1998) 185
	MSK1	Mitogen and stress-activated kinase 1	Deak et al. (1998) 160
	MAPKAP2	MAP kinase activated protein kinase 2	Rouse et al (1994) 186
	CCND1	Cyclin D1	Lavoie et al (1996) 129
	HSP27	Heat shock protein	Freshney et al (1992) 187
	p53	p53 tumor suppressor	Sanchez-Prieto et al (2000) 188

Table 2. MAPK substrates potentially regulating hematopoiesis. Substrates include various transcription factors, kinases as well as other factors that can be directly regulated by MAPK-mediated phosphorylation. The list of indicated substrates is not exhaustive, but illustrates many of the important substrates that have currently been identified.

cytokines that may act as autocrine growth factors to activate signalling pathways implicated in regulating expansion and survival of hematopoietic progenitor cells. Although found to be an essential regulator of expansion and survival of myeloid progenitors, conditional activation of MEK signalling did not affect terminal differentiation of neutrophil progenitors. However, a recent study indicates that ERK signalling may actually be involved in the regulation of early myeloid commitment of hematopoietic stem cells (HSCs) (44). *In vitro* Granulocyte-Macrophage (GM) colony formation of murine HSCs in presence of MEK-ERK pharmacological inhibitors was inhibited, whereas HSCs ectopically expressing active MEK1 showed increased percentages of Mac-1^{+} myeloid cells.

In addition, it has been recently demonstrated that ERK-mediated phosphorylation is implicated in the degradation of active globin transcription factor 1 (GATA-1), a transcription factor that plays a critical role in regulating erythroid, megakaryocytic and eosinophil

MAPK	Functions/Phenotypes	Lethality	References
ERK1	Defective T-cell development	No	Pagès et al (1999) 30
ERK2	Defective mesoderm development; Defects in placenta function and throphobast formation	Yes	Saba-El-Leil et al (2003) 31 Yao et al (2003) 33
JNK1	Defects in Th2 differentiation; Defects in T cell activation and apoptosis of immature thymocytes	No	Sabapathy et al (2001) 60 Dong et al (1998) 189
JNK2	Defects in Th1 differentiation; Defects in T cell activation and apoptosis of immature thymocytes	No	Sabapathy et al (2001) 60 Yang et al (1998) 151
JNK1/2	Defective neural tube closure	Yes	Kuan et al (1999) 190
JNK3	Resistant to kainate-induced neural damage	No	Yang et al (1997) 62
p38α	Defects in EPO production; Defects in placental angiogenesis	Yes	Mudgett et al (2000) 83 Tamura et al (2000) 84
p38β	No phenotype	No	Beardmore et al (2005) 87
p38γ	No phenotype	No	Sabio et al (2005) 88
p38δ	No phenotype	No	Krens et al (2006) 191

Table 3. Phenotypes of MAPK knockout mice. The functions, obtained by gene-targeting studies are described for each MAPK. The indicated lethality shows if gene-disruption resulted in a lethal phenotype.

development (45)(46). Acetylation of GATA-1 was found to both stimulate DNA binding and enhance GATA-1-dependent transcription as well as target GATA-1 for degradation by the ubiquitin–proteasome pathway (45). However, acetylation of GATA-1 alone was not sufficient to cause degradation but rather additional phosphorylation on ERK-specific residues was required. Together, this suggests that acetylated active GATA-1 remains at promoters untilits degradation is triggered in response to ERK-mediated phosphorylation. Furthermore, it has been reported that ERK mediates erythropoietin-induced phosphorylation of TAL1/SCL in murine proerythroblasts, a transcription factor belonging to the family of the helix-loop-helix class of transcription factors which is essential for erythropoiesis (47)(48).

In contrast to most experimental cell systems, undifferentiated embryonic stem (ES) cells do not require the ERK pathway for proliferation and self-renewal (49)(50). Remarkably, inhibition of MEK-ERK MAPK actually promotes self-renewal of murine ES cells and dependency on ERK pathway only becomes apparent upon lineage commitment. Although the molecular basis how ERK potentiates lineage commitment is poorly defined, it has been proposed that ERK1/2 activation may somehow act as a stimulus for naïve ES cells to exit the self-renewal program and facilitate transition to a state that is responsive to inductive stimuli (51). Murine embryos deficient for MAP3K B-Raf indeed have a quantitative defect in fetal liver hematopoietic progenitor cell development, however, the relative progenitor frequency was not affected in the absence of B-Raf, suggesting that B-Raf plays a critical role in establishing the proper number of myeloid progenitors in the fetal liver (52). Additional data suggest that this phenotype is due to the loss of associated ERK1/2 activation during hematopoietic progenitor cell formation. In addition to its effect on hematopoietic progenitor development, thrombopoietin (TPO)-induced expansion of ES cell-derived megakaryocytic

progenitors and suppression of mast cell development are abrogated in the absence of B-Raf. Ourselves and others have recently demonstrated that ERK MAPK activity is essential for survival of erythroid $CD34^+$ progenitors (53), (Geest *et al*, submitted). Inhibition of ERK1/2 activity completely abolished expansion and subsequent differentiation of $CD34^+$ erythrocyte progenitors, due to induction of apoptosis. This was further found to correlate with abrogation of anti-apoptotic Bcl-xL levels in $CD34^+$ cells treated with MEK1/2 inhibitor, indicating that MEK-ERK activity is essential for survival and development of erythroid progenitors. Furthermore, recently it has been reported that uncoupling protein 2 (UCP2), an inner membrane mitochondrial protein which has been implicated in the regulation of erythropoiesis, modulates expansion of erythrocyte progenitors in an MEK-ERK pathway dependent manner. Analysis of progenitor cells from bone marrow and fetal liver from UCP2 deficient mice revealed that UCP2 deficiency results in a significant decrease in cell proliferation during the erythropoietin-dependent early phase of erythropoiesis. This was accompanied by reduction in the phosphorylated form of ERK, suggesting that UCP2 is modulating proliferation during erythropoiesis through regulation of ERK activation (54). Consistently with a key role of ERK1/2 in regulating erythropoiesis, tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) was found to negatively regulate erythroid maturation of $CD34^+$ derived erythroid progenitors through an ERK-dependent pathway (55). Remarkably, although previous studies showed that ERK MAPK activity is indispensable during erythropoiesis, inhibitory effects of TRAIL on erythroid maturation were reverted upon addition of a pharmacologic inhibitor of the ERK pathway. Thus, this suggests that anti-differentiative effects of TRAIL on erythroblasts are indeed mediated through activation of the MEK-ERK pathway.

Taken together, these results suggest that correct activation of the ERK pathway is required for normal hematopoiesis and modulation of the duration and extent of ERK activation seems to be an important factor in regulating the ultimate balance between expansion, survival and differentiation of hematopoietic progenitors.

The JNK pathway is essential for normal erythropoiesis

The JNK MAPKs, also known as stress-activated protein kinases (SAPKs) were originally discovered by their ability to phosphorylate the N-terminal transactivating domain of the transcription factor c-Jun (56). Three distinct genes, JNK1, JNK2, and JNK3 encoding ten isoforms have now been identified in mammals (57-59). JNK1 and JNK2 are ubiquitously expressed, while expression of the JNK3 isoform is primarily restricted to the brain, testis and heart (5). JNKs are activated in response to inflammatory cytokines, growth factors, DNA and protein synthesis inhibition, and environmental stresses, such as heat, ionizing radiation, DNA damage and oxidant stress. Downstream substrates of JNKs include the transcription factors c-Jun, Elk-1, p53, ATF-2 and NFAT, microtubule-associated proteins (MAPs), and pro-apoptotic Bcl-2 family members, including Bid, Bax and Bim (Table 2) (15)(60).

The generation of mice deficient in one or more of the JNK genes has facilitated the functional analysis of the role of JNKs (Table 3). Mice lacking individual members of the JNK family are viable, however, both JNK1 and JNK2 knockouts exhibit defects in T cell activation and apoptosis of immature thymocytes (61). In contrast, embryos deficient of both JNK1 and JNK2 die early during embryonic development (E11.5-12.5) due to neural tube closure and

brain defects. In addition, JNK1-/- JNK2-/- embryonic fibroblasts are resistant to stress-induced apoptosis, suggesting a role for JNK1 and JNK2 in regulating apoptosis in response to environmental stress (62). Mice lacking JNK3, which is mainly expressed in the nervous system, are more resistant to glutamate-receptor agonist kainate-induced apoptosis of neurons in the hippocampus (63). The same phenotype is exhibited by “knock-in” mice with mutations in the two c-Jun phosphorylation sites (Ser-63/73), indicating that JNK3 may exert its effect through c-Jun (64). In addition, the early embryonic death caused by the targeted deletion of the MKK4 or MKK7 genes in mice further supports an essential role for JNK MAPK signalling pathway in the regulation of developmental processes (60).

Although JNK proteins were first identified as kinases that were activated by stress and apoptosis-inducing agents, JNKs are now known to be activated by a variety growth factors that regulate proliferation, differentiation and survival of hematopoietic cells, including EPO and stem cell factor (SCF), as well as thrombopoietin, interleukin (IL)-3, and GM-CSF (65-67). Furthermore, several studies focussing on JNK function in hematopoietic cell systems revealed the importance of the JNK signalling module in regulating erythropoiesis (68-70). Homologous deletion of either JNK1 or JNK2 in mice has no apparent effect on hematopoiesis, which might be explained by redundancy of the different JNK isoforms. In contrast, activation of JNK signalling has been implicated in the regulation of erythroid proliferation and survival. Treatment with the JNK1/2/3 inhibitor SP600125 inhibited EPO-dependent proliferation of several differentiation-competent erythroid cell lines (68). Furthermore, inhibition of JNK activity in primary mouse bone marrow cells reduced the number of erythroid burst-forming units (BFU-E), whereas the more differentiated erythroid colony-forming units (CFU-E) were not affected. In addition, SP600125 protected the BFU-E's from apoptosis induced by cytosine arabinoside, demonstrating that JNK activity appears to be an important regulator of proliferation in immature, primary erythroid cells and these erythroid cell lines, but presumably may not be required for the survival or proliferation of CFU-E’s or proerythroblasts (69). Moreover, it has recently been reported that disruption of the upstream JNK regulator MEKK1 causes embryonic death due to defects in erythrocyte differentiation, further indicating that the MEKK1-JNK signalling pathway is indeed essential for erythropoiesis (70).

It has also been demonstrated that the activator protein-1 (AP-1) transcription factor complex, which is comprised of members of the Jun and Fos families of phosphoproteins, is implicated in the regulation of proliferation and apoptosis of the murine erythroleukemia cell line HCD57 (71). AP-1 DNA-binding activity was induced upon both growth factor stimulation and withdrawal, however different AP-1 factors were found to be involved in the two processes. c-Jun DNA-binding activity and JNK activity were induced in the presence of EPO, whereas EPO withdrawal resulted in a decrease in JNK activity and an increase in JunB DNA-binding activity.

Besides involvement in the regulation of erythropoiesis, the importance of JNK signalling in the regulation of myeloid differentiation was illustrated by a recent study showing that JNK1 can physically interact with the DNA-binding domain (DBD) of C/EBPα *in vitro* and *in vivo* (72). C/EBPα is a leucine zipper transcription factor that plays a critical role in regulating myelopoeisis and mice deficient for C/EBPα lack mature granulocytes, and accumulate immature myeloblasts in the bone marrow (73)(74). It was reported that active JNK1 inhibits

ubiquitination of C/EBPα possibly by phosphorylating the DBD, increasing C/EBPα protein stability and altering its conformation, contributing to enhanced C/EBPα transcriptional activation. Thus, it is possible that JNK1 acts as positive regulator of C/EBPα and that JNK activity is required for C/EBPα activation in myeloid progenitors. In contrast, co-immunoprecipitation experiments demonstrated that C/EBPα can also interact with c-Jun and JunB (75), thereby competing with c-Jun for interaction with PU.1, an important transcriptional regulator during monopoiesis (76). In addition, in murine myeloid progenitors, C/EBPα:c-Jun or C/EBPα:c-Fos heterodimers induced monocyte in favour of granulocyte lineage commitment (75), suggesting that c-Jun or c-Fos interaction may prevent C/EBPα mediated induction of granulocyte differentiation.

In mast cells lacking MKK7, JNK activation is completely abolished and results in hyperproliferation in response to IL-3 and SCF stimulation (77). Furthermore, MKK7 deficiency reduced expression of JunB, the cell cycle inhibitor p16INK4a and resulted in upregulation of cyclin D1. Re-expression of p16INK4a in MKK7-/- mast cells abrogated the hyperproliferative response, indicating that the MKK7-JNK signalling pathway functions as negative regulator of cell growth in mast cells. Furthermore, *gadd45b*, a gene rapidly induced in response to genotoxic stress or cytokines, has also been demonstrated to stimulate JNK activity, thereby sensitizing cells for growth arrest and apoptosis. Takekawa and Saito reported that Gadd45-like proteins can activate JNK signalling through binding and activation of MTK1 MAP3K, which is upstream of both p38 and JNK MAPKs (78). However, exposure of Gadd45b-deficient bone marrow cells to UV-light was found to result in prolonged JNK activation and increased sensitivity to UV-induced apoptosis, suggesting that in murine bone marrow cells Gadd45b suppresses JNK activity in response to UV-radiation (79). Although several lines of evidence suggest that in hematopoietic cells JNK activation is linked to cell death, the exact function of Gadd45 and JNK in the regulation of apoptosis remains unclear. During monocytic differentiation of primary mouse progenitor cells, sustained JNK activation by hematopoietic progenitor kinase 1 (HPK1), a potent activator of the JNK signalling pathway, was found to actually confer progenitor cell survival independent of the growth factor IL-3 (80). Together, these studies indicate that JNK signalling is involved in the regulation of normal erythropoiesis and myelopoiesis, however, the precise role of the different JNK family members is currently incompletely defined.

p38MAPK activity is indispensable during erythroid development but detrimental for myelopoiesis

p38α was first isolated as a tyrosine-phosphorylated protein in macrophages in response to LPS stimulation (26). To date, four splice variants of the p38 family have been identified: p38α, p38β, p38γ, and p38δ. p38α and p38β are ubiquitously expressed, whereas expression of p38γ and p38δ appears to be more tissue restricted (7). Sequence comparisons further revealed that the four p38 isoforms share only about 60% identity, suggesting distinct cellular functions. p38MAPKs respond to a variety of extracellular stimuli, particularly cellular stress, such as osmotic shock, UV radiation, hypoxia, inflammatory cytokines and growth factors (15). Upon activation, p38MAPKs phosphorylate and regulate a broad variety of substrates, which include (1) transcription factors ATF-1/2, C/EBPβ, STAT1 and STAT3, (2) protein kinases such as p90 ribosomal S6 kinase (RSK), MAPKAP2 and -3 as well as (3) a variety

of other substrates including heat shock proteins (Table 2) (7)(81)(82)(83).
Although members of the p38 family of MAP kinases were also originally identified as mediators of cellular stress, gene-disruption and silencing experiments in mice revealed central roles for p38MAPK in multiple developmental processes. p38α deficiency is embryonic lethal and most embryos die between days E10.5 and E12.5 due to defects in placental angiogenesis (Table 3) (84)(85). However, some mice deficient of p38α can survive until E16.5 and these embryos exhibit a normal morphology, but are anemic due to stress-induced abnormal erythropoiesis. Lack of p38 activity was found to interfere with stabilization of Epo mRNA in human hepatoma cells undergoing hypoxic stress, resulting in diminished erythropoietin gene expression (85)(86). Despite proposed roles for p38MAPK isoforms in various cellular processes, mice deficient of either p38β, p38γ or p38δ survive normally and exhibit no pathological changes, indicating redundancy between the various isoforms (87-89). In addition, it has been demonstrated that p38MAPK is implicated in the regulation of Translocation-Ets-Leukemia/ETV6 (TEL), a member of the ETS family transcription factors that plays a critical role in establishment of post-natal hematopoiesis for all lineages in the transition of hematopoietic activity from fetal liver to bone marrow (90)(91). p38MAPK-dependent phosphorylation of TEL was found to reduce the transcriptional repression abilities of TEL, suggesting that p38MAPK might indeed play a role in regulating neo-natal hematopoiesis through suppression of TEL function.
So far, experiments focussing on p38 function *in vivo* have revealed a critical role for p38 in the regulation of erythropoiesis. Additional ex-*vivo* studies using differentiation-competent cells further established the importance of the p38 signalling module in regulating both erythroid and myeloid differentiation (92-96)(Geest *et al*, submitted). Furthermore, functional analysis of p38α and p38β MAPKs in these cellular processes has been accelerated by the availability of pyridinyl imidazole compounds, such as SB203580, which inhibit both p38 isoforms (97). However, since many studies have relied solely on using these pyridinyl imidazole inhibitors, the relative contribution of the different isoforms in most cellular processes remains unknown.
p38 activation was reported to be induced by EPO (67)(65)(98) and several reports have suggested that p38 is necessary for the initiation of erythroid differentiation. Pharmacological inhibition of p38 suppressed EPO-induced differentiation of SKT6 cells, and p38 anti-sense oligonucleotides inhibited Epo-induced hemoglobinization (92). In contrast, activation of p38 by ectopic expression of a constitutively active MKK6 mutant induced hemoglobinization of SKT6 cells in absence of Epo, indicating that activation of p38 is indeed required for Epo-induced erythroid differentiation. Furthermore, proliferation of human $CD34^+$ derived erythroid progenitors and several erythroid cell lines was found to be positively regulated by p38MAPK (93-95). Interestingly, distinct stage-specific expression patterns of the four p38MAPK isoforms have been described for primary erythroid progenitors during erythroid differentiation (99). Examination of the mRNA expression patterns of each of the four p38 isoforms during erythroid differentiation of primary erythroid progenitors revealed that p38α and p38γ transcripts are expressed in early hematopoietic progenitors as well as in late differentiating erythroblasts, whereas p38δ mRNA is only expressed and active during the terminal phase of erythroid differentiation (99). In contrast, p38β was found to be minimally expressed in early $CD34^+$ hematopoietic progenitors and completely absent in lineage-committed erythroid

progenitors. These distinct stage-specific expression patterns suggest that the p38MAPK isoforms are not redundant and may have distinct functions during erythrocyte maturation.
Besides involvement in the regulation of erythropoiesis, the importance of p38MAPK activity in regulating myeloid differentiation is illustrated by a recent study showing that p38 plays an important role in regulating expansion and differentiation of myeloid progenitors during granulopoiesis (Geest *et al*, submitted). Inhibition of p38MAPK was found to enhance neutrophil development, whereas constitutive activation of p38 completely abrogated neutrophil differentiation. Furthermore, *in vivo* experiments using NOD/SCID mice transplanted with $CD34^+$ cells ectopically expressing constitutively active MKK3 also resulted in reduced neutrophil differentiation. The underlying molecular mechanism was found to involve inhibitory phosphorylation of C/EBPα on serine 21 in response to p38MAPK activation.
Besides being activated by cytokines that promote proliferation, differentiation and survival of hematopoietic progenitors, the p38MAPK signalling pathway is also activated by cellular stimuli that exert negative regulatory effects on hematopoiesis. p38MAPK appears to be activated by myelosuppressive cytokines, such as interferon (IFN)-α and -β, transforming growth factor-β (TGFβ), and tumor necrosis factor-α (TNFα) (100-102). In human hematopoietic progenitor cells, treatment with IFN-α and -β, TGFβ, and TNFα resulted in dose-dependent inhibition of both myeloid and erythroid colonies in methylcellulose colony-forming assays (100)(102). In addition, the p38MAPK pharmacological inhibitors SB203580 and SB202190 were both found to reverse the growth inhibitory effects of these cytokines. Additional studies to clarify the molecular mechanism of these observations have indicated that p38 activation indeed is required for IFN-α dependent transcriptional activation (103). On the hand, a study by Kale *et al* reported that TGFβ, when used at very low doses, stimulated colony formation from $CD34^+$ cells (104). Low doses were found to strongly induce ERK phosphorylation, whereas high doses induced p38 activation. Furthermore, use of p38 MAPK inhibitor SB 203580 along with low TGFβ concentrations exhibited a synergistic effect on stimulation of colony formation. These data suggest that dose-dependent bidirectional effects of TGFβ on hematopoiesis may be result from differential activation of the p38 and ERK MAPK signalling pathways by TGFβ.
Furthermore, bone marrow stromal cells have been demonstrated to play an essential role in the regulation of proliferation and differentiation of hematopoietic stem and progenitor cells (105). Long-term bone marrow cultures were found to produce essential cytokines, chemokines and growth factors such as IL-6, IL-11, G-CSF and GM-CSF. Interestingly, pharmacological inhibition of p38MAPK was found to disrupt the cytokine network between stromal cells and hematopoietic cells through dose-related inhibition of hematopoietic cytokine production, suggesting that p38MAPK mediated regulation of stromal-derived cytokines might be involved in supporting normal hematopoiesis (106).
Taken together, these studies suggest an important role for p38MAPK signalling in the regulation of erythropoiesis and myelopoiesis.

Role of ERK and p38MAPK signalling in HSC maintainance

Throughout the lifetime of an organism, hematopoietic stem cells (HSCs) are required to both self-renew as well as differentiate into the various hematopoietic lineages (107).

Therefore, appropriate control of hematopoietic stem cell self-renewal is essential for the maintainance of hematopoietic homeostasis. Recently MAPK signalling has been demonstrated to play a key role in the maintenance of HSC quiescence (108)(109). The p38MAPK pathway was found to be constitutively activated in HSCs obtained from mice deficient in the gene encoding *Atm*, a cell-cycle checkpoint regulator that is also thought to be involved in regulating oxidant levels (108). Deletion of *Atm* resulting in elevated levels of reactive oxygen species (ROS) and HSC-specific phosphorylation of p38MAPK was accompanied by loss of self-renewal capacity and defects in HSC maintainance. Treatment with an antioxidant or p38MAPK inhibitor rescued these mice from bone marrow failure, suggesting that the p38MAPK signalling pathway contributes to HSC exhaustion in response to ROS-mediated oxidative stress. Examination of normal HSCs revealed that a significant fraction of the stem cells were quiescent and in the G0 phase of the cell cycle, whereas ROS-mediated activation of p38 resulted abrogation of quiescence in HSCs. The connection between oxidants and stem cell aging was further supported by a study reporting that ROS-related oxidative stress abrogates the reconstituting capacity of HSCs leading to defective self-renewal of HSCs (109). Recent experiments showed that the ROS^{low} HSC population has a higher self-renewal potential, whereas significant HSC exhaustion was observed in the ROS^{high} population following serial transplantation. p38MAPK activity was higher in the ROS^{high} compared to the ROS^{low} population. Importantly, prolonged treatment of HSCs with anti-oxidant or p38MAPK inhibitor extended the lifespan of HSCs in serial transplantation experiments and was able to restore HSC function in the ROS^{high} population, indicating that inactivation of p38MAPK protects HSCs against loss of self-renewal capacity (109).
In addition, the ERK MAPK signalling pathway has also been demonstrated to play an important role in the maintenance of HSC quiescence. A recent report by Oostendorp *et al* showed that stimulation of cord blood-derived $Lin^{-}CD34^{+}CD133^{+}$ cells with SCF resulted in strong activation of ERK and rapid induction of proliferation (110). However, as a single factor SCF was not able to promote long-term colony-forming activity, indicating that self-renewal requires additional signals. Expansion of $Lin^{-}CD34^{+}CD133^{+}$ cells was further increased by the addition of the growth factor oncostatin M (OSM) and OSM was found to cooperate with SCF not only to promote mitogenic activity, but also to preserve the repopulating activity of $CD34^{+}$ cells expanded with SCF. Interestingly, OSM was found to specifically modulate the duration of SCF-induced activation of ERK, indicating there was indeed a role for ERK signalling in the regulation of HSC maintenance. Importantly, the effects of OSM could be mimicked by specific inhibition of the MEK-ERK pathway, indicating that the loss of repopulating activity in response to SCF stimulation is counteracted by OSM through down-regulation of ERK. Together, these experiments support a role for both p38MAPK and ERK MAPK signalling in the regulation of HSC self-renewal and homeostasis.

MAPK signalling and hematological malignancies

Since MAPK activation plays critical roles in the regulation of proliferation, survival and differentiation (Figure 2), aberrant activation of this pathway could be considered as a likely cause of hematopoietic disease. Over the last decade, extensive work has confirmed the hypothesis that MAPK pathways play critical roles in the pathogenesis of various hematological malignancies. The importance of MEK-ERK signalling in the regulation of

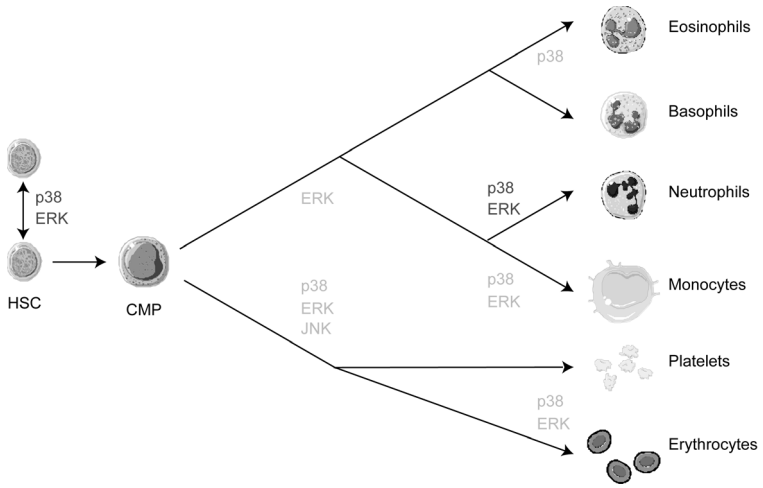

Figure 2. A model summarizing the lineage stage-specific requirement for MAPK activity during hematopoiesis. Inhibition of p38 is essential for neutrophil development, whereas activation is required for eosinophil, monocyte and erythrocyte differentiation. In addition, activation of ERK is essential for final maturation of erythrocytes and monocytes, whereas initial ERK activity is required for optimal expansion of progenitors during myelopoiesis. Furthermore both p38MAPK and ERK MAPK signalling negatively regulate HSC self-renewal and homeostasis. Although the precise role of the different JNK family members is currently incompletely defined, activation of JNK is important for erythrocyte development. Positive effects on lineage development are depicted in grey and inhibitory effects are depicted in black.

hematopoiesis is underscored by constitutive activation of the ERK MAPK pathway in a wide variety of myeloid malignancies (111)(112). Sustained activation of the MEK-ERK axis has been reported in a majority of acute myeloid leukemias (AML) and chronic myeloid leukemias (CML) (113). For example, Morgan *et al* have shown activation of the MEK-ERK pathway in 9 of 14 AML cell lines and 2 of 5 CML cell lines studied. Expression of a constitutively active form of Raf kinase in hematopoietic cell lines results in MEK activation and growth factor independence (114). Moreover, inhibition of MEK-ERK activation by pharmacological inhibitors inhibits proliferation and induces apoptosis in AML blasts (115-118). Mutations constitutively activating the FLT3 receptor tyrosine kinase gene, which are found in up to 30% of AML patients, are also believed to mediate their effects through activation of the downstream MEK-ERK signalling pathway (119). Activation of FLT3 was found to inhibit C/EBPα function by ERK1/2-mediated phosphorylation on serine 21, which may contribute to the observed differentiation block in leukemic blast cells. In human FLT3 mutant AML cells, pharmacological inhibition of MEK1 resulted in granulocytic differentiation, whereas there was no effect when serine 21 was mutated to aspartate, which mimics phosphorylation of C/EBPα (119).

Previously, it has also been reported that MEK1/2 inhibitors markedly enhanced the lethality of imatinib mesylate, a tyrosine kinase inhibitor that inhibits Bcr/Abl, in Bcr/Abl+ leukemia cells, including some that were resistant to imatinib mesylate due to increased Bcr/Abl expression (120). Recently, it has been suggested that these agents interact in a highly synergistic manner to induce mitochondrial injury and apoptosis in resistant CML cells in

association with multiple perturbations in survival signalling pathways, including inactivation of Bcr/Abl, ERK1/2 and down-regulation of Bcl-xL, Mcl-1 and activation of Bim (121). Consequently, the combination of dasatinib, second-generation Bcr/Abl kinase inhibitor that is more active than imatinib mesylate, and MEK inhibitor PD184352 may have reduced Bcl-xL expression by disrupting activation of the MEK-ERK signalling axis (122). In addition, expression of Mcl-1 is known to be regulated by MEK-ERK, which therefore may account for the observed down-regulation of Mcl-1 in dasatinib/PD184352-treated cells (122). Furthermore, recent studies suggest that Mcl-1 cooperates with Bcl-xL to inactivate pro-apoptotic Bak (123). Thus, disruption of both Mcl-1 and Bcl-xL expression in cells exposed to the combination of dasatinib and MEK inhibitor PD184352 could release Bak, thereby triggering mitochondrial dysfunction culminating in cell death. In addition, recent reports further suggest that ERK1/2-dependent phosphorylation of Bim can inhibit interactions between Bim and Bax, thereby inhibiting its pro-apoptotic activity and leading to enhanced survival (124).

Interestingly, our recent observations have revealed that modulating the duration and extent of ERK activation is essential in regulating the balance between expansion and survival of myeloid progenitors (Geest *et al*, submitted). Activation of MEK1 in $CD34^+$ cells resulted in increased progenitor expansion, however in contrast to the effects of constitutive MEK activation in AML blast cells, prolonged MEK1 activation prevented myeloid differentiation due to induction of cell death. This suggests that while MEK-ERK signalling may indeed be a critical factor in the generation of myeloid leukemia, additional mechanisms are required for cellular transformation to occur. The central role of the MEK-ERK pathway in sustaining proliferation in a large percentage of myeloid leukemias, makes this signalling module a potential target for the molecular therapy. Various studies have demonstrated the beneficial effects of chemotherapeutic agents that inhibit MEK-ERK activation in inducing growth suppression and apoptosis of AML cells (113)(115)(125).

Although a role for the MEK-ERK signalling pathway in mediating mitogenic and anti-apoptotic signals in hematological malignancies has been well established, defects in p38 and JNK MAPK signalling that play a specific role in the regulation of hematological disorders are not well defined. It has been recently demonstrated that p38MAPK regulates cell-cycle checkpoint controls, suggesting that defective p38MAPK function may contribute to cell cycle defects and tumorigenesis (126-128). In various cell types p38MAPK has been implicated in the suppression of tumorigenesis through inhibition of cell growth by regulating cyclin D levels and inhibiting the activity of Cdc25 (127)(128). In contrast, p38MAPK was found to be constitutively activated in the bone marrows of patients with myelodysplastic syndromes (MDS) (101)(100)(129). Inhibition of p38MAPK decreased apoptosis and stimulated colony formation of primary MDS progenitors, suggesting that aberrant activation of the p38MAPK pathway may cause differentiation defects. Furthermore, we have recently demonstrated that inhibition of p38MAPK in $CD34^+$ progenitor cells isolated from G-CSF unresponsive severe congenital neutropenia (SCN) patients restored neutrophil differentiation, supporting the idea that aberrant activation of the p38MAPK pathway can be involved in the development of specific bone marrow failure syndromes (Geest *et al*, submitted).

Despite increasing evidence implicating the JNK pathway in the development of cancer, the mechanistic basis for these findings remains unclear. For example, the BCR-ABL fusion

gene, encoding a cytoplasmic tyrosine kinase that is the underlying molecular cause of CML, has been demonstrated to activate the JNK signalling pathway (130). Dominant-negative mutants of its downstream effector, the transcription factor c-Jun, were found to impair the transforming capacity of BCR-ABL, suggesting a potential role for the JNK/c-Jun axis in BCR-ABL-mediated transformation of hematopoietic cells. Furthermore, analysis of AML patient cells with specific translocations t(8;21), t(15;17) or inv(16) revealed increased expression levels of c-Jun (131). In t(8;21)-positive AML cells, the AML1-ETO fusion gene, resulting from the t(8;21) translocation was found to induce upregulation of c-Jun expression in a JNK-dependent manner, indicating that activation of c-Jun or JNK may promote cellular transformation.

Aberrant activation of Flt3 signalling through mutation or overexpression is also believed to mediate constitutive activation of JNK in a significant number of AML cases (132). Moreover, another study showed a relationship between constitutive activation of JNK in leukemic blast cells and treatment failure in AML to anthracyclines (133). Anthracycline compounds are widely used to treat leukemias and resistance to anthracyclines in AML has been mainly attributed to P-glycoprotein (P-gP), a glycoprotein expressed at the cell surface that confer resistance to the drug by effluxing it out of the cell. Analysis of 67 AML samples revealed a positive correlation between JNK activity and increased P-gP efflux, suggesting a potential role for the JNK signalling in AML (133).

However, *in vitro* exposure of primary AML cells to the anthracycline compound daunorubicin demonstrated a strong correlation between high JNK activity and the response of patients to standard induction chemotherapy. In this study, failure of JNK activation was found to confer resistance to anthracycline in AML, indicating that daunorubicin-induced apoptosis in AML cells is mediated through JNK activation, which when abrogated, contributes to drug resistance (134). Taken together, these studies suggest that MAPK pathways play critical roles in the pathogenesis of various hematologic malignancies, providing new possible molecular targets for future therapeutic approaches.

Concluding Remarks

It is clear that correct regulation of MAPK signalling is essential in the regulation of multiple processes involved in blood cell production. Extensive work over the last decade has increased our understanding of the mechanisms that are involved in the regulation of normal hematopoiesis. Several studies also support the idea that the ERK MAPK pathway participates in mitogenic responses in various hematological malignancies, whereas the regulatory roles of the JNK and p38 MAPK pathways are less well defined and may vary depending on the specific cellular context. Although less is known of the functional roles of p38 and JNK in the regulation of normal and malignant hematopoiesis, they appear to mediate the processes involved in regulating the sensitivity or resistance to various chemotherapeutic agents. While the precise molecular mechanisms underlying the development of different hematopoietic malignancies upon aberrant activation of the different MAPK signalling modules remains to be investigated, increased understanding of their specific functions has accelerated the development of specific inhibitors that are being clinically evaluated. Further identification specific MAPK substrates and their role in differentiation, proliferation and survival of hematopoietic stem and progenitor cells should

provide novel therapeutic strategies for the treatment of hematological malignancies and bone marrow failure.

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Chapter 3

p38 MAP kinase inhibits neutrophil development through phosphorylation of C/EBPα on serine 21

Christian R Geest[1], Miranda Buitenhuis[1], Annemieke G Laarhoven[1], Marc B Bierings[2], Marrie CA Bruin[2], Edo Vellenga[3], and Paul J Coffer[1,4]

[1] Molecular Immunology Lab, Department of Immunology, University Medical Center, Utrecht, The Netherlands, [2] Department of Pediatric Hematology, Wilhelmina Children's Hospital, University Medical Center, Utrecht, The Netherlands, [3] Department of Hematology, University Medical Center, Groningen, The Netherlands, [4] Department of Pediatric Immunology, University Medical Center, Utrecht, The Netherlands

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ABSTRACT

Many extracellular stimuli regulate growth, survival and differentiation responses through activation of the dual specificity kinase MAPK kinase 3 (MKK3) and its downstream effector p38 Mitogen-Activated Protein Kinase (MAPK). Using $CD34^+$ hematopoietic progenitor cells, here we describe a novel role for MKK3-p38MAPK in the regulation of myelopoiesis. Inhibition of p38MAPK utilising the pharmacological inhibitor SB203580, enhanced neutrophil development ex-*vivo*, but conversely reduced eosinophil differentiation. In contrast, constitutive activation of MKK3 dramatically inhibited neutrophil differentiation. Transplantation of β2-microglobulin$^{-/-}$ NOD/SCID mice with $CD34^+$ cells ectopically expressing constitutively active MKK3 resulted in reduced neutrophil differentiation *in vivo*, whereas eosinophil development was enhanced. Inhibitory phosphorylation of C/EBPα on serine 21 was induced upon activation of p38MAPK. Moreover, ectopic expression of a non-phosphorylatable C/EBPα mutant was sufficient to abrogate MKK3-induced inhibition of neutrophil development. Furthermore, treatment of $CD34^+$ progenitors from patients with severe congenital neutropenia with SB203580 restored neutrophil development. These results establish a novel role for MKK3-p38MAPK in the regulation of lineage choices during myelopoiesis through modulation of C/EBPα activity. This signaling module may thus provide an important therapeutic target in the treatment of bone marrow failure.

INTRODUCTION

Hematopoiesis is a highly regulated process resulting in the formation of different types of blood cells (1). Appropriate control of hematopoietic stem cell (HSC) self-renewal and differentiation is crucial for the maintenance of hematopoietic homeostasis. p38 Mitogen-Activated Protein Kinase (MAPK) is a widely expressed MAP kinase family member that is well known for its important roles in the response to inflammation and environmental stresses, although it can also regulate other cellular processes in a cell type-specific manner (2)(3). p38MAPK has also been demonstrated to play a key role in the development of a plethora of cell types, including the maintenance of HSC quiescence (4). Aberrant activation of the p38MAPK pathway has been linked to bone marrow failure while inhibition of p38MAPK restored defective hematopoiesis in some individuals with aplastic anemia and myelodysplastic syndromes (5)(6). Although these studies demonstrate a role of p38MAPK in the maintenance of HSC homeostasis, they have not addressed the question whether p38MAPK may also play a role in the regulation of hematopoietic lineage choice decisions.
A plethora of extracellular stimuli mediate their intracellular responses through activation of MAPK (7)(8). Three major groups of MAPKs have been characterized in mammals, including extracellular signal-regulated protein kinases (ERKs), c-Jun NH2-terminal kinases (JNKs), and p38MAPKs (9)(10). Activation of MAPK is regulated by an evolutionary conserved kinase cascade. MAPKKKs are serine/threonine kinases capable of phosphorylating and activating MAPKKs, which in turn stimulate MAPK activity through dual phosphorylation on threonine and tyrosine residues within a tripeptide motif (Thr-X-Tyr). Upon activation, MAPK itself can phosphorylate specific target substrates on serine and threonine residues (11)(12).
p38 MAP kinases have been studied extensively during the last few years (3). To date, four different p38 kinases with overlapping substrate specificity have been described (p38α, p38β, p38γ and p38δ). Both p38α and p38β are ubiquitously expressed, whereas expression of p38δ is restricted to lung, pancreas, kidney, testis, and small intestine, and expression of p38γ is limited to skeletal muscle (2)(13). It has been demonstrated that activation of p38MAPK is predominantly regulated by the MAPKKs MKK3 and MKK6. MKK6 is capable of phosphorylating all four isoforms, whereas MKK3 is more restricted and can only activate p38α, p38β and p38δ (14)(15). Substrates of p38MAPK include various transcription factors such as ATF-1/2, C/EBPβ, STAT1 and STAT3 (13)(16). In addition, several kinases including p90 ribosomal S6 kinase (RSK), MAPKAP2 and -3 as well as heat shock proteins can be directly regulated by p38MAPK-mediated phosphorylation (2)(3).
Analysis of p38MAPK-deficient mice has revealed that p38MAPK plays an important role in multiple developmental processes. p38α(-/-) mice are embryonic lethal and most embryos die between days E10.5 and E12.5 due to defects in placental angiogenesis. Some p38 deficient mice, however, can survive until E16.5. These embryos exhibit a normal morphology, but are anemic due to stress-induced abnormal erythropoiesis (17)(18). Lack of p38 activity was found to interfere with stabilization of Epo mRNA in human hepatoma cells undergoing hypoxic stress, resulting in diminished erythropoietin gene expression (19)(18). In contrast to p38α(-/-) null mutant mice, mice deficient of either p38β, p38γ or p38δ survived normally and showed no obvious phenotypes (20)(21). Deletion of p38αMAPK in adult mice, resulted in enhanced proliferation and defective differentiation of lung stem and progenitor cells (22). Furthermore, p38MAPK has been demonstrated to regulate the

differentiation and proliferation of various cell types, including myocytes, cardiomyocytes, neurons, adipocytes and immune cells (23-30). A role for p38MAPK in regulation of myelopoiesis, however, remains to be investigated.
In this study, we have investigated the role of the MKK3-p38MAPK signal transduction module in the regulation of myelopoiesis, utilizing a human ex-*vivo* granulocyte differentiation system as well as an *in vivo* mouse transplantation model. MKK3/p38MAPK activity was found to be important in regulation of hematopoietic progenitor expansion as well as lineage choice decisions during myelopoiesis. This was found to involve regulation of C/EBPα phosphorylation. In addition, treatment of $CD34^+$ progenitors from patients with severe congenital neutropenia with the p38MAPK inhibitor SB203580 resulted in greatly improved neutrophil production. These results identify a novel role for p38MAPK in regulation of lineage choice decisions during myelopoiesis and suggest that modulation of this pathway may lead to the development of novel therapies for the treatment of bone marrow failures.

MATERIALS AND METHODS

Isolation and culture of human $CD34^+$ cells
Mononuclear cells were isolated from umbilical cord blood by density centrifugation over a ficoll-paque solution (density 1.077 g/mL). MACS immunomagnetic cell separation (Miltenyi Biotech, Auburn, CA) using a hapten conjugated antibody against CD34, which was coupled to beads, was used to isolate $CD34^+$ cells. $CD34^+$ cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco, Paisley, UK) supplemented with 9% fetal calf serum (FCS) (Hyclone, Logan, UT), 50 μM β-mercaptoethanol, 10 U/mL penicillin, 10 μg/mL streptomycin, and 2mM glutamine at a density of 0.3×10^6 cells/mL. Cells were differentiated towards eosinophils upon addition of stem cell factor (SCF) (50 ng/mL), FLT-3 ligand (50 ng/mL), granulocyte-macrophage colony-stimulating factor (GM-CSF) (0.1 nmol/L), interleukin 3 (IL-3) (0.1 nmol/L), and IL-5 (0.1 nmol/L). Every three days, cells were counted and fresh medium was added to a density of 0.5×10^6 cells/ mL. After three days of differentiation, only IL-3 and IL-5 were added to the cells. Neutrophil differentiation was induced upon addition of SCF (50 ng/mL), FLT-3 ligand (50 ng/mL), GM-CSF (0.1 nmol/L), IL-3 (0.1 nmol/L) and G-CSF (30 ng/mL). After 6 days of culture only G-CSF was added to the cells. $CD34^+$ cells used in transplantation studies were cultured for three days in IMDM containing the cytokines SCF (50 ng/mL), FLT-3 ligand (50 ng/mL) and thrombopoietin (TPO) (10 ng/mL). Pharmacological inhibitors were freshly added to the cells every 3 or 4 days. 10 μM SB203580 (Alexis Corporation, San Diego, CA) was used to inhibit p38MAPK activity during granulopoiesis. Cord blood samples were collected from healthy donors after informed consent was provided according to the Declaration of Helsinki. Protocols were approved by the local ethics committee of the University Medical Center in Utrecht.

Patients
Pediatric patients diagnosed with SCN in the Wilhelmina Children's Hospital Utrecht were included in the study (Figure 7A). Diagnosis of SCN has been confirmed by using criteria from the Severe Chronic Neutropenia International Registry (SCNIR). Ideally, only SCN patients resistant to G-CSF treatment and without known mutations to the G-CSF receptor would have been included. Due to the rarity of the syndrome, we also included a patient with a low response to G-CSF as well. This patient had a known ELA-2 mutation. Written informed consent was obtained from parents.

Viral transduction of $CD34^+$ cells
Bicistronic retroviral DNA constructs were utilized, expressing the gene of interest and an Internal Ribosomal Entry Site (IRES) followed by the gene encoding for either enhanced green fluorescent protein (eGFP) (LZRS-eGFP) or ΔNGFR. Retrovirus was produced by transfection of the Phoenix-ampho packaging cell line by calcium-phosphate co-precipitation. After 2 weeks of selection with puromycin, cells were grown in a minimal amount of medium for 24 hours. Viral supernatants were collected and filtered through a 0.2 μm filter. $CD34^+$ cells were transduced in 24-well dishes precoated with 1.25 $\mu g/cm^2$ recombinant human fibronectin fragment CH-296 (RetroNectin; Takara, Otsu, Japan) overnight at 4°C. Transduction was performed by addition of 0.5 mL viral supernatant to 0.5 mL cell suspension. Twenty-four hours after transduction, 0.7 mL medium was removed from the cells, and 0.5 mL fresh virus supernatant was added together with 0.5 mL medium.

Histochemical staining of hematopoietic cells
May-Grunwald Giemsa staining was used to analyze maturation. Cytospins were prepared from 50.000 differentiating granulocytes. After fixation in methanol for 3 minutes, cytospins were stained in a 50% Eosin Methylene Blue solution according to May-Grunwald (Sigma-Aldrich GmbH, Seelze, Germany) for 20 minutes, rinsed in water for 5 seconds, and stained with 10% Giemsa solution

(Merck kGaA, Darmstadt, Germany) for 15 minutes. Differentiated neutrophils were characterized as cells containing banded or segmented nuclei. A minimum of 100 cells per cytospin were counted in multiple randomly selected microscopy fields.

Immunohistochemical staining of hematopoietic cells
Neutrophil differentiation was also analyzed by intracellular staining of lactoferrin. Cells were first washed in phosphate-buffered saline (PBS) and resuspended in 100 µL 0.5% formaldehyde. After 15 minutes incubation at 37°C, 900 µL of ice-cold methanol was added to the cells. Cells were washed with PBS after 30 minutes of incubation on ice and resuspended in PBS/0.5% BSA. After 10 minutes incubation at room temperature, cells were washed and neutrophil progenitors were resuspended in phycoerythrin (PE)-conjugated lactoferrin antibody (Immunotech, Marseille, France) and incubated for another 25 minutes. Cells were again washed and lactoferrin positive cells were detected by FACS analysis (FACS Calibur, Beckton Dickinson, Alphen a/d Rijn, The Netherlands).

Measurement of apoptosis
Apoptotic and necrotic cells were measured by staining with Annexin V and propidium iodide (Alexis, Leiden, The Netherlands) according to the manufacturer's protocol.

Respiratory burst measurement
Production of H_2O_2 was measured as described by Mardiney *et al* (31). Briefly, neutrophils (2.5 x 10^6 cells/mL) were incubated with DHR123 for 15 minutes and stimulated with 1 µM fMLP for 30 minutes. Subsequently, cells were washed with ice-cold PBS and oxidation of DHR123 to fluorescent Rhodamine 123 was measured by FACS analysis (FACS Canto, Beckton Dickinson, Alphen a/d Rijn, The Netherlands).

Western blot analysis
Western blot analysis was performed using standard techniques. In brief, differentiating granulocytes were lysed in Laemmli buffer (0.12 M Tris HCl pH 6.8, 4% SDS, 20% glycerol, 0.05 µg/µL bromophenol blue, and 35 mM β-mercaptoethanol) and boiled for 5 minutes. Equal amounts of total lysate were analyzed by 12% SDS-polyacrylamide gel electrophoresis. Proteins were transferred to Immobilon-P and incubated with blocking buffer (Tris buffered saline/Tween-20) containing 5% low-fat milk for 1 hour before incubating with antibodies against C/EBPα (Santa Cruz Biotechnology, Santa Cruz, CA) JunB (Santa Cruz Biotechnology, Santa Cruz, CA), p38 (Cell Signaling Technology, Beverly, MA, USA), MMP-9 (Cell Signaling Technology, Beverly, MA, USA) and an antibody against β-actin (Santa Cruz Biotechnology) overnight at 4°C in the same buffer. Before incubation with an antibody against phosphorylated C/EBPα (Cell Signaling Technology, Beverly, MA, USA) or phosphorylated p38 (Cell Signaling Technology, Beverly, MA, USA) for 16 hours at 4°C, blots were incubated for 1 hour in blocking buffer containing 5% bovine serum albumin (BSA). Blots were subsequently incubated with peroxidase conjugated secondary antibodies for 1 hour. Enhanced chemical luminescence (ECL) was used as a detection method according to the manufacturer's protocol (Amersham Pharmacia, Amersham, UK).

Transplantation of human CD34$^+$ cells into β2-microglobulin$^{-/-}$ NOD/SCID mice
The β2-microglulin$^{-/-}$ nonobese diabetic/severe combined immune deficient (NOD/SCID) mice were bred and maintained under sterile conditions in micro-isolator cages and provided with autoclaved food and acidified water containing 111 mg/l Ciprofloxacin (Ciproxin®). Eight- to ten-week-old mice, sublethally irradiated with 300 cGy Röntgen were transplanted via tail vein injections with approximately 500.000 unsorted retrovirally transduced cord blood derived hematopoietic progenitors along with 1 x 10^6 irradiated (1500 cGy) CD34-depleted cord blood derived accessory cells. After 6 weeks, the mice were sacrificed and both tibiae and femora were flushed. eGFP positive bone marrow cells were sorted on a FACS Aria (Beckton Dickinson, Alphen a/d Rijn, The Netherlands) and cytospins prepared. May-Grunwald Giemsa staining was used to analyze lineage development. All animal procedures were performed with consent from the local ethics committee at Utrecht University.

Production of C/EBPα-GST fusion protein
A fusion protein of glutathione S-transferase (GST) and C/EBPα was generated and transformed into Escherichia coli BL21 cells. Expression of recombinant protein was induced by addition of 0.1 mM isopropylthiogalactoside for 3 hours. Cells were lysed utilizing a lysis buffer (50 mM Tris-HCl, pH 8, 2 mM $MgCl_2$, 0.2 mM Na_2S_2O, 10% glycerol, 20% sucrose), containing a freshly added protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), and sonication. Cell lysates were centrifuged at 4°C for 20 minutes after which the supernatant was incubated with glutathione-sepharose beads (GE Healthcare) for 30 minutes at 4°C. The beads were washed three times in lysis buffer to reduce aspecific binding and the amount of fusion protein bound to the beads was estimated using Coomassie-stained SDS gels.

Kinase assay
The GST fusion proteins bound to glutathione-agarose beads were resuspended in kinase buffer (20mM HEPES pH 7.5, 5 mM MgCl2, 1mM DTT, 2mM ATP), 200 ng active recombinant p38α MAPK (Cell Signaling Technology, Beverly, MA, USA) was added to the glutathione-agarose beads and the samples were incubated for 30 minutes at 37°C. After incubation, sample buffer (60mM Tris pH 6.8, 2% SDS, 10% glycerol, 2% β-mercaptoethanol and bromophenol blue) was added to the beads upon which the sample

was boiled for 5 minutes, and the proteins were separated utilizing SDS-PAGE.

Statistics

A sample t test for was performed to compare the differences in proliferation, differentiation, and annexin positive cells between the controls and transduced cells. The same assay was performed to compare cells cultured either in absence of presence of the pharmacological inhibitor SB203580 (Alexis Corporation, San Diego, CA). For mouse transplantation studies, an independent sample t test was performed to compare the differences in differentiation between cells transduced with either eGFP or MKK3. A p value of 0.05 or less was considered significant (*).

RESULTS

p38MAPK activity differentially regulates myeloid progenitor expansion during myelopoiesis

In order to determine whether p38MAPK activity plays a critical role in regulation of myelopoiesis, an ex-*vivo* differentiation system was utilized (32)(33). Human $CD34^+$ hematopoietic progenitor cells, isolated from umbilical cord blood, were cultured in the presence of either G-CSF or IL-3 and IL-5 to induce neutrophil and eosinophil differentiation respectively. Cells were cultured either in absence or presence of SB203580, a specific pharmacological inhibitor of p38α and p38β, and differences in expansion, survival and differentiation were analyzed. SB203580 significantly enhanced expansion during neutrophil differentiation (Figure 1A). To determine whether SB203580 mediated induction of expansion was due to decreased levels of apoptosis, the percentage of Annexin-V positive cells was analyzed. Inhibition of p38MAPK did not significantly affect the levels of annexin-V positive cells during neutrophil differentiation (Figure 1B). Interestingly, in contrast to neutrophil differentiation, inhibition of p38MAPK significantly reduced progenitor expansion during eosinophil development (Figure 1C), which was again not due to altered progenitor survival (Figure 1D). These results demonstrate that p38MAPK plays a differential role in regulating neutrophil and eosinophil progenitor expansion.

p38MAPK activity differentially regulates neutrophil and eosinophil differentiation

To determine whether p38MAPK also plays a role in regulating myeloid development, $CD34^+$ progenitor cells were differentiated towards neutrophils or eosinophils for 17 days either in the presence or absence of SB203580. After 17 days of culture, cytospins were prepared and the morphology of the differentiating granulocytes was analyzed as described in Materials and Methods (Figure 2B). Inhibition of p38MAPK activity resulted in a significant increase in both the percentage and absolute number of mature neutrophils with banded or segmented nuclei (Figure 2A, 2D). Western blot analysis was also performed to support the morphological observations and showed that pharmacological inhibition of p38MAPK increased the expression of MMP-9, a neutrophil specific granule protein (Figure 2C). Production of reactive oxygen species (ROS) production, one of the most important neutrophil effector functions, was also found to be increased in $CD34^+$ derived neutrophils that had been cultured in the presence of SB230580 (Figure S1). This demonstrates the increased functionality of these cells.

In contrast, inhibition of p38MAPK activity resulted in a dramatic reduction of eosinophil differentiation (Figures 2E-G). These results demonstrated that p38MAPK also plays an important but differential role in regulation of lineage development during granulopoiesis.

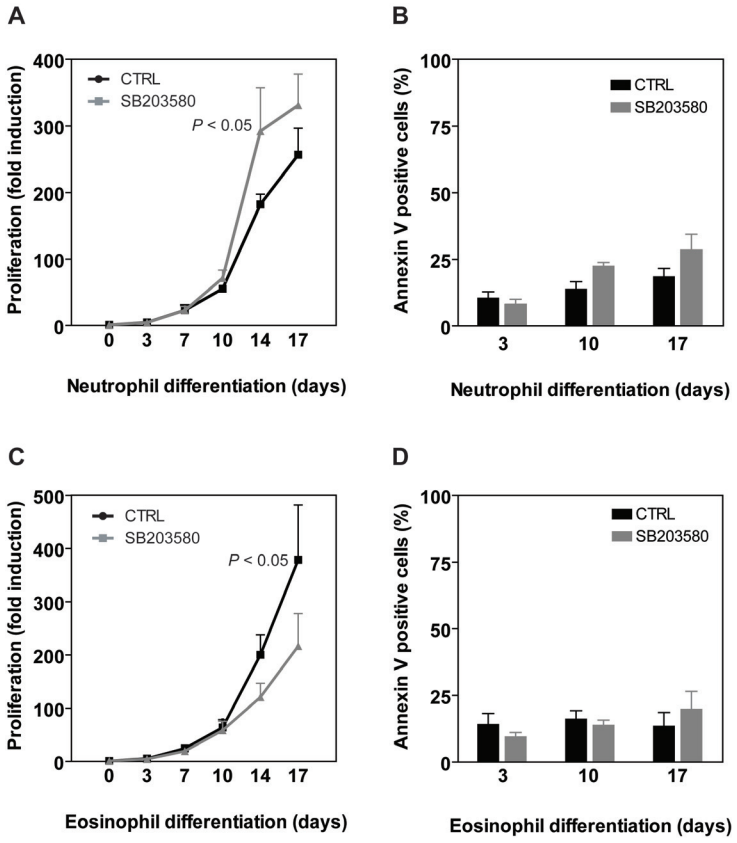


Figure 1. p38MAPK activity regulates progenitor expansion during myelopoiesis in a lineage specific manner. CD34$^+$ cells were cultured for 17 days either in presence of (A) G-CSF or (C) IL-5 and IL-3 to induce neutrophil differentiation or eosinophil differentiation, respectively. Cells were cultured in absence or presence of 10 μM SB203580. Expansion was determined by counting the trypane blue-negative cells. (B/D) During the 17-day culture period the percentage apoptotic cells was determined by Annexin V staining. Results are presented as means of 5 independent experiments. Error bars represent SEM.

MKK3 activity is detrimental for neutrophil development

To further validate the results obtained utilizing a pharmacological inhibitor of p38MAPK, a bicistronic retroviral DNA construct was utilized co-expressing eGFP and a constitutively active form of MKK3 (CA-MKK3), a kinase known to activate p38α, p38β and p38δ isoforms. Retrovirus was generated and used to transduce CD34$^+$ cells, which were cultured either in presence of G-CSF or IL-3 and IL-5 to induce neutrophil or eosinophil differentiation respectively. Three days after transduction, eGFP positive cells were sorted by FACS from the non-transduced cells. After 17 days of differentiation, cytospins were prepared and the morphology of the cells was analyzed by May-Grunwald Giemsa staining. Although eosinophil development was not affected by ectopic expression of CA-MKK3 (data not shown), neutrophil differentiation was dramatically inhibited (Figure 3A-C). Flow cytometric

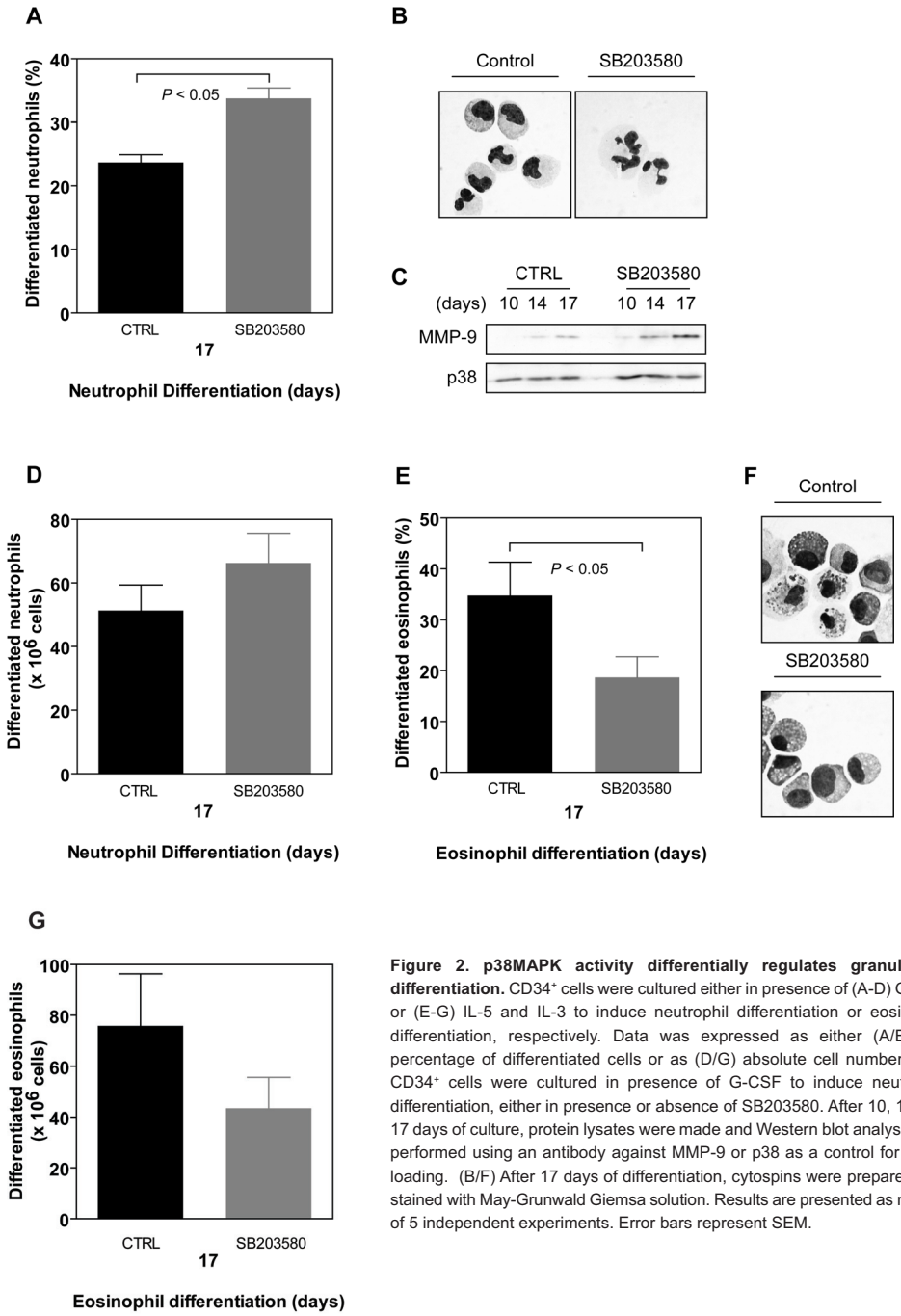


Figure 2. p38MAPK activity differentially regulates granulocyte differentiation. CD34$^+$ cells were cultured either in presence of (A-D) G-CSF or (E-G) IL-5 and IL-3 to induce neutrophil differentiation or eosinophil differentiation, respectively. Data was expressed as either (A/E) the percentage of differentiated cells or as (D/G) absolute cell numbers. (C) CD34$^+$ cells were cultured in presence of G-CSF to induce neutrophil differentiation, either in presence or absence of SB203580. After 10, 14 and 17 days of culture, protein lysates were made and Western blot analysis was performed using an antibody against MMP-9 or p38 as a control for equal loading. (B/F) After 17 days of differentiation, cytospins were prepared and stained with May-Grunwald Giemsa solution. Results are presented as means of 5 independent experiments. Error bars represent SEM.

analysis was also performed to support the morphological observations and showed that constitutive activation of MKK3 reduced the expression of lactoferrin, a neutrophil specific granule protein (Figure 3D). To determine whether the observed block in neutrophil differentiation was due to enhanced levels of apoptosis, the percentage of Annexin-V positive cells was analyzed. Ectopic expression of CA-MKK3 did not affect the levels of annexin-V positive cells during neutrophil differentiation (Figure 3E). These results demonstrate that enhanced MKK3 activity is detrimental for neutrophil maturation.

Activation of MKK3 regulates granulopoiesis *in vivo*

The results presented so far demonstrate that MKK3 and its downstream effector p38MAPK can modulate the expansion and differentiation of myeloid progenitors ex-*vivo*. To investigate whether the p38MAPK signalling pathway also plays a significant role in regulation of lineage development *in vivo*, sublethally irradiated β2-microglobulin$^{-/-}$ NOD/SCID mice were transplanted with CD34^{+} progenitor cells transduced with either CA-MKK3 or eGFP as a control. Six weeks after injection, eGFP-positive cells were sorted and cytospins prepared. The morphology of cells was analyzed by May-Grunwald Giemsa staining (Figure 4E). Transplantation of mice with human CD34^{+} cells ectopically expressing CA-MKK3 resulted in a significant inhibition of neutrophil differentiation and enhanced eosinophil development, whereas erythrocyte and monocyte differentiation was not significantly altered (Figures 4A-D). Taken together, these results confirm that MKK3 plays an important role in regulation of lineage choice decisions during granulopoiesis *in vivo*.

p38MAPK phosphorylates C/EBPα on serine 21 in hematopoietic progenitors

Although our data demonstrates that p38MAPK plays an important role in the regulation of myelopoiesis, the molecular mechanisms underlying these observations remain to be defined. One of the key transcriptional regulators involved in lineage choice decisions during myeloid differentiation is CCAATT/enhancer binding protein α (C/EBPα) (34)(35). It has recently been demonstrated that phosphorylation of C/EBPα on serine 21 inhibits the activity of C/EBPα and its ability to induce granulocyte differentiation (36). It was therefore of interest to investigate whether p38MAPK could also regulate C/EBPα phosphorylation in primary human hematopoietic progenitors.

To determine whether p38MAPK can indeed directly phosphorylate C/EBPα on serine residue 21, wild-type C/EBPα or C/EBPα serine 21 mutant GST-fusion proteins were incubated with active p38α MAPK. Addition of active p38α induced phosphorylation of WT C/EBPα on serine 21, whereas mutation of this residue completely abolished C/EBPα phosphorylation (Figure 5A). Inhibition of p38MAPK with SB203580 blocked C/EBPα serine 21 phosphorylation in neutrophil progenitor cells (Figure 5B). In addition, inhibition of p38MAPK also reduced the expression level of JunB, a transcriptional target of C/EBPα, suggesting that phosphorylation of C/EBPα indeed inhibits its transcriptional activity. Taken together, these results suggest that p38MAPK may regulate lineage choice decisions during myelopoiesis through modulating C/EBPα transcriptional activity.

p38MAPK regulates myelopoiesis by phosphorylation of C/EBPα

To investigate whether p38MAPK indeed regulates myelopoiesis through a C/EBPα-

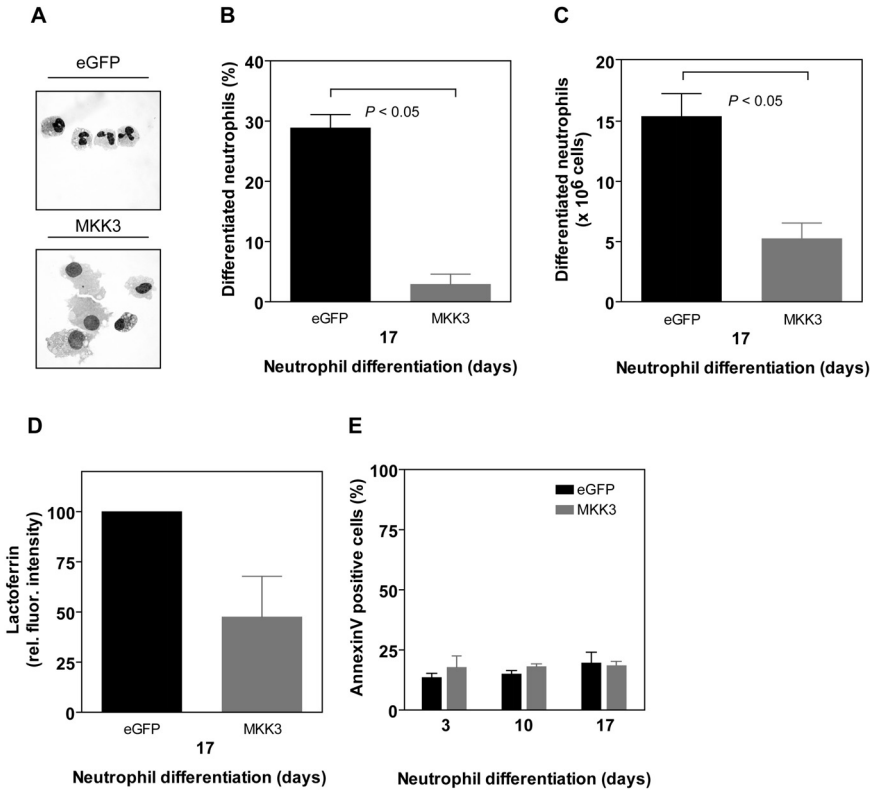


Figure 3. Ectopic expression of MKK3 inhibits neutrophil differentiation. CD34⁺ cells were retrovirally transduced with MKK3 or eGFP alone and cultured in presence of G-CSF to induce neutrophil differentiation. After 17 days of culture, transduced cells were separated from non-transduced cells by FACS, and cytospins were prepared. (A) Cytospins were stained with May-Grunwald Giemsa solution. (B) Data were expressed as the percentage of differentiated neutrophils or as (C) absolute cell numbers. (D) Lactoferrin expression was analyzed by FACS to determine neutrophil development. (E) During the 17-day culture period the percentage apoptotic cells was determined by Annexin V staining. Results are presented as means of 4 independent experiments. Error bars represent SEM.

dependent mechanism, bicistronic retroviral constructs were used co-expressing ΔNGFR and wildtype C/EBPα (C/EBPαWT) or a non-phosphorylatable C/EBPα mutant (C/EBPαS21A). Retrovirus was generated and used to infect CD34⁺ cells cultured in presence of G-CSF to induce neutrophil differentiation. After isolation of the transduced, ΔNGFR-positive cells utlizing MACS immunomagnetic cell separation, these cells were again retrovirally transduced resulting in ectopic expression of CA-MKK3. After 17 days of differentiation, eGFP and ΔNGFR double positive cells were sorted by FACS and cytospins were prepared and analyzed. Ectopic expression of CA-MKK3 resulted in low levels of differentiated neutrophils, which could not be enhanced by co-expression of wild type C/EBPα. In contrast, ectopic expression of the non-phosphorylatable C/EBPαS21A mutant was sufficient to abrogate the CA-MKK3 induced inhibition of neutrophil development (Figure 6). These data clearly demonstrate that MKK3/p38MAPK can inhibit neutrophil differentiation,

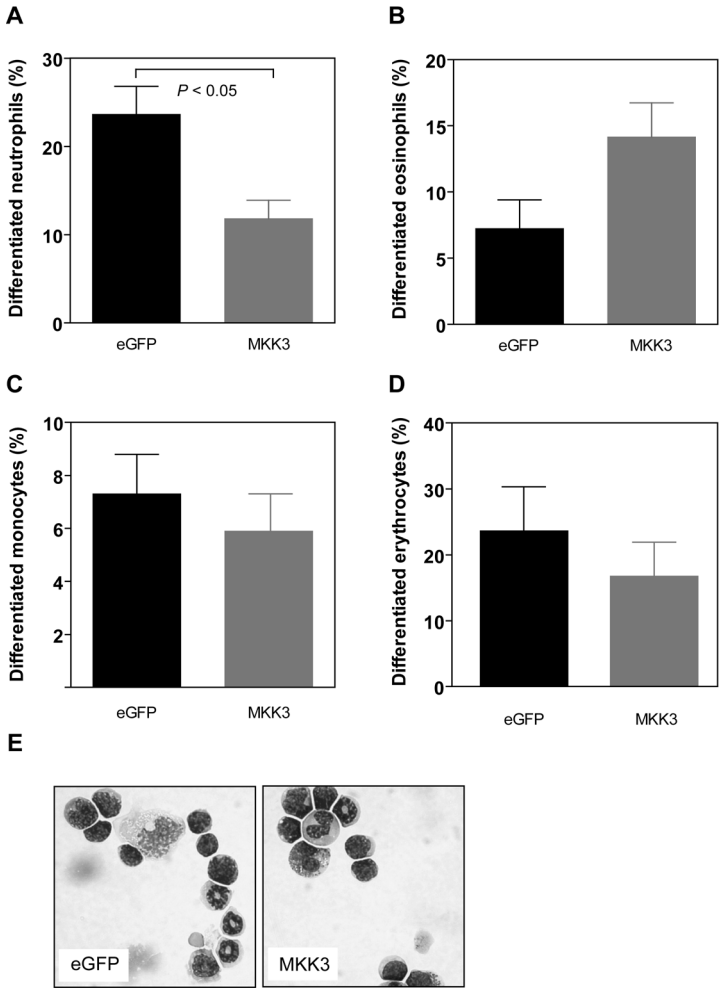


Figure 4. Modulation of p38MAPK activity in human CD34⁺ hematopoietic progenitors affects lineage development in β2-microglobulin$^{-/-}$ NOD/SCID mice. CD34⁺ cells cultured in the presence of SCF, Flt3-ligand and TPO were transduced with MKK3 or eGFP alone. After 3 days of culture, cells were injected into β2-microglobulin$^{-/-}$ NOD/SCID mice. Six weeks after injection, mice were sacrificed, eGFP-positive human cells were sorted, and cytospins were prepared. **(E)** Cytospins were stained with May-Grunwald Giemsa solution. Lineage development was depicted as the percentage of **(A)** neutrophils, **(B)** eosinophils, **(C)** monocytes and **(D)** erythrocytes,. Results are presented as means of 6 independent experiments. Error bars represent SEM.

at least in part, through regulation of C/EBPα activity.

Inhibition of p38MAPK restores neutrophil development of SCN hematopoietic progenitors

Having established the beneficial effects of p38MAPK inhibition on neutrophil development, we were interested to test the potential of p38 inhibition as a therapeutic strategy in

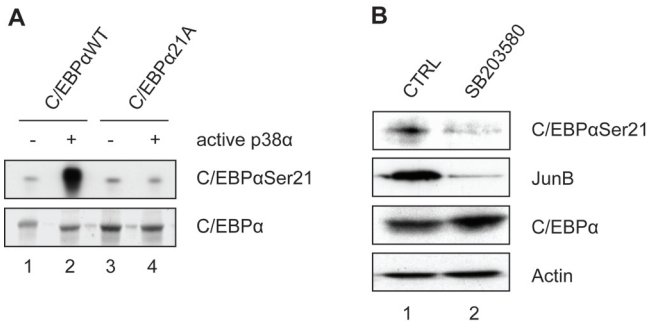


Figure 5. p38MAPK phosphorylates C/EBPα on serine 21. (A) GST fusion proteins expressing either wild type C/EBPα (lane 1 and 2) or C/EBPα, in which serine 21 was mutated (lane 3 and 4) were incubated with or without active p38α. Western blot analysis was performed using an antibody against phosphorylated C/EBPα. Input protein was stained demonstrating equal loading. (B) $CD34^+$ cells were cultured in presence of G-CSF to induce neutrophil differentiation in absence (lane 1) or presence of 10 μM SB203580 (lane 2). After 6 days of culture, protein lysates were made. Western blot analysis was performed using an antibody against phosphorylated C/EBPα, JunB, C/EBPα or β-actin as a control for equal loading. Similar results were obtained in 4 independent experiments.

severe congenital neutropenia (SCN). SCN is a rare bone marrow failure syndrome characterized by severe chronic neutropenia due to an arrest of granulocytic differentiation (37). Although most SCN patients respond well to G-CSF administration with increased neutrophil numbers, about 10% of all SCN patients are unresponsive to G-CSF and have no alternative treatment options (38). To investigate whether inhibition of p38MAPK can result in improved neutrophil development of SCN progenitors, $CD34^+$ hematopoietic progenitor cells isolated from bone marrow of SCN patients (n=2) unresponsive to G-CSF, were cultured in the presence of G-CSF to induce neutrophil differentiation. Cells were also cultured either in absence or presence of SB203580 and differences in proliferation, survival and differentiation were analyzed. After 17 days of differentiation, cytospins were prepared and the morphology of the differentiating granulocytes was analyzed by May-Grunwald Giemsa staining. Treatment of $CD34^+$ progenitor cells from SCN patients with SB203580 resulted in greatly increased percentages of mature neutrophils with banded or segmented nuclei (Figure 7B, 7C). Taken together, these data demonstrate that inhibition of p38MAPK can overcome the block in neutrophil differentiation observed in SCN and might provide a novel therapeutic option for SCN patients who are unresponsive to G-CSF treatment.

DISCUSSION

Although p38MAPK has been implicated in playing an important role in a plethora of cellular processes, a role in the regulation of myelopoiesis remains relatively unexplored. In this study we have investigated this issue utilizing a human ex-*vivo* granulocyte differentiation system as well as an *in vivo* mouse transplantation model. Our results show that p38MAPK plays an important role in expansion and differentiation during granulopoiesis, which was found to involve regulation of C/EBPα phosphorylation. Furthermore, modulation of this pathway was sufficient to improve neutrophil development in SCN patients.

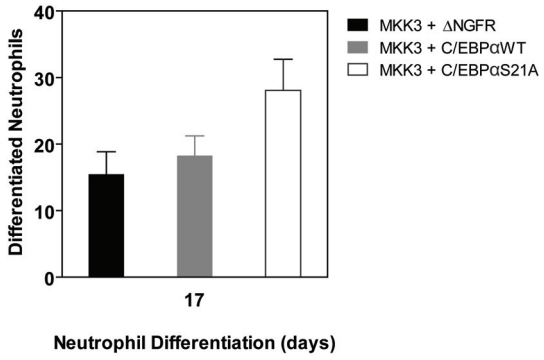


Figure 6. p38MAPK regulates myelopoiesis through C/EBPα serine 21 phosphorylation. CD34⁺ cells were retrovirally transduced with C/EBPαWT, C/EBPα21A or ΔNGFR alone after which transduced cells were separated from the non transduced cells utilizing MACS immunomagnetic cell separation. ΔNGFR-positive cells were retrovirally transduced to ectopically express MKK3 and subsequently differentiated towards neutrophils. After 17 days of differentiation, eGFP and ΔNGFR double positive cells were sorted by FACS and cytospins were prepared. Data was expressed as the percentage of differentiated neutrophils. Results are presented as means of 4 independent experiments. Error bars represent SEM.

p38MAPK has been demonstrated to play an important role in the regulation of proliferation of various cell types. For example, proliferation of human CD34⁺ derived erythroid progenitors and several erythroid cell lines is positively regulated by p38MAPK (39-41), whereas proliferation of myoblasts, fibroblasts, and chondrocytes is inhibited (42-45). Our data demonstrate that p38MAPK activity inhibits proliferation during neutrophil differentiation, but conversely enhances proliferation during eosinophil development. Although there is no consensus as to how p38MAPK regulates proliferation, multiple, putative molecular mechanisms have been proposed. Perdiguera *et al* demonstrated that c-Jun transcriptional activity was enhanced in p38α-deficient myoblasts resulting in increased cyclin D1 expression (44). Furthermore, a more recent study showed that p38MAPK can regulate the expression of genes required for mitosis in cardiomyocytes, including cdc2, cdc25B, cyclin D and cyclin B (24). In addition, we showed that inhibition of p38MAPK resulted in reduced expression levels of JunB (Figure 5B), which is known for both its role as an inhibitor of proliferation and as a tumor suppressor. Mice lacking JunB expression in hematopoietic cells, develop increased granulocyte numbers, which may represent loss of the anti-proliferative effect of JunB (46).

The differential effect of p38MAPK on proliferation of neutrophil and eosinophil progenitors might be explained by the observation that different p38MAPK isoforms are not redundant and can actually have opposing functions. For example, AP-1 dependent transcription of the vitamin D receptor and cellular proliferation are induced by p38β, whereas both p38δ and p38γ inhibit these processes (47). Similarly, p38α can induce apoptosis in Hela cells, whereas p38β induces survival in the same cells (48). Furthermore, it has been demonstrated that transcription of certain genes can be regulated by specific p38 isoforms. During myogenesis, expression of the E2F transcription factor 2 is induced by activation of p38α, p38β as well as p38γ, whereas cyclin D3 is uniquely induced by p38β (49-51). Together, this indicates that the expression pattern of p38MAPK isoforms may determine whether activation of p38MAPK results in either enhanced or decreased proliferation. Distinct stage-specific expression patterns of the four p38MAPK isoforms have been described for primary erythroid progenitors during erythroid differentiation (52). In addition, studies performed in inflammatory cell lineages such as monocytes, macrophages, neutrophils and T lymphocytes have shown differential expression patterns for all isoforms (53). Although the expression profile of the p38 isoforms in granulocyte progenitors and during hematopoietic differentiation has not thus

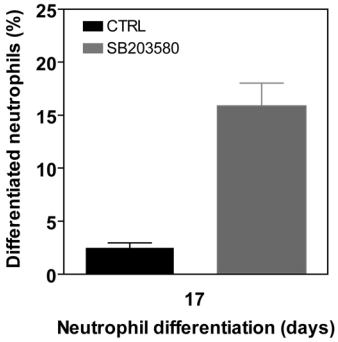
A

Patient characteristics

Patient no.	Age (y)	Sex	Leuco. (10^9/L)	Gran. (10^9/L)	Respons to G-CSF therapy	Mutations
1	4	M	9.2	1.48	low	Ela-2
2	7	M	3.3	0.15	none	none

Figure 7. Inhibition of p38MAPK restores neutrophil development of SCN hematopoietic progenitors. (A) Characteristics of studied SCN patients (B) $CD34^+$ cells, isolated from bone marrow of SCN patients, were cultured in presence of G-CSF to induce neutrophil differentiation. Data was expressed as the percentage of differentiated neutrophils. (C) After 17 days of differentiation, cytospins were prepared and stained with May-Grunwald Giemsa solution. Results are presented as means of 2 independent experiments. Error bars represent SEM.

B



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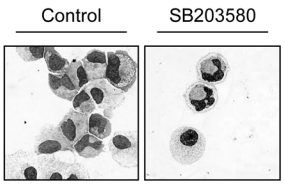

far been investigated, it is tempting to speculate that the combination of p38MAPK isoforms expressed in neutrophil progenitors is different compared to eosinophil precursors.

In addition, p38MAPK has been implicated in regulation of the development of various cell types, including myocytes, chondrocytes, endothelial cells, adipocytes and keratinocytes. It has been demonstrated that p38MAPK activity is essential for differentiation of adipocytes, myocytes and bronchioalveolar stem cells and progenitors in the lungs, whereas differentiation of endothelial cells and chondrocytes is inhibited upon p38MAPK activation (22)(25)(44)(45)(51)(54-56). Moreover, p38MAPK activity has been shown to constitute a switch in mouse embryonic stem cell commitment from neurogenesis to cardiogenesis (54). Our results show that p38MAPK activity is detrimental for neutrophil development, but required for eosinophil differentiation, suggesting a “switch-like” function in regulating hematopoietic lineage choice decisions.

To delineate the molecular mechanism underlying p38MAPK mediated regulation of myelopoiesis, we focussed on the transcription factor C/EBPα. C/EBPα is a leucine zipper transcription factor that plays a critical role in myelopoiesis. Expression of C/EBPα is detectable in early myeloid precursors and is upregulated upon commitment to granulocytes (34)(57). Consistent with this expression pattern, mice deficient for C/EBPα lack mature neutrophils and eosinophils, and accumulate immature myeloblasts in the bone marrow (35). Conversely, ectopic expression of C/EBPα in precursor cell lines triggers neutrophil differentiation (58)(59). Several recent studies demonstrated that, in addition to the importance of correct regulation of the expression levels, the activity of C/EBPα can be

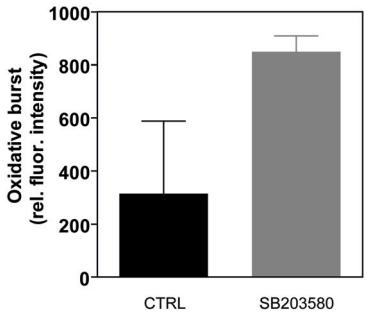

Figure S1. ROS production is increased in SB203580 treated neutrophil progenitors. $CD34^+$ cells were cultured in presence of G-CSF to induce neutrophil differentiation. Cells were cultured either in absence or presence of SB203580. After 14 days of differentiation, neutrophil progenitors were incubated with DHR123, stimulated with 1 μM fMLP for 30 minutes and subsequently, ROS production was measured by FACS analysis. Results are presented as means of two independent experiments. Error bars represent SEM.

regulated at the level of post-translational modification. We have previously demonstrated that GSK-3 mediated phosphorylation C/EBPα on threonine residues 222 and 226 can modulate lineage choice decisions during hematopoiesis (60). Furthermore, Ross and colleagues have found that C/EBPα can be directly phosphorylated by ERK1/2 on serine residue 21, which regulates the activity of C/EBPα and its ability to induce neutrophil differentiation (36). Specifically, only the dephosphorylated form of C/EBPα was able to induce neutrophil differentiation. This was further illustrated by the observation that ectopic expression of the phosphomimetic C/EBPα mutant inhibited neutrophil differentiation. Although the precise effect of C/EBPα phosphorylation of serine 21 is not completely understood, it has been suggested that phosphorylation induces a conformational change in C/EBPα thereby increasing the distance between the transactivation domains of the two, dimerized C/EBP molecules (36). Alternatively, phosphorylation may also regulate the association of C/EBPα with specific binding partners, such as other transcription factors, coactivators or corepressors.

Using a serine 21 phosphorylation deficient mutant of C/EBPα, we were able to demonstrate that MKK3/p38MAPK can inhibit neutrophil differentiation, at least in part, through phosphorylation of C/EBPα on serine 21 (Figure 6). Furthermore, treatment of progenitors with SB203580 resulted in reduced JunB expression (Figure 5B). These data support the idea that phosphorylation of C/EBPα leads to inactivation of its transcriptional activity. However, Qiao *et al* recently demonstrated that in liver cells phosphorylation of C/EBPα on serine 21 by p38MAPK enhanced C/EBPα transactivation and thereby increased expression of genes involved in hepatic gluconeogenesis (61). It is thus likely that this phosphorylation event might regulate C/EBPα activity in a tissue-restricted manner, for example, through interaction with specific binding partners expressed in certain lineages.

p38MAPK was originally identified as a stress-signaling kinase, and various studies have implicated p38MAPK as an important mediator of apoptosis in various cell types including cardiac, neuronal and immune cells (62-65). A recent study by Ito *et al* demonstrates that activation of p38MAPK in response to increased levels of reactive oxygen species limits the lifespan of hematopoietic stem cells *in vivo*, emphasizing the important role of p38MAPK in regulation of hematopoiesis (4). However, our data showed that survival of hematopoietic progenitors was not altered upon ectopic expression of MKK3, suggesting a decreased sensitivity to activation of p38MAPK of the more differentiated progenitor populations

compared to HSCs. Moreover, recently it was shown that p38MAPK is constitutively activated in the bone marrow of patients with myelodysplastic syndromes (MDS). Inhibition of p38MAPK activity, decreased apoptosis and stimulated colony formation of primary MDS progenitors, suggesting that aberrant activation of the p38MAPK pathway is associated with differentiation defects (5)(6). Ineffective neutrophil differentiation is also believed to be the major cause of chronic neutropenia observed in patients with SCN. Despite successful supportive treatment using recombinant human G-CSF, 10% of all SCN patients do not respond to G-CSF and have no alternative treatment options other than allogeneic bone marrow transplantation (38). Although new therapeutic approaches are required for treatment of these unresponsive variants of severe congenital neutropenia, the genetic and epigenetic changes that contribute to SCN are still largely unknown. Based on our data that inhibition of p38MAPK activity can positively modulate neutrophil development, it was of interest to test whether SB203580 could restore neutrophil differentiation in SCN patients. Treatment of $CD34^+$ progenitor cells isolated from G-CSF unresponsive SCN patients with SB203580 indeed resulted in enhanced neutrophil production. These findings confirm the idea that aberrant activation of the p38MAPK pathway is involved in the development of specific hematopoietic malignancies. Taken together, these data have identified an important, novel mechanism regulating neutrophil production and have shown that pharmacological modulation of the p38MAPK could play an important role in novel therapeutic options for bone marrow failure or hematological malignancies.

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Chapter 4

Tight control of MEK-ERK activation is essential in regulating proliferation, survival and cytokine production of CD34$^+$ derived neutrophil progenitors

Christian R Geest[1], Miranda Buitenhuis[1], Marian JA Groot-Koerkamp[2], Frank CP Holstege[2], Edo Vellenga[3], and Paul J Coffer[1,4]

[1] Molecular Immunology Lab, Department of Immunology, University Medical Center, Utrecht, The Netherlands, [2] Genomics Laboratory, Department of Physiological Chemistry, University Medical Center, Utrecht, The Netherlands, [3] Department of Hematology, University Medical Center, Groningen, The Netherlands, [4] Department of Pediatric Immunology, University Medical Center, Utrecht, The Netherlands

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ABSTRACT

A plethora of extracellular stimuli regulate growth, survival and differentiation responses through activation of the MEK-ERK MAPK signaling module. Using $CD34^+$ hematopoietic progenitor cells, we describe a novel role for the MEK-ERK signalling module in the regulation of proliferation, survival and cytokine production during neutrophil differentiation. Addition of the specific MEK1/2 inhibitor U0126 resulted in decreased proliferation of neutrophil progenitors. Conversely, transient activation of a conditionally active MEK1 mutant resulted in expansion of progenitor cells which thereafter differentiated normally into mature neutrophils. In contrast, chronic MEK1 activation was found to induce cell death of $CD34^+$ neutrophil progenitors. Microarray analysis of $CD34^+$ progenitor cells revealed that activation of MEK1 resulted in changes in expression of a variety of cell-cycle modulating genes. Furthermore, conditional activation of MEK1 resulted in a dramatic increase in the expression of mRNA transcripts encoding a large number of hematopoietic cytokines, chemokines and growth factors. These findings identify a novel role for MEK-ERK signalling in regulating the balance between proliferation and apoptosis during neutrophil differentiation, and suggest the need for tight control of MEK-ERK activation to prevent the development of bone marrow failure.

INTRODUCTION

Cytokines play an important role in the maintainance of hematopoietic homeostasis by regulating hematopoietic cell growth, survival and differentiation through the activation of a number of intracellular signaling pathways that ultimately results in the activation of specific transcription factors and subsequent modulation of target gene expression (1). The Mitogen-activated protein kinase (MAPK) cascade is one of the key signaling pathways that couples the signals from cell surface cytokine receptors to trigger downstream pathways (2)(3). Three major groups of MAPKs have been characterized in mammals, including extracellular signal-regulated protein kinases (ERKs), c-Jun NH2-terminal kinases (JNKs), and p38MAPKs (4)(5). Activation of MAPK is regulated by an evolutionary conserved kinase cascade and MAPKKKs are serine/threonine kinases capable of phosphorylating and activating MAPKKs, which in turn stimulate MAPK activity through dual phosphorylation on threonine and tyrosine residues within a tripeptide motif (Thr-X-Tyr). Upon activation, MAPK itself can phosphorylate specific target substrates on serine or threonine residues (6)(7).

The ERK MAP kinase is the most extensively studied MAPK family member that is best known for its role in the regulation of proliferation and survival of a plethora of cell types (9-10). To date, two ERK kinases have been described, p44 ERK1 and p42 ERK2, that share 83% homology (11). They are ubiquitously expressed and are directly activated by the MAPKKs MEK1 and -2, whose only known substrates are ERK1 and ERK2 (4). Activation of ERKs occurs as a result of cytokine or growth factor stimulation, but they are also activated in response to hydrogen peroxide, UV light, and ionizing radiation (12-15). Upon activation, ERK1 and -2 translocate to the nucleus, where they phosphorylate and regulate a variety of nuclear targets, such as STAT3, Elk-1, c-fos, c-myc and Ets transcription factors. Although the majority of ERK substrates are nuclear proteins, others are found in the cytoplasm and organelles. The primary cytoplasmic target of the ERK kinases is p90 ribosomal S6 kinase, but a wide array of other targets are known to exist, including various membrane proteins, such as CD120a, Syk, and calnexin (3)(16)(17).

Analysis of ERK MAPK-deficient mice has revealed that ERK MAPK signalling plays an important role in the regulation of multiple cellular processes. ERK1(-/-) mice are viable, fertile, and of normal size, indicating that ERK2 may compensate for its loss (18). However, although ERK1 apparently is dispensable during embryonic stages, ERK1(-/-) mice do exhibit impaired thymocyte development. In contrast, deletion of ERK2 was found to be embryonic lethal due to defects in throphoblast formation, mesoderm differentiation and placental function, showing distinct biological functions for ERK1 and ERK2 (19-21).

Evidence has accumulated showing that ERK signalling plays a fundamental role in regulating cellular proliferation at the G1/S transition (22). ERK1/2 activation is required for cyclin D1 expression and sustained activity of ERK is also required for the down-regulation of many anti-proliferative genes throughout the G1 phase of the cell cycle (23). In addition, the ERK MAPK pathway has profound effects on the regulation of apoptosis by the post-translational phosphorylation of apoptotic regulatory molecules including Bad, Bim, Mcl-1, caspase 8 (9).

Although the ability of the ERK MAPK signaling module to promote cellular proliferation and survival has been well established, emerging evidence now suggests that it is also important

for the differentiation of a variety of cell types. ERK MAPK has been demonstrated to play a key role in the development of neuronal cells (24), myoblasts (25), and cells in the visual cortex (26). In the hematopoietic system, ERK MAPK signalling has been shown to be essential for transition of double-negative thymocytes to double-positive stages (27), as well as for megakaryocyte and erythrocyte differentiation (28)(29). In addition, a recent study indicates that ERK signalling may also play a role in lineage conversion of murine common lymphoid progenitors towards the myeloid lineage (30).
Since the ERK MAPK signaling pathway plays an important role in the regulation of various cellular processes, it could be hypothesized that aberrant activation of this pathway may also cause hematopoietic disease in humans. The ERK MAPK pathway has been reported to be activated in over 50% of acute myeloid leukemias and acute lymphocytic leukemias and is also frequently activated in other cancer types (31)(32).
In this study, we have investigated the role of the MEK-ERK MAPK signalling pathway in $CD34^+$ hematopoietic progenitor cell function, focussing on neutrophil development, utilizing a human ex-*vivo* differentiation system. MEK-ERK activity was found to be an important regulator of both hematopoietic progenitor expansion and survival. Subsequent microarray analysis of $CD34^+$ cells revealed that activation of MEK1 resulted in changes in expression of a variety of cytokine, chemokine and cell-cycle genes, clarifying these observations. These findings identify a novel role for MEK-ERK signalling in regulating the balance between proliferation and apoptosis during neutrophil development, and suggest the need for tight control of ERK activation as aberrant activation of this pathway could lead to the development of bone marrow failure.

MATERIALS AND METHODS

Isolation and culture of human $CD34^+$ cells
Mononuclear cells were isolated from umbilical cord blood by density centrifugation over a ficoll-paque solution (density 1.077 g/mL). MACS immunomagnetic cell separation (Miltenyi Biotech, Auburn, CA) was used to isolate $CD34^+$ cells. $CD34^+$ cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco, Paisley, UK) supplemented with 9% fetal calf serum (FCS) (Hyclone, Logan, UT), 50 µM β-mercaptoethanol, 10 U/mL penicillin, 10 µg/mL streptomycin, and 2mM glutamine. Cells were differentiated towards neutrophils upon addition of stem cell factor (SCF) (50 ng/mL), FLT-3 ligand (50 ng/mL), granulocyte-macrophage colony-stimulating factor (GM-CSF) (0.1 nmol/L), interleukin 3 (IL-3) (0.1 nmol/L), and G-CSF (30 ng/mL). After 6 days of culture, only G-CSF was added to the cells. Erythrocytes were generated as described by Giarratana *et al* (33). Briefly, $CD34^+$ were cultured in IMDM supplemented with 1% BSA, 120 µg/ml iron-saturated human transferrin, 900 ng/ml ferrous sulfate, 90 ng/mL ferric nitrate and 10 µg/ml insulin (Sigma-Aldrich GmbH, Seelze, Germany). Erythrocyte differentiation was induced upon addition of 100 ng/mL SCF, 10^{-6} M hydrocortisone (Sigma-Aldrich GmbH, Seelze, Germany), 5 ng/mL IL-3 and 3 IU/mL erythropoietin (EPO) (Eprex, Janssen-Cilag, Tilburg, The Netherlands). After 8 days of culture, erythroblasts were co-cultured with MS-5 stromal cells in presence of EPO for 3 days. Finally, cells were cultured on MS-5 cells in absence of cytokines until day 18. 5 µM U0126 (Biomol International LP, Hamburg, Germany) was added to the cells every 3 or 4 days to inhibit MEK1/2 activity. Cord blood was collected after informed consent was provided according to the Declaration of Helsinki. Protocols were approved by the ethics committee of the University Medical Center in Utrecht.

Colony-forming unit (CFU) assay
500 freshly isolated $CD34^+$ cells or retrovirally transduced cells were plated in IMDM supplemented with 35.3% FCS, 44.4% Methocult medium (StemCell Technologies, Vancouver, Canada), β-mercaptoethanol (11.1 µmol/L), penicillin (2.2 units/mL), streptomycin (2.2 µg/mL), and glutamine (0.44 mmol/L). CFU assays were performed in the presence of SCF (50 ng/mL), FLT-3L (50 ng/mL), GM-CSF (0.1 nmol/L), IL-3 (0.1 nmol/L), and G-CSF (0.2 nmol/L). Colonies were scored after 12 days of culture.

Viral transduction of $CD34^+$ cells
Bicistronic retroviral constructs were utilized co-expressing a 4-hydroxytamoxifen (4-OHT) inducible active MEK1 mutant (MEK1:ER*) and enhanced green fluorescent protein (eGFP). Retrovirus was produced by transfection of the Phoenix-ampho

packaging cell line by calcium-phosphate co-precipitation. After 2 weeks of selection with puromycin, cells were grown in a minimal amount of medium for 24 hours. Viral supernatants were collected and filtered through a 0.2 µm filter. $CD34^+$ cells were transduced in 24-well dishes precoated with 1.25 µg/cm^2 recombinant human fibronectin fragment CH-296 (RetroNectin; Takara, Otsu, Japan) overnight at 4°C. Transduction was performed by addition of 0.5 mL viral supernatant to 0.5 mL cell suspension. Twenty-four hours after transduction, 0.7 mL medium was removed from the cells, and 0.5 mL fresh virus supernatant was added together with 0.5 mL medium.

Histochemical staining of hematopoietic cells
May-Grunwald Giemsa staining was used to analyze maturation. Cytospins were prepared from 50.000 differentiating granulocytes. After fixation in methanol for 3 minutes, cytospins were stained in a 50% Eosin Methylene Blue solution according to May-Grunwald (Sigma-Aldrich GmbH, Seelze, Germany) for 20 minutes, rinsed in water for 5 seconds, and stained with 10% Giemsa solution (Merck kGaA, Darmstadt, Germany) for 15 minutes. Differentiated neutrophils were characterized as cells containing banded or segmented nuclei. A minimum of 100 cells per cytospin were counted in multiple randomly selected microscopy fields.

^{3}H-thymidine incorporation assays
80.000 $CD34^+$ cells were incubated with 1 µCi/mL ^{3}H-thymidine for 72 hours at day 8. The amount of ^{3}H-thymidine incorporated over a 3-day period was measured, and data were depicted as percentage of ^{3}H-thymidine incorporation compared with control cells.

Single-cell proliferation assay
$CD34^+$ progenitor cells, ectopically expressing MEK1:ER* were seeded in Terasaki plates (Nunc, Kamstrup, Denmark) at a density of 1 cell per well in 20 µL of IMDM containing SCF, FLT-3L, GM-CSF, IL-3, and G-CSF in presence or absence of 20 nM 4-OHT. The wells were scored for colonies after 12 days of incubation.

Lactoferrin staining of hematopoietic cells
Neutrophil differentiation was analyzed by intracellular staining of lactoferrin. Cells were fixed in 100 µL 0.5% formaldehyde for 15 minutes at 37°C, after which the cells were permeabilised in 900 µL of ice-cold methanol for 30 minutes on ice. Cells were subsequently washed with PBS, resuspended in phycoerythrin (PE)-conjugated lactoferrin antibody (Immunotech, Marseille, France) and incubated for 25 minutes. Cells were again washed and FACS analysis was performed (FACS Calibur, Beckton Dickinson, Alphen a/d Rijn, The Netherlands).

Measurement of apoptosis
Apoptotic and necrotic cells were measured by staining with Annexin V and propidium iodide (Alexis, Leiden, The Netherlands) according to the manufacturer's protocol.

Multiplex immunoassay
Neutrophil progenitors, ectopically expressing MEK:ER*, were cultured either in absence or presence of 4-OHT for 12 hours before collecting supernatants. The IL-8 (Sanquin, Amsterdam, The Netherlands), CCL-2 (R&D Systems, Abingdon, United Kingdom) and CCL-3 (R&D Systems) capture antibodies were covalently coupled to carboxylated polystyrene microspheres (Luminex Corporation, Austin, Tx) (34)(35). To block any residual of interfering antibodies, the samples were diluted with 10% v/v normal rat and mouse serum (1:1 ratio; Rockland, Gilbertsville, P). Samples were run undiluted and diluted 1:50 in high-performance ELISA buffer (Sanquin, Amsterdam, The Netherlands). Samples were measured and blank values were subtracted from all readings (34)(35). Measurements and data analysis were performed using the Bio-Plex system in combination with the Bio-Plex Manager software V.4.0 using five parametric curve fitting (Bio-Rad Laboratories, Hercules, Ca).

Western blot analysis
Cells were lysed in Laemmli buffer (0.12 M Tris HCl pH 6.8, 4% SDS, 20% glycerol, 0.05 µg/µL bromophenol blue, and 35 mM β-mercaptoethanol) and boiled for 5 minutes. Equal amounts of lysate were analyzed by SDS-polyacrylamide gel electrophoresis. Proteins were transferred to Immobilon-P and incubated with blocking buffer (Tris buffered saline/Tween-20) containing 5% low-fat milk for 1 hour before incubating with antibodies against Bcl-xL, p21Cip1, β-actin (all from Santa Cruz Biotechnology) or ERK1/2 (Cell Signaling Technology, Beverly, MA) overnight at 4°C. Before incubation with antibodies against phosphorylated ERK1/2 and phosphorylated p38 (both from Cell Signaling Technology, Beverly, MA), blots were incubated in blocking buffer containing 5% BSA. Blots were subsequently incubated with peroxidase conjugated secondary antibodies for 1 hour. Enhanced chemical luminescence was used as a detection method (Amersham Pharmacia, Amersham, UK).

RNA isolation
Neutrophil progenitors, ectopically expressing MEK1:ER*, were cultured either in absence or presence of 4-OHT for 12 hours before lysing the cells in 1 mL Trizol (Invitrogen, Breda, The Netherlands). Samples were subsequently incubated for 3 minutes at room temperature, 0.2 mL chloroform was added, vortexed and incubated for 3 minutes at room temperature, followed by 15

minutes of centrifugation at 8000 rpm at 4°C. Subsequently, 0.5 mL isopropanol was added to the aqueous phase and incubated for 30 minutes at -20°C. Samples were centrifuged at 14000 rpm for 10 minutes at 4°C. The pellet was washed with 70% ethanol and dissolved in water. DNAse treatment and purification was performed using Qiagen's RNeasy kit (Qiagen Inc., Valencia, CA).

Microarray analysis

500 ng of cRNA coupled to Cy3 and Cy5 fluorophores (Amersham) was hybridized onto Codelink activated slides (GE Healthcare) containing the Operon Human Genome Oligo Set V2. Hybridized slides were scanned on an Agilent scanner (G2565AA) at 100% laser power, 100% PMT. After data extraction using Imagene 7.5 (BioDiscovery), printtip Loess normalization was performed. Data was analysed using ANOVA (R2.2.1/MAANOVA version 0.98-3) (http://www.rproject.org/). In a fixed effect analysis, sample, array and dye effects were modelled. P-values were determined by a permutation F2-test, in which residuals were shuffled 5000 times globally. Genes with $p < 0.05$ after family wise error correction were considered significantly changed. Visualisation and cluster-analysis was done using GeneSpring 7.2 (Agilent).

Statistics

An independent sample t test for was performed to compare the differences in proliferation, differentiation, and colony forming capacity. A p value of 0.05 or less was considered significant (*).

RESULTS

MEK-ERK signalling is required for expansion of CD34$^+$-derived neutrophil progenitors

In order to determine whether the MEK-ERK signalling pathway plays a critical role in regulation of neutrophil development, an ex-*vivo* differentiation system was utilized. Human CD34$^+$ hematopoietic progenitor cells, isolated from umbilical cord blood, were cultured in the presence of G-CSF to induce neutrophil differentiation (36). Cells were cultured either in absence or presence of U0126, a specific pharmacological inhibitor of MEK1/2, and differences in expansion and survival were analyzed. The specificity of U0126 was confirmed by its ability to inhibit the phosphorylation of ERK1/2 in CD34$^+$ cells, but not p38MAPK phosphorylation (Figure 1A). U0126 significantly inhibited progenitor expansion during neutrophil differentiation as determined by both cell counting as well as by performing ^{3}H-thymidine incorporation assays. (Figure 1B, 1C). To determine whether U0126 mediated inhibition of expansion was due to increased levels of apoptosis, the percentage of Annexin-V positive cells was analyzed. Inhibition of ERK MAPK activity did not significantly alter the levels of annexin-V positive cells during the first two weeks, however an increase in apoptosis was observed after 14 days of neutrophil differentiation (Figure 1D).

To address the question as to whether MEK-ERK activity also plays similar role in regulating the expansion of other non-myeloid lineages, CD34$^+$ progenitors were differentiated towards erythrocytes for 18 days either in the presence or absence of U0126 and differences in expansion, survival and differentiation were analyzed. In contrast to neutrophil development, addition of U0126 completely abrogated expansion and subsequent differentiation of CD34$^+$ erythrocyte progenitors (Figure 2A, 2B), due to induction of apoptosis (Figure 2C). This was found to correlate with abrogation of anti-apoptotic Bcl-xL levels in CD34$^+$ cells treated with U0126 (Figure 2D).

Together, these results show that MEK-ERK activity is essential for survival and development of erythroid progenitors and expansion of neutrophil progenitors.

MAPK activity is not essential for neutrophil differentiation of hematopoietic progenitor cells

Having established the effects of MEK inhibition on neutrophil progenitor expansion, we were interested to determine whether MEK-ERK also plays a role in regulating neutrophil

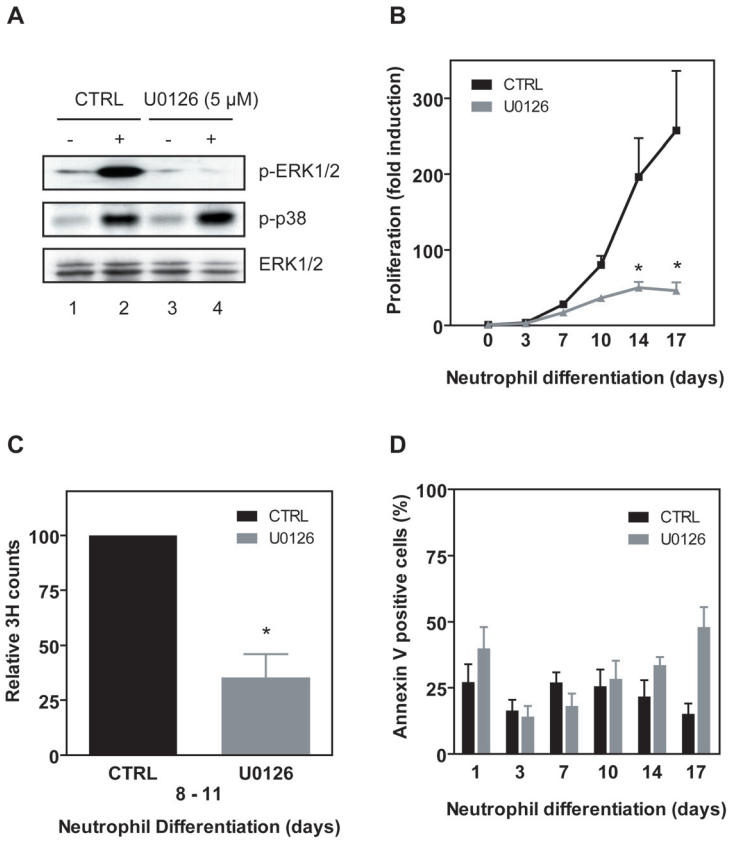

Figure 1. MEK-ERK activation is required for expansion of hematopoietic progenitor cells. (A) $CD34^+$ cells were starved overnight in absence of cytokines and in presence of 0.5% FCS. Cells were left untreated (lane 1, 2) or treated with 5 µM U0126 (lane 3, 4) for 1 hour before stimulation with G-CSF (lane 2, 4) for 15 minutes. Protein lysates were prepared and Western blot analysis was performed with an antibody against phosphorylated ERK1/2, phosphorylated p38MAPK, and as a control for equal loading an antibody against total ERK1/2. (B/C) $CD34^+$ cells were cultured for 17 days in presence of G-CSF to induce neutrophil differentiation in absence or presence of 5 µM U0126. Expansion was determined by (B) counting the trypane blue-negative cells or by (C) performing ^{3}H-thymidine incorporation assays. Data was depicted as a ratio between control and cells treated with U0126. (D) During the 17-day culture period the percentage apoptotic cells was determined by Annexin V staining. Results are presented as means of 4 independent experiments. Error bars represent SEM.

differentiation. $CD34^+$ cells, isolated from umbilical cord blood, were differentiated towards neutrophils for 17 days either in the presence or absence of U0126. After 14 and 17 days of culture, cytospins were prepared and the morphology of the differentiating granulocytes was analyzed by May-Grunwald Giemsa staining. Treatment of hematopoietic $CD34^+$ cells with U0126 did not alter the total percentage of differentiated neutrophils (Figure 3A, 3B). However, since U0126 inhibited progenitor expansion during neutrophil development, the absolute numbers of mature neutrophils after 14 and 17 days of culture were reduced (Figure 3C). Furthermore, flow cytometric analysis was performed to support the morphological observations and showed that treatment with U0126 did not affect the expression of

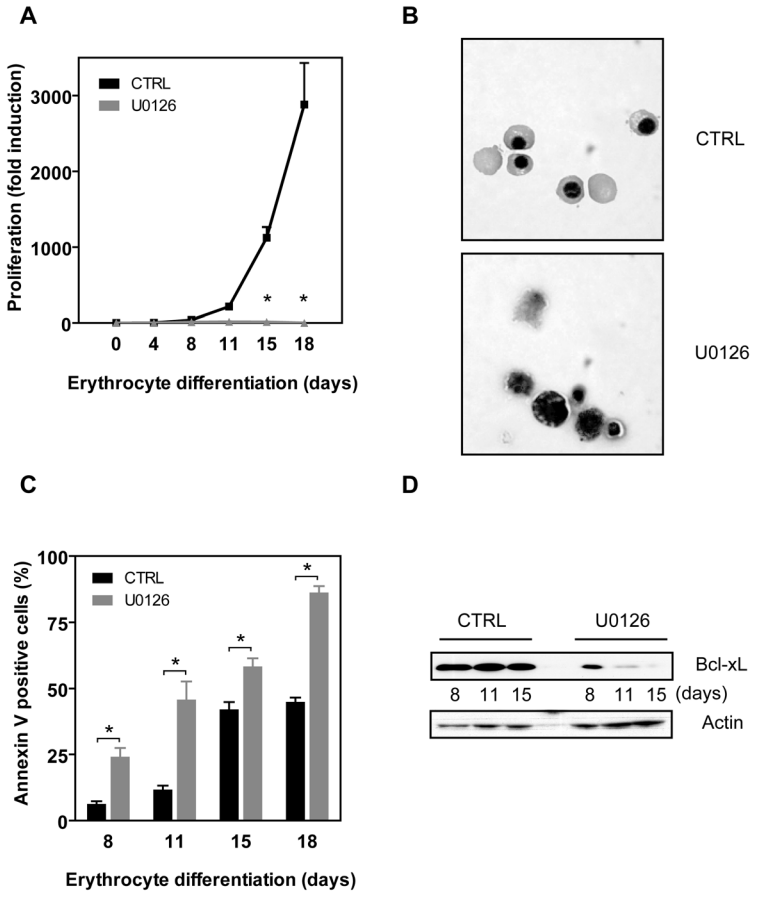

Figure 2. MEK activity is essential for survival of hematopoietic progenitors during erythropoiesis. CD34$^+$ progenitors were differentiated towards erythrocytes for 18 days either in the presence or absence of 5 μM U0126. (A) Expansion was determined by counting the trypane blue-negative cells. (B) After 18 days of culture, cytospins were prepared and stained with May-Grunwald Giemsa solution. (C) During the 18-day culture period the percentage apoptotic cells was determined by Annexin V staining. Results are presented as means of 4 independent experiments. Error bars represent SEM. (D) Protein lysates were prepared from CD34$^+$ cells differentiated toward erythrocytes for 8, 11 and 15 days in the presence or absence of 5 μM U0126. Western blot analysis was performed with an antibody against Bcl-xL (upper panel) or β-actin (bottom panel) as a control for equal loading. Similar results were obtained in 3 independent experiments.

lactoferrin, a neutrophil specific granule protein (Figure 3D).

In order to assess the development of single hematopoietic progenitors, CD34$^+$ cells were plated in CFU assays, either in presence or absence of U0126, and colony formation was analyzed after 12 days. Similarly, inhibition of MEK-ERK signalling did not significantly affect the Granulocyte-Macrofage (GM) colony formation capacity of hematopoietic progenitor cells (Figure 3E). However, absolute cell numbers of total GM colonies were significantly decreased in presence of U0126, indicating that MEK-ERK activity is indeed required for

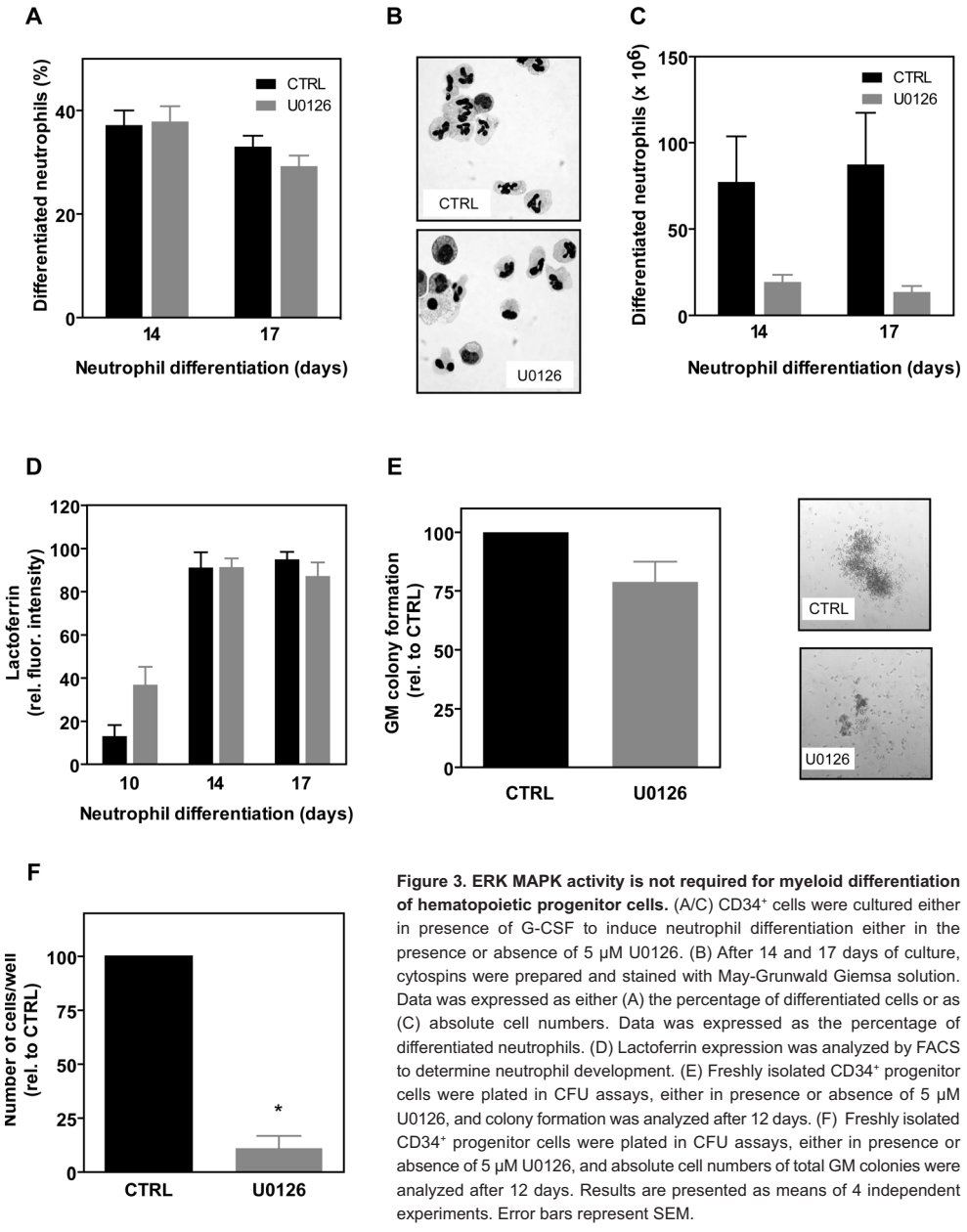

Figure 3. ERK MAPK activity is not required for myeloid differentiation of hematopoietic progenitor cells. (A/C) CD34$^+$ cells were cultured either in presence of G-CSF to induce neutrophil differentiation either in the presence or absence of 5 μM U0126. (B) After 14 and 17 days of culture, cytospins were prepared and stained with May-Grunwald Giemsa solution. Data was expressed as either (A) the percentage of differentiated cells or as (C) absolute cell numbers. Data was expressed as the percentage of differentiated neutrophils. (D) Lactoferrin expression was analyzed by FACS to determine neutrophil development. (E) Freshly isolated CD34$^+$ progenitor cells were plated in CFU assays, either in presence or absence of 5 μM U0126, and colony formation was analyzed after 12 days. (F) Freshly isolated CD34$^+$ progenitor cells were plated in CFU assays, either in presence or absence of 5 μM U0126, and absolute cell numbers of total GM colonies were analyzed after 12 days. Results are presented as means of 4 independent experiments. Error bars represent SEM.

expansion of hematopoietic progenitors during myeloid development (Figure 3F). Together these results show that MEK-ERK activity, although essential for expansion, is not required for differentiation of CD34$^+$ progenitor cells during neutrophil development.

Conditional activation of MEK1 promotes expansion of hematopoietic progenitors during neutrophil differentiation

To further validate and extend the results obtained utilizing the MEK1/2 pharmacological inhibitor, $CD34^+$ cells, cultured in presence of G-CSF, were retrovirally transduced to ectopically express a 4-hydroxytamoxifen (4-OHT) inducible active form of MEK1 (MEK:ER*) (37). In the absence of 4-OHT, activation of MEK1:ER* is inhibited by heat-shock and chaperone proteins that associate with the fused ER hormone-binding domain. Upon addition of 4-OHT, these proteins dissociate, resulting in the release of active MEK1. Three days after transduction, eGFP positive cells were sorted from the non-transduced cells and resuspended in culture medium containing G-CSF in presence or absence of 20 nM 4-OHT. Activation of ERK1/2 in $CD34^+$ cells transduced with MEK:ER* upon 4-OHT stimulation was confirmed by Western blotting (Figure 4A). MEK1 activation resulted in significant expansion of progenitor cells between day 3 and 7, however, prolonged MEK activation prevented neutrophil differentiation due to massive apoptosis (Figure 4B, 4C).

In addition, in order to assess expansion of single hematopoietic progenitors, $CD34^+$ cells transduced with MEK1:ER* were seeded in Terasaki plates at a density of 1 cell per well in culture medium containing G-CSF in presence or absence of 20 nM 4-OHT. After 12 days, positive wells were scored. MEK1 activation resulted in a significant increase in the proliferative capacity of $CD34^+$ cells (Figure 4D). To further understand how MEK-ERK regulates progenitor numbers over time, $CD34^+$ progenitor cells transduced with MEK1:ER* were differentiated towards neutrophils in the presence of 4-OHT. After either 3 or 7 days of culture, cells were plated in CFU assays in absence of 4-OHT, and colony formation was further analyzed after 12 days of culture. Remarkably, GM colony formation of $CD34^+$ cells, ectopically expressing MEK1:ER* that had been cultured in presence of 4-OHT for 3 and 7 days, was significantly decreased compared to controls (Figure 4E). These data suggest that constitutive activation of MEK-ERK is not sufficient to overcome the natural loss of colony forming capacity, or to retain the early immature progenitor phenotype in presence of lineage-directing cytokines, such as G-CSF. Together, these results suggest that tight control of MEK-ERK activity is an important factor in regulating the balance between expansion and survival of hematopoietic progenitors during neutrophil differentiation.

Conditional activation of MEK1 does not affect terminal differentiation of neutrophil progenitors

To further evaluate the effect of MEK1 on neutrophil development, $CD34^+$ cells were transduced with MEK1:ER*, sorted from non-transduced cells and differentiated to neutrophils in presence or absence of 4-OHT. After three days of culture, cells were washed and resuspended in culture medium containing G-CSF. After 14 and 17 days of differentiation, cytospins were prepared and the morphology of the cells was analyzed. Although prolonged activation of MEK1 prevented neutrophil differentiation due to induction of cell death, addition of 4-OHT for three days resulted in an increase in progenitor cell numbers, that thereafter differentiated normally to mature neutrophils (Figure 5A). However, since MEK1 activation resulted in significant expansion of progenitor cells between day 3 and 7, the absolute numbers of mature neutrophils after 17 days of culture were also increased (Figure 5B). In

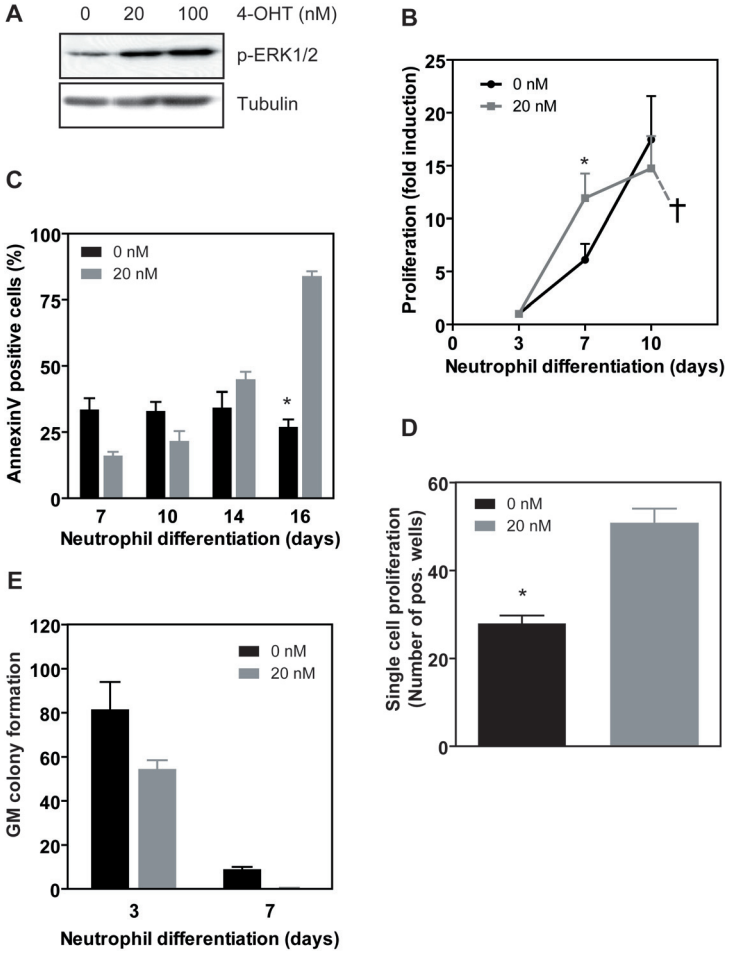

Figure 4. Conditional activation of MEK1 promotes expansion of myeloid progenitors. (A) Protein lysates were prepared from CD34$^+$ cells transduced with MEK:ER* and treated with solvent, 20 or 100 nM of 4-OHT for 30 minutes. Western blot analysis was performed with an antibody against phosphorylated ERK1/2 or tubulin as a control for equal loading. Similar results were obtained in 4 independent experiments. (B) CD34$^+$ cells were transduced with MEK1:ER*, sorted by FACS from the non-transduced cells and differentiated to neutrophils in presence or absence of 20 nM 4-OHT. Expansion was determined by counting the trypane blue-negative cells. (C) CD34$^+$ cells were transduced with MEK1:ER*, sorted by FACS from the non-transduced cells and differentiated to neutrophils in presence or absence of 20 nM 4-OHT. During the 17-day culture period the percentage apoptotic cells was determined by Annexin V staining.(D) CD34+ cells, transduced with MEK:ER and sorted by FACS were seeded in 60-wells plates at a density of 1 cell per well in normal culture medium containing G-CSF in presence or absence of 20 nM 4-OHT. After 12 days, wells with colonies were scored. (E) CD34$^+$ progenitor cells transduced with MEK1:ER* were differentiated towards neutrophils in the presence 4-OHT. After either 3 and 7 days of culture, cells were plated in CFU assays in absence of 4-OHT, and colony formation was analyzed after 12 days of culture. Results are presented as means of 4 independent experiments. Error bars represent SEM.

addition, flow cytometric analysis was also performed to support the morphological observations and conditional MEK1 activation had no significant effect on the expression of lactoferrin, a neutrophil specific granule protein (Figure 5C).

To assess the development of single hematopoietic progenitors, CD34$^+$ cells transduced with MEK1:ER* were plated in CFU assays, either in presence or absence of 4-OHT, and colony formation was analyzed after 12 days. Similarly, activation of MEK1 by addition of 4-OHT did not affect the Granulocyte-Macrophage (GM) colony formation capacity of hematopoietic progenitor cells (Figure 5D). Taken together, although found to be an essential regulator of expansion and survival of myeloid progenitors, these results show that MEK-ERK signalling does not modulate terminal differentiation of neutrophil progenitors.

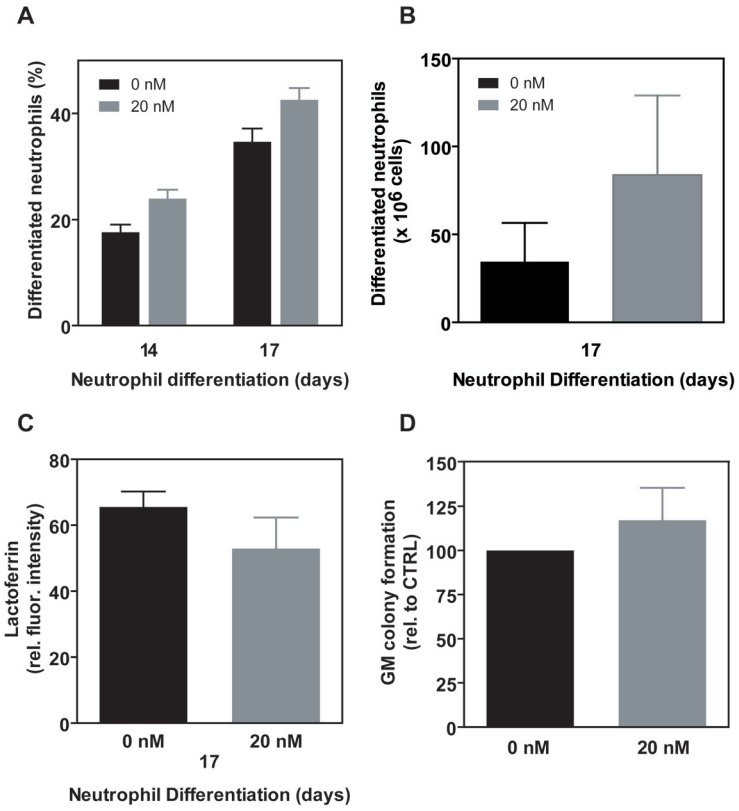

Figure 5. Conditional activation of MEK1 does not affect terminal differentiation of neutrophil progenitors. (A/B) CD34$^+$ cells were transduced with MEK1:ER*, sorted by FACS from the non-transduced cells and differentiated to neutrophils in presence or absence of 4-OHT. After three days of culture, cells were washed and resuspended in normal culture medium containing G-CSF. After 14 and 17 days of differentiation, cytospins were prepared and the morphology of the cells was analyzed by May-Grunwald Giemsa staining. Data was expressed as either (A) the percentage of differentiated cells or as (B) absolute cell numbers. (C) Lactoferrin expression was analyzed by FACS to determine neutrophil development. (D) CD34$^+$ cells, transduced with MEK1:ER*, were sorted by FACS from the non-transduced cells and plated in CFU assays, either in presence or absence of 20 nM 4-OHT, and colony formation was analyzed after 12 days. Results are presented as means of 4 independent experiments. Error bars represent SEM.

Analysis of differential gene expression in response to MEK1 activation

Taken together, these results demonstrate that tight control of MEK-ERK signalling plays an important role in the regulation of progenitor expansion and survival. To identify changes in gene expression that might underly these observations, a microarray analysis was performed. $CD34^+$ cells retrovirally transduced to ectopically express MEK1:ER* were cultured in the presence of G-CSF and IL-3 to induce neutrophil differentiation. After 6 days of culture, cells were starved overnight in absence of cytokines. Cells were subsequently stimulated with 20 nM 4-OHT or carrier for 12 hours before RNA was isolated. Results obtained from four independent donors were subjected to both a filter query and statistical analysis, allowing only those data sets to pass that were increased or decreased and showed a fold change of two or more in all four independent sets when compared to the carrier control. The resulting gene set representing 181 differentially expressed genes in response to MEK1 activation were classed into functional groups of which are shown in Table 1 and Supplementary Figure S1. The number of genes induced by MEK1 activation is much larger than the number of genes that show down-regulation, consistent with MAPK being primarily an inducer of transcription. c-myc, c-fos, p21CIP1, cyclin D1 and cyclin D3, all of which are genes involved in regulation of cell-cycle, show strong up-regulation upon MEK1 activation. Interestingly, transcripts coding for hematopoietic cytokines and growth factors, such as IL-8,

Table 1. Differential gene expression induced by MEK1 activation in $CD34^+$ cells

CYTOKINE-CYTOKINE RECEPTOR INTERACTION

Gene	Fold change	Definition
IL8	31.67	interleukin 8
IL1B	19.99	interleukin 1 beta
CCL4	18.89	chemokine (C-C motif) ligand 4
CCL2	17.88	chemokine (C-C motif) ligand 2
CCL7	9.97	chemokine (C-C motif) ligand 7
CXCL3	5.73	chemokine (C-X-C motif) ligand 3
CXCL2	4.2	chemokine (C-X-C motif) ligand 2
IL1A	2.78	interleukin 1alpha
CCL5	2.76	chemokine (C-C motif) ligand 5
IFNGR2	2.61	interferon gamma receptor 2
TGFB1	2.25	transforming growth factor beta 1
G-CSFR	0.42	granulocyte colony-stimulating factor receptor precursor
CD70	0.48	CD70 molecule
NGFR	0.5	nerve growth factor receptor

CELL CYCLE AND APOPTOSIS

Gene	Fold change	Definition
EGR1	9.76	early growth response protein 1
p21Cip1	3.63	cyclin-dependent kinase inhibitor 1A
CCND1	2.96	cyclin D1
BIRC3	2.85	baculoviral IAP repeat-containing 3
NFKBIA	2.63	NFKB inhibitor alpha
CCND3	2.41	cyclin D3
MYC	2.39	Myc transcription factor
FOS	2.15	Fos gene family transcription factor
NOXA	2.03	Bcl-2 protein family member Noxa
XRCC3	0.48	DNA-repair protein XRCC1
GADD45B	0.54	growth arrest and DNA-damage-inducible beta

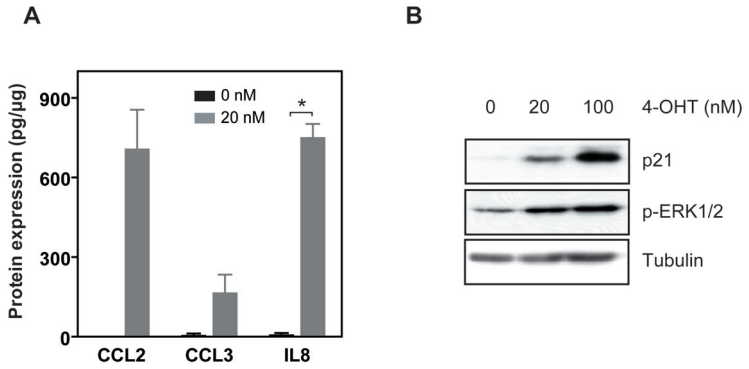

Figure 6. Differential expression of MEK1 target genes. (A) CD34⁺ hematopoietic progenitor cells, transduced with MEK1:ER* were cultured in presence of G-CSF to induce neutrophil differentiation. Two days after transduction, eGFP positive cells were separated from the non-transduced cells by FACS and resuspended in culture medium containing G-CSF and IL-3. After 6 days of differentiation, cells were washed twice with PBS and starved overnight in absence of cytokines and in presence of 0.5% FCS. Subsequently, cells were incubated with 0 or 20 nM 4-OHT for 12 hours, supernatants were collected and IL-8, CCL2 and CCL3 protein expression was determined using Luminex multiplex analysis as described in Materials and Methods. Results are presented as means of 2 or 3 independent experiments. Error bars represent SEM. (B) Protein lysates were prepared from CD34⁺ cells transduced with MEK:ER* and treated with solvent, 20 or 100 nM of 4-OHT overnight. Western blot analysis was performed with an antibody against p21Cip1, phosphorylated ERK1/2 or tubulin as a control for equal loading. Similar results were obtained in 4 independent experiments.

IL1ß and various members of the CCL and CXCL family were strongly up-regulated in response to MEK1 activation (Table 1). To validate changes in gene expression, a subset of the identified MEK1 target genes was verified using Luminex multiplex analysis (Figure 6A). IL-8, CCL2 and CCL3 showed dramatic upregulation in response to MEK1. Furthermore, whole cell lysates prepared of MEK1:ER* transduced cells stimulated with 0, 20 or 100 nM 4-OHT were used for detection of p21Cip1, which also showed significant upregulation in response to MEK1 activation (Fig. 6B). These data clearly demonstrate that conditional activation of MEK1 in CD34⁺ progenitor cells results in changes in expression of a variety of proliferation modulating genes, consistent with the MEK-ERK module being an important regulator of progenitor expansion.

DISCUSSION

In this study, we have investigated the role of the MEK-ERK MAPK signalling pathway in CD34⁺ hematopoietic progenitor function, focussing on neutrophil development. The data presented here demonstrate that ERK MAPK activity is required for the initial expansion of CD34⁺ hematopoietic progenitors during both neutrophil and erythroid differentiation. Subsequent microarray analysis of CD34⁺ cells revealed that activation of MEK1 resulted in changes in expression of a variety of genes encoding hematopoietic cytokines and growth factors. In addition, we observed up-regulation of genes involved in regulating cell-cycle progression including c-myc, c-fos, cyclin D1 and cyclin D3.
During G1 phase of the cell-cycle, D-type cyclins assemble with cyclin-dependent kinases

(CDKs) facilitating cell cycle progression through phosphorylation of several key substrates, such as retinoblastoma protein (38). Previously, it has been shown that activation of the ERK pathway in epithelial cells by expression of an conditionally active Raf1 mutant resulted in increased cyclin D1 expression, whereas blocking ERK activity by expressing a dominant negative form of MEK led to decreased cyclin D1 expression (39). However, cyclin D1 was not induced immediately after growth factor stimulation, but only after sustained ERK activity, suggesting that this was not directly due to ERK activation (40). Candidates potentially mediating ERK transcriptional induction of cyclin D1 gene are the c-fos and c-myc transcription factors. Seth *et al* demonstrated that direct phosphorylation of c-myc on serine 62 by ERK MAPK enhanced its stability, resulting in subsequent transcriptional induction of the cyclin D1 gene (41)(42). Furthermore, it has been demonstrated that activated ERK can phosphorylate pre-existing transcription factors, such as Elk-1, involved in the upregulation of immediate-early gene c-fos. Increased expression of many other genes through upregulation of c-fos, including fra-1, ultimately results in expression of cyclin D1 (43). Thus, expression of immediate-early genes has been shown to regulate subsequent induction of delayed early genes, which include cyclin D1. Since we also observe increased expression of c-fos and c-myc, it is likely that the MEK-ERK pathway regulates expansion of $CD34^+$ progenitors through this mechanism, resulting in upregulation of cyclin D1 and subsequent assembly of active cyclinD–CDK complexes, facilitating cell-cycle progression.

Interestingly, Furukawa *et al* demonstrated that expression of cell cycle control genes, including D-type cyclins, is differentially regulated in a lineage-specific manner during hematopoiesis, suggesting that these genes are not only involved in cell cycle regulation but also play a role in lineage-restricted functions of hematopoietic cells (44). Lineage-specific changes, for example include sustained elevation of cdc2 and cyclin A during erythrocyte differentiation and selective induction of cyclin D1 in myeloid progenitors. However, little is known about the function of cyclin D1 in terminally differentiated myeloid cells. Zwijsen *et al* reported that cyclin D1 directly interacts with the oestrogen receptor and enhances its binding to oestrogen-responsive elements, thereby activating oestrogen-mediated transcription (45). Through a similar mechanism, cyclin D1 may thus be involved in the regulation of myeloid-specific genes. Our data support the idea that the MEK-ERK signaling module plays a critical role in regulation of expansion of myeloid precursor cells through up-regulation of genes that are required for cell-cycle progression, such as cyclin D1. However, maturation of granulocyte progenitors was unaffected, suggesting that cyclin D1 is acting predominantly as a regulator of proliferation in $CD34^+$ progenitors.

Besides transcriptional induction of cyclin D1, various inhibitors of signalling were up-regulated, such as MAP kinase phosphatases DUSP2, 5 and 6, a subclass of protein tyrosine phosphatases that specifically dephosporylate threonine and tyrosine residues on MAPKs and are thought to be part of a negative feedback loop (46). Moreover, p21Cip1 was found to be up-regulated by MEK1 activation in $CD34^+$ cells (Figure 6B). p21Cip1 is best known for its function as a cell cycle inhibitor and was demonstrated to associate and inhibit cyclin-CDK complexes during the G1 phase of the cell cycle (47). Interestingly, using epithelial cells expressing a conditionally active Raf mutant, it was demonstrated that strong and sustained Raf activity throughout the G1 phase of the cell-cycle triggered cell cycle arrest by inducing massive expression of the cell cycle inhibitor p21Cip1, whereas modest activation resulted

in enhanced cell cycle entry (48)(49). It is thus also possible that in $CD34^+$ progenitors the p21Cip1 expression observed at low 4-OHT concentrations could also be partly responsible for increased proliferation.

Several studies have provided evidence that the MEK-ERK signaling module is often constitutively activated in AML (50). For example, Morgan *et al* have shown activation of the MEK-ERK pathway in 9 of 14 AML cell lines studied, suggesting that aberrant activation of ERK kinases is indeed contributing to the abnormal cell growth in acute leukemias (51). Similarly, expression of a constitutively active form of Raf kinase in hematopoietic cell lines results in MEK activation and growth factor independence (52). These properties make the MEK-ERK signaling module a potential target for the molecular therapy in AML and various studies have demonstrated the beneficial effects of chemotherapeutic agents that inhibit MEK-ERK activation in inducing growth suppression and apoptosis of AML cells (51)(53). Interestingly, our data also demonstrates that modulating the duration and extent of ERK activation is essential in regulating the balance between expansion and survival of hematopoietic progenitors during neutrophil differentiation. Inducible activation of MEK1 in $CD34^+$ cells through addition of 4-OHT resulted in dramatic progenitor expansion during the first three days, however in contrast to the effects of constitutive MEK activation in AML blast cells, prolonged MEK1 activation prevented neutrophil differentiation due to induction of cell death. Together, this suggests that while MEK-ERK signalling may indeed be a critical factor in the generation of myeloid leukemia, additional mechanisms are required for cellular transformation to occur. Thus dysregulation of MEK-ERK signalling alone is insufficient for $CD34^+$ progenitor transformation and cells are “culled” if this signalling module is inappropriately activated.

In addition to down-regulation of many anti-proliferative genes, the ERK MAPK pathway is involved in the regulation of apoptosis through controlling expression of apoptotic regulatory molecules including Bad, Bim, Mcl-1, caspase 9 (8). Our results also demonstrated that ERK MAPK activity is essential for survival of erythroid $CD34^+$ progenitors. Inhibition of ERK activity prevented erythropoiesis due to induction of apoptosis in $CD34^+$ cells, which was found to correlate with a dramatic decrease in anti-apoptotic Bcl-xL levels. In contrast, our results also show that MEK-ERK activity, although essential for expansion, is not required for development of $CD34^+$ progenitor cells during neutrophil development. Although it is beyond the scope of this manuscript to describe the lineage-specific effects of the MEK-ERK pathway, these results suggest that the MEK-ERK pathway plays a specific and differential role in regulating the development of myeloid and erythroid lineages.

Although our results demonstrate that MEK/ERK pathway is not essential for maturation of neutrophil progenitors, many of the 180 differentially expressed genes in response to MEK1 activation, were genes coding for hematopoietic cytokines and chemokines, such as IL-8, IL1ß and various members of the CCL and CXCL family. Computational analysis of the IL-8, IL1ß, CCL2, CCL-3 and CCL-7 genes using the MAPPER Search Engine (http://mapper.chip.org) indeed identified putative transcription factor binding sites for MEK-ERK-induced transcriptional regulators, including the AP-1 transcription factor complex. Chemokines are involved in migration, homing, mobilization, proliferation and survival of HSCs and hematopoietic progenitor cells, and are also implicated in the development of malignancies (54-56). It is plausible that they act as autocrine growth factors to activate

signalling pathways implicated in the regulation of progenitor survival and proliferation (57)(58). For example, autocrine stimulation of the IL-8 receptor pathway has already been demonstrated in human myeloid and lymphoid leukemia (59). Furthermore, IL-8 in synergy with M-CSF was found to promote the clonogenic potential of $CD34^+$ cells, which involved an autocrine regulatory loop (60). Our microarray data suggest that cytokines and chemokines production through MEK-ERK activation could contribute to hematopoietic homeostasis by regulating expansion and survival of hematopoietic progenitor cells.
Taken together, we have identified a novel role for MEK-ERK signalling in regulating the balance between proliferation and apoptosis of $CD34^+$derived neutrophil progenitor cells. This indicates the need for tight control of this signalling module since aberrant MEK-ERK activation could result in the development of bone marrow failure or hematological malignancies.

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Supplementary Figure S1

Gene	*Fold change*	*Definition*
31.67	IL8	Interleukin-8 precursor
22.21	CCL3	Small inducible cytokine A3 precursor
19.99	IL1B	Interleukin-1 beta precursor
18.89	CCL4	Small inducible cytokine A4 precursor
17.88	CCL2	Small inducible cytokine A2 precursor
9.97	CCL7	Small inducible cytokine A7 precursor
9.76	EGR1	Early growth response protein 1
7.55	DUSP6	Dual specificity protein phosphatase 6
6.57	THBD	Thrombomodulin precursor
6.4	IER3	Radiation-inducible immediate-early gene IEX-1
6.11	MMP1	Interstitial collagenase precursor
5.84	SERPINB2	Plasminogen activator inhibitor 2 precursor
5.78	TIMP1	Metalloproteinase inhibitor 1 precursor
5.73	CXCL3	Macrophage inflammatory protein 2-beta precursor
5.58	GTR3	Solute carrier family 2, facilitated glucose transporter member 3
5.17	TFPI2	Tissue factor pathway inhibitor 2 precursor
5.15	CXCL1	Growth-regulated protein alpha precursor
4.74	MMP9	Matrix metalloproteinase-9 precursor
4.2	CXCL2	Macrophage inflammatory protein 2-alpha precursor
4.14	PLAUR	Urokinase plasminogen activator surface receptor precursor
4.12	CCL20	Small inducible cytokine A20 precursor
3.9	EMP1	Epithelial membrane protein 1
3.89	FGFRL1	Fibroblast growth factor receptor-like 1 precursor
3.77	SH3BGRL3	SH3 domain-binding glutamic acid-rich-like protein 3
3.75	HSP90AA1	Heat shock protein HSP 90-alpha
3.74	PBEF1	Nicotinamide phosphoribosyltransferase
3.73	MAFB	Transcription factor MafB
3.68	STIM1	Stromal interaction molecule 1 precursor.
3.67	CLEC5A	C-type lectin domain family 5 member A
3.63	CDKN1A	Cyclin-dependent kinase inhibitor 1
3.57	HSPH1	Heat-shock protein 105 kDa
3.48	RPN1	Ribophorin I
3.47	KRT121P	Hemoglobin subunit beta
3.37	ST6GAL2	beta-galactoside alpha-2,6-sialyltransferase II
3.34	DUSP2	Dual specificity protein phosphatase 2
3.29	HNRPL	Heterogeneous nuclear ribonucleoprotein L
3.29	CD69	Early activation antigen CD69
3.22	NME1	Nucleoside diphosphate kinase B
3.19	BTG2	BTG2 protein
3.19	GDF15	Growth/differentiation factor 15 precursor
3.19	BCM:PHLDA1	Pleckstrin homology-like domain, family A, member 1
3.18	TRIB3	Tribbles homolog 3
3.16	DNAJA1	DnaJ homolog subfamily A member 1
3.14	DHRS9	Dehydrogenase/reductase SDR family member 9 precursor
3.09	PKM2	Pyruvate kinase isozymes M1/M2
3.08	THSD1	Thrombospondin type-1 domain-containing protein 1 precursor
3.08	CD44	CD44 antigen precursor
3.08	TFEC	Transcription factor EC isoform b
2.97	NMES1	Normal mucosa of esophagus-specific gene 1 protein
2.97	INSIG1	Insulin-induced gene 1 protein
2.96	CCND1	G1/S-specific cyclin-D1
2.95	FHL2	Four and a half LIM domains protein 2
2.93	HSPA1A	Heat shock 70 kDa protein 1
2.92	LAT1	Large neutral amino acids transporter small subunit 1
2.92	VSIG4	V-set and immunoglobulin domain-containing protein 4 precursor

2.85	cIAP2	Baculoviral IAP repeat-containing protein 3
2.8	GZMB	Granzyme B precursor
2.78	IL1A	Interleukin-1 alpha precursor
2.77	UPP1	Uridine phosphorylase 1
2.76	CCL5	Small inducible cytokine A5 precursor
2.73	KBTBD8	Kelch repeat and BTB domain-containing protein 8
2.73	KLF6	Krueppel-like factor 6
2.72	PSMD1	26S proteasome non-ATPase regulatory subunit 1
2.7	FABP5	E-FABP Fatty acid-binding protein, epidermal
2.7	CD55	Complement decay-accelerating factor precursor
2.68	BZW1	Basic leucine zipper and W2 domain-containing protein 1
2.63	NFKBIA	NF-kappa-B inhibitor alpha
2.61	IFNGR2	Interferon-gamma receptor beta chain precursor
2.6	RAMP1	Receptor activity-modifying protein 1 precursor
2.59	MTSS1	Metastasis suppressor protein 1
2.58	EREG ER	Epiregulin precursor
2.52	POMP	Proteasome maturation protein
2.51	SH2B3 LNK	SH2B adapter protein 3
2.48	DDX21	Nucleolar RNA helicase 2
2.48	ATP13A3	Probable cation-transporting ATPase 13A3
2.47	SPCS3	Signal peptidase complex subunit 3
2.42	BHLHB2	Class B basic helix-loop-helix protein 2
2.42	CKLF	Chemokine-like factor (C32).
2.41	GLO1	Lactoylglutathione lyase
2.41	CCND3	G1/S-specific cyclin-D3
2.4	FCGR2B	Low affinity immunoglobulin gamma Fc region receptor II-b precursor
2.39	PRDX4	Peroxiredoxin-4
2.39	MYC	Myc proto-oncogene protein
2.39	CD52	CAMPATH-1 antigen precursor
2.33	DHCR24	24-dehydrocholesterol reductase precursor
2.31	RGC-32	Response gene to complement 32 protein
2.3	VGF	Neurosecretory protein VGF precursor
2.3	UBE3C	Ubiquitin-protein ligase E3C
2.28	LGALS1	Galectin-1
2.27	STARD4	StAR-related lipid transfer protein 4
2.27	KIFC3	Kinesin-like protein KIFC3
2.26	ARMET	ARMET protein precursor
2.26	SQLE	Squalene monooxygenase
2.25	TGFB1	Transforming growth factor beta-1 precursor
2.25	SPINK1	Pancreatic secretory trypsin inhibitor precursor
2.23	EIF4G2	Eukaryotic translation initiation factor 4 gamma 2
2.22	ITGAX	Integrin alpha-X precursor
2.18	CHORDC1	cysteine and histidine-rich domain (CHORD)-containing, zinc binding protein 1
2.18	TUBB6	Tubulin beta-6 chain
2.17	MCT4	Monocarboxylate transporter 4
2.17	RBM13	MAK16-like protein RBM13
2.17	HNRPA2B1	Heterogeneous nuclear ribonucleoproteins A2/B1
2.17	MMP12	Macrophage metalloelastase precursor
2.15	FOS	Proto-oncogene protein c-fos
2.14	CLDND1	Claudin domain-containing protein 1
2.14	VIL2	Ezrin
2.13	CCL3L1	Small inducible cytokine A3-like 1 precursor
2.13	TXNRD1	Thioredoxin reductase 1, cytoplasmic precursor
2.12	RANBP1	Ran-specific GTPase-activating protein
2.12	PPIF	Peptidyl-prolyl cis-trans isomerase, mitochondrial precursor
2.11	PHLDA2	Pleckstrin homology-like domain family A member 2
2.1	NELF	Nasal embryonic luteinizing hormone-releasing hormone factor
2.1	DUSP5	Dual specificity protein phosphatase 5

2.1	TUBG1	Tubulin gamma-1 chain
2.09	TNF	Tumor necrosis factor precursor
2.09	SQSTM1	Sequestosome-1
2.09	FDPS	Farnesyl pyrophosphate synthetase
2.07	CD300A	CMRF35-H antigen precursor
2.06	LDLR	Low-density lipoprotein receptor precursor
2.05	G6PD	Glucose-6-phosphate 1-dehydrogenase
2.05	TM4SF19	Transmembrane 4 L6 family member 19
2.05	ACTB	Actin
2.03	PMAIP1	NOXA
2.02	B3GNT2	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase
2.02	MGAT4B	mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N acetylglucosaminyltransferase, isoenzyme B isoform 1
2.02	PDXK	Pyridoxal kinase
2.01	PLEC1	Plectin-1
2.01	MAPK6	Mitogen-activated protein kinase 6
2.01	NCOR2	Nuclear receptor corepressor 2
2.01	PRNP	Major prion protein precursor
2	HSPE1	10 kDa heat shock protein, mitochondrial
2	STIP1	Stress-induced-phosphoprotein 1 (STI1) (Hsc70/Hsp90-organizing

0.29	FUCA1	Tissue alpha-L-fucosidase precursor
0.31	SORL1	Sortilin-related receptor precursor
0.32	GPNMB	Transmembrane glycoprotein NMB precursor
0.32	HSD17B11	Dehydrogenase/reductase SDR family member 8 precursor
0.33	CAT	Catalase
0.33	CD24	Signal transducer CD24 precursor
0.35	onzin	Placenta-specific gene 8 protein
0.36	LMO4	LIM domain transcription factor LMO4
0.37	SERPINB1	Leukocyte elastase inhibitor
0.38	ZNF521	zinc finger protein 521
0.41	MPO	Myeloperoxidase precursor
0.41	NR1D2	Orphan nuclear receptor NR1D2
0.42	RAB37	Ras-related protein Rab-37
0.42	TKT	Transketolase
0.42	GCSFR	Granulocyte colony-stimulating factor receptor precursor
0.42	KCNH2	Potassium voltage-gated channel subfamily H member 2
0.42	ASAH1	Acid ceramidase precursor
0.43	KBTBD11	Kelch repeat and BTB domain-containing protein 11
0.43	AZU1	Azurocidin precursor
0.43	CEACAM6	Carcinoembryonic antigen-related cell adhesion molecule 6
0.44	OPCML	Opioid-binding protein/cell adhesion molecule precursor
0.45	CAMLG	Calcium signal-modulating cyclophilin ligand
0.45	CDT1	DNA replication factor Cdt1
0.45	ADA	Adenosine deaminase
0.46	GLUL	Glutamine synthetase
0.46	HCP5	HLA complex P5
0.46	CFD	Complement factor D precursor
0.47	FGF4	Fibroblast growth factor 4 precursor
0.47	NPY	Neuropeptide Y precursor
0.47	AOAH	Acyloxyacyl hydrolase precursor
0.47	FAM46A	Protein FAM46A
0.47	SELPLG	P-selectin glycoprotein ligand 1 precursor
0.47	RhoGAP4	Rho GTPase-activating protein 4
0.47	CALB2	Calretinin
0.48	CD70	Tumor necrosis factor ligand superfamily member 7
0.48	ELA2	Leukocyte elastase precursor

0.48	GNG7	Guanine nucleotide-binding protein G(I)/G(S)/G(O) gamma-7 subunit precursor
0.48	LRRC40	Leucine-rich repeat-containing protein 40
0.48	XRCC3	DNA-repair protein XRCC3
0.49	FPRL1	FMLP-related receptor I
0.49	DPEP2	Dipeptidase 2 precursor
0.49	BNIP3L	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3-like
0.49	LCK	Proto-oncogene tyrosine-protein kinase LCK
0.49	AIG1	Androgen-induced protein 1
0.5	NCOA4	Nuclear receptor coactivator 4
0.5	KLHDC8B	Kelch domain-containing protein 8B
0.5	MAS1	MAS proto-oncogene
0.5	NGFR	Tumor necrosis factor receptor superfamily member 16 precursor
0.5	ATG16L2	ATG16 autophagy related 16-like 2

Chapter 5

mTOR activity is required for expansion of CD34+ hematopoietic progenitor cells

Christian R Geest[1], Fried J Zwartkruis[2], Edo Vellenga[3], Paul J Coffer[1,4] and Miranda Buitenhuis[1]

[1] Molecular Immunology Lab, Department of Immunology, University Medical Center, Utrecht, The Netherlands, [2] Department of Physiological Chemistry, Centre for Biomedical Genetics, University Medical Center, Utrecht, The Netherlands, [3] Department of Hematology, University Medical Center, Groningen, The Netherlands, [4] Department of Pediatric Immunology, University Medical Center, Utrecht, The Netherlands

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ABSTRACT

The mammalian target of rapamycin (mTOR) is a conserved protein kinase known to regulate protein synthesis, cell size and proliferation. Aberrant regulation of mTOR activity has been observed in hematopoietic malignancies, including acute leukemias and myelodysplastic syndromes, suggesting that correct regulation of mTOR is critical for normal hematopoiesis. An ex-*vivo* granulocyte differentiation system was utilized to investigate the role of mTOR in regulation of myelopoiesis. Inhibition of mTOR activity with the pharmacological inhibitor rapamycin, dramatically reduced hematopoietic progenitor expansion, without altering levels of apoptosis or maturation. Moreover, analysis of distinct hematopoietic progenitor populations revealed that rapamycin treatment inhibits the expansion potential of committed $CD34^+$ lineage-positive progenitors, but does not affect early hematopoietic progenitors. Further examinations showed that these effects of rapamycin on progenitor expansion might involve differential regulation of PKB and mTOR signalling. Together, these results indicate that mTOR activity is essential for expansion of $CD34^+$ hematopoietic progenitor cells during myelopoiesis. Modulation of the mTOR pathway may be of benefit in the design of new therapies to control hematological malignancies.

INTRODUCTION

Hematopoiesis is a highly regulated process resulting in the formation of blood cells which predominantly occurs in the bone marrow, and is regulated at the level of proliferation, survival and differentiation (1). Although it is evident that these complex processes are regulated by cytokines and depend on correct function of the bone marrow micro-environment, the cytokine-mediated intracellular signal transduction pathways regulating hematopoietic stem cell (HSC) function are thus far incompletely understood. The phosphatidylinositol 3-kinase (PI3K) signal transduction pathway has been demonstrated to play an important role in survival and proliferation of a plethora of cell types (2). It has, for example, been demonstrated that PI3K plays an essential role in regulation of hematopoietic progenitor survival and expansion during myelopoiesis (3). Furthermore, impaired regulation of PI3K and its downstream effector protein kinase B (PKB/c-akt) has been implicated in carcinogenesis. In particular, activation of the PI3K/PKB signalling module is observed in a variety hematopoietic malignancies, including acute leukemias and high-risk myelodysplastic syndromes (4-6). One of the downstream targets of PI3K/PKB is the mammalian target of rapamycin (mTOR), which is a conserved serine/threonine kinase that has been demonstrated to regulate cell size and cell cycle progression in various cell types (7). Activation of mTOR is mediated by PKB through direct phosphorylation (8). In addition, PKB inhibitory phosphorylates the GTPase activating protein tuberous sclerosis protein 2 (TSC2), which results in accumulation of GTP-bound Rheb and subsequent activation of mTOR (9). mTOR exists in two distinct multiprotein complexes: mTORC1 and mTORC2. mTORC1 consists of the regulatory-associated protein of mTOR (Raptor) adaptor protein, mLST8 and mTOR.

The mammalian translational initiation machinery governs the recruitment of ribosomes to mRNA to commence the production of protein synthesis. This machinery consists of various eukaryotic initiation factors (eIFs) that tightly regulate protein synthesis. Importantly, activation of mTORC1 positively stimulates mRNA translation via its downstream substrates p70S6 kinase and 4E-BP1/eIF4E (10)(11). Phosphorylation of 4E-BP1 by mTORC1 results in its dissociation from eIF4E, promoting assembly of the eIF4F complex, a protein complex that mediates recruitment of ribosomes to mRNA (12). The mTORC2 complex consists of mTOR, Rapamycin-insensitive companion of mTOR (Rictor) and mLST8 (13), and is involved in activation of PKB. In contrast to mTORC1, mTORC2 activity, is not abrogated by treatment with rapamycin, a potent pharmacological inhibitor of mTOR activity (14).

Analysis of mTOR-deficient mice has revealed that mTOR is essential for embryonal development. mTOR-deficient mice are embryonic lethal due to impaired cell proliferation (15). Moreover, exposure of mouse embryos to rapamycin results in a block in cell proliferation, indicating that the rapamycin-sensitive mTORC1 complex plays an essential role in regulation of developmental processes (16). Rapamycin has also been demonstrated to regulate the differentiation and proliferation of various adult cell types, including human endothelial progenitors, epithelial cells, chondrocytes, osteoblasts and myoblasts (17-24). Although these data demonstrate the importance of mTOR signalling in the regulation of multiple cellular processes, a role for mTOR in regulation of myelopoiesis remains to be investigated.

In this study, we have investigated the role of the mTOR signal transduction pathway in regulation of myelopoiesis utilizing a human ex-*vivo* granulocyte differentiation system. mTOR activity was found to be important in regulation of hematopoietic progenitor expansion, without altering the level of apoptosis or maturation. Analysis of hematopoietic progenitor populations revealed that rapamycin inhibits the expansion potential of committed $CD34^+$ lineage-positive progenitors, but does not affect $CD34^+CD38^-$ hematopoietic stem cells, suggesting that the response of hematopoietic progenitors is dependent on the stage of differentiation. Together, these results indicate that mTOR activity is essential for expansion of $CD34^+$ hematopoietic progenitor cells during myelopoiesis. Therefore, modulation of mTOR activity could play an important role in future therapies to control hematological malignancies.

MATERIALS AND METHODS

Isolation and culture of human $CD34^+$ cells

Mononuclear cells were isolated from umbilical cord blood by density centrifugation over a ficoll-paque solution (density 1.077 g/mL). MACS immunomagnetic cell separation (Miltenyi Biotech, Auburn, CA) using a hapten conjugated antibody against CD34, which was coupled to beads, was used to isolate $CD34^+$ cells. $CD34^+$ cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco, Paisley, UK) supplemented with 9% fetal calf serum (FCS) (Hyclone, Logan, UT), 50 µM β-mercaptoethanol, 10 U/mL penicillin, 10 µg/mL streptomycin, and 2mM glutamine at a density of 0.3×10^6 cells/mL. Cells were differentiated towards eosinophils upon addition of stem cell factor (SCF) (50 ng/mL), FLT-3 ligand (FTL-3L)(50 ng/mL), granulocyte-macrophage colony-stimulating factor (GM-CSF) (0.1 nmol/L), interleukin 3 (IL-3) (0.1 nmol/L), and IL-5 (0.1 nmol/L). Every three days, cells were counted and fresh medium was added to a density of 0.5×10^6 cells/ mL. After three days of differentiation, only IL-3 and IL-5 were added to the cells. Neutrophil differentiation was induced upon addition of SCF (50 ng/mL), FLT-3L (50 ng/mL), GM-CSF (0.1 nmol/L), IL-3 (0.1 nmol/L) and G-CSF (30 ng/mL). After 6 days of culture only G-CSF was added to the cells. 20 ng/mL of rapamycin (Biomol International LP, Hamburg, Germany) used to inhibit mTOR activity was freshly added to the cells every 3 or 4 days. Cord blood samples were collected from healthy donors after informed consent was provided according to the Declaration of Helsinki. Protocols were approved by the local ethics committee of the University Medical Center in Utrecht.

Flow cytometric analysis of myeloid progenitors

Hematopoietic progenitors were isolated as described by Manz *et al* (25). In short, $CD34^+$ cells were isolated as described above and cultured for 2 days in the presence of SCF, FLT-3L, GM-CSF, IL-3 and G-CSF in absence or presence of rapamycin. Cells were subsequently washed and resuspended in PBS/5% FCS and incubated for 30 minutes on ice with a mixture of antibodies (Becton Dickinson, Alphen a/d Rijn, the Netherlands). Lineage markers included CD2, CD3, CD4, CD7, CD8, CD14, CD19, CD20 and CD235a. Myeloid progenitors are negative for these lineage markers. The lineage negative (Lin^-), $CD34^+$, and $CD38^-$ populations consist of hematopoietic stem cells (HSC). Lin^-, $CD34^+$, $CD38^+$, $CD123^+$, and $CD45RA^-$ cells are common myeloid progenitors (CMP), whereas Lin^-, $CD34^+$, $CD38^+$, $CD123^+$, and $CD45RA^+$ cells are granulocyte-macrophage progenitors (GMP) and Lin^-, $CD34^+$, $CD38^+$, $CD123^-$, and $CD45RA^-$ cells are megakaryocyte-erythrocyte progenitors (MEP). Different HSC, CMP, and GMP populations were analysed using a FACS ARIA (from Becton Dickinson). Appropriate isotype-matched, control antibody staining was used to determine the level of background staining.

Measurement of apoptosis

Apoptotic cells were measured by staining with Annexin V (Alexis, Leiden, The Netherlands) according to the manufacturer's protocol. Necrotic cells were visualized in the same assay by staining with propidium iodide.

Colony-forming unit assay

Freshly isolated $CD34^+$ cells were used in colony-forming unit (CFU) assays. Cells were plated in IMDM supplemented with 35.3% FCS, 44.4% methylcellulose-based medium called Methocult (StemCell Technologies, Vancouver, Canada), 11.1 µmol/L of β-mercaptoethanol, 2.2 units/mL of penicillin, 2.2 µg/mL of streptomycin, and 0.44 mmol/L of glutamine at a density of 500 cells/well. CFU assays were done in the presence of SCF (50 ng/mL), FLT-3L (50 ng/mL), GM-CSF (0.1 nmol/L), IL-3 (0.1 nmol/L), and G-CSF (0.2 nmol/L). Colonies were scored after 7 days of culture.

Histochemical staining of hematopoietic cells

May-Grunwald Giemsa staining was used to analyze myeloid differentiation. Cytospins were prepared from 5×10^4 differentiating granulocytes and were fixed in methanol for 3 minutes. After fixation, cytospins were stained in a 50% Eosin Methylene Blue solution according to May-Grunwald (Sigma-Aldrich GmbH, Seelze, Germany) for 20 minutes, rinsed in water for 5 seconds, and the nuclei were counterstained with 10% Giemsa solution (Merck kGaA, Darmstadt, Germany) for 15 minutes. During eosinophil

differentiation, cells could be characterized as differentiating from myeloblasts towards pro-myelocyte type I, pro-myelocyte type II, myelocyte, meta-myelocyte, and finally mature eosinophils with segmented nuclei. These stages can be distinguished by the size of the cells, ratio of cytoplasm versus nucleus present, presence of azurophilic granules, appearance of eosinophilic granules and the shape of the nuclei. Differentiated eosinophils were characterized as cells belonging to the stages of myelocyte, metamyelocyte and mature eosinophils. Neutrophil differentiation can also be characterized by distinct stages from myeloblast, promyelocyte I, promyelocyte II, myelocyte, and metamyelocytes towards neutrophils with banded or segmented nuclei. Differentiated neutrophils were characterized as cells containing either banded or segmented nuclei. Micrographs were acquired with an Axiostar plus microscope (Carl Zeiss, Sliedrecht, the Netherlands) fitted with a 100x/1.3 NA EC Plan Neofluor oil objective using Immersol 518F oil (Carl Zeiss), a Canon Powershot G5 camera (Canon Nederland, Hoofddorp, the Netherlands), and Canon Zoombrowser EX image acquisition software. Photoshop CS3 was used for image processing (Adobe Systems Benelux, Amsterdam, the Netherlands). A minimum of 100 cells per cytospin were counted in two to three randomly selected microscopy fields.

Western blot analysis

Western blot analysis was performed using standard techniques. In brief, differentiating granulocytes were lysed in Laemmli buffer (0.12 M Tris HCl pH 6.8, 4% SDS, 20% glycerol, 0.05 µg/µL bromophenol blue, and 35 mM β-mercaptoethanol) and boiled for 5 minutes. Equal amounts of total lysate were analyzed by 12% SDS-polyacrylamide gel electrophoresis. Proteins were transferred to Immobilon-P and incubated with blocking buffer (Tris buffered saline/Tween-20) containing 5% low-fat milk for 1 hour before incubating with antibodies against PKB (Cell Signaling Technology, Beverly, MA, USA) or Tubulin (Sigma) overnight at 4°C in the same buffer. Before incubation with an antibody against phosphorylated PKB, phosphorylated S6, phosphorylated p70S6kinase or phosphorylated eIF4B (all obtained from Cell Signaling Technology, Beverly, MA, USA) for 16 hours at 4°C, blots were incubated for 1 hour in blocking buffer containing 5% bovine serum albumin (BSA). Blots were subsequently incubated with peroxidase conjugated secondary antibodies for 1 hour. Enhanced chemical luminescence (ECL) was used as a detection method according to the manufacturer's protocol (Amersham Pharmacia, Amersham, UK).

^{3}H-thymidine incorporation assays

A quantity of 80.000 CD34⁺ cells, resuspended in normal culture medium (see "Isolation and culture of human CD34⁺ cells), were incubated with 1 µCi/mL ^{3}H-thymidine for 72 hours at days 0, 7 and 10. The amount of ^{3}H-thymidine incorporated was analyzed after 3 days of culture.

Single-cell proliferation assay

For single-cell proliferation assays, freshly isolated CD34⁺ cells were seeded in 60-wells plates (Nunc, Kamstrup, Denmark) by limiting dilution at a density of 1 cell per well in 20 µL of normal culture medium containing SCF, FLT-3L, GM-CSF, IL-3, and G-CSF in presence or absence of 20 ng/mL rapamycin. Per condition, a total number of 120 wells was scored for both colony size and number of colonies after 7 days of incubation.

Proliferation assays

For proliferation assays, freshly isolated CD34⁺ cells were first resuspended in normal culture medium containing 2 µM of LavaCell (Active Motif, Rixensart, Belgium), a commercially available non-toxic fluorescent cell stain, and stained for 15 minutes at 37°C. Subsequently cells were washed twice and resuspended in normal culture medium containing SCF, FLT-3L, GM-CSF, IL-3 and G-CSF in absence or presence of rapamycin. Proliferation was visualised as the decrease of fluorescent LavaCell-probe per cell as measured by flow cytometric analysis.

Statistics

An independent sample t test for was performed to compare the differences in proliferation, differentiation, and annexin positive cells between the controls and rapamycin treated cells. A p value of 0.05 or less was considered significant (*).

RESULTS

Inhibition of rapamycin-sensitive mTOR signalling decreases cellular expansion of granulocyte progenitors during myelopoiesis

In order to determine whether mTOR activity plays a critical role in regulation of myelopoiesis, an ex-*vivo* differentiation system was utilized. Human CD34⁺ hematopoietic progenitor cells, isolated from umbilical cord blood, were cultured in the presence of either granulocyte colony-stimulating factor (G-CSF) or Interleukin (IL)-3 and IL-5 to induce neutrophil and eosinophil differentiation, respectively. Cells were cultured either in absence or presence of rapamycin,

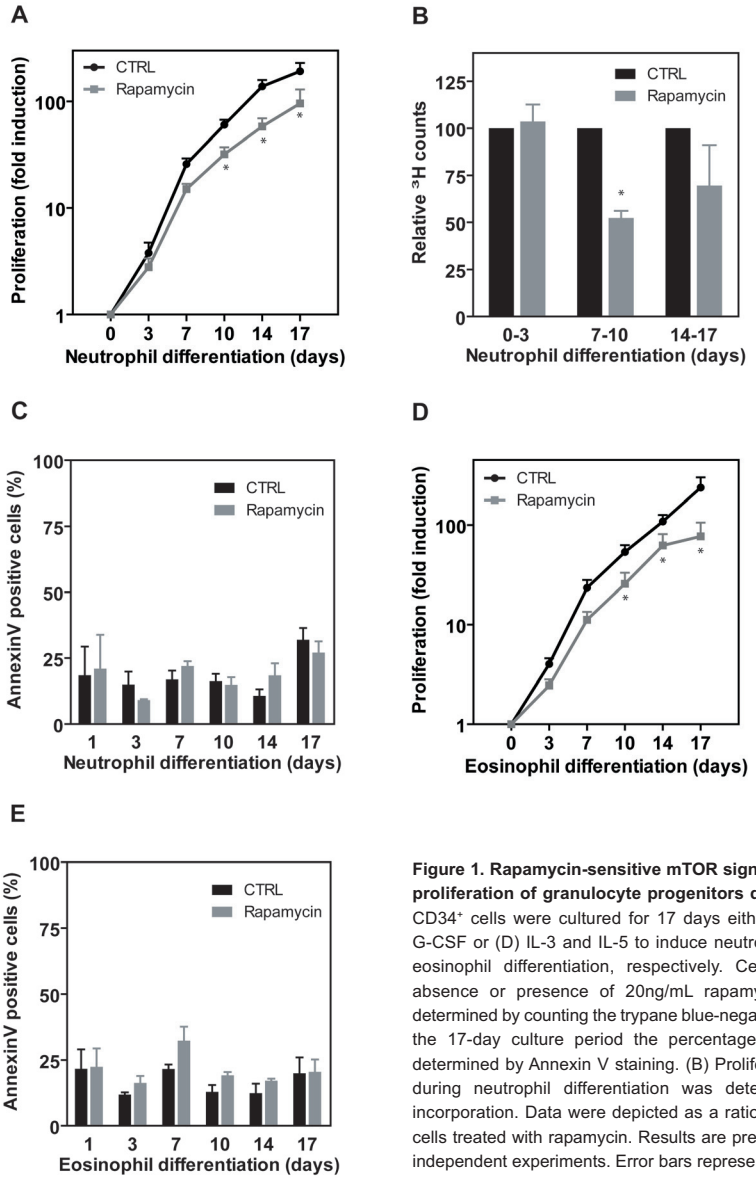

Figure 1. Rapamycin-sensitive mTOR signalling is required for proliferation of granulocyte progenitors during myelopoiesis. $CD34^+$ cells were cultured for 17 days either in presence of (A) G-CSF or (D) IL-3 and IL-5 to induce neutrophil differentiation or eosinophil differentiation, respectively. Cells were cultured in absence or presence of 20ng/mL rapamycin. Expansion was determined by counting the trypane blue-negative cells. (C/E) During the 17-day culture period the percentage apoptotic cells was determined by Annexin V staining. (B) Proliferation of $CD34^+$ cells during neutrophil differentiation was determined ^{3}H-thymidine incorporation. Data were depicted as a ratio between control and cells treated with rapamycin. Results are presented as means of 4 independent experiments. Error bars represent SEM.

a specific pharmacological inhibitor of mTOR, and differences in expansion were determined both by analysis of the number trypan blue negative cells as well as by performing ^{3}H-thymidine incorporation assays. Rapamycin reduced expansion during neutrophil differentiation (Figure 1A, 1B). To determine whether rapamycin mediated inhibition of expansion was due to enhanced levels of apoptosis, the percentage of Annexin-V positive cells was analyzed. Inhibition of mTOR activity did not significantly affect the levels of

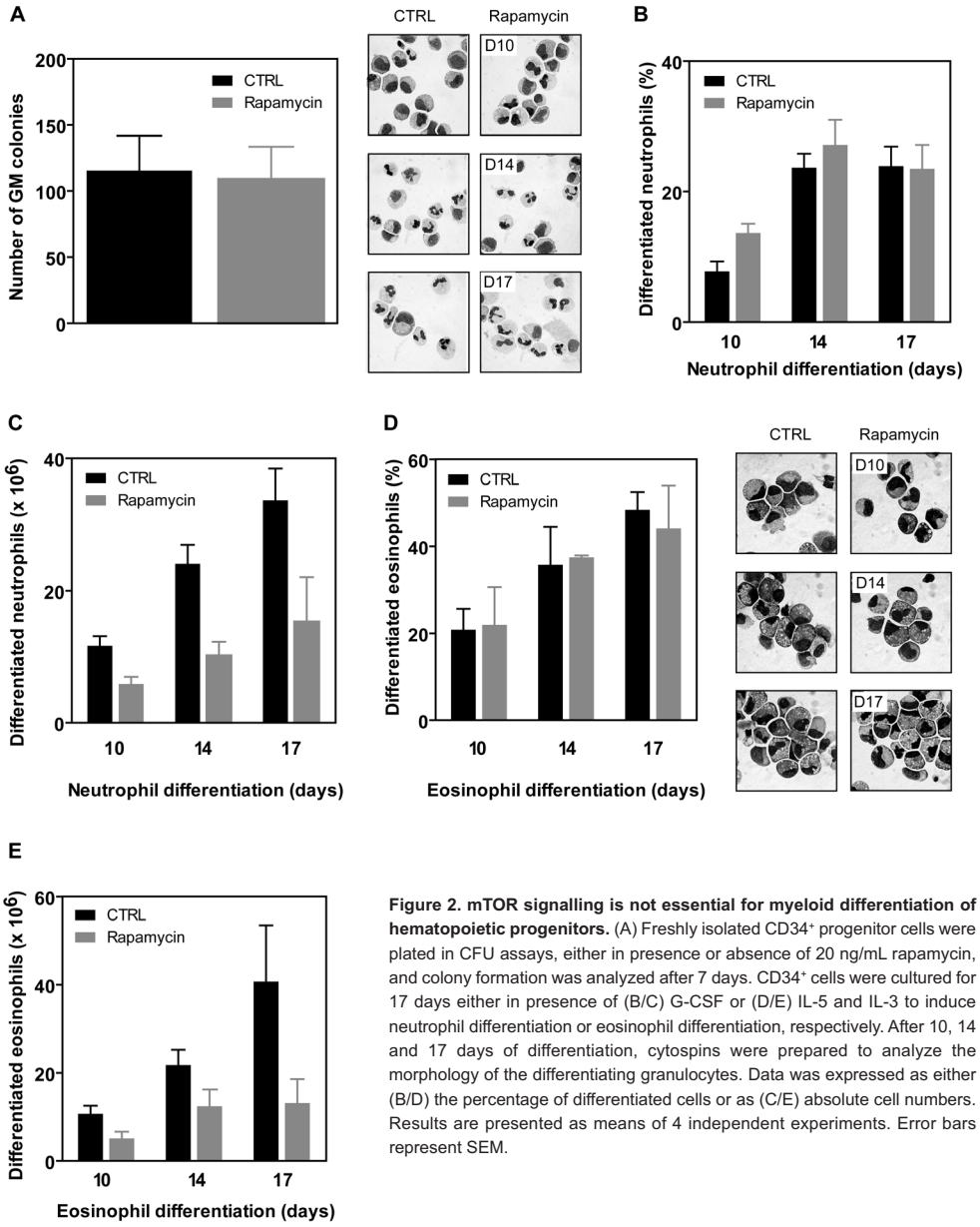

Figure 2. mTOR signalling is not essential for myeloid differentiation of hematopoietic progenitors. (A) Freshly isolated CD34⁺ progenitor cells were plated in CFU assays, either in presence or absence of 20 ng/mL rapamycin, and colony formation was analyzed after 7 days. CD34⁺ cells were cultured for 17 days either in presence of (B/C) G-CSF or (D/E) IL-5 and IL-3 to induce neutrophil differentiation or eosinophil differentiation, respectively. After 10, 14 and 17 days of differentiation, cytospins were prepared to analyze the morphology of the differentiating granulocytes. Data was expressed as either (B/D) the percentage of differentiated cells or as (C/E) absolute cell numbers. Results are presented as means of 4 independent experiments. Error bars represent SEM.

Annexin-V positive cells during neutrophil differentiation (Figure 1C). Similar to neutrophil differentiation, inhibition of mTOR reduced progenitor expansion during eosinophil development (Figure 1D), without altering progenitor survival (Figure 1E). These results demonstrate that inhibition of mTOR decreases cellular expansion of neutrophil and

eosinophil progenitors during differentiation.

mTOR signalling is not essential for myeloid differentiation of hematopoietic progenitors

In order to investigate the clonogenicity of distinct hematopoietic progenitor populations, CFU assays were performed, either in absence or presence of rapamycin, and colony formation was analyzed after 7 days. Inhibition of mTOR did not affect the Granulocyte-Macrophage (GM) colony formation capacity of hematopoietic progenitor cells (Figure 2A). To determine whether mTOR plays a role in regulating terminal maturation, $CD34^+$ progenitor cells were differentiated towards neutrophils or eosinophils for 17 days either in the presence or absence of rapamycin. After 10, 14 and 17 days of culture, cytospins were prepared and the morphology of the differentiating granulocytes was analyzed as described in Materials and Methods. Treatment of hematopoietic progenitor cells with rapamycin did not significantly alter the percentages of mature neutrophils or eosinophils (Figure 2B, 2D). However, since rapamycin inhibited progenitor expansion during both neutrophil and eosinophil development, the absolute numbers of mature neutrophils and eosinophils were also reduced (Figure 2C, 2E). Together these results show that rapamycin-sensitive mTOR signaling is not involved in regulation of early lineage development or terminal maturation.

Rapamycin-sensitive mTOR is not implicated in the regulation of cell-size of myeloid progenitors

mTOR activity has been implicated in the regulation of cell-size in a variety of cell lineages (26). In order to investigate whether mTOR is also involved in regulation of the cell size of hematopoietic progenitors, human $CD34^+$ progenitors were cultured for 2 days in the presence of SCF, FLT-3L, GM-CSF, IL-3 and G-CSF either in absence or presence of rapamycin and myeloid progenitors were analyzed by multilineage flow cytometry. Different hematopoietic progenitor populations, including HSC, CMP, GMP, MEP, and Lineage positive (Lin^+) cells can be distinguished by a combination of extra-cellular lineage markers as described in Material and Methods (Figure 3A). Differences in cell-size were determined by analysis of the forward scatter (FSC-H). Analysis of distinct myeloid progenitor populations revealed that inhibition of mTOR by rapamycin did not result in a significant change in overall cell-size (Figure 3B, 3C). These results suggest that rapamycin-sensitive mTOR is not implicated in the regulation of cell-size of myeloid progenitors.

mTOR activity regulates expansion of hematopoietic progenitor cells during myeloid differentiation in a stage-specific manner

To further investigate the role of mTOR in regulating expansion during myelopoiesis, single-cell proliferation assays were performed to evaluate the expansion capacity of individual hematopoietic progenitors. Freshly isolated $CD34^+$ cells were single cell plated in culture medium containing G-CSF in presence or absence of rapamycin. After 7 days, wells with colonies were scored (Figure 4). Treatment with rapamycin resulted in a decreased number of colonies, which were also reduced in size, showing that mTOR is involved in the regulation of expansion of myeloid progenitors.

To further dissect how the mTOR pathway regulates progenitor expansion, $CD34^+$ progenitor

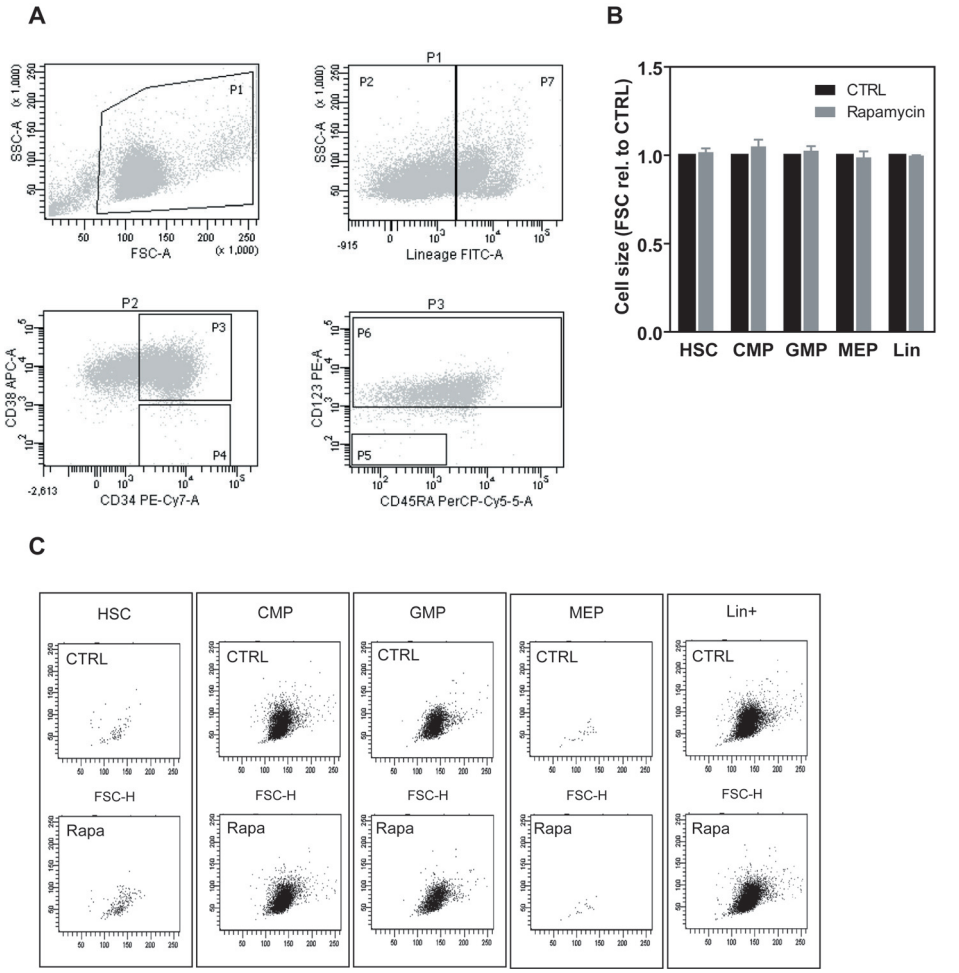

Figure 3. Rapamycin-sensitive mTOR is not implicated in the regulation of cell-size of myeloid progenitors. (A) CD34⁺ cells were cultured for 2 days in the presence of SCF, FLT-3 ligand, GM-CSF, IL-3 and G-CSF, either in absence or presence of rapamycin and different hematopoietic progenitor populations, including HSC, CMP, GMP, MEP, and Lin⁺ cells were analyzed by multilineage flow cytometry. Analysis of the distinct progenitor populations was performed as depicted in panel A. (B) Differences in cell-size were determined by analysis of the value of the FSC-H in all individual progenitor populations. Data was expressed as a ratio between controls and cells treated with rapamycin. Results are presented as means of 4 independent experiments. Error bars represent SEM. (C) The FSC-H plots of all individual hematopoietic progenitor populations are depicted. One representative experiment is shown.

cells were differentiated towards neutrophils in the presence or absence of rapamycin. After 3 and 7 days of culture, cells were plated in CFU assays, and colony formation was analyzed after 7 days of culture. Interestingly, GM colony formation of CD34⁺ cells that had been cultured in presence of rapamycin for 7 days was significantly increased compared to control CD34⁺ cells (Figure 5A). These data suggest that rapamycin selectively inhibits expansion

and thereby preserves the clonogenic capacity of a specific population of progenitors.

To define what cell population is the target of rapamycin, freshly isolated CD34$^+$ cells were stained with LavaCell, a fluorescent dye, and cultured either in presence or absence of rapamycin in the presence of SCF, FLT-3L, GM-CSF, IL-3 and G-CSF. Subsequently, after 3 days of culture, myeloid progenitor populations were analyzed by flow cytometry and the level of proliferation was visualised by the decrease in the mean fluorescent intensity of LavaCell. Inhibition of mTOR activity significantly decreased proliferation of Lin$^+$ CD34$^+$ hematopoietic cells (Figure 5B), while expansion of HSC and CMP/GMP populations (Figure 5D, 5E) and the more differentiated Lin$^+$ progeny that no longer expresses the CD34 (Figure 5C) was not altered upon treatment with rapamycin. Together, these observations show that rapamycin selectively inhibits expansion of the most-committed CD34$^+$ progenitors, indicating that rapamycin-sensitive mTOR activity regulates proliferation of hematopoietic progenitor cells during myeloid differentiation in a stage-specific manner.

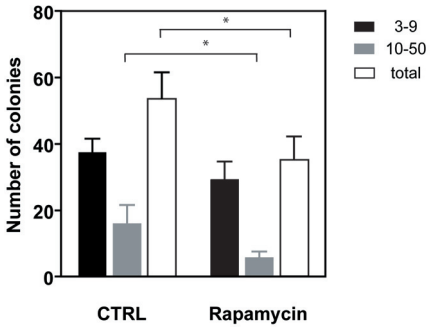

Figure 4. mTOR activity regulates expansion of myeloid progenitor cells. Freshly isolated CD34$^+$ cells were seeded in 60-wells plates at a density of 1 cell per well (120 wells in total) in normal culture medium containing G-CSF in presence or absence of 20 ng/mL rapamycin. After 7 days, wells with colonies were scored. Results are presented as means of 4 independent experiments. Error bars represent SEM.

Inhibition of mTOR activity by rapamycin differentially regulates PKB and mTOR signalling during granulopoiesis

To investigate the effect of rapamycin on mTOR signalling during granulopoiesis, CD34$^+$ cells were cultured in presence of G-CSF to induce neutrophil differentiation in presence or absence of rapamycin. After 3, 10 and 17 days of culture, protein lysates were made and Western blot analysis was performed using an antibody against phosphorylated p70S6K and phosphorylated S6, a direct substrate of the mTOR substrate p70S6K (Figure 6A). S6 phosphorylation levels were low early during neutrophil differentiation, but increased after 10 to 17 days of differentiation. However, phosporylation of both p70SK and S6 was inhibited or completely blocked in cells cultured in presence of rapamycin for 10 or 17 days, respectively. However, no effect of rapamycin on S6 phosphorylation could be detected after 3 days of culture. In addition, rapamycin treatment resulted in a block in PKB phosphorylation at day 10, while phosphorylation of PKB at day 3 of culture in the presence of rapamycin was induced compared to control cells and was unaffected at day 17. In addition, rapamycin treatment resulted in a decrease in phosphorylation of eIF4B, a downstream target of PKB (27), at day 10 (Figure 6B). In contrast, G-CSF-induced PKB phosphorylation is not inhibited upon short exposure to rapamycin at the same time point (Figure 6C). These observations suggest that the effects of rapamycin might be due to differential regulation of PKB and mTOR signalling.

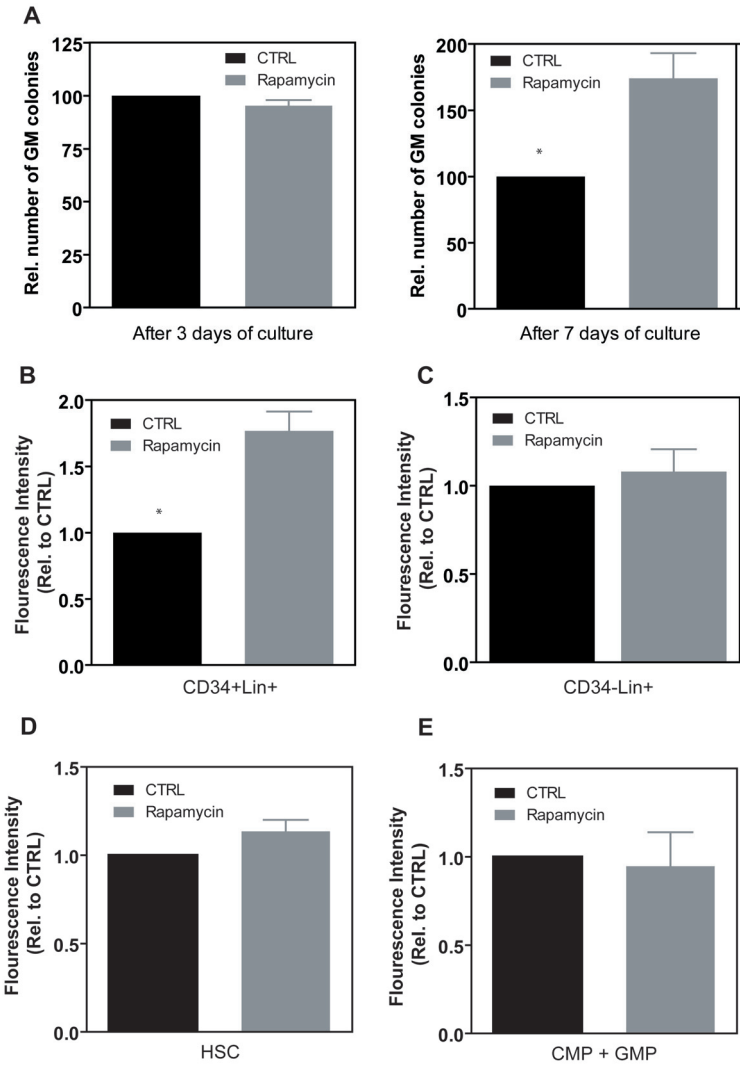

Figure 5. mTOR activity regulates proliferation of hematopoietic progenitor cells during myeloid differentiation in a stage-specific manner. (A) CD34⁺ progenitor cells were differentiated towards neutrophils in the presence or absence of rapamycin. After 3 and 7 days of culture, cells were plated in CFU assays, and colony formation was analyzed after another 7 days of culture. (B-E) Freshly isolated CD34⁺ cells were stained with LavaCell, a fluorescent dye, and cultured either in presence or absence of rapamycin in the presence of SCF, FLT-3 ligand, GM-CSF, IL-3 and G-CSF for 3 days. Different myeloid progenitor populations, including (B) $CD34^+Lin^+$, (C) $CD34^-Lin^+$, (D) HSC, and (E) CMP/GMP cells were analyzed by flow cytometry. Proliferation was visualised by the decrease in the mean fluorescent intensity per cell of LavaCell. Results are presented as means of 4 independent experiments. Error bars represent SEM.

DISCUSSION

Although mTOR signalling has been demonstrated to play an important role in a plethora of cellular processes, a role in the regulation of myelopoiesis remains relatively unexplored. In this study, we have investigated the role of mTOR in regulation of myelopoiesis utilizing a human ex-*vivo* granulocyte differentiation system. Our results show that rapamycin-sensitive mTOR signalling plays an important role in the regulation of expansion of hematopoietic progenitors during myelopoiesis in a stage-specific manner.

mTOR is known as a regulator of cell cycle progression and proliferation (28). In B and T lymphocytes, rapamycin induces a G1-phase arrest and can therefore be used as a potent anti-proliferative drug (29). In most other cell types, however, rapamycin reduces the proliferation rate by a delay in cell cycle progression. For example, proliferation of human endothelial progenitors, epithelial cells, osteoblasts and myoblasts is inhibited by rapamycin (19-22). Our data demonstrates that mTOR activity is also required for proliferation of hematopoietic progenitors during myelopoiesis. Progenitor expansion in the presence of rapamycin was significantly reduced as measured by performing ^{3}H-thymidine incorporation experiments (Figure 1B) and single cell proliferation assays (Figure 4). Cell cycle analysis of myeloid progenitors showed that rapamycin does not significantly alter cell cycle distribution (data not shown), suggesting that the observed inhibition of proliferation was due to a delay

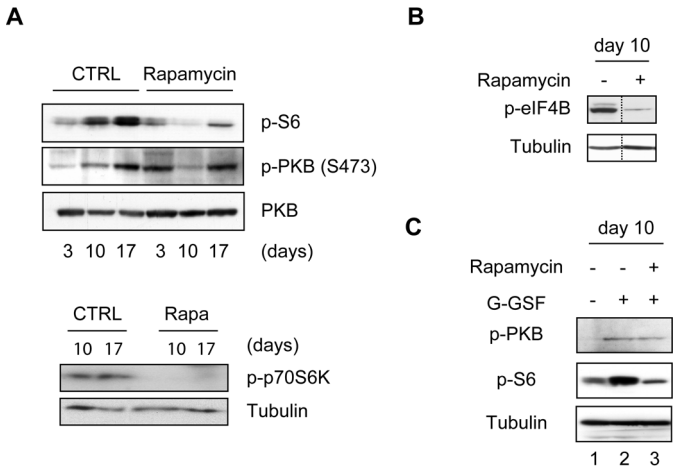

Figure 6. Inhibition of mTOR activity by rapamycin differentially regulates of PKB and mTOR signalling during granulopoiesis. (A) CD34^{+} cells were cultured in presence of G-CSF to induce neutrophil differentiation, either in presence or absence of rapamycin. After 3, 10 and 17 days of culture, protein lysates were made and Western blot analysis was performed using an antibody against phosphorylated p70S6K, phosphorylated S6, phosphorylated PKB or total PKB as a control for equal loading. (B) CD34^{+} cells were cultured in presence of G-CSF to induce neutrophil differentiation, either in presence or absence of rapamycin. After 10 days of culture, protein lysates were made and Western blot analysis was performed using an antibody against phosphorylated eIF4B or tubulin as a control for equal loading. (C) CD34^{+} cells were in presence of G-CSF to induce neutrophil differentiation. After 10 days of culture, cells were left untreated (lane 1, 2) or treated with rapamycin (lane 3) for 3 hours before stimulation with G-CSF (lane 2, 3) for 15 minutes. Protein lysates were prepared and Western blot analysis was performed with an antibody against phosphorylated PKB, phosphorylated S6, and as a control for equal loading an antibody against tubulin. Similar results were obtained in 3 independent experiments.

in cell cycle progression rather than arrest in G0/G1 phase. mTOR mediates cell growth at the translational level through phosphorylation of p70S6K and 4E-BP1, two essential regulators of ribosome biogenesis and translation initiation. Ectopic expression of p70S6K and eIF4E in quiescent U2OS osteosarcoma cells, which are stimulated with serum to enter G1 phase, accelerates entry into S phase, whereas reduction of p70S6K expression with RNAi or expression of a dominant-negative 4E-BP1 mutant inhibits the rate of S phase entry (30). In addition, ectopic expression of eIF4E or rapamycin-resistant mutants of p70S6K partially rescues the rapamycin-induced delay in cell cycle progression, indicating that p70S6K and eIF4E are important mediators of mTOR-dependent cell division (30). It has been demonstrated that mTOR regulates the translation of proteins involved in G1/S transition during cell cycle progression, including retinoblastoma protein, cell-cycle inhibitors of the Cip/Kip family p21, p27, and cyclin D or E (31-36). Thus, it is likely that mTOR also regulates hematopoietic progenitor expansion at the translational level by regulating translation of cell-cycle modulating proteins.

Recently, Fingar *et al* demonstrated that rapamycin not only delays proliferation, but is also involved in regulation of cell-size. Although rapamycin treated osteosarcoma cells are significantly smaller in size, proliferation is not completely blocked (37), indicating that cell division does not require a fixed size. It has been suggested that both cell size and proliferation can be regulated by the same mTOR-dependent downstream effectors (38). A reduction of, for example, S6K1 expression in osteosarcoma cells has been demonstrated to both delay proliferation and to reduce cell-size (30). However, a reduction in energy sources results in an inhibition of cell-size in yeast, but does not block cell division (39). In addition, the cell-size of rat neuronal cells varies depending on the level of extracellular growth factors, whereas proliferation is unaltered (40). Moreover, deletion of p70S6K in mouse myoblasts mimics the inhibitory effect of rapamycin on cell size but not on proliferation (22). Our data demonstrates that mTOR activity is essential for proliferation of myeloid progenitors, whereas cell-size appears not to be regulated by mTOR (Figure 3), indicating that in certain cell lineages cell cycle and cell size are controlled by multiple independent signal transduction pathways.

Analysis of different progenitor cells revealed that rapamycin differentially affects the diverse populations. Inhibition of mTOR activity significantly decreased proliferation of $CD34^+$ Lin^+ hematopoietic cells, whereas proliferation of $CD34^-$ Lin^+, HSC, CMP and GMP populations was unaffected (Figure 5B-E). These observations suggest that the response of hematopoietic progenitors to rapamycin is dependent on the stage of differentiation. It is likely that the observed resistance to rapamycin of the early hematopoietic progenitors is due to a reduced dependency on the mTOR pathway compared to the more committed $CD34^+Lin^+$ hematopoietic cells. Correspondingly, recent analysis of TSC1 deficient mice revealed that constitutive mTORC1 activation induces severe multi-lineage defects including anemia and progenitor expansion (41) These data, combined with our results, suggest that correct regulation of mTOR activity is critical for optimal progenitor expansion. Zeiser *et al* recently showed that regulatory T cells and conventional T cells also display differential expansion kinetics upon exposure to rapamycin. Expression of phosphatase and tensin homolog deleted on chromosome 10 (PTEN), a negative regulator of the PI3K/PKB/mTOR pathway, was found to remain high in regulatory T cells but not in conventional T cells during

stimulation, which may explain the difference in susceptibility to rapamycin between these T cell populations (42). Furthermore, our results suggest that the effect of rapamycin on progenitor expansion during granulopoiesis might be due to differential regulation of PKB and mTOR activity (Figure 6). It has, for example, been demonstrated that modulation of PKB activity regulates the sensitivity of glioblastoma cells to the mTOR inhibitors rapamycin and CCI-779 by expression of cyclin D1 and c-myc, two proteins found to be essential for cell cycle transit (43). Furthermore, we have previously demonstrated that PKB activity is indeed critical for expansion of hematopoietic progenitors during myelopoiesis (3). Although regulation of PKB activity is believed to be mediated by mTORC2, which is insensitive to rapamycin, Sarbassov *et al* demonstrated that treatment with rapamycin can result in either increased or decreased levels of PKB phosphorylation, depending on the cell type (44). A possible explanation for this could be that, although rapamycin cannot inhibit mTORC2, it can associate with free mTOR, thereby preventing the association with Rictor and assembly of the mTORC2 complex (45). Alternatively, differential expression of PTEN or other components of the PI3K/PKB/mTOR pathway, such as Raptor, may explain the difference in susceptibility of distinct hematopoietic progenitors to rapamycin.

Constitutive activation of PI3K and its downstream effectors PKB and mTOR have also been implicated in the pathogenesis of a variety of hematopoietic malignancies, including acute myeloid leukemia (46)(47). It has been demonstrated that tumors displaying enhanced expression or activation of the PI3K signalling module, are highly sensitive to rapamycin and its analogs CCI-779 and RAD001 (48)(49). Low doses of rapamycin were found to inhibit colony formation of AML progenitors, suggesting that inhibition of the mTOR pathway could be of clinical interest in AML (50)(51). However, recent studies also demonstrated that in AML samples with constitutive PI3K-PKB activation, rapamycin treatment resulted in increased PKB phosphorylation, suggesting the need for dual inhibition of both the mTORC and the PI3K-PKB pathway in treatment of this disease (52)(53).

Our results demonstrate that mTOR selectively regulates proliferation of $CD34^{+}Lin^{+}$ hematopoietic progenitors, while expansion of HSC and CMP/GMP populations is not affected. In addition, mTOR signalling appears not to be essential for differentiation of myeloid progenitors. Taken together, our data suggest that modulation of the mTOR pathway could play an important role in future therapies to control hematological malignancies.

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Chapter 6

Ectopic expression of C/EBPα and ID1 is sufficient to restore defective neutrophil development in low-risk myelodysplasia

Christian R Geest[1], Miranda Buitenhuis[1], Edo Vellenga[2], and Paul J Coffer[1,3]

[1] Molecular Immunology Lab, Department of Immunology, University Medical Center, Utrecht, The Netherlands, [2] Department of Hematology, University Medical Center, Groningen, The Netherlands, [3] Department of Pediatric Immunology, University Medical Center, Utrecht, The Netherlands

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ABSTRACT

In patients with myelodysplasia (MDS), a general defect in the multipotent stem-cell compartment results in a disturbed proliferation and differentiation of the erythroid, megakaryocytic and myeloid lineages. Although a number of genetic defects in MDS progenitors have been described, the intracellular signalling pathways underlying aberrant regulation of myelopoiesis remain relatively undefined. Here, an ex-*vivo* differentiation system was used to selectively screen for molecules improving defective hematopoiesis in MDS $CD34^+$ progenitor cells. Bone marrow derived $CD34^+$ cells isolated from low-risk MDS patients showed impaired capacity to proliferate and differentiate as well as increased levels of apoptosis. In an attempt to improve the expansion and differentiation of MDS $CD34^+$ progenitors, cells were treated with the p38MAPK pharmacological inhibitor SB203580, or retrovirally transduced to ectopically express active protein kinase B (PKB/c-akt), or the transcriptional regulators STAT5, C/EBPα or ID1. Wheras treatment of progenitors with SB203580, PKB or STAT5 did not enhance neutrophil development, ID1 and C/EBPα transduced cells exhibited increased granulocyte/macrophage (GM) colony formation. Furthermore, ectopic expression of C/EBPα resulted in improved neutrophil maturation.These data suggest that targeting the ID1 and C/EBPα transcriptional regulators may be of benefit in the design of novel therapies for low risk MDS.

INTRODUCTION

Myelodysplastic syndromes (MDS) are defined as clonal stem-cell disorders characterized by ineffective hematopoiesis and with an increased risk of transformation to acute myeloid leukemia (AML) (1). Leukemic transformation from normal stem cells is believed to be a multistep process during which a normal hematopoietic stem cell acquires multiple genetic and epigenetic abnormalities that ultimately lead to malignant transformation and clonal expansion (2)(3). Expansion of the aberrant clone is characterized by morphological dysplasia, impaired differentiation and defective cellular functions, resulting in peripheral cytopenias that frequently involve the erythroid, myeloid, and megakaryocytic lineages (4). Programmed cell death is often up-regulated in early MDS, due to the enhanced proliferative capacity of MDS clones and may also contribute to the peripheral cytopenias. Progression to leukemia is usually associated with abrogation of apoptosis (3). The clinical symptoms resulting from these cellular defects are transfusion-dependent anemia, an increased risk of infection or hemorrhage, and a potential progression to AML (5).

The myelodysplastic syndromes can be classified into high-risk or low-risk groups according to the French American British (FAB) or the more recently established World Health Organisation (WHO) classification systems (6)(7). These classifications are based on the number of blast cells in the bone marrow and peripheral blood, the morphology of cells and cytogenetic abnormalities. Progression towards leukemia is rare in low-risk MDS and life expectancy is relatively high, whereas in the high-risk groups progression rates towards AML are significantly higher (8).

Cytogenetic abnormalities are common and are observed in about half the cases of primary MDS and in 90% of secondary, therapy-related MDS (9). The abnormalities observed in MDS are predominantly specific chromosomal deletions, suggesting a pathogenic mechanism based on loss of tumor suppressor genes or of genes necessary for normal hematopoiesis. The most frequently observed cytogenetic abnormalities in MDS include loss of chromosome 7 or partial deletions of chromosome arms 5q, 20q, or 7q (10-12). While the majority of putative tumor suppressors in MDS remain unknown, several chromosomal translocation-mediated oncogenes and tumor suppressors have been identified. Gene inactivation comprises a relatively small number of MDS cases and includes p53, RB, NF1, C/EBPα, and nucleophosmin (13-19). In addition, activating mutations in the RAS proto-oncogene, FLT3 duplications, loss-of-function point mutations in the gene encoding the AML1/RUNX1 transcription factor and p15 promoter hypermethylation have also been associated with disease progression to AML (20-23). However, none of these observed alterations is specific for MDS and the underlying molecular causes of the disease remain poorly understood.

Although a number of genetic defects in MDS progenitors have been described, the intracellular signalling pathways underlying deregulation of myelopoiesis have scarcely been investigated thus far. Through identification of the intracellular components responsible for dysfunctional hematopoiesis it will be possible to develop novel treatment strategies for MDS and AML.

To investigate defects in intracellular signaling pathways in MDS $CD34^+$ hematopoietic progenitor cells, we developed an ex-*vivo* hematopoiesis culture system. Bone marrow derived $CD34^+$ cells isolated from low-risk MDS donors showed impaired capacity to

proliferate and differentiate as well as increased levels of apoptosis, thus mimicking published observations in MDS bone marrow *in vivo*. In an attempt to improve the proliferation, survival and differentiation of MDS CD34$^+$ progenitors, cells were treated with the p38MAPK pharmacological inhibitor SB203580, or retrovirally transduced to ectopically express active protein kinase B (PKB/c-akt), or the transcriptional regulators STAT5, C/EBPα or ID1. In contrast to treatment of progenitors with either SB203580, PKB or STAT5 treated progenitors, which was not sufficient to induce hematopoiesis, cells ectopically expressing either ID1 or C/EBPα exhibited increased granulocyte/macrophage (GM) colony formation. In addition, ectopic expression of C/EBPα dramatically improved neutrophil maturation. These data suggest that targeting the transcriptional regulators ID1 and C/EBPα may be of benefit in the design of novel therapies for low risk MDS.

MATERIALS AND METHODS

Isolation and culture of human CD34$^+$ cells

Mononuclear cells were isolated from bone marrow of healthy subjects and MDS patients by density centrifugation over a ficoll-paque solution (density 1.077 g/mL). MACS immunomagnetic cell separation (Miltenyi Biotech, Auburn, CA) using a hapten conjugated antibody against CD34, which was coupled to beads, was used to isolate CD34$^+$ cells. CD34$^+$ cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco, Paisley, UK) supplemented with 9% fetal calf serum (FCS) (Hyclone, Logan, UT), 50 μM β-mercaptoethanol, 10 U/mL penicillin, 10 μg/mL streptomycin, and 2mM glutamine at a density of 0.3 x 10^6 cells/mL. Cells were differentiated towards neutrophils upon addition of stem cell factor (SCF) (50 ng/mL), FLT-3 ligand (50 ng/mL), granulocyte-macrophage colony-stimulating factor (GM-CSF) (0.1 nmol/L), and G-CSF (30 ng/mL). Every three days, cells were counted and fresh medium was added to a density of 0.5 x 10^6 cells/ mL. After three days of differentiation, only G-CSF was added to the cells. Pharmacological inhibitors were freshly added to the cells every 3 or 4 days. 10 μM SB203580 (Alexis Corporation, San Diego, CA) was used to inhibit p38MAPK activity during granulopoiesis.

Patients

Heparinized human bone marrow cells were collected from MDS patients with a mean age of 62 years (range, 37-78 years) after informed consent was obtained in accordance with the Declaration of Helsinki. Bone marrow specimens were obtained at diagnosis before treatment. According to the FAB classification, the patients were categorized as RA (n=3), RARS (n=6) or RAEB (n=1). Patient characteristics are described in Table 1. None of these patients were treated with granulocyte-colony stimulating factor. Normal bone marrow was obtained from orthopaedic surgery patients, after informed consent, before the surgery. The protocols were approved by the human subject review board of the University Medical Center Groningen.

Histochemical staining of hematopoietic cells

May-Grunwald Giemsa staining was used to analyze myeloid differentiation. Cytospins were prepared from 5 x 10^4 differentiating granulocytes and were fixed in methanol for 3 minutes. After fixation cytospins were stained in a 50% Eosin Methylene Blue solution according to May-Grunwald (Sigma-Aldrich GmbH, Seelze, Germany) for 20 minutes, rinsed in water for 5 seconds, and the nuclei were counterstained with 10% Giemsa solution (Merck kGaA, Darmstadt, Germany) for 15 minutes. During neutrophil differentiation, cells could be characterized as differentiating from myeloblast, promyelocyte I, promyelocyte II, myelocyte, and metamyelocytes towards neutrophils with banded or segmented nuclei. These stages can be distinguished by the size of the cells, ratio of cytoplasm versus nucleus present, presence of azurophilic granules and the shape of the nuclei. Differentiated neutrophils were characterized as cells containing either banded or segmented nuclei.

Viral transduction of CD34$^+$ cells

Bicistronic retroviral DNA constructs were utilized, expressing the gene of interest and an Internal Ribosomal Entry Site (IRES) followed by the gene encoding for enhanced green fluorescent protein (eGFP) (LZRS-eGFP) (24-26). Retrovirus was produced by stable transfection of the retroviral packaging cell line, Phoenix-ampho by calcium-phosphate co-precipitation. Cells were plated in 6-cm dishes, 24 hours before transfection. Ten micrograms DNA was used per transfection. Medium was refreshed, 16 hours after transfection. After an additional 24 hours, cells were split into 75 cm^2 culture flasks (Greiner, Frickenhausen, Germany), and 2 μg/mL puromycin was added to the cells. After 2 weeks of selection, cells were grown to a confluence of 90%. Subsequently, cells were grown in a minimal amount of medium for 24 hours. Viral supernatants were collected and filtered through a 0.2 μm filter. CD34$^+$ cells were transduced in 24-well dishes precoated with 1.25 μg/cm^2 recombinant human fibronectin fragment CH-296 (RetroNectin; Takara, Otsu, Japan) overnight at 4°C. Transduction was performed by addition of 0.5 mL viral supernatant to 0.5 mL medium containing 0.3 x 10^6 cells. 24 hours after transduction, 0.7 mL medium was removed from the cells, and 0.5 fresh virus

supernatant was added together with 0.5 mL fresh medium.

Colony-forming unit assay

Freshly isolated CD34$^+$ cells or retrovirally transduced cells, separated from non-transduced cells by flow cytometry, were used in colony-forming unit (CFU) assays. Cells were plated in IMDM supplemented with 35.3% FCS (Hyclone, Logan, UT), 44.4% methylcellulose-based medium called Methocult (StemCell Technologies, Vancouver, Canada), 11.1 µmol/L of β-mercaptoethanol, 2.2 units/mL of penicillin, 2.2 µg/mL of streptomycin, and 0.44 mmol/L of glutamine at a density of 500 cells/well. CFU assays were done in the presence of SCF (50 ng/mL), FLT-3 ligand (50 ng/mL), GM-CSF (0.1 nmol/L), IL-3 (0.1 nmol/L), and G-CSF (0.2 nmol/L). Colonies were scored after 12 days of culture.

Measurement of apoptosis

Apoptotic cells were measured by staining with Annexin V (Alexis, Leiden, The Netherlands) according to the manufacturer's protocol. Necrotic cells were visualized in the same assay by staining with propidium iodide.

Western blot analysis

Western blot analysis was performed using standard techniques. In brief, differentiating granulocytes were lysed in Laemmli buffer (0.12 M Tris HCl pH 6.8, 4% SDS, 20% glycerol, 0.05 µg/µL bromophenol blue, and 35 mM β-mercaptoethanol) and boiled for 5 minutes. Equal amounts of total lysate were analyzed by 12% SDS-polyacrylamide gel electrophoresis. Proteins were transferred to Immobilon-P and incubated with blocking buffer (Tris buffered saline/Tween-20) containing 5% low-fat milk for 1 hour before incubating with an antibody against p38MAPK (Cell Signaling Technology, Beverly, MA, USA) overnight at 4°C in the same buffer. Before incubation with antibodies against phosphorylated p38MAPK or phosphorylated MAPKAPK-2 (Cell Signaling Technology, Beverly, MA, USA) for 16 hours at 4°C, blots were incubated for 1 hour in blocking buffer containing 5% bovine serum albumin (BSA). Blots were subsequently incubated with peroxidase conjugated secondary antibodies for 1 hour. Enhanced chemical luminescence (ECL) was used as a detection method according to the manufacturer's protocol (Amersham Pharmacia, Amersham, UK).

Immunohistochemical staining of hematopoietic cells

Cells were first washed in phosphate-buffered saline (PBS) and resuspended in 100 µL 0.5% formaldehyde. After 15 minutes incubation at 37°C, 900 µL of ice-cold methanol was added to the cells. Cells were washed with PBS after 30 minutes of incubation on ice and resuspended in PBS/5% FCS (Hyclone, Logan, UT). After 10 minutes incubation at room temperature, cells were washed and neutrophil progenitors were incubated with an antibody against phosphorylated p38MAPK (Cell Signaling Technology, Beverly, MA, USA) in PBS containing 5% FCS and incubated for another 30 minutes at 4°C. Cells were washed and subsequently incubated with a phycoerythrin (PE)-conjugated anti-rabbit antibody (Southern Biotechnology Associates, Inc., Birmingham, Ala) for another 30 minutes at 4°C. Cells were again washed cells and were analyzed by FACS (FACS Canto II, Beckton Dickinson, Alphen a/d Rijn, The Netherlands).

Statistics

An independent sample t test for was performed to compare the differences in proliferation, differentiation, and annexin positive cells between the controls and cells transduced with STAT5, myrPKB, ID1, or C/EBPα. The same assay was performed to compare cells cultured either in absence or presence of the pharmacological inhibitor SB203580. A p value of 0.05 or less was considered significant (*).

RESULTS

MDS bone marrow progenitor cells show impaired progenitor expansion and increased levels of apoptosis during neutrophil differentiation

To investigate defects in intracellular signaling pathways in MDS CD34$^+$ hematopoietic progenitor cells, an ex-*vivo* differentiation system was utilized as described in Materials and Methods. Human CD34$^+$ hematopoietic progenitor cells, isolated from bone marrow of healthy subjects and low-risk MDS patients (Table 1), were cultured in the presence of Granulocyte colony-stimulating factor (G-CSF) to induce neutrophil differentiation. Cells were cultured for 17 days and differences in expansion, survival and differentiation were analyzed. Expansion of MDS CD34$^+$ cells was dramatically decreased during neutrophil differentiation compared to healthy controls (Figure 1A). To determine whether this impaired capacity to expand could be due to increased levels of apoptosis, the percentage of Annexin-V positive cells was

Table 1: Patient Characteristics

Patient no.	Age (years)	MDS type	Hb (mmol/l)	Leuco. ($x10^9$/l)	Plat. ($x10^9$/l)	Gran.
1	37	RA	Transf.	7.4	30	40%
2	77	RARS	5.5	6.4	402	38%
3	78	RARS	6.0	5.8	200	40%
4	70	RARS	6.0	4.8	297	42%
5	60	RARS	Transf.	2.9	153	66%
6	58	RARS	Transf.	3.0	173	62%
7	50	RAEB	5.7	3.9	93	22%
8	68	RA	6.5	6.5	218	30%
9	62	RA	6.6	3.9	63	18%
10	78	RARS	6.6	4.8	475	25%

R.A.: refractory anemia; RARS: refractory anemia with ring sideroblasts; RAEB: refractory anemia with excess blasts; Transf: transfusion dependent; Gran: granulocyte percentage in the peripheral blood. None of the patients had cytogenetic abnormalities.

analyzed. Survival of MDS hematopoietic progenitor cells was significantly lower compared to control $CD34^+$ cells during neutrophil development (Figure 1B). These results demonstrate that MDS hematopoietic progenitors have an impaired capacity to proliferate and show decreased survival during neutrophil development ex-*vivo*.

Impaired differentiation and decreased colony formation capacity of MDS bone marrow progenitor cells during myelopoiesis

To determine whether MDS hematopoietic progenitor cells were also impaired in their capacity to differentiate, $CD34^+$ cells were isolated from the bone marrow of healthy subjects and low-risk MDS patients and differentiated towards neutrophils for 17 days. After 14 and 17 days of differentiation, cytospins were prepared to analyze the morphology of the differentiating granulocytes. As expected, MDS bone marrow progenitor cells showed significantly lower percentages of mature neutrophils with banded or segmented nuclei and increased numbers of undifferentiated myeloblasts after 14 and 17 days of neutrophil differentiation (Figure 2A, 2B). In addition, to assess the clonogenic potential of progenitor cells, $CD34^+$ cells of either controls or low-risk MDS patients were plated in CFU assays, and colony formation was analyzed after 12 days of culture. Granulocyte-Macrophage (GM) colony forming units (CFU-GM) of MDS $CD34^+$ cells were reduced compared to control $CD34^+$ cells (Figure 2C). Together these results demonstrate that $CD34^+$ bone marrow cells of low-risk MDS patients have an impaired capacity to proliferate as well as to differentiate and have increased levels of apoptosis during neutrophil development. Importantly, this ex-*vivo* system is representative of published observations in MDS bone marrow *in vivo*.

Inhibition of p38 MAPK does not improve maturation or increase clonogenic capacity of MDS hematopoietic progenitor cells

A recent study reported that p38MAPK is constitutively activated in hematopoietic cells of patients with low-risk MDS (27). Inhibition of p38MAPK activity resulted in decreased apoptosis and stimulated colony formation of primary MDS progenitors, indicating a role for

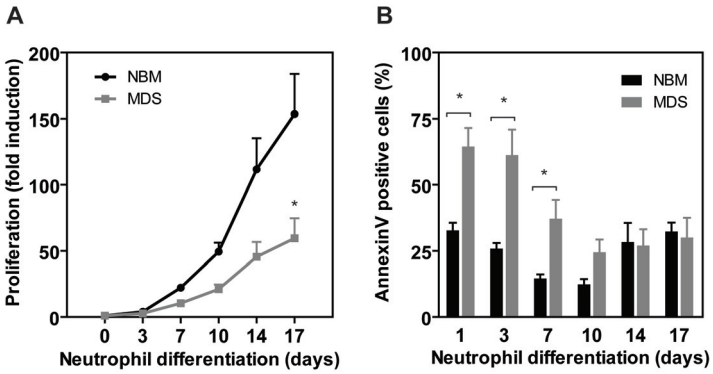

Figure 1. MDS progenitor cells show impaired expansion and increased levels of apoptosis during neutrophil differentiation. CD34⁺ hematopoietic progenitor cells, isolated from the bone marrow of healthy subjects and low-risk MDS patients were cultured for 17 days in presence of G-CSF to induce neutrophil differentiation. (A) Expansion was determined by counting the trypane blue-negative cells. (B) During the 17-day culture period the percentage apoptotic cells was determined by Annexin V staining. Results are presented as means of 5 independent experiments. Error bars represent SEM.

p38MAPK in the pathogenesis of MDS (27). To determine whether aberrant p38MAPK activation may be involved in the defective neutrophil differentiation observed in MDS primary progenitors, CD34⁺ hematopoietic progenitor cells isolated from either controls or patients with MDS, were cultured in presence of G-CSF to induce neutrophil differentiation. After 6 days of culture, protein lysates were made and Western blot analysis was performed using an antibody against phosphorylated p38MAPK or p38MAPK as a control for equal loading (Figure 3A). In contrast to the previous report, CD34⁺ cells from low-risk MDS patients did not show elevated levels of phosphorylated p38MAPK compared to controls. In addition, flow cytometric analysis using an antibody against phosphorylated p38MAPK was performed to further support these observations (Figure 3B).

In order to investigate whether inhibition of p38MAPK can indeed improve final maturation of MDS progenitors during myelopoiesis, CD34⁺ cells, isolated from patients with MDS, were differentiated towards neutrophils either in the presence or absence of the specific p38MAPK inhibitor SB203580. The efficacy of SB203580 was confirmed by its ability to inhibit the phosphorylation of MAPKAPK-2 (MK2) in CD34⁺ cells, a direct target of p38MAPK (Figure 3C). After 17 days of differentiation, cytospins were prepared to analyze the morphology of the differentiating granulocytes. Inhibition of p38MAPK activity did not improve neutrophil development of MDS CD34⁺ cells (Figure 3D). In addition, to determine whether inhibition of p38MAPK can improve the clonogenic capacity of primary MDS progenitor cells, CD34⁺ cells of low-risk MDS patients were plated in CFU assays, and colony formation was analyzed after 12 days. Treatment of MDS hematopoietic progenitor cells with SB203580 did not result in significantly increased CFU-GM (Figure 3E). Together these results suggest that inhibition of p38MAPK is not sufficient to restore final maturation or improve the clonogenic capacity of low-risk MDS hematopoietic progenitor cells.

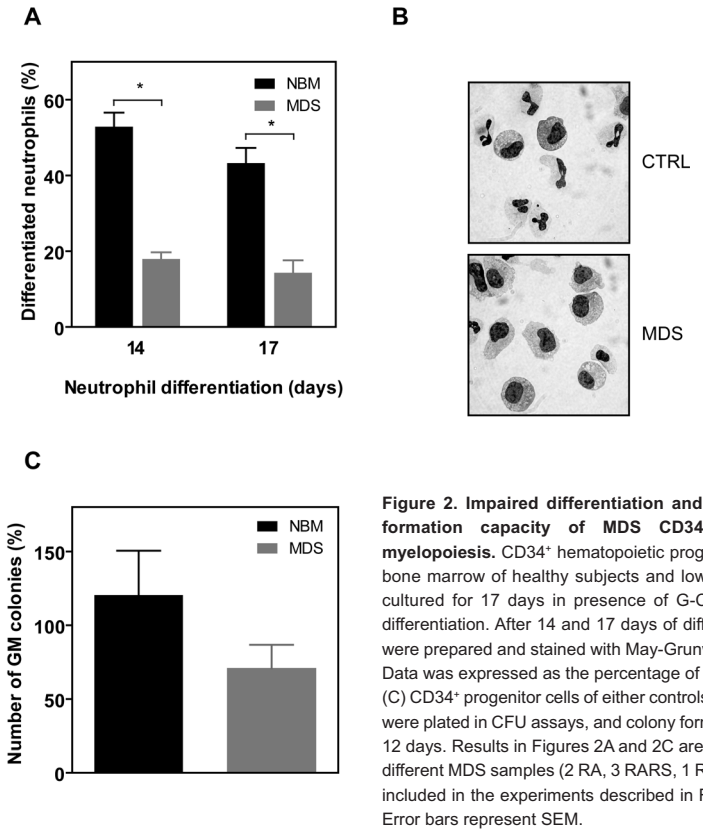

Figure 2. Impaired differentiation and decreased GM colony formation capacity of MDS CD34⁺ progenitors during myelopoiesis. CD34⁺ hematopoietic progenitor cells, isolated from bone marrow of healthy subjects and low-risk MDS patients were cultured for 17 days in presence of G-CSF to induce neutrophil differentiation. After 14 and 17 days of differentiation, (B) cytospins were prepared and stained with May-Grunwald Giemsa solution. (A) Data was expressed as the percentage of differentiated neutrophils. (C) CD34⁺ progenitor cells of either controls or low-risk MDS patients were plated in CFU assays, and colony formation was analyzed after 12 days. Results in Figures 2A and 2C are presented as means of 6 different MDS samples (2 RA, 3 RARS, 1 RAEB), of which 4 are also included in the experiments described in Figure 3 (2 RA, 2 RARS). Error bars represent SEM.

Increased PKB or STAT5a activity is not sufficient to restore neutrophil development in MDS

Hematopoietic cytokines can activate several signal transduction pathways, which have shown to be involved in the regulation of myeloid differentiation including, p38MAPK, phosphatidylinositol 3 kinase (PI3K), and Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway (28). Recently we have demonstrated that during myelopoiesis PKB activity is essential for both hematopoietic progenitor survival and neutrophil development (26). We also observed that CD34⁺ cells from low-risk MDS patients show decreased PKB activation in response to the chemo-attractant SDF-1 (29). Furthermore, neutrophils isolated from low-risk MDS patients also exhibited decreased PKB phosphorylation upon stimulation with fMLP (30). However, CD34⁺ derived neutrophil progenitor cells from low-risk MDS patients did not show significantly altered levels of phosphorylated PKB compared to controls (Figure S1). To further determine whether aberrant PKB activation may be involved in the defective neutrophil differentiation observed in MDS primary progenitors, we utilized of a bicistronic retroviral DNA construct co-expressing eGFP and a constitutively active form of PKBα (myrPKB). Retrovirus was generated and used to transduce low-risk MDS CD34⁺ cells, which were cultured in presence of G-CSF to induce

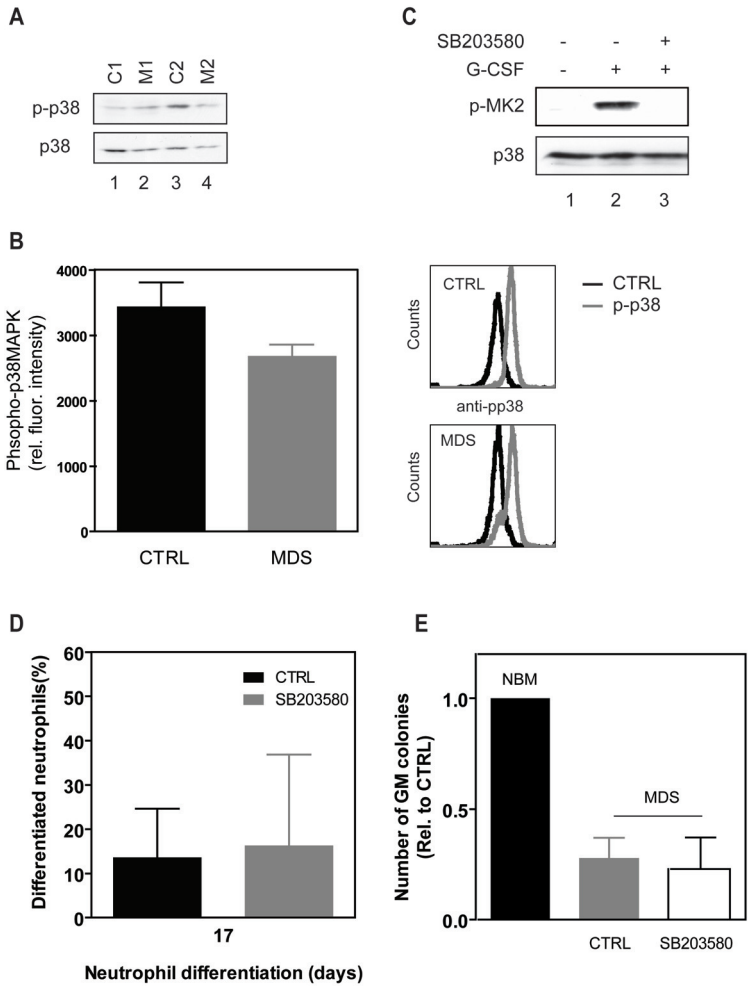

Figure 3. Inhibition of p38MAPK does not improve maturation or increase clonogenic capacity of MDS progenitor cells. (A) $CD34^+$ cells, isolated from bone marrow of healthy subjects (lane 1, 3) and low-risk MDS patients (lane 2, 4) were cultured in presence of G-CSF to induce neutrophil differentiation. After 6 days of culture, protein lysates were made and Western blot analysis was performed using an antibody against phosphorylated p38MAPK, or p38MAPK as a control for equal loading. (B) Activation of p38MAPK was further analyzed by flow cytometric analysis using an antibody against phosphorylated p38MAPK. (C) $CD34^+$ cells were cultured in presence of G-CSF induce neutrophil differentiation. After 6 days of culture, cells were left untreated (lane 1, 2) or treated with SB230580 (lane 3) for 45 minutes before stimulation with G-CSF (lane 2, 3) for 15 minutes. Protein lysates were prepared and Western blot analysis was performed with an antibody against phosphorylated MAPKAPK-2 or p38MAPK as a control for equal loading. (D) After 17 days of differentiation, cytospins were prepared to analyze the morphology of the differentiating granulocytes. Data were expressed as the percentage of differentiated neutrophils. (E) $CD34^+$ progenitor cells, isolated from low-risk MDS patients were plated in CFU assays, either in the presence or absence of 10 μM SB203580 and colony formation was analyzed after 12 days. Results are presented as means of 3 independent experiments. Error bars represent SEM.

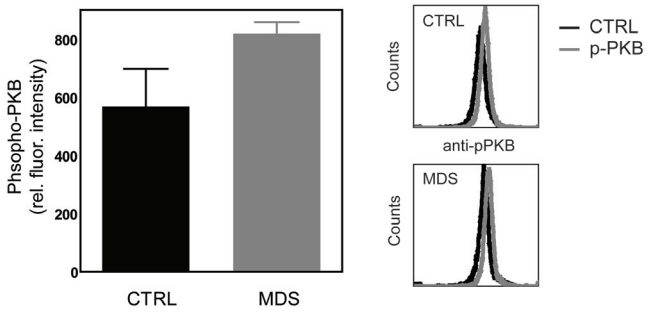

Figure S1. CD34+ cells, isolated from bone marrow of healthy subjects and low-risk MDS patients were cultured in presence of G-CSF to induce neutrophil differentiation. After 6 days of culture, activation of PKB was analyzed by flow cytometric analysis using an antibody against phosphorylated PKB.

neutrophil differentiation. Three days after transduction, eGFP positive cells were sorted by FACS from the non-transduced cells. After 14 and 17 days of differentiation, cytospins were prepared and the morphology of the cells was analyzed after May-Grunwald Giemsa staining. Activation of PKB did not improve the reduced neutrophil development from hematopoietic progenitors isolated from patients with MDS (Figure 4A), indicating that activation of PKB alone is not sufficient to rescue neutrophil development in MDS.

Ourselves and others have also shown that activation of the transcription factor STAT5 regulate proliferation, apoptosis and differentiation during erythroid and myeloid development (24)(31). To address the question whether STAT5 signalling plays a critical role in regulating neutrophil differentiation in MDS, a bicistronic retroviral DNA construct co-expressing STAT5a and eGFP was used to transduce CD34+ cells isolated from MDS patients. After 17 days of differentiation, eGFP positive cells were sorted by FACS from the non-transduced cells and cytospins were prepared. Ectopic expression of STAT5a resulted in a modest increase in the percentage of mature neutrophils with banded or segmented nuclei compared to cells transduced with eGFP alone (Figure 4B). Together these data indicate that despite playing essential roles in granulopoiesis, ectopic expression of myrPKB or STAT5a alone is not sufficient to restore neutrophil differentiation in MDS.

Increased C/EBPα and ID1 expression results in improved neutrophil production in low-risk MDS patients

One of the key transcriptional regulators involved in lineage choice decisions during myeloid differentiation is CCAATT/enhancer binding protein α (C/EBPα). Genetic alterations and reduced expression of the C/EBPα gene have been found in both AML and MDS, which supports the involvement of deregulated C/EBPα expression in the inefficient granulopoiesis characteristic for MDS (18)(32-34). In addition, another transcriptional regulator that has been demonstrated to play an important role in the regulation of proliferation and differentiation during myelopoiesis is inhibitor of DNA binding protein 1 (ID1) (25). To investigate whether ectopic expression of ID1 or C/EBPα can indeed improve myeloid maturation of MDS progenitors, CD34+ hematopoietic progenitor cells, isolated from patients with MDS, were transduced with ID1, C/EBPα or eGFP as a control and were cultured in presence of G-CSF to induce neutrophil differentiation. Three days after transduction, eGFP positive cells were sorted by FACS from the non-transduced cells. After 17 days of differentiation, cytospins were prepared and the morphology of the cells was subsequently analyzed after May-

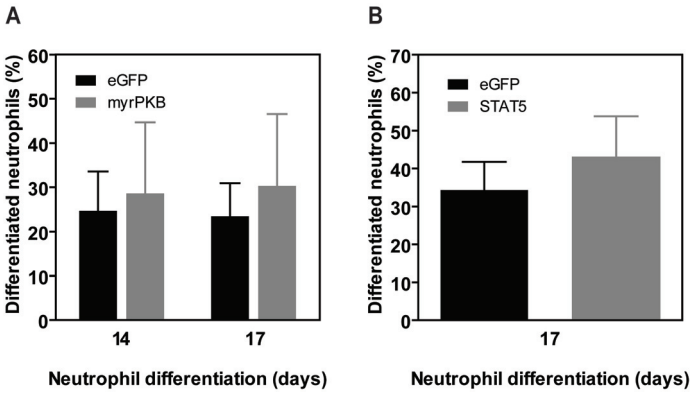

Figure 4. Increased PKB or STAT5a activity is not sufficient to restore neutrophil development in MDS. CD34$^+$ cells, isolated from patients with low-risk MDS were retrovirally transduced with (A) myrPKB, (B) STAT5a or eGFP as a control and cultured in presence of G-CSF to induce neutrophil differentiation. After 17 days of culture, transduced cells were separated from non-transduced cells by FACS, and cytospins were prepared. Data were expressed as the percentage of differentiated neutrophils. Results are presented as means of 4 independent experiments. Error bars represent SEM.

Grunwald Giemsa staining (Figure 5A). Ectopic expression of ID1 did not improve the reduced neutrophil development of MDS CD34$^+$ cells. However, transduction of MDS CD34$^+$ cells with C/EBPα resulted in a dramatic increase in the percentage of neutrophils with banded or segmented nuclei (Figure 5B). These results demonstrate that ectopic expression of C/EBPα is sufficient to restore neutrophil development of MDS hematopoietic progenitors. In addition, to determine whether ID1 or C/EBPα can improve the clonogenic capacity of MDS CD34$^+$ cells, CFU assays were performed, and colony formation was analyzed after 12 days of culture. Interestingly, ectopic expression of both ID1 and C/EBPα resulted in an increased GM colony formation (Figure 5C). Together these data suggest that targeting the ID1 and C/EBPα transcriptional regulators may be of benefit in the design of novel therapies for low-risk MDS.

DISCUSSION

Although myelodysplastic syndromes are some of the most prevalent hematological disorders, the defects in the intracellular signalling pathways responsible for aberrant hematopoiesis remain largely undefined. In the present study, we have investigated whether known regulators of myeloid differentiation can improve neutrophil development of MDS CD34$^+$ hematopoietic progenitor cells utilizing a human ex-*vivo* granulocyte differentiation system. Our data demonstrate that ID1 and C/EBPα both play a role in expansion and differentiation during myeloid development of MDS hematopoietic progenitors. Ectopic expression of ID1 and C/EBPα resulted in enhanced GM colony formation, whereas treatment of progenitors with SB203580, PKB or STAT5 did not improve colony formation. In addition, C/EBPα transduced MDS progenitors exhibited greatly improved neutrophil maturation.

Here we describe an ex-*vivo* differentiation system to study the defects in intracellular

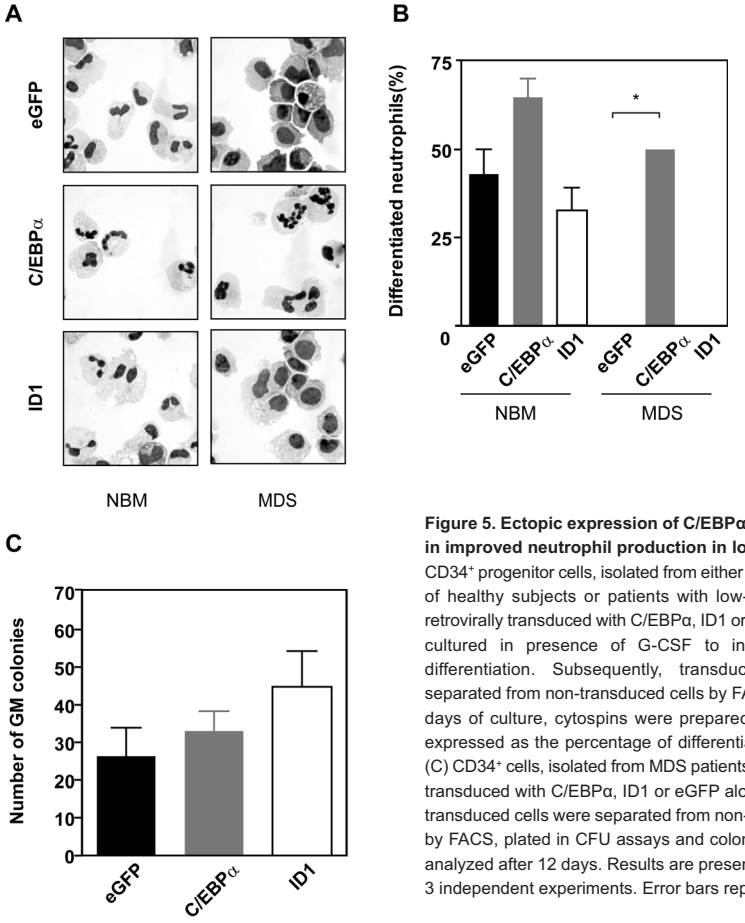

Figure 5. Ectopic expression of C/EBPα and ID1 results in improved neutrophil production in low-risk MDS. (A) CD34⁺ progenitor cells, isolated from either the bone marrow of healthy subjects or patients with low-risk MDS, were retrovirally transduced with C/EBPα, ID1 or eGFP alone and cultured in presence of G-CSF to induce neutrophil differentiation. Subsequently, transduced cells were separated from non-transduced cells by FACS and after 17 days of culture, cytospins were prepared. (B) Data were expressed as the percentage of differentiated neutrophils. (C) CD34⁺ cells, isolated from MDS patients were retrovirally transduced with C/EBPα, ID1 or eGFP alone. After 3 days, transduced cells were separated from non-transduced cells by FACS, plated in CFU assays and colony formation was analyzed after 12 days. Results are presented as means of 3 independent experiments. Error bars represent SEM.

signaling pathways in MDS CD34⁺ hematopoietic progenitor cells. Using this differentiation protocol we were able to demonstrate that CD34⁺ bone marrow cells of low-risk MDS patients have both an impaired capacity to proliferate and show increased levels of apoptosis during neutrophil development. Importantly, this ex-*vivo* system thus mimics the published observations in MDS bone marrow in vivo (35).

p38MAPK is a serine-threonine kinase, originally discovered as a stress-activated kinase, that has been demonstrated to be involved in the regulation of differentiation of various cell types, including granulocytes, with its effects being cell type and context specific (36). Inhibition of p38MAPK activity was shown to enhance neutrophil development, while constitutive activation of MKK3/p38MAPK signalling module dramatically inhibited neutrophil differentiation (Geest *et al*, submitted). In addition, it was recently shown that p38MAPK is constitutively activated in the bone marrow of patients with low-risk MDS. Inhibition of p38MAPK activity was found to decrease apoptosis and stimulate GM colony formation in primary MDS progenitors, suggesting a role for p38MAPK in the pathogenesis of this

syndrome (27)(37). However, our data indicate that inhibition of p38MAPK is not sufficient to restore final maturation or improve the clonogenic capacity of low-risk MDS hematopoietic progenitors (Figure 3D, 3E). Moreover, $CD34^+$ cells from low-risk MDS patients did not show elevated levels of phosphorylated p38MAPK compared to controls (Figure 3A, 3B). These differences might be explained by donor variations between MDS patient samples as MDS comprise a heterogeneous group of stem cell disorders. In addition, although Navas *et al* demonstrated that inhibition of p38MAPK stimulates GM colony formation in primary low-risk MDS progenitors (27), they have not characterized the morphology of the differentiating neutrophils.

PI3K has been demonstrated to play a critical role in the survival and proliferation of a variety of cell types and recent evidence showed that PI3K and its downstream effector PKB also play an important role in regulating hematopoiesis (26). Previously, we have demonstrated that $CD34^+$ cells from low-risk MDS patients show decreased PKB phosphorylation in response to the chemo-attractant SDF-1 (29). In addition, constitutive activation of PKB in bone marrow mononuclear cells from high risk MDS patients was reported, while mononuclear cells from normal bone marrow and low-risk MDS patients demonstrated low or absent levels of PKB activation (38). Taken together, this suggests that aberrant PKB activation might be one of the factors contributing to the ineffective hematopoiesis observed in MDS. However, activation of PKB in MDS $CD34^+$ hematopoietic progenitor cells did not improve aberrant neutrophil development, indicating that activation of PKB alone is also insufficient to rescue neutrophil development in low-risk MDS (Figure 4A).

Several studies suggest that STAT5 may play a critical role in neutrophil development. Loss of STAT5 function in primary bone marrow cells, for example, leads to a reduction in CFU-G colony formation, while bone marrow cells from mice lacking STAT5 are unable to repopulate the myeloid lineage of lethally irradiated wild-type recipient mice (39-42). Furthermore, it has been demonstrated that STAT5 favors the survival of myeloid progenitors by inducing expression of the anti-apoptotic protein Bcl-xL (43). However, although STAT5 expression has shown to be essential during myelopoiesis, our data indicate that expression of STAT5a is again not sufficient to restore neutrophil development in low-risk MDS (Figure 4B).

ID proteins function as inhibitors of members of the basic helix-loop-helix family of transcription factors and have been demonstrated to play an important role in regulating proliferation and differentiation of a variety of cell lineages (44). It has been shown that ID1 mRNA levels are often high in proliferating cells, but are down-regulated in differentiating cells (45). Previously we have shown that during early granulopoiesis, ID1 levels were found to be upregulated, which was followed by a decrease in expression during final maturation (25). Our data suggest that ectopic expression of ID1 is not sufficient to improve neutrophil differentiation in MDS, however GM colony formation of MDS hematopoietic progenitors was significantly increased, suggesting that ID1 may exert its major effects on progenitor expansion during the early phase of granulopoiesis. Previous studies have demonstrated that aberrant activation of ID proteins can contribute to tumorigenesis by stimulating proliferation and facilitating neovascularization. In addition, analysis of diverse solid and leukemic human tumors have revealed that the expression level of ID proteins is often elevated (46-49). While this suggests that targeting ID1 may be of benefit in the design of novel therapies for low-risk MDS, manipulation for therapeutic purposes will not be without

risk.

C/EBPα is a leucine zipper transcription factor that plays a critical role in normal myelopoiesis. Expression of C/EBPα is detectable in early myeloid precursors and is upregulated upon commitment to granulocytes (50)(51). Consistent with this expression pattern, mice deficient for C/EBPα lack mature neutrophils and accumulate immature myeloblasts in the bone marrow (52). Conversely, ectopic expression of C/EBPα in precursor cell lines triggers neutrophil differentiation (53)(54). Mutations within the C/EBPα gene are found in approximately 9% of patients with AML, leading to production of C/EBPα mutants deficient in DNA binding (32-34). C/EBPα levels are also affected by various leukemic fusion proteins through mechanisms that involve transcriptional as well as translational repression (55-57). Although, alterations in the C/EBPα gene have been found in AML cases, they seem to be less frequently observed in MDS patients (17). However, in patients with 5q- syndrome, a distinct clinical subgroup of MDS, the gene encoding C/EBPα was found to be extensively down-regulated in MDS progenitor cells (18). Besides mutations in the CEBPα gene itself, C/EBPα transcription may be repressed by DNA promoter hypermethylation. Methylation of DNA is a commmon epigenetic modification, which plays an important role in correct regulation of gene expression in mammalian cells. Hypermethylation of promoter residues and consequent inactivation of regulatory genes has been found to play a pathogenetic role in MDS development (58). Recent data have shown that in a specific subgroup of AML, which phenotypically resembles AML with mutations in C/EBPα, the CEBPα gene indeed was silenced due to promoter hypermethylation (59). Our results demonstrate that ectopic expression of C/EBPα is sufficient to restore neutrophil development of MDS hematopoietic progenitors, supporting the hypothesis that abrogation of granulopoiesis in MDS patients is, at least in part, due to aberrant C/EBPα expression or functionality in the bone marrow. Interestingly, the major effect of C/EBPα was observed on neutrophil differentiation rather than CFU-GM growth, arguing for a key role in granulocytic differentiation. Consistently, previous studies showing that expression of C/EBPα is detectable in early myeloid precursors and is upregulated upon commitment to granulocytes, indicate that C/EBPα may indeed exert its major effects on progenitor maturation during the late phase of granulopoiesis (50)(51).

G-CSF is an essential cytokine for both the proliferation of myeloid precursors and their differentiation into mature neutrophils. It is tempting to speculate that the number of G-CSF receptors expressed on the membrane of progenitors may play a critical role in the maturation defect in myelodysplastic patients. Previous studies demonstrated that CEBPα plays an important role in transcriptionally regulating G-CSF receptor expression, by direct interaction with the G-CSFR promoter (60)(61). Moreover, decreased G-CSF receptor expression on $CD34^+$ cells was found in a significant proportion of both low-risk and high-risk myelodysplastic patients (62). Interestingly, MDS patients with a low receptor expression had a high predisposition to develop neutropenia and a poor or absent response to G-CSF administration. It could therefore be hypothesized that increased expression of C/EBPα in low-risk MDS $CD34^+$ cells may result in enhanced G-CSF receptor expression, leading to more efficient signalling in response to G-CSF, ultimately resulting in improved neutrophil development.

In conclusion, while a variety of genetic alterations have been reported to be involved in the pathogenesis of MDS, our data suggest that targeting C/EBPα may be sufficient in the design

of novel therapies for low risk MDS.

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Chapter 7

HDAC inhibition modulates cell fate decisions during myeloid differentiation

Marije Bartels[1,2], Christian R Geest[1], Marc B Bierings[2], Miranda Buitenhuis[1], Paul J Coffer[1,3]

[1] Molecular Immunology Lab, Department of Immunology, University Medical Center, Utrecht, The Netherlands, [2] Department of Pediatric Hematology/Oncology, Wilhelmina Children's Hospital, University Medical Centre, Utrecht, The Netherlands, [3] Department of Pediatric Immunology, University Medical Center, Utrecht, The Netherlands

Submitted

ABSTRACT

While the clinical use of histone deacetylase inhibitors (HDACi) is increasing, little is known concerning their effects on myelopoiesis. To investigate this, we utilized an ex-*vivo* differentiation system in which umbilical cord blood derived $CD34^+$ cells were treated with trichostatin A (TSA), sodium butyrate (SB) and valproic acid (VPA). We evaluated the effect of HDACi treatment on myeloid lineage development, colony-forming potential, proliferation, and terminal neutrophil differentiation. TSA treatment modestly reduced progenitor proliferation, while SB and VPA resulted in concentration dependent effects on proliferation and apoptosis. Addition of 100 μM VPA uniquely stimulated $CD34^+$ proliferation. SB treatment both quantitatively and qualitatively inhibited terminal neutrophil differentiation. Addition of 100 μM resulted in increased numbers of mature neutrophils with a block in differentiation at increasing concentrations. In contrast to TSA, both VPA and SB treatment resulted in increased histone (H)-3- and H4-acetylation. Since HDACi induced effects on $CD34^+$ progenitor proliferation and differentiation do not correlate with differences in H3- and H4-acetylation, this suggest a role for acetylation of non-histone targets. These data provide novel insights in the effects of HDAC inhibitors on regulation of normal hematopoiesis, which is of importance when considering utilizing these compounds for the treatment of myeloid malignancies and bone marrow failure syndromes.

INTRODUCTION

Hematopoiesis is complex, dynamic and carefully orchestrated series of events involving self renewal and differentiation of primitive pluripotent stem cells (1). Differentiation of Common Myeloid Progenitors (CMP) generates cells of both the granulocyte/macrophage lineage, leading to the formation of granulocytes, monocytes and macrophages, as well as the megakaryocyte/erythroid lineage, leading to the formation of megakaryocytes, platelets and erythrocytes (2)(3). Dysregulation of hematopoietic differentiation can result in the development of a variety of pathological conditions ranging from aplasia of the bone marrow to aberrant differentiation of myeloid progenitors in diseases such as myelodysplastic syndromes and leukemia (4). This process is tightly regulated by signal transduction pathways and transcriptional networks regulating gene expression. In recent years a further level of epigenetic regulation has been revealed involving both DNA methylation and histone post-translational modifications (5). Many malignancies are characterized by such epigenetic modifications, which include alterations in cellular acetylation and methylation profiles (6-8). Abnormal activity of histone acetyltransferases (HAT) and histone deacetylases (HDAC), leads to transcriptional dysregulation of key genes involved in the control of cell-cycle progression, differentiation and apoptosis (9). For acute promyelocytic leukemia (APL), abnormal recruitment of HDAC by PML-RAR is a key pathogenetic mechanism, and the HDAC itself is the molecular target in differentiation therapy for APL (10). Moreover, distinct histone modifications, such as the loss of monoacetylated and trimethylated forms of histone 4 have been found in a large number of tumours (6). These findings have led to a dramatic increase research focusing on the use of chromatin modulating drugs, including histone deacetylase (HDAC) inhibitors for the treatment of malignancies (11)(12). Treatment of cells with HDAC inhibitors can, to a variable extent, induce growth arrest, differentiation and apoptosis both in vitro and in vivo by modulation of both transcription-dependent and transcription-independent mechanisms. cDNA microarray studies have revealed that broad specificity HDAC inhibitors can alter the expression levels of 7-10% of all genes (13-17). However recent studies have also suggested that the primary activity of HDACs may also be directed towards non-histone substrates, of which to date almost 200 proteins have been identified (18). These include transcription factors, such as GATA-1 and p53 and STAT-3, but also structural and chaperone proteins such as HSP90 (19).

Valproic acid (VPA), a short chain fatty acid and potent class I/IIa HDAC inhibitor, has been extensively studied in preclinical and clinical trials involving hematological malignancies (20). Studies in patients with advanced myeloid leukemia and myelodysplastic syndromes have shown that treatment with VPA, as monotherapy or in combination with retinoic acid, results in a reduction of malignant blast cells and hematological improvement (21)(23). In addition, in an ex-*vivo* acute leukemia model, it has been demonstrated that VPA has the potential to relieve transcriptional repression resulting in cellular differentiation of leukemic blast cells (24). While the clinical use of HDAC inhibitors for treatment of hematological malignancies is increasing rapidly, surprisingly little is known concerning the specific effects of different HDAC inhibitors and their molecular targets in normal hematopoietic cells.

In this study, we have investigated the effects of three different HDAC inhibitors on myeloid development. Utilizing an ex-*vivo* primary human $CD34^+$ culture system, we show that

specific HDAC inhibitors have differential effects on myelopoiesis, often in a concentration dependent manner. These HDAC inhibitor specific phenotypic changes observed in myeloid differentiation do not coincide with differences in histone (H)3- and H4-acetylation. This suggests that the differential effects of HDAC inhibitors on $CD34^+$ progenitor cells may be due to regulation of non-histone targets. These data provide novel insights in the effects of HDAC inhibitors on regulation of normal hematopoiesis, which is of importance when considering utilizing these inhibitors for the treatment of myeloid malignancies and bone marrow failure syndromes.

MATERIALS AND METHODS

Isolation and culture of human $CD34^+$ cells.
Mononuclear cells were isolated from human umbilical cord blood by density centrifugation over a Ficoll-Paque solution (density, 1.077 g/mL). MACS immunomagnetic cell separation (Miltenyi Biotech, Auburn, CA) using a hapten-conjugated antibody against CD34, which was coupled to beads, was used to isolate $CD34^+$ cells. $CD34^+$ cells were cultured in Iscove's modified Dulbecco's medium (Gibco, Paisley, United Kingdom) supplemented with 8% fetal calf serum (FCS) (Hyclone, South Logan, Utah, USA), 50 µmol/L of ß-mercaptoethanol, 10 units/mL of penicillin, 10 µg/mL of streptomycin, and 2 mM glutamine at a density of 0.3×10^6 cells/mL. Cells were differentiated towards neutrophils in 17 days upon addition of stem cell factor (SCF) (50 ng/mL), FLT-3 ligand (50 ng/mL), granulocyte macrophage colony-stimulating factor (GM-CSF) (0.1 nmol/L), interleukin 3 (IL-3) (0.1 nmol/L), and granulocyte colony-stimulating factor (G-CSF) (30 ng/mL). Every 3 days, cells were counted with trypan blue, and fresh medium was added to a density of 0.5×10^6 cells/mL. After 3 days of differentiation,only G-CSF was added to the cells. The HDAC inhibitors Trichostatin A (TSA), sodium butyrate (SB) and valproic acid (VPA) (Alexis chemicals, Lausen, Switzerland) were added to the fresh medium every 3 days.

Annexin V staining
Cells were harvested at the indicated time points and washed with PBS. Samples were subsequently incubated for 15 minutes with Annexin V-FITC (Bender MedSystems, Vienna, Austria) in binding buffer (10 mmol/L HEPES-NaOH (pH 7.4), 150 mmol/L NaCl, 2.5 mmol/L $CaCl_2$). Cells were washed and resuspended in binding buffer containing 1 µg/mL propidium iodide (Bender MedSystems, Vienna, Austria). Percentages of early apoptotic (Annexin V-positive, propidium iodide negative) and late apoptotic (Annexin V and propidium iodide-positive) cells were determined by FACS analysis (FACS Canto, Becton Dickinson, Alphen a/d Rijn, the Netherlands).

Histochemical staining of hematopoietic cells
May-Grunwald Giemsa staining was used to analyze myeloid differentiation. Cytospins were prepared from 5×10^4 differentiating granulocytes and were fixed in methanol for 3 minutes. After fixation, cytospins were stained in a 50% eosin methylene blue solution according to May-Grunwald (Sigma Aldrich, Seelze, Germany) for 15 minutes, rinsed in water for 5 seconds, and nuclei were counterstained with 10% Giemsa solution (Merck kGaA, Darmstadt, Germany) for 20 minutes. Neutrophil differentiation can be characterized by distinct stages from myeloblast, promyelocyte I, promyelocyte II, myelocyte and metamyelocyte towards neutrophils with banded or segmented nuclei. Mature neutrophils were characterized as cells containing either banded or segmented nuclei. Micrographs were acquired, after staining with May-Grunwald Giemsa solution, with an Axiostar plus microscope (Carl Zeiss, Sliedrecht, the Netherlands) fitted with an 100x/1.3 NA EC Plan Neofluor oil objective using Immersol 518F oil (Carl Zeiss), a Canon Powershot G5 camera (Canon Nederland, Hoofddorp, the Netherlands), and Canon Zoombrowser EX image acquisition software. Photoshop CS3 was used for image processing (Adobe Systems Benelux, Amsterdam, the Netherlands.

Western blot analysis.
Western blot analysis was performed using standard techniques. In brief, differentiating $CD34^+$ progenitors were lysed in Laemmli buffer [0.12 mol/L Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 0.05 µg/µL bromophenol blue, and 35 mmol/L β-mercaptoethanol], sonicated, and boiled for 5 minutes. Equal amounts of total lysate were analyzed by 15% SDS-PAGE. Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA), incubated with blocking buffer (Tris buffered saline/Tween 20) containing 5% low-fat milk for 1 hour at room temperature before incubating with antibodies against either acetyl-histone 4 (lysine 16), acetyl-histone 3 (lysine 9) (Millipore, Billerica, MA), acetyl-lysine (Cell Signaling Technology, Danvers, MA), or an antibody against tubulin (Sigma-Aldrich, Zwijndrecht, the Netherlands) overnight at 4°C in a buffer containing Tris buffered saline/Tween 20 with 5% bovine serum albumine (BSA) (Sigma-Aldrich, Zwijndrecht, The Netherlands). Blots were subsequently incubated with peroxidase-conjugated secondary antibodies (Dako, Glostrup, Denmark) for 1 hour at room temperature. Chemiluminescence was used as a detection method according to the protocol of the manufacturer (Amersham Pharmacia, Amersham, United Kingdom).

Immunohistochemical staining of CD34$^+$ hematopoietic progenitor cells

CD34$^+$ cells, isolated from umbilical cord blood, were cultured in presence of G-CSF to induce neutrophil differentiation as described above. After 7 days of culture, cells were washed and resuspended in PBS/5% FCS (Hyclone, South Logan, Utah, USA) and subsequently incubated for 30 minutes on ice with a phytoerythrin conjugated CD34 antibody (Becton Dickinson, Alphen a/d Rijn, the Netherlands). After incubation, cells were again washed and the percentage of CD34 positive cells was determined by FACS analysis (FACS Calibur, Becton Dickinson, Alphen a/d Rijn, the Netherlands).

Myeloid progenitor staining

Hematopoietic progenitors were characterised as described by Manz *et al* (25). Briefly, CD34$^+$ cells were isolated and cultured to induce neutrophil differentiation as described above. Cells were subsequently washed and resuspended in PBS/5% FCS (Hyclone, South Logan, Utah, USA) and incubated for 30 minutes on ice with a mixture of antibodies (all from Becton Dickinson, Alphen a/d Rijn, the Netherlands). Lineage markers included CD2, CD3, CD4, CD7, CD8, CD14, and CD235a and myeloid progenitors are negative for these markers. The lineage negative (Lin$^-$), CD34$^+$, and CD38$^-$ populations consists of hematopoietic stem cells (HSC). Lin$^-$, CD34$^+$, CD38$^+$, CD123$^+$, and CD45RA$^-$ cells are common myeloid progenitors (CMP), whereas Lin$^-$, CD34$^+$, CD38$^+$, CD123$^+$, and CD45RA$^+$ cells are granulocyte-macrophage progenitors (GMP). The Lin$^-$ CD34$^+$, CD38$^+$, CD123$^-$ and CD45RA$^-$ cell population contains the megakaryocyte-erythroid progenitors (MEP). Cell populations containing HSCs, CMPs, GMPs and MEPs were characterised by FACS analysis (FACS Canto, Becton Dickinson, Alphen a/d Rijn, the Netherlands). Isotype antibody staining was used to ensure gating of the correct population.

Colony-forming unit (CFU) assay

Five hundred CD34$^+$ cells were plated in Iscove's modified Dulbecco's medium (Gibco, Paisley, United Kingdom) supplemented with 35.3% FCS (Hyclone, South Logan, Utah, USA), 44.4% methylcellulose-based medium called Methocult (StemCell Technologies, Vancouver, Canada), 11.1 µmol/L of β-mercaptoethanol, 2.2 units/mL of penicillin, 2.2 µg/mL of streptomycin, and 0.44 mmol/L of glutamine at a density of 1,250 cells/well. CFU assays were performed in the presence of SCF (50 ng/mL), FLT-3 ligand (50 ng/mL), GM-CSF (0.1 nmol/L), IL-3 (0.1 nmol/L), G-CSF (60 ng/mL) and erythropoietin (EPO) (6IE/mL). TSA, SB and VPA were added to the medium in a single dose. CFU-GM (granulocyte/macrophage) and CFU-E (erythrocyte) colonies were scored after 10 days of culture.

Statistics

An one-way ANOVA was performed in all experiments, followed by an unpaired t-test. A P-value of <0.05 (*) was considered significant.

RESULTS

HDAC inhibitors have differential effects on CD34$^+$ progenitor proliferation

As previously discussed, little is currently known concerning the effects of HDAC inhibition on the normal hematopoietic compartment. In particular, the effects of HDAC inhibitor treatment on myeloid progenitor cell functionality have not been well characterized. To determine the effect of HDAC inhibition on human CD34$^+$ hematopoietic progenitor functionality, umbilical cord blood (UCB) derived CD34$^+$ hematopoietic progenitors were cultured in the presence of G-CSF to induce neutrophil differentiation. Cells were cultured in the absence or presence of increasing concentrations of trichostatin A (TSA), sodium butyrate (SB) and valproic acid (VPA), and proliferation and survival were analysed. TSA treatment modestly reduced progenitor proliferation in a concentration dependent manner (Figure 1A), and this was not accompanied by decreased cell survival (Figure 1B). Addition of SB to CD34$^+$ cultures resulted in a significant reduction of progenitor expansion, which was accompanied by increased apoptosis (Figure 1C-D). While 100 µM VPA significantly increased progenitor proliferation, addition of 500 µM VPA resulted in decreased proliferation and a significant increase in the percentage of apoptotic cells. These data demonstrate concentration-dependent HDAC inhibitor specific effects on CD34$^+$ progenitor expansion during neutrophil development.

To further analyze the effects of HDAC inhibition on CD34$^+$ progenitors specifically, cells were

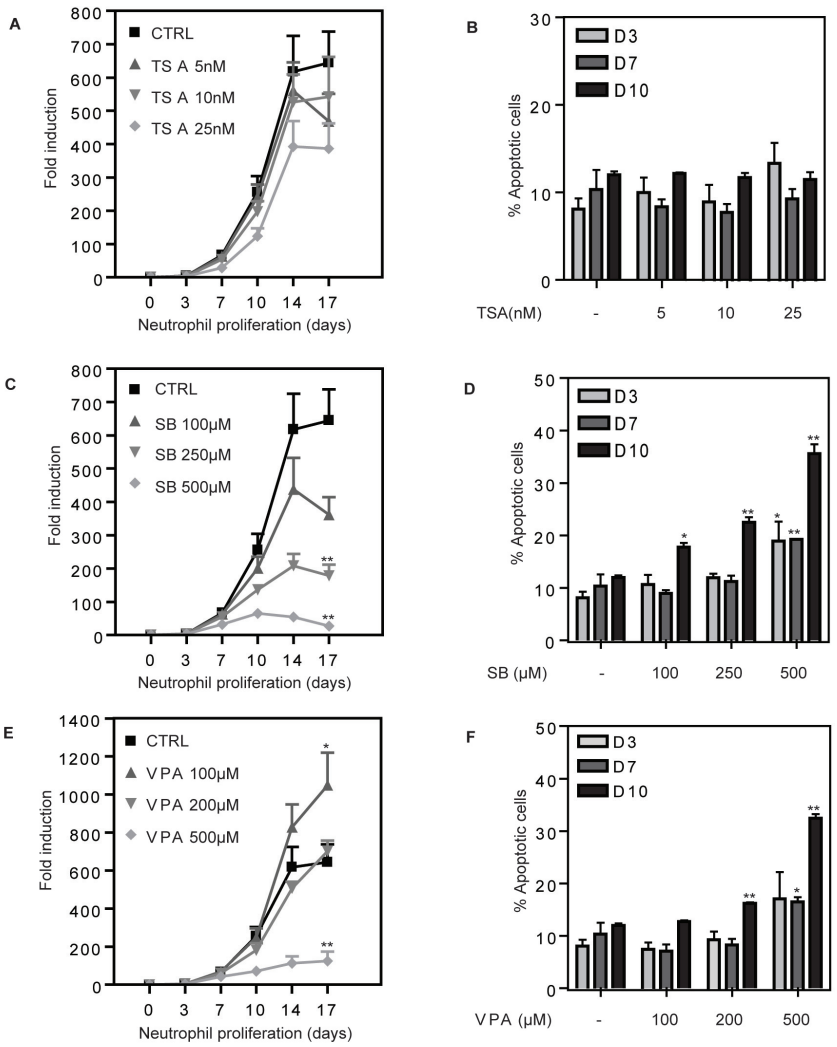

Figure 1. HDAC inhibitors differentially modulate myeloid progenitor proliferation and viability. CD34⁺ cells were cultured in presence of G-CSF to induce neutrophil differentiation during 17 days. Cells were cultured either in absence or presence of TSA (5-25 nM) (A-B), SB (100-500 µM) (C-D) or VPA (100-500 µM) (E-F). Proliferation was determined by counting the tryphan blue negative cell population and data were expressed as fold induction (A, C, E). Apoptosis was determined by Annexin-V/PI staining at day 3, 7 and 10 of differentiation. Data were expressed as percentages of apoptotic cells (B, D, F). Error bars represent SEM. * p = <0.05, ** p = <0.01.

cultured as previously described and both the percentage and absolute number of CD34⁺ cells was analyzed. Addition of 25 nM TSA resulted in a small but significant increase in the percentage of CD34⁺ cells at day 3, which was no longer apparent at day 7 (p=0.063) (Figure 2A), and was not accompanied by an increase in absolute cell numbers (Figure 2B). Addition

of SB to cultures resulted in no significant changes in the percentage or absolute numbers of $CD34^+$ progenitors at all time points (Figure 2A,B). Since we previously observed a significant increase in apoptosis after SB treatment of differentiating progenitors (Figure 1D), this suggests that it is the CD34 negative cell population that was susceptible to SB-induced apoptosis. In contrast, treatment with 200-500 μM VPA resulted in a significant increase in the percentage (Figure 2A) and absolute number (Figure 2B) of $CD34^+$ progenitors in a concentration dependent manner. This effect was particularly clear at the later time points suggesting that VPA is capable of inducing expansion of $CD34^+$ progenitors that then retain their undifferentiated phenotype.

Taken together, these data demonstrate that TSA, SB and VPA differentially affect $CD34^+$ progenitors, and that VPA can uniquely induce $CD34^+$ progenitor proliferation in a concentration dependent manner.

SB and VPA inhibit the transition from common myeloid progenitor to granulocyte/macrophage progenitor

To determine the effect of HDAC inhibition on distinct myeloid progenitor populations within the $CD34^+$ compartment, we analysed progenitor populations as previously described by Manz *et al* (25). Based on the expression of CD123 and CD45RA, myeloid progenitors can be divided in common myeloid progenitors (CMP, $CD123^+/CD45RA^-$), granulocyte/macrophage progenitors (GMP, $CD123^+/CD45RA^+$) and megakaryocyte/erythroid progenitors (MEP, $CD123^-/CD45RA^-$). In addition to the effect on the percentage of $CD34^+$ progenitors early in neutrophil development (Figure 2A), we observed no effects of treatment with TSA on the percentage of CMP. However, at day 3, the percentage of GMP was decreased, which was accompanied by an increase in the percentage of MEP (Figure 3). This effect was not observed at day 7, suggesting a transient block of CMP differentiation into GMP.

Treatment with SB significantly increased the percentage of CMP at day 7 (Figure 3B), which was accompanied by a corresponding decrease in the percentage of GMP (Figure 3D). This suggests that SB inhibits the differentiation of CMP towards the GMP lineage. Finally, in addition to the effects on $CD34^+$ progenitor expansion (Figure 2), addition of VPA resulted in a concentration dependent increase in the percentage of CMP at day 7 (Figure 3B), accompanied by a significant and concentration dependent inhibition of the percentage of GMP (Figure 3D). Unexpectedly, in the absence of EPO and TPO, all HDAC inhibitors increased the percentage of $CD123^-/CD45RA^-$ progenitors at day 3 (Figure 3E). Since we observed no significant differences in the numbers of $CD34^+$ cells at day 3 (Figure 2B), this suggest that HDAC inhibition increases the number of MEP, which was most prominent after addition of 25 nM TSA and 500 μM VPA. With the exception of treatment with 500 μM VPA, this effect was absent at day 7 (Figure 3F), suggesting a transient effect on immature non-committed progenitors.

Taken together, these data demonstrate that specific HDAC inhibitors differentially modulate both the composition and differentiation capacity of the $CD34^+$ progenitor compartment.

SB and VPA treatment inhibits GM-colony formation

To investigate the effect of HDAC inhibition on colony forming potential and lineage

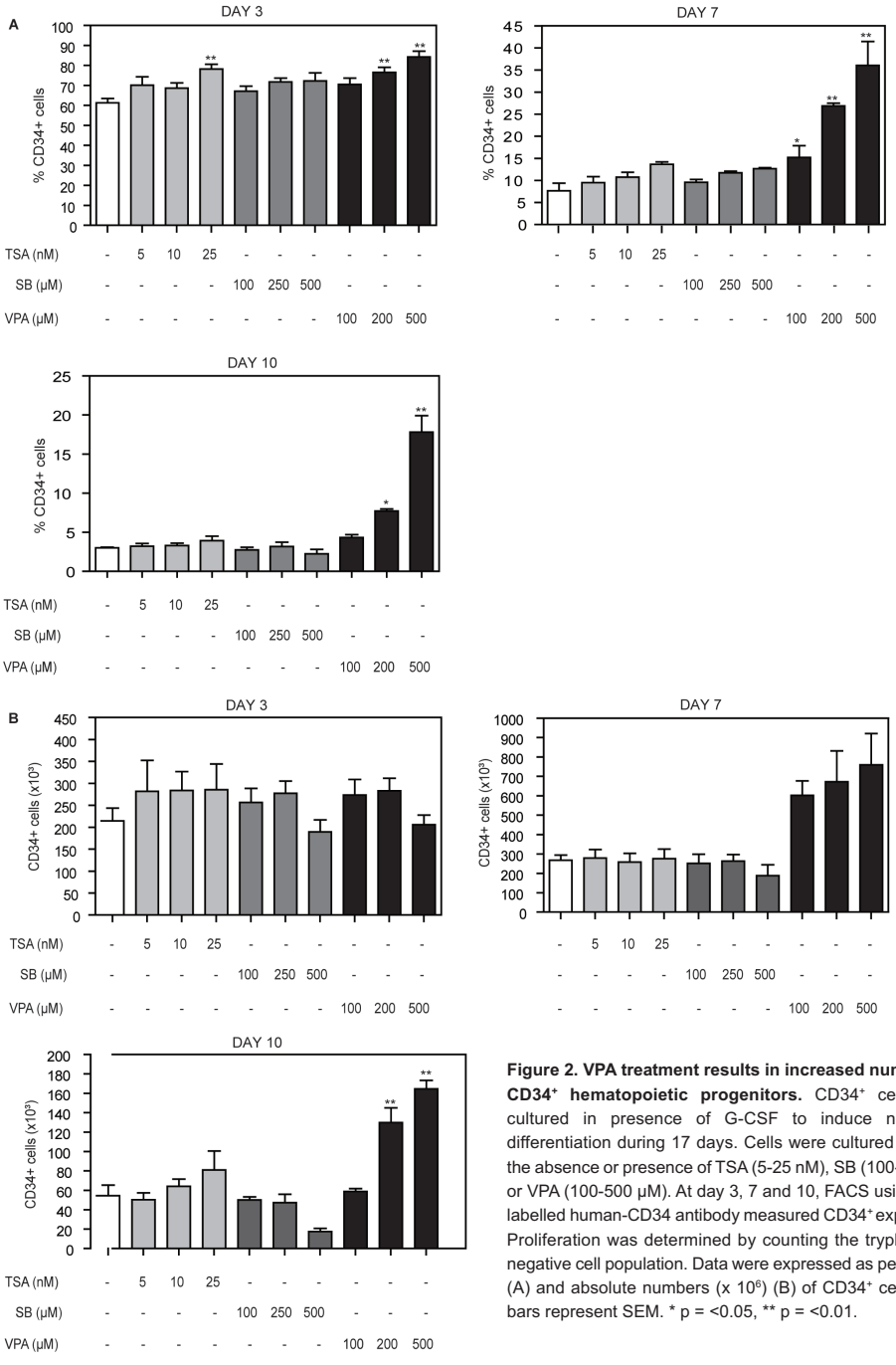

Figure 2. VPA treatment results in increased numbers of CD34⁺ hematopoietic progenitors. CD34⁺ cells were cultured in presence of G-CSF to induce neutrophil differentiation during 17 days. Cells were cultured either in the absence or presence of TSA (5-25 nM), SB (100-500 µM) or VPA (100-500 µM). At day 3, 7 and 10, FACS using a PE-labelled human-CD34 antibody measured CD34⁺ expression. Proliferation was determined by counting the tryphan blue negative cell population. Data were expressed as percentage (A) and absolute numbers (x 10^6) (B) of CD34⁺ cells. Error bars represent SEM. * p = <0.05, ** p = <0.01.

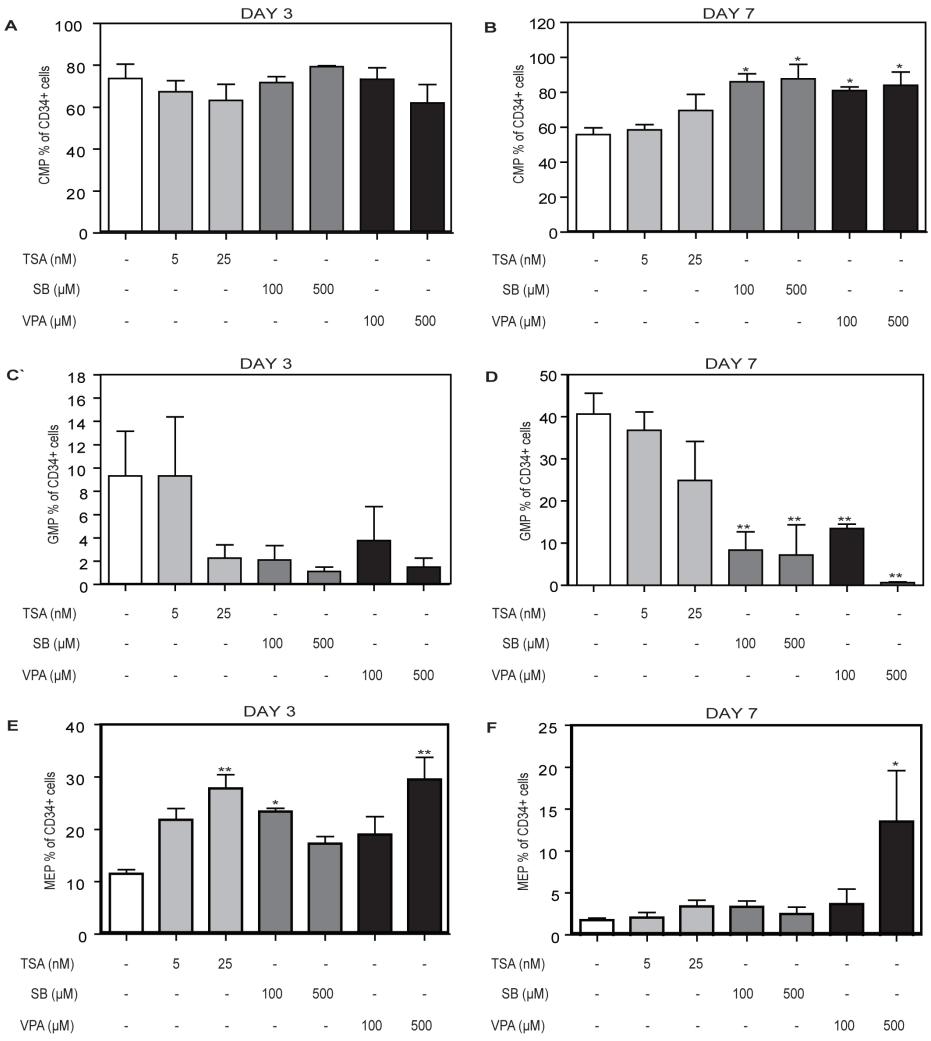

Figure 3. SB and VPA treatment results in inhibition of CMP differentiation. CD34$^+$ cells were cultured in the presence of G-CSF to induce neutrophil differentiation during 17 days. Cells were cultured either in the absence or presence of TSA (5-25 nM), SB (100-500 µM) or VPA 100-500 µM). At day 3 and 7, a progenitor staining was performed. CD34$^+$CD38$^+$ cells were characterized by FACS based on CD123 and CD45RA expression as CMP (CD123$^+$/CD45RA$^-$), GMP (CD123$^+$/CD45RA$^-$) or MEP (CD123$^-$/CD45RA$^-$). Data were expressed as the percentage of CMP (A-B), percentage of GMP (C-D) and percentage of MEP (E-F) at day 3 (A, C, E) and day 7 (B, D, F). CMP, GMP and MEP represent a percentage of total CD34$^+$ cells. Error bars represent SEM. * p = <0.05, ** p = <0.01.

commitment of CD34+ progenitors, we utilised a semi-solid culture system. CD34+ cells were cultured in the presence of a cytokine cocktail including G-CSF and EPO to stimulate production of both granulocyte/macrophage and erythroid colonies. Addition of all HDAC inhibitors resulted in a concentration dependent reduction in the total number of colonies (Figure 4A). In agreement with the progenitor analysis (Figure 3), this suggests that in the presence of HDAC inhibitors, the differentiation of CD34+ progenitors is inhibited. In contrast to the addition of TSA and VPA, SB-treated progenitor colonies appeared less differentiated and smaller (Figure 4B). This is in agreement with our previous data showing inhibition of proliferation in liquid culture (Figure 1).
Treatment with all three HDAC inhibitors resulted in an increase in the percentage of GM colonies (Figure 4C) and concentration dependent decrease in the percentage of erythroid colonies (Figure 4D), which was primarily responsible for the decrease in the total number of CFU (data not shown). Taken together with the data from the progenitor analysis (Figure 3), this suggests that after HDAC inhibition, CMP and GMP still have the potential to differentiate towards the GM-lineage, while further differentiation of MEP towards the erythroid lineage is inhibited.

HDAC inhibitors have differential effects on terminal neutrophil differentiation
To determine the effect of HDAC inhibition on terminal neutrophil differentiation, CD34+ progenitors were cultured for 17 days in the presence of G-CSF and cytospins were prepared to analyse neutrophil differentiation. Differentiated neutrophils were characterised as cells containing either banded or segmented nuclei (see Materials and Methods). Treatment with 5 nM TSA had no effect on the percentage and absolute number of mature neutrophils, but we observed increased segmentation at day 17 (Figure 5A, C). Addition of 25 nM TSA resulted in modest decrease in the percentage of mature neutrophils and significant decrease in the absolute number of mature neutrophils, which can be explained by reduced progenitor proliferation (Figure 1A). Since this effect was not accompanied by a significant increase in metamyelocytes (Figure 5D) or immature precursors (data not shown), this suggests that the addition of 25 nM TSA inhibits neutrophil differentiation. Treatment with SB impaired neutrophil differentiation in a concentration dependent manner, resulting in neutrophils with dysplastic features (Figure 5A-C), increased numbers of morphologically apoptotic cells and a relative increase of monocytic cells (data not shown). Addition of 100 μM SB resulted in a slight decrease in the percentage of mature neutrophils, which was accompanied by an increased percentage of metamyelocytes, which suggests terminal neutrophil differentiation was not affected. However, mature neutrophils showed delicate dysplastic features such as hypergranulation and ringshaped nuclei (Figure 5A, arrows). Taken together, this suggests that treatment with SB impairs neutrophil differentiation both quantitatively and qualitatively. Addition of 100 μM VPA resulted in a modest decrease in the percentage of mature neutrophils (Figure 5C). This effect was accompanied by a slight increase in the absolute number of mature neutrophils, but significant increase in the absolute number of metamyelocytes (Figure 5D). However, after addition of 500 μM VPA, we observed a significant decrease in both the percentage and absolute number of mature neutrophils (Figure 5C), which was accompanied by an increase in young precursors (data not shown) and no increase in metamyelocytes (Figure 5D). This suggests a differentiation block at the

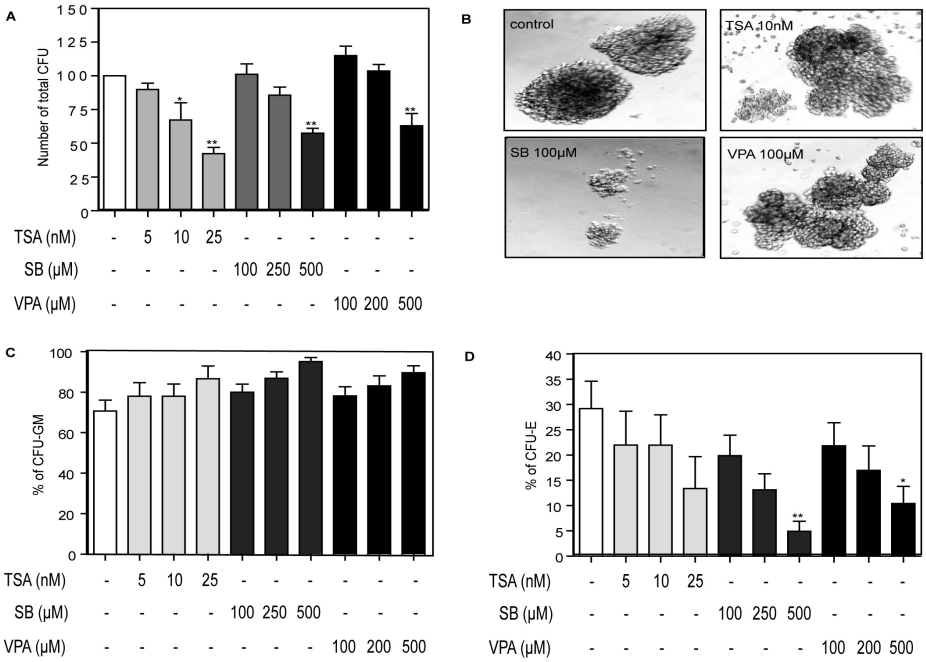

Figure 4. HDAC inhibition affects CD34⁺ progenitor differentiation and lineage commitment. CD34⁺ cells were cultured in the presence of G-CSF and EPO to induce CFU-GM and CFU-E for 11 days. Cells were cultured either in the absence or presence of TSA (5-25 nM), SB (100-500 μM) or VPA (100-500 μM). Each plate was scored for granulocyte/macrophage colony-forming unit and erythroid burst/colony forming unit growth. Data were expressed as the number of total colonies (A), or percentage of granulocyte/macrophage colonies (C) and erythroid colonies (D). Photos were taken to demonstrate GM-colony size (B). Error bars represent SEM. * p = <0.05, ** p = <0.01.

promyelocytic stage and in contrast to treatment with SB these cells appeared morphologically normal (Fig. 5A).

Treatment with SB and VPA leads to hyperacetylation of H3 and H4

To determine whether the observed differential effects of HDAC inhibitors on CD34⁺ progenitor expansion and neutrophil development were accompanied by differences in histone acetylation, we analysed protein lysates prepared after 3 and 7 days of neutrophil differentiation. Treatment with TSA resulted in no reproducible changes in total H3- or H4-acetylation (Figure 6). In contrast, addition of SB or VPA resulted in a concentration dependent hyperacetylation of both total H3 and total H4. We further analysed the effects of HDAC inhibition on histone 3 lysine 9 (H3K9) and histone 4 lysine 16, whose acetylation has been implicated in the regulation of neutrophil development (26)(27). While addition of TSA had no reproducible effect on H3K9 or H4K16 acetylation, treatment with SB or VPA resulted in a concentration dependent increase in acetyation of H3K9 and H4K16. These data demonstrate that SB and VPA have similar, concentration dependent effects on histone

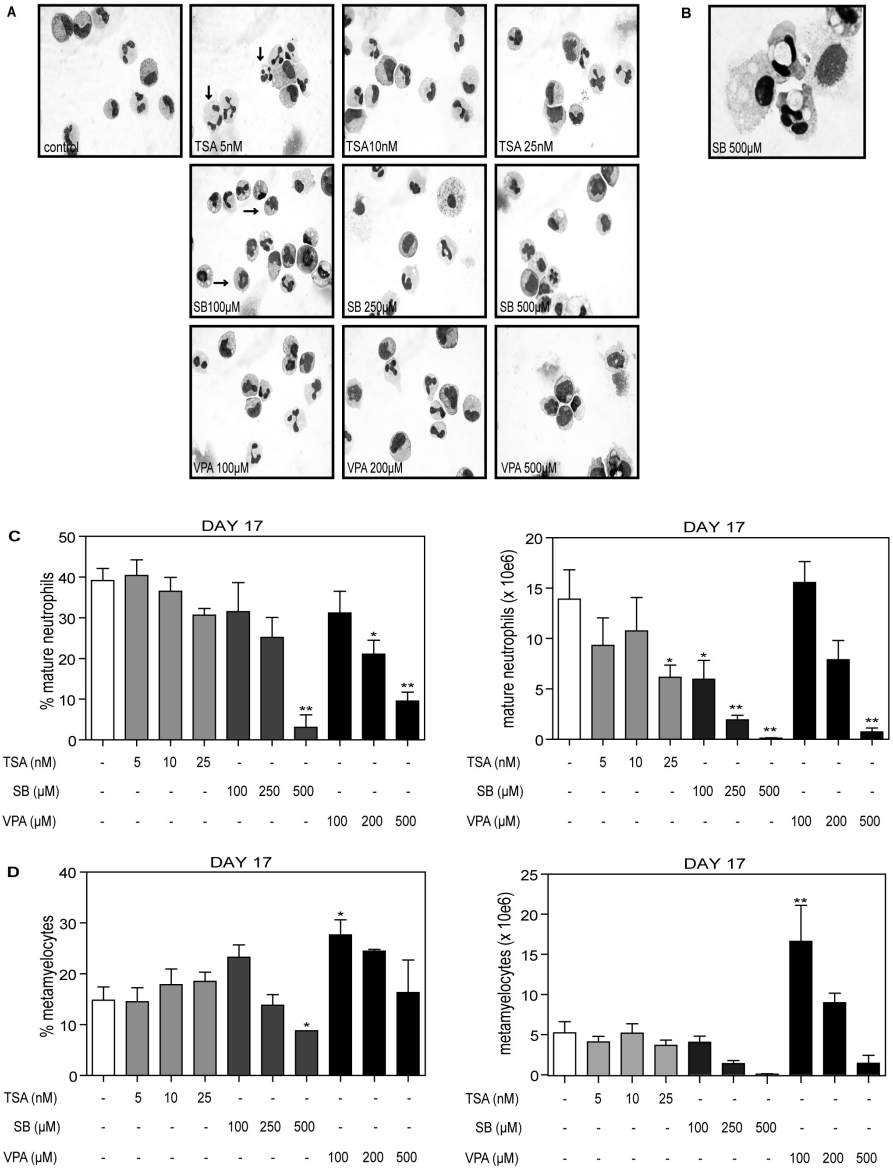

Figure 5. Differential effects of HDAC inhibitors on terminal neutrophil differentiation. $CD34^+$ cells were cultured in the presence of G-CSF to induce neutrophil differentiation during 17 days. Cells were cultured either in the absence or presence of TSA (5-25 nM), SB (100-500 µM) or VPA (100-500 µM). After 17 days of neutrophil differentiation, cytospins were made and stained with May-Grunwald Giemsa solution (A-B). Data were expressed as the percentage and absolute numbers ($x10^6$) of mature neutrophils (banded or segmented nuclei) (C) and metamyelocytes (D) on day 17. Error bars represent SEM. * p = <0.05, ** p = <0.01.

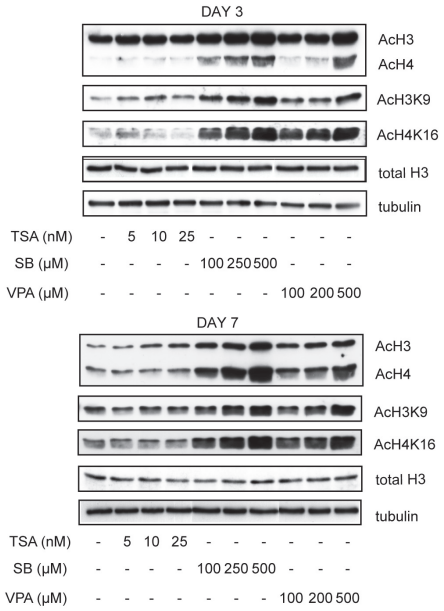

Figure 6. Differential effects of HDAC inhibitor treatment on H3- and H4- acetylation. CD34$^+$ cells were cultured in the presence of G-CSF to induce neutrophil differentiation during 17 days. Cells were treated overnight with TSA (5-25 nM), SB (100-500 µM) or VPA (100-500 µM) at day 3 and day 7. Lysates were made and Western Blot analysis was performed with antibodies against acetylated lysines, acetylated H3K9 and acetylated H4K16. As a control for equal loading an antibody against total H3 and an antibody against tubulin was used.

acetylation in early neutrophil development, while these effects are not observed after addition of TSA. While SB and VPA demonstrate similar effects in terms of histone acetylation, their effects on CD34$^+$ progenitor expansion are distinct (Figure 1, 2), suggesting that these effects may be due to acetylation of non-histone proteins.

DISCUSSION

The use of chromatin-modulating drugs, such as HDAC inhibitors, for the treatment of hematological malignancies has increased dramatically in recent years (12)(28). Although these agents have shown to improve hematological outcome in patients with myelodysplastic syndromes and acute myeloid leukemia (29)(32), little is known concerning their specific effects and molecular targets. In order to investigate this we utilized an ex-*vivo* hematopoiesis culture system to study the effects of specific HDAC inhibitors on the proliferation and differentiation of human myeloid progenitors focussing on neutrophil development.
Our data demonstrate that TSA, SB and VPA, despite the large overlap in HDAC specificity, have differential effects on neutrophil progenitor proliferation. While treatment with TSA has no significant effects on progenitor proliferation, addition of SB dramatically reduces

proliferation, which is accompanied by increased apoptosis (Figure 1). Moreover our data show distinct concentration dependent effects. This is most clearly demonstrated by cells treated with VPA in which 100 µM significantly increases proliferation while 500 µM induces apoptosis of differentiating $CD34^+$ progenitor cells (Figure 1E). In agreement with recent papers, we show that VPA stimulates expansion of $CD34^+$ cells. Bug *et al* showed that this effect on human hematopoietic stem cells was accompanied by reduced expression of the cell cycle inhibitor p21, resulting in an accelerated cell cycle progression (33). In contrast De Felice *et al* suggested that VPA-induced HSC expansion was the result of self-renewal accompanied by an increased duration of the cell cycle and reduced cell death (34). In our experiments, treatment of neutrophil progenitors with increasing concentrations of VPA was accompanied by a decrease in cells in the G2/M phase of the cell cycle (data not shown) together with a significant increase in apoptosis (Figure 1C). However $CD34^+$ progenitors were less susceptible to VPA-induced apoptosis (Figure 2). Taken together, this suggests that VPA treatment has a concentration limiting effect on the proliferation of distinct progenitor populations throughout neutrophil development.

We show that HDAC inhibitors have the potential to induce apoptosis in neutrophil progenitors (Figure 1). This is surprising since it has been previously suggested that untransformed cells are generally more resistant to HDAC inhibitor-induced cell death than transformed cells (35-37). Furthermore, results of phase I clinical trials with HDAC inhibitors suggest no unfavorable effects on the normal progenitor compartment (21)(29)(31)(32). In agreement with these observations, our data suggest that $CD34^+$ progenitors are less susceptible to SB- and VPA-induced apoptosis than more mature neutrophil precursors (Figure 2). This suggests specific effects of HDAC inhibition on distinctive progenitor subsets. Interestingly, while the treatment of leukemic blast cells with HDAC inhibitors generally results in the induction of apoptosis (38-40), Bug and colleagues demonstrated that in $CD34^+CD38^-$ leukemic stem cells from AML patients, VPA treatment resulted in amplification and thereby stabilization of the leukemic stem cell compartment (41). We further investigated the effect of HDAC inhibition on progenitor differentiation using colony-forming assays and by analysing progenitor populations during neutrophil development. In agreement with De Felice *et al* (34) we demonstrated that VPA, and also SB have the potential to inhibit myeloid differentiation towards the granulocyte/macrophage lineage, illustrated by an increased percentage of CMP and corresponding decrease in the percentage of GMP (Figure 3). Furthermore, we observed a transient increase in the number of $CD123^-/CD45RA^-$ cells, candidate megakaryocyte/erythroid progenitors (MEP), after addition of all HDAC inhibitors (Figure 3). However, in contrast we observed no increase in the percentage or number of erythroid colonies (Figure 4), suggesting that MEP differentiation is thereafter inhibited. An alternative explanation could be that MEP are redirected towards the GM-lineage, since it has been shown that lineage commitment of progenitors is a dynamic process in which over-expression of lineage specific players can force committed progenitors towards another lineage (42-44). In addition to the data on neutrophil progenitor differentiation, we have demonstrated that specific HDAC inhibitors differentially affect terminal neutrophil differentiation. Interestingly, Skokowa *et al* recently published that nicotinamide, a functionally different, specific class III (sirtuin) HDAC inhibitor, stimulates neutrophil development in congenital neutropenia patients and healthy individuals (45). Our data show that, while TSA-treatment had no significant

effects on neutrophil differentiation, addition of SB impaired differentiation both qualitatively and quantitatively (Figure 5). Treatment with VPA resulted in specific concentration dependent effects. While 100 μM VPA improved neutrophil differentiation, our data suggest that treatment with increasing concentrations finally leads to a differentiation block, in which precursors loose their potential to terminally differentiate. Intriguingly, this inhibitory effect is in contrast to the effect on differentiation of leukemic precursors (24)(30) and together with other signs of dysmyelopoiesis, has also been described in patients who received long-term treatment with VPA for epilepsy (46)(47). In general, the differential effects of HDAC inihibition described in normal hematopoietic progenitors and leukemic precursors may be partially due to distinctive HDAC expression profiles (48).

The phenotypic effects on proliferation and differentiation observed after HDAC treatment cannot be explained by changes in histone acetylation alone (Figure 6). For example, while both VPA and SB exhibit almost identical changes in histone acetylation, VPA-treatment, unlike SB, results in $CD34^+$ expansion. This suggests that these differential effects may be explained by effects on non-histone protein targets of which to date more than 200 have been defined (18). Myeloid development is tightly regulated by key transcription factors including PU.1, GATA-1 and C/EBPα (49)(50). In recent years it has become clear that the expression and function of these key transcription factors is regulated by post-translational modifications, including acetylation (5). In murine myeloid cell lines it has been shown that acetylation of PU.1 by TSA treatment leads to reduced expression of PU.1 and its target genes (51). Furthermore, PU.1 itself inhibits acetylation and thereby functional activity of GATA-1 (52). It is tempting to speculate that the differential effects of HDAC inhibitors observed during $CD34^+$ cultures are the direct effect of the acetylation of key regulators of myeloid differentiation.

In summary, we describe differential effects of HDAC inhibitors on myeloid progenitors during neutrophil development. Despite the published overlap in HDAC inhibitor specificity, we clearly demonstrate that these inhibitors have specific effects on $CD34^+$ progenitor expansion and neutrophil development. Since the clinical use of HDAC inhibitors is increasing, it is important to define the effects of this family of compounds on the normal hematopoietic compartment. Our data clearly show that both the choice and concentration of HDAC inhibitor can greatly affect the proliferative and differentiation capacity of $CD34^+$ hematopoietic progenitor cells. Furthermore, our results suggest that these effects may be mediated by non-histone targets. Identification of these acetylated proteins may lead to the development of novel, more targeted therapies for the treatment of bone marrow failure and hematological malignancies.

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Chapter 8

General Discussion

Blood cell production or hematopoiesis involves a complex series of events which are precisely regulated by the interplay of cytokines and the bone marrow micro-environment. This requires the coordinated expression of many genes that directly or indirectly govern hematopoietic stem cell (HSC) and progenitor cell maintenance, lineage commitment, differentiation, migration and mature blood cell function. The correct regulation of proliferation, survival and differentiation is critical for normal homeostasis within the bone marrow and aberrant regulation of hematopoiesis can lead to bone marrow failure ranging from hematological malignancies to severe immune deficiencies. The work described here was performed to characterize the intracellular signalling pathways and molecular components involved in the regulation of hematopoiesis to further enhance our understanding on the regulation of the cytokine-mediated intracellular signal transduction pathways regulating HSC and myeloid progenitor function. To investigate the mechanisms underlying myeloid differentiation in human $CD34^+$ cells, an ex-*vivo* hematopoiesis culture system as well as an *in vivo* mouse transplantation model was utilized.

Regulation of hematopoiesis involves differential expression of transcription factors

The hematopoietic system produces appropriate levels of all types of blood cells over an individual's lifetime through a tightly regulated balance of differentiation, proliferation and self-renewal (1). The importance of correct regulation of proliferation, survival and differentiation for hematopoietic homeostasis is underscored by several reports showing that disruption of transcriptional regulators by chromosomal translocations are involved in the etiology of hematopoietic disease (2). Lineage commitment and differentiation programs of multipotent cells involves the selective activation and silencing of a set of genes, which are ultimately controlled by transcription factors (3)(4). The production of myeloid cells has been demonstrated to be orchestrated by a relatively small number of transcription factors, among them are PU.1, CCAAT/enhancer binding proteins, including C/EBPα, C/EBPβ and C/EBPε, growth-factor independent 1 (GFI1) and interferon-regulatory factor 8 (IRF8) (5) (see Chapter 1, Figure 2). Thus, lineage-restricted expression and activation patterns of these specific transcription factors at different stages of blood cell development are required for specification and formation of a cell type of a certain lineage (6-9). For example, GATA-1 is highly expressed in megakaryocytic/erythroid progenitors (MEP) that give rise to megakaryocyte and red blood cell precursors, while C/EBPα, an essential transcription factor in the development of granulocytes is present in granulocyte/macrophage progenitors (GMP). Furthermore, studies in mice have shown that common myeloid progenitor (CMP) population comprises two separate subpopulations. One subpopulation was found to express high levels of PU.1, an essential transcriptional mediator during myeloid development, while the other CMP population lacked PU.1 expression (10)(11). Interestingly, PU.1-expressing CMPs clearly showed myeloid potential, whereas PU.1-deficient CMPs exhibited erythroid potential, suggesting that PU.1 expression determines the developmental fate of CMPs.

Modulation of C/EBPα, PU.1 and c-Jun activity is regulated through MKK3-p38MAPK signalling

While these and several other transcription factors have been demonstrated to play an important role in directing the development of hematopoietic lineages, the signal transduction

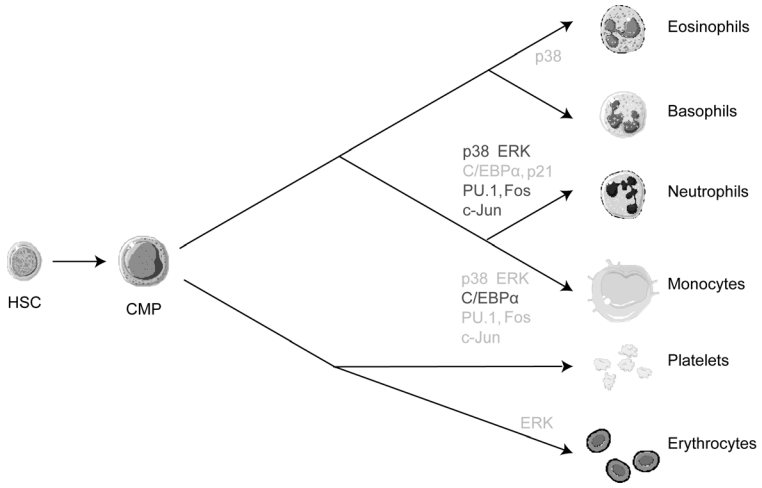

Figure 1. Regulation of myeloid differentiation by ERK and p38MAPK signalling. Inhibition of p38 is essential for neutrophil development, whereas activation is required for both monocyte and eosinophil differentiation. In addition, activation of ERK is essential for final maturation of erythrocytes and monocytes, whereas initial ERK activity is required for optimal expansion of progenitors during neutropoiesis. Positive effects on lineage development are depicted in grey and inhibitory effects are depicted in black.

pathways responsible for the regulation of transcription factor activity are less well understood (Figure 1). In chapter 3, we demonstrated that the MKK3-p38MAPK signalling pathway plays an essential role in regulation of differentiation of hematopoietic progenitors during myelopoiesis. Inhibition of p38MAPK activity enhanced neutrophil differentiation, while high levels of p38MAPK activity were found to abolish final maturation of neutrophils. Furthermore, this was shown to involve MKK3-p38MAPK dependent regulation of C/EBPα activity through phosphorylation of C/EBPα on serine 21. Inhibitory phosphorylation of C/EBPα on serine 21 was induced upon activation of p38MAPK, whereas ectopic expression of a non-phosphorylatable C/EBPα mutant was sufficient to abrogate MKK3-induced inhibition of neutrophil development.

Besides its role in regulating C/EBPα activity, p38MAPK has been demonstrated to indirectly induce phosphorylation and thereby regulate activity of the hematopoietic transcription factor PU.1 (12). PU.1 is a member of the large family of ETS transcription factors, and its expression is restricted to hematopoietic cells (13). PU.1 deletion in mice leads to a lethal defects in fetal liver and post natal hematopoiesis, including the complete absence of B cells, macrophages and greatly reduced neutrophils (14)(15). Recently it has been reported that absence of PU.1 impairs HSC repopulation capacity and abolishes differentiation into CMPs and common lymphoid progenitors (CLP) (16-18), suggesting a key role for PU.1 as a transcriptional regulator of myeloid and lymphoid cells. In addition, expression of low levels of PU.1 in PU.1 deficient cells was found to induce granulopoiesis, whereas high levels induced monocyte differentiation (6)(8). In addition, c-Jun can directly interact with and enhance the ability of PU.1 to activate the M-CSF receptor and IL-1β promoters, thereby

favouring monopoiesis over granulopoiesis (19)(20). Interestingly, p38MAPK has also been suggested to induce transcription of c-Jun, indicating that inhibition of p38MAPK activity could reduce activity of both PU.1 and its cofactor c-Jun and their ability to inhibit granulopoiesis (21). As a consequence, activation of MKK3-p38MAPK in neutrophil progenitors could, besides inactivation of C/EBPα, also result in enhanced PU.1 activity and thereby leading to inhibition of terminal neutrophil maturation.
The activator protein-1 (AP-1) transcription factor complex, which is comprised of members of the Jun and Fos families of phosphoproteins, including c-Fos, c-Jun and JunB, has also been implicated in the regulation of myeloid differentiation. For example, c-Jun, JunB and JunD levels increase during monocytic maturation, whereas ectopic expression of both c-Fos and c-Jun has been demonstrated to induce monocytic differentiation of myeloid cells (22-24). Furthermore, mice deficient for JunB in the hematopoietic system develop increased numbers of granulocytes, however it is unclear whether this represents an effect on lineage commitment rather than loss of the anti-proliferative effect of JunB (25). Besides direct interaction of c-Jun with PU.1, co-immunoprecipitation experiments demonstrated that C/EBPα can interacts with c-Jun, JunB or c-Fos (26), thereby competing with c-Jun for interaction with PU.1 (27). In addition, in murine myeloid progenitors, C/EBPα:c-Jun or C/EBPα:c-Fos heterodimers induced monocyte in favour of granulocyte lineage commitment (26), suggesting that c-Jun or c-Fos interaction may prevent C/EBPα to induce granulocyte differentiation. Functional analysis of C/EBPalpha:cJun dimer revealed that this complex was able to activate the endogenous PU.1 promoter, providing a plausible mechanism for induction of monopoiesis. Thus, besides inhibition of C/EBPα activity through phosphorylation on serine 21, it is possible that MKK3-p38MAPK mediated upregulation of c-Jun is responsible for the observed block in neutrophil differentiation upon ectopic expression of MKK3.

MEK-ERK: an important regulator of various aspects of neutrophil development
In addition to p38MAPK, Ross and colleagues have found that C/EBPα can be directly phosphorylated by ERK1/2 on serine residue 21, which regulates the activity of C/EBPα and its ability to induce neutrophil differentiation (28). Specifically, only the dephosphorylated form of C/EBPα was able to induce neutrophil differentiation, whereas ectopic expression of the phosphomimetic C/EBPα mutant inhibited neutrophil differentiation. Mutations constitutively activating the FLT3 receptor tyrosine kinase gene, which are found in up to 30% of AML patients, are also believed to mediate their effects through activation of the downstream MEK-ERK signalling pathway (29). Aberrant constitutive activation of FLT3 was found to inhibit C/EBPα function by ERK1/2-mediated phosphorylation on serine 21, contributing to the observed differentiation block in leukemic blast cells. In human FLT3 mutant AML cells, pharmacological inhibition of MEK1 resulted in granulocytic differentiation, whereas there was no effect when serine 21 was mutated to aspartate, which mimics phosphorylation of C/EBPα (29). Moreover, activation of RAS signalling has been demonstrated to increase binding of C/EBPα to the G-CSFR promoter upon phosphorylation of C/EBPα on serine 248. Mutation of serine 248 to an alanine completely abrogated the ability of C/EBPα to induce granulocytic differentiation, suggesting that RAS-dependent modulation of C/EBPα activity on serine 248 is involved in the regulation of granulocytic

differentiation (30). In addition, in 32D myeloid cells, DNA binding activity of PU.1 was found to be partially attenuated upon pharmcalogical inhibition of MEK, suggesting a role for MEK-ERK signalling in the regulation of PU.1 activity (31). Furthermore, it has been demonstrated that activated ERK can phosphorylate pre-existing transcription factors, such as Elk-1, involved in the upregulation of immediate-early gene c-Fos (32). Microarray analysis of $CD34^+$ progenitor cells further revealed that activation of MEK1 can result in upregulation of c-Fos, which besides its role as regulator of the cell-cycle, can directly interact with C/EBPα and thereby its inhibit its DNA binding capacity and its ability to induce neutrophil differentiation (Chapter 4). Although our results demonstrate that MEK/ERK pathway is not essential for final maturation of neutrophil progenitors, these above mentioned studies clearly suggest a role for MEK-ERK signalling in the regulation of neutrophil development. Interestingly, our data demonstrate that modulating the duration of ERK activation is essential in regulating the balance between expansion and survival of hematopoietic progenitors during neutrophil differentiation. Inducible activation of MEK1 in $CD34^+$ cells through addition of 4-OHT resulted in dramatic progenitor expansion during the first three days, however, prolonged MEK1 activation prevented neutrophil differentiation due to induction of cell death. It is tempting to speculate that levels of MEK-ERK activation are often high in proliferating hematopoietic progenitor cells to facilitate progenitor expansion, but are down-regulated in differentiating cells to relieve its inhibitory effects on neutrophil maturation, suggesting that ERK signalling may exert its major effects on progenitor expansion during the early phase of granulopoiesis.

p21Cip1, an important cell-cycle regulator, plays a crucial role in the regulation of hematopoiesis

Surprisingly, p21Cip1 was found to be up-regulated by MEK1 activation in $CD34^+$ cells. p21 is best known for its function as a cell cycle inhibitor and was demonstrated to associate and inhibit cyclin-CDK complexes during the G1 phase of the cell cycle (33). Interestingly, using epithelial cells expressing a conditionally active Raf mutant, it was also demonstrated that strong and sustained Raf activity throughout the G1 phase of the cell-cycle triggered cell cycle arrest by inducing massive expression of the cell cycle inhibitor p21, whereas modest activation resulted in enhanced cell cycle entry (34)(35). Since our data also demonstrate that regulation of the duration and extent of ERK activation is essential in regulating the balance between expansion and survival of hematopoietic progenitors during neutrophil differentiation, a possible mechanism explaining the induction of p21 might be through the accumulation of cyclin D1. p21 is a short-lived protein that is degraded through association with the a subunit of the 20S proteasome catalytic complex (36). However, upon high ERK activation, cyclin D1 protein was found to accumulate and associate with p21, thereby preventing routing to the proteasome and subsequent degradation (37). Furthermore, Park *et al* demonstrated that in primary hepatocytes, ERK activation increased phosphorylation of transcription factors such as Ets2 and C/EBPα, which results in enhanced transcription of p21 through Ets- and C/EBP binding elements within the p21 promoter (38).

Several studies have suggested that p21 plays a crucial role in the regulation of hematopoiesis. For example, it has been demonstrated that in absence of p21, expansion of of hematopoietic stem cells is dramatically increased (39). In addition, serial transplantation

of p21-deficient cells resulted in premature stem cell exhaustion, suggesting that p21 is involved in regulating hematopoietic stem and progenitor pool size. Furthermore, upregulation of p21 has been shown in multiple cell lines induced to differentiate, indicating an association between differentiation commitment and arrest in the G1 phase of the cell cycle. For example, Steinman *et al* demonstrated that myeloid maturation of $CD34^+$ progenitor cells is associated with increased p21 expression (40). Taken together, these studies support the idea that p21 may serve additional roles in hematopoiesis either dependent or independent of its primary role as cell-cycle regulator.

Myelosuppressive effects on hematopoiesis are mediated by MAPK signalling

Besides being activated by cytokines that promote proliferation, differentiation and survival of hematopoietic progenitors, p38MAPK signalling pathway is also activated by cellular stimuli that exert negative regulatory effects on hematopoiesis. p38MAPK appears to be activated by myelosuppressive cytokines (41-43) and treatment of human hematopoietic progenitor cells with IFN-α and -β, TGFβ, and TNFα resulted in dose-dependent inhibition of myeloid colonies in methylcellulose colony-forming assays (41)(43). In addition, constitutive activation of MAPK pathways have been reported in a variety of myeloid malignancies, including AML, CML and MDS (44)(45). Although in contrast with our own observations, p38MAPK was found to be constitutively activated in the bone marrows of patients with myelodysplastic syndromes (MDS) (41)(42)(46). Inhibition of p38MAPK decreased apoptosis and stimulated colony formation of primary MDS progenitors, suggesting that aberrant activation of the p38MAPK pathway may cause differentiation defects. In Chapter 3 we have demonstrated that inhibition of p38MAPK in $CD34^+$ progenitor cells isolated from G-CSF unresponsive severe congenital neutropenia (SCN) patients restored neutrophil differentiation. These data support the idea that aberrant activation of the p38MAPK pathway could, for example through inhibitory phosphorylation of C/EBPα on serine 21, be involved in the development of specific bone marrow failure syndromes. Furthermore, in patients with 5q- syndrome, a distinct clinical subgroup of MDS, the gene encoding C/EBPα was found to be extensively down-regulated in MDS progenitor cells (47). Besides mutations in the CEBPα gene itself, C/EBPα transcription may be repressed by DNA promoter hypermethylation (48). Recent data have shown that in a specific subgroup of AML, which phenotypically resembles AML with mutations in C/EBPα, the CEBPα gene was indeed silenced due to, promoter hypermethylation (49). In Chapter 5 we showed that ectopic expression of C/EBPα is sufficient to restore neutrophil development of MDS hematopoietic progenitors, supporting the hypothesis that abrogation of granulopoiesis in MDS patients is, at least in part, due to aberrant C/EBPα expression or functionality in the bone marrow.

Interestingly, in addition to lineage-specific myeloid genes, such as myeloperoxidase, neutrophil elastase and G-CSF receptor (50-52), C/EBPε is one of the regulatory transcription factors whose mRNA has been shown to be rapidly induced by CEBPα in 32D myeloid cells (53). C/EBPε deficient mice develop all of the hematopoietic lineages, but have a defect in terminal neutrophil maturation and increased numbers of immature myeloid cells, indicating that C/EBPε is crucial for late-stage neutrophil production (54)(55). It is interesting to speculate that increased expression of C/EBPα in low-risk MDS $CD34^+$ cells may exert its beneficial effects on neutrophil differentiation through enhanced expression of genes critically

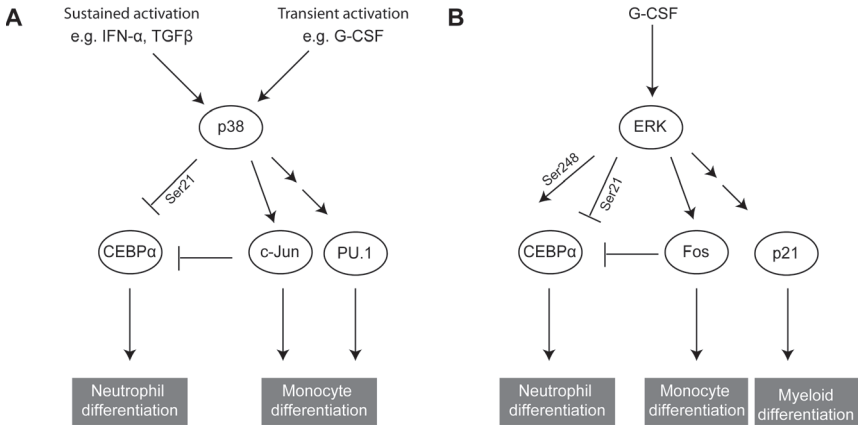

Figure 2. MAPK activity plays a critical role in the regulation of myeloid differentiation. Besides its role in regulating C/EBPα activity, p38MAPK activity has been demonstrated to regulate PU.1 function and its co-factor c-Jun. (A) Transient activation of p38MAPK by cytokines, such as G-CSF promote neutrophil differentiation, whereas sustained activation p38MAPK signalling by myelosuppressive cytokines is believed to inhibit neutrophil development. Inhibition of p38MAPK results in low levels of PU.1 and c-Jun, but increased C/EBPα activity, enabling neutrophil differentiation. (B) Activation of ERK inhibits C/EBPα function by phosphorylation on serine 21, whereas RAS-dependent modulation of C/EBPα activity on serine 248 favours granulocytic differentiation. Furthermore, activated ERK can phosphorylate pre-existing transcription factors, involved in the upregulation of c-Fos which can directly interact with C/EBPα and thereby its inhibit its DNA binding capacity and its ability to induce neutrophil differentiation.

involved in neutrophil production such as C/EBPε.

Regulation of myeloid differentiation *in vivo* is mediated through complex interplay between cytokines

Normal differentiation of myeloid cells *in vivo* is regulated by a complex interplay between different hematopoietic cytokines. For example, IL-3 and G-CSF both play key roles in the regulation of hematopoiesis through modulation of common signal transduction pathways, suggesting need for additional regulatory mechanisms to confer signalling specificity (56)(57). Previous studies have demonstrated that while IL-3 stimulates proliferation of myeloid progenitors, G-CSF-induced granulocytic differentiation is inhibited, suggesting that the action of these cytokines is mutually exclusive at specific stages of differentiation. Furthermore, selective loss of ERK activation in mature neutrophils by G-CSF but not GM-CSF was found to involve upregulation of SHP-1 during G-CSF-induced granulocytic differentiation of the 32D myeloid cells (58). Consistent with our observations that ERK activation is associated with proliferative, but not differentiative signalling in neutrophil progenitors, G-CSF activation of SHP-1 may be a regulatory mechanism for downregulating ERK activation permitting optimal terminal differentiation during neutrophil production. Traditionally, MAPK pathways have been depicted as linear signalling pathways with scaffold proteins providing means to separate pathways from each other. However, it is more plausible that mechanisms facilitating crosstalk between MAPK pathways in mammalian cells exist to increase the possibilities to specify cellular responses without increasing the amount of components in the pathways. This concept is illustrated by studies showing that MEKK1-3, MAP3Ks involved

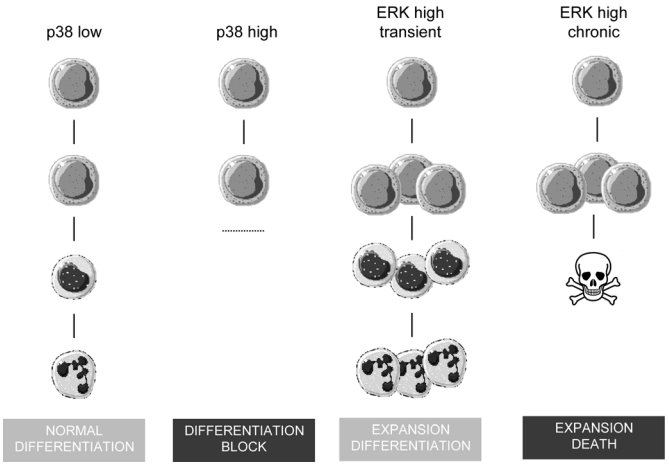

Figure 3. Possible mechanisms of MAPK function involved in the regulation of normal and pathological myelopoiesis. Inhibition of p38 activity is required for terminal differentiation of $CD34^+$ neutrophil progenitor cells, whereas initial ERK activity is required for optimal expansion of progenitors during neutropoiesis. Modulation of the duration of ERK activation is essential in regulating the balance between expansion and survival of hematopoietic progenitors during neutrophil differentiation.

in direct activation of JNK and p38 have been shown to interact with and activate MEK1/2 by direct phosphorylation (59-61). Furthermore, p38MAPK and ERK1/2 were shown to directly interact and further evidence was provided that binding of p38MAPK and ERK1/2 can directly modulate ERK1/2 activity. Because inhibition of ERK1/2 phosphorylation by activated p38MAPK was observed after several hours, it is also possible that ERK1/2 dephosphorylation is mediated indirectly by inducing expression of phosphatases, such as MKP-1 (62)(63). Several reports further suggest that the relative amplitude of ERK1/2 activation can be coupled to specific biological outcomes (64-66). In rat pheochromocytoma PC12 cells, sustained activation of ERK by NGF is required for the induction of neuronal differentiation and growth arrest, whereas transient ERK activation induced by epidermal growth factor (EGF) results in proliferation.

Altogether, these data support the idea that cytokine-mediated differential regulation of signalling components through cross talk of different signalling pathways may play a key role in attenuating certain signalling routes and thereby dictating the ultimate differentiation status of hematopoietic cells.

In addition, bone marrow stromal cells have been demonstrated to play an essential role in the regulation of proliferation and differentiation of hematopoietic stem and progenitor cells (67). Long-term bone marrow cultures were found to produce essential cytokines, chemokines and growth factors such as IL-6, IL-11, G-CSF and GM-CSF. For example, pharmacological inhibition of p38MAPK was found to disrupt the cytokine network between stromal cells and hematopoietic cells through dose-related inhibition of hematopoietic cytokine production, suggesting that MAPK mediated regulation through stromal-derived cytokines might be involved in regulation of normal hematopoiesis (68).

Concluding Remarks

Regulation of myeloid differentiation *in vivo* is mediated through complex interplay between cytokines, modulating the activity of downstream signalling cascades. The experiments described in this thesis have focussed on the roles of several intracellular signalling pathways and transcription factors in the regulation of myeloid differentiation. Together, our data imply distinct roles for both ERK and p38 MAP kinases in regulating neutrophil development (Figure 2). Inhibition of p38 activity is necessary for terminal differentiation of $CD34^+$ neutrophil progenitor cells, whereas initial ERK activity is required for optimal expansion of progenitors during neutropoiesis. Moreover, aberrant regulation of hematopoiesis can lead to bone marrow failure and by identifying the molecular mechanisms underlying the function of these protein kinases in hematopoietic progenitor cells, it will be possible to design therapies modulating MAPK activities for the treatment of bone marrow failure or hematological malignancies. As already discussed, inhibition of p38MAPK in $CD34^+$ progenitor cells isolated from G-CSF unresponsive SCN patients restored neutrophil differentiation, whereas pharmacological inhibition of MEK-ERK pathway inhibits proliferation and induces apoptosis in primary AML blasts (69). Furthermore, therapies targeting constitutive activation of MEK-ERK or p38MAPK may be effective in treatment of neutropenias resulting from inactivation of C/EBPα. Although these results enhance our understanding regarding the mechanisms involved in the regulation of normal and pathological myelopoiesis (Figure 3), future experiments will be necessary to further dissect the signalling pathways involved in determining lineage choices and how these can confer specificity.

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Het bloed bestaat uit verschillende typen cellen die allemaal een specifieke functie vervullen. De “rode bloedcellen” verzorgen bijvoorbeeld het zuurstoftransport vanuit de longen naar de verschillende weefsels. De “megakaryocyten”, die bloedplaatjes produceren, zijn betrokken bij het herstel van bloedvaten na verwonding, terwijl de “witte bloedcellen” een belangrijke rol spelen bij de afweer van het lichaam tegen ziekteverwekkers. Witte bloedcellen kunnen verder worden onderscheiden in, onder andere, B-cellen, T-cellen, monocyten en neutrofiele granulocyten. De ontwikkeling van bloedcellen, ook wel “haematopoiese” genoemd, is een complex proces dat plaatsvindt in het beenmerg. In het beenmerg bevinden zich voorlopercellen, of “haematopoietische stamcellen”, die zich vermenigvuldigen en kunnen uitrijpen tot de verschillende typen volwassen bloedcellen. Daarbij is het instandhouden van deze voorlopercellen essentieel om de ontwikkeling van bloedcellen te waarborgen gedurende het gehele leven. Verstoring van dit proces kan leiden tot verkeerde uitrijping van bloedcellen wat als gevolg kan hebben dat sommige volwassen bloedcellen niet volledig kunnen functioneren. Mensen met Myelodysplasie (MDS) hebben bijvoorbeeld een tekort aan neutrofielen, rode bloedcellen en bloedplaatjes door een verstoorde uitrijping van de voorlopercellen in het beenmerg. Voor de ontwikkeling van therapieën is het daarom van belang om inzicht te verkrijgen in de processen die betrokken zijn bij de normale ontwikkeling van bloedcellen.

De studies beschreven in dit proefschrift zijn gericht op de vraag hoe de ontwikkeling van neutrofiele granulocyten uit voorlopercellen gereguleerd is. Om de ontwikkeling van neutrofielen tijdens de verschillende stadia te kunnen bestuderen, hebben we een kweeksyteem gebruikt wat het mogelijk maakt om geïsoleerde haematopoietische stamcellen, uit zowel beenmerg als navelstrengbloed, buiten het lichaam te laten ontwikkelen tot volwassen bloedcellen met behulp van specifieke groeifactoren. Omdat alle verschillende typen bloedcellen zich ontwikkelen uit dezelfde voorlopercel, is het belangrijk om te begrijpen hoe de beslissing genomen wordt in welke richting een cel zich gaat ontwikkelen. Daarnaast is het van belang om te begrijpen welke van de vele verschillende genen en eiwitten in een cel een rol spelen in de ontwikkeling van een vroege voorlopercel tot de volwassen neutrofiel. DNA is de drager van erfelijke informatie en bestaat uit afzonderlijke genen. Elk gen bevat de instructies voor het synthetiseren van een specifiek eiwit. Deze eiwitten bepalen uiteindelijk de activiteiten van een cel. Om de DNA code te vertalen in eiwitten, moet eerst een afdruk van het DNA gemaakt worden tijdens een proces genaamd “transcriptie”. Het proces van transcriptie wordt gereguleerd door verschillende eiwitten die transcriptiefactoren genoemd worden. De aanmaak van specifieke eiwitten in de cel door transcriptiefactoren kan worden gereguleerd door signalen van buiten de cel, zoals groeifactoren. Deze externe signaalmoleculen communiceren met de cel door receptoren op de buitenkant van de cel waaraan deze groeifactoren kunnen binden. Binding van een groeifactor aan zijn receptor activeert vervolgens een cascade van eiwitten en transcriptiefactoren, wat uiteindelijk resulteert in een verandering in de expressie van specifieke eiwitten. Het vertalen van externe signalen naar verandering in de aanmaak van specifieke eiwitten in de cel wordt “signaal transductie” genoemd. Om te kunnen onderzoeken welke signaaltransductie routes en welke specifieke eiwitten van belang zijn voor neutrofielontwikkeling, kunnen eiwitten van interesse geïntroduceerd worden in haematopoietische cellen met behulp van retrovirussen. Door gebruik te maken van deze techniek hebben we in Hoofdstuk 3 een signaaltransductie route

die zorgt voor de activatie van het eiwit p38MAPK onderzocht, waarvan bekend is dat deze een belangrijke rol speelt tijdens de ontwikkeling van andere bloedcellen. Tevens kan, door gebruik te maken van een specifieke farmacologische remmer, de activiteit van p38MAPK worden stilgelegd en onderzocht worden of dit eiwit een rol speelt tijdens de ontwikkeling van neutrofiele granulocyten. Uit deze experimenten is gebleken dat remming van p38MAPK nodig is voor de uitrijping van hematopoietische voorlopercellen naar volwassen neutrofielen, terwijl verhoogde activatie van p38MAPK ervoor zorgt dat de ontwikkeling van neutrofiele granulocyten geremd wordt. Dit duidt erop dat correcte regulatie van de activiteit van p38MAPK belangrijk is voor de normale ontwikkeling van neutrofiele granulocyten. Verder hebben we in hoofdstuk 3 laten zien dat p38MAPK zijn functie vervult door de activiteit van een transcriptiefactor C/EBPα te reguleren. Uit de experimenten beschreven in Hoofdstuk 4 is gebleken dat, behalve p38MAPK ook, ERK MAPK, een ander eiwit uit dezelfde familie, een belangrijke rol speelt tijdens de ontwikkeling van neutrofielen. Tijdelijke activatie van de ERK MAPK signaaltransductie route in een vroeg stadium van ontwikkeling is belangrijk voor een optimale neutrofielproductie, terwijl langdurige activatie van ERK MAPK de uiteindelijke uitrijping van de neutrofiele granulocyt blijkt te remmen door een verlaagde overleving van de cellen. In Hoofdstuk 5 hebben we een andere signaaltransductie route onderzocht waarvan al bekend was dat deze betrokken is bij de regulatie van celdeling in andere soorten cellen. We laten zien dat remming van de activiteit van het eiwit mTOR de uitgroei van neutrofielvoorlopercellen remt in een specifiek stadium: de vroege haematopoietische voorlopercellen worden niet geremd, terwijl productie van de latere, meer rijpe cellen negatief wordt beïnvloed. De mogelijke, klinische toepassing van de verkregen inzichten in de regulatie van neutrofielproductie wordt geillustreerd in Hoofdstuk 6. In dit hoofdstuk laten we zien dat verhoogde expressie van de transcriptiefactor C/EBPα de geblokkeerde uitrijping van neutrofiele granulocyten in beenmergcellen, die afkomstig zijn van patiënten met myelodysplasie, kan corrigeren. Deze transcriptiefactor wordt normaal gesproken tijdens de ontwikkeling van neutrofielen aangemaakt en geactiveerd. Uit eerdere studies in muismodellen is gebleken dat tekorten in de expressie van C/EBPα kunnen leiden tot een verminderde neutrofielproductie. Onze experimenten laten zien dat inactivatie of afwezigheid van C/EBPα kan leiden tot een verlaagde uitrijping van de neutrofiele granulocyt in patienten met myelodysplasie.

Experimenten beschreven in dit proefschrift hebben er toe bijgedragen dat ons inzicht in de moleculaire mechanismen die betrokken zijn bij de regulatie van neutrofielontwikkeling verder is toegenomen. In de toekomst zal nog meer onderzoek nodig zijn om de ontwikkeling van nieuwe therapieën tegen ziekten als MDS mogelijk te maken.

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-Christian

Christian Geest werd op 21 februari 1979 geboren te Mijdrecht. Na het behalen van zijn Atheneum diploma, begon hij in 1997 met de studie Medische Biologie aan de Vrije Universiteit in Amsterdam. Het doctoraal examen werd behaald na afstudeeronderwerpen gevolgd te hebben aan de afdelingen Tumor Cel Biologie (Vrije Universiteit Amsterdam) en Genetica en Celbiologie (Erasmus Universiteit Rotterdam). In 2003 begon hij als assistent in opleiding bij de afdeling Immunologie van het Universitair Medisch Centrum te Utrecht. Het in dit proefschrift beschreven onderzoek vond plaats onder begeleiding van Prof. Paul Coffer, Prof. Edo Vellenga en Dr. Miranda Buitenhuis.