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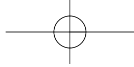

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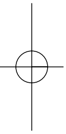

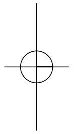

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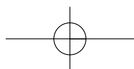

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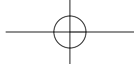

The Role of the Forkhead Transcription Factor Foxo3a in Erythropoiesis

De Rol van de Forkhead Transcriptie Factor Foxo3a in Erythropoïese

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de Rector Magnificus Prof.dr. S.W.J. Lamberts
en volgens het besluit van het College voor Promoties.

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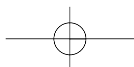

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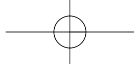

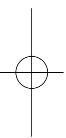

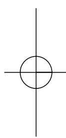

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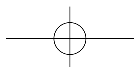

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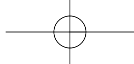

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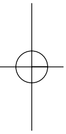
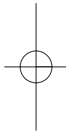

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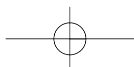

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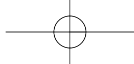

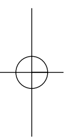

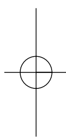

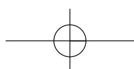

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Introduction

1. Hematopoiesis

Hematopoiesis, or blood cell formation, is the process in which a limited set of hematopoietic stem cells is able to give rise to all types of functional blood cells via commitment to specific hematopoietic lineages (Figure 1.1). The majority of the stem cells are quiescent, leaving only a small population of actively cycling hematopoietic stem cells (181). When a stem cell divides, it again generates a stem cell, a proces called self-renewal, and a cell that is committed to develop into either a myeloid or lymphoid restricted stem cell (120). Differentiation is the process in which a hematopoietic stem cell matures into a functional blood cell. This process is irreversible and the more differentiated a progenitor cell is, the less blood cells it can produce (175). Blood cells can be classified into three groups. Red cells, or erythrocytes, which constitute about 45% of the blood volume. They are responsible for transport of oxygen (O_2) from the lungs throughout the organism, and of CO_2 from the tissues to the lungs. Platelets, which are important in blood clotting, constitute 0.1 % of the blood volume whereas white blood cells have a role in defense against infections and constitute less than 0.1% of the total blood volume.

During murine embryonic development, hematopoiesis starts in the blood islands of the yolk sac 7-11 days post coitum (69). Yolk sac hematopoiesis is referred to as primitive hematopoiesis. It is believed that definitive erythropoiesis starts in the aorta-gonad-mesonephros region (AGM-region), where the first definitive hematopoietic stem cells develop (55, 68, 171, 200). From the AGM, the hematopoietic stem cells migrate to the fetal liver and to a lesser extent to the fetal spleen, where definitive hematopoiesis continues at about day 11 of development. In neonates, hematopoiesis is finally established in the bone marrow. Hematopoiesis is a tightly regulated process that has to respond and adjust to a variety of conditions such as blood cell turnover, infection and blood loss (168, 174, 175). Hematopoiesis is regulated by a variety of factors presented by stromal cells or present in the extracellular matrix. However, regulation by hematopoietic growth factors is a dominant and certainly the best studied aspect of hematopoiesis.

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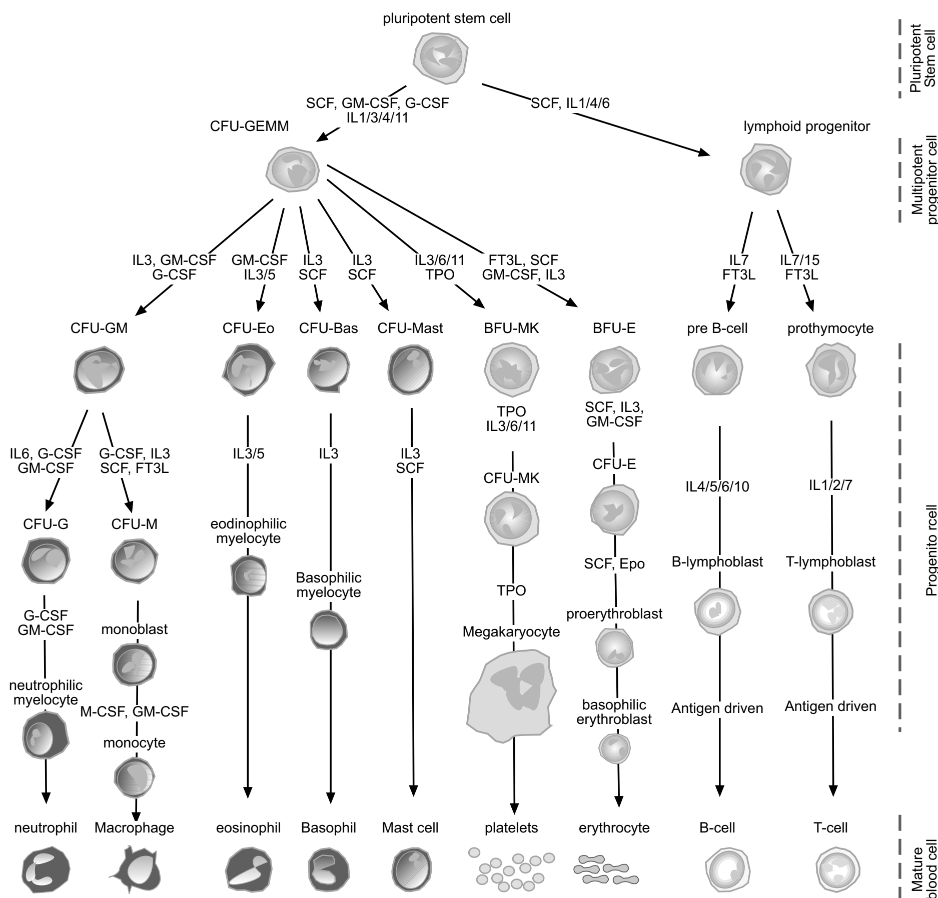

Figure 1.1. **Schematic picture of hematopoiesis**. Stem cells are capable of self-renewal and differentiation. Upon differentiation into the common myeloid or lymphoid progenitor the self-renewal capability is lost. During hematopoiesis differentiation is assumed to be irreversible. Via distinct progenitor stages, multipotent progenitor cells differentiate in mature, fuctional blood cells. Differentiation into the separated hematopoietic lineages requires the presence of certain hematopoietic growth factors and cytokines. SCF (Stem cell factor), IL3 (Interleukin 3), FT3L (Fms-like tyrosine kinase-3 ligand), Epo (erythropoietin), GM-CSF (granulocyte/macrophage-colony stimulating factor), G-CSF (granulocyte -colony stimulating factor), M-CSF (macrophage-colony stimulating factor), TPO (thrombopoietin), CFU-GEMM (colony forming unit-granulocyte-erythroid-monocyte-megakaryocyte), CFU-Bas (colony forming unit-basophilic granulocyte), CFU-Mast (colony forming unit-mast cell), CFU-Eo(colony forming unit-eosinophilic-granulocyte), BFU-MK (burst forming unit-megakaryocyte), BFU-E (burst forming unit-erythroid).

1.1. Regulation of Hematopoiesis by hematopoietic growth factors

Many extrinsic and intrinsic factors determine lineage commitment, expansion and survival of hematopoietic stem cells. Two models have been proposed for regulation of hematopoiesis. The stochastic model (267, 288) implies that commitment is a random process in which growth factors only determine the outgrowth of certain committed progenitors. The instructive model claims a deterministic role in lineage commitment for extracellular factors, such as hematopoietic growth factors (270, 282). For example, certain transcription factors which determine commitment of hematopoietic stem cells, are regulated by growth factors (326). Although additional research is needed to address the validity of these models in its full scope, hematopoietic growth factors such as cytokines are of great importance for regulation of the differentiation process (60, 266). Hematopoietic growth factors are produced by stromal cells in the bone marrow and by monocytes, macrophages, endothelial cells, and various organs such as the pancreas, kidney and liver. In general, the receptors for the hematopoietic growth factors can be classified in two groups: the tyrosine kinase family with an intrinsic kinase domain (276), and the cytokine family lacking an intrinsic kinase domain (44). Members of the latter family couple to the Janus kinase family members, which phosphorylate the receptor and downstream targets (113).

As shown in Figure 1.1, certain growth factors and cytokines have a broad target cell specificity and act on a wide variety of hematopoietic progenitor cells, including multilineage progenitors. Examples of this category are stem cell factor (SCF; 169), Interleukin-3 (IL-3; 162), and Fms-like tyrosine kinase-3 ligand (FLT3-L; 229). The target cell spectrum of other growth factors is more restricted. They regulate the proliferation and differentiation of particular, committed subsets of hematopoietic cells only, as exemplified by erythropoietin (Epo; 98, 106, 141), thrombopoietin (TPO; 166), and granulocyte-colony stimulating factor (G-CSF; 59). Hematopoietic growth factors may also act in concert to support proliferation of a certain hematopoietic cells. For example, SCF cooperates with GM-CSF, G-CSF, IL-3 or Epo to stimulate the proliferation of myeloid and erythroid progenitor cells (169). In addition, a series of growth inhibiting factors have been described. Examples of cytokines with a negative effect on growth are transforming-growth factor-β (124, 125), and tumor necrosis factor-α (29).

1.2. Regulation of hematopoiesis by transcription factors

Transcription factors are important intrinsic factors that regulate hematopoiesis. This is shown by the fact that deregulation of the transcription factors PU-1, GATA-1, AML-1 or C/EBPα contributes to development of human acute myeloid leukemia (259). Transcription factors in the hematopoietic system can be classified in two categories: (1) general factors, which play a role in multiple hematopoietic lineages and mostly in multipotent progenitors, e.g. GATA-2 (273), AML-1 (196), or SCL (223), and (2) specific transcription factors, which are expressed in distinct lineages, such as PAX5, involved in early B-cell development (194), and erythroid Krüppel-like factor, involved in erythroid development (193). Except for lineage specific gene expression, some lineage-specific transcription factors play a deterministic role in lineage commitment. A

typical example is GATA-1. This factor is required for erythroid and megakaryocytic differentiation (189, 241, 292) and has been shown to regulate lineage commitment at two different levels. First, the ratio GATA-1 *versus* PU-1, a transcription factor required for development of early multipotent myeloid progenitors (30, 57), determines development of the myeloid progenitor into either the myeloid or the erythroid lineage. Second, differential binding to friend of GATA (FOG) to GATA-1 regulates megakaryocytic versus erythroid commitment (37). A similar role in lineage choice has been proposed for C/EBPα and PU-1 in granulocytic/macrophage progenitors (215, 218).

These examples highlight the importance of transcriptional regulation in normal hematopoietic development. Interestingly, a category of transcription factors is regulated by hematopoietic growth factors. Because hematopoietic growth factors are dominant in the regulation of hematopoiesis, they may exert their function at least in part through transcriptional interference.

1.3. Signaling regulated gene expression

Transcription factors and growth factors act in concert to determine commitment, expansion, survival and differentiation of hematopoietic cells. Several classes of transcription factors have been described that integrate growth factor signaling with regulation of gene transcription in the hematopoietic system. By doing so, signaling regulated transcription factors change the gene expression profile to allow adaptation of a certain hematopoietic cell to its environment. This group of transcription factors comprises Signal transducers and activator of transcription (Stats), Forkhead box containing transcription factors sub class O (Foxo), cAMP response element binding proteins and the NFκB family. Stats are activated upon cytokine induction via Janus kinases (128), and mice lacking Stat5a and Sta5b suffer from severe anemia during fetal liver erythropoiesis (46, 245). Foxo family members are inactivated upon cytokine or growth factor treatment via direct phosphorylation by protein kinase B (PKB; 30).

2. Erythropoiesis

Erythropoiesis is the process by which erythrocytes are produced. As outlined in Figure 1.1, the earliest known progenitor cell with the potential to form erythrocytes, the CFU_{GEMM}, originates from the common, multipotent progenitor cell, and differentiates via multiple intermediate stages (BFU_E, CFU_E, see below) into a functional erythrocyte.

2.1. Erythroid development

Progenitors can be measured by various *in vivo* and *in vitro* methods. Often used *in vivo* tests are the colony forming unit-spleen assay (CFU_S) and repopulation assays, whereas colony cultures in semi-solid medium, liquid cultures, flow cytometry and cytology represent commonly used *in vitro* tests. These methods have been used to study the multipotency and the proliferative capacity of erythroid progenitor cells and have contributed to the identification of discrete stages of erythroid development. The *in vivo* colony forming Unit-spleen assay (CFU_S) was for a long time the leading assay for studying stem cells (265). In this assay, bone marrow progenitor cells that are transplanted in irradiated mice

produce colonies on the surface of the mouse spleen 7 to 13 days after transplantation. The assay identifies multipotent progenitors (CFU_S) from which erythrocytes can be grown (172). This multipotent progenitor also showed similarities towards the burst forming unit-erythroid (BFU_E, described later; 161). A very early erythroid progenitor can be identified using an *in vitro* erythroid colony assay in semisolid medium. This progenitor is called CFU_{GEMM} for colony-forming-unit granulocytes, erythrocytes, megakaryocytes, and macrophages (173). An immature, fully erythroid committed progenitor is the burst forming unit-erythroid (BFU_E). *In vitro*, when grown in the presence of Epo, SCF and interleukin-3 (IL-3), the BFU_E is able to form a colony consisting of about 5000 erythrocytes in about 10 days (176, 310). A more mature, phenotypically distinct, erythroid progenitor is the colony forming unit-erythroid (CFU_E). *In vitro*, this progenitor is able to give rise to colonies containing 8 to 64 erythrocytes in the presence of Epo (309). The CFU_{GEMM}, BFU_E, and CFU_E can be discriminated in the same culture by analyzing colony size, color and morphological characteristics. SCF has been shown to act mainly on the BFU_E stage and has little effect on the CFU_E progenitor. Epo however, is required and sufficient for survival and proliferation from the CFU_E stage towards the mature erythrocytes (98, 106, 115). During maturation of a CFU_E, several morphological distinctive stages can be discriminated (108). The first is the pro-erythroblast. In humans this cell has a size of 14 - 19 µm, contains visible nucleoli, and is able to store iron required for hemoglobin synthesis later on in differentiation. The basophilic erythroblast represents the next morphological distinctive stage and can be characterized by disappearing nucleoli, a cell size of 12 - 17 µm, the start of hemoglobin synthesis and condensation of DNA. It is called basophilic because of large amount of alkaline ribosomal and globin mRNA molecules present in the cytoplasm. Further differentiation gives rise to the polychromatic erythroblast. This progenitor has already high levels of the hemoglobin protein, and has decreased its size to 12 - 15 µm. Further condensation of the DNA then yields the orthochromatic erythroblast (8 - 12 µm). Extrusion of the nucleus results in a reticulocyte. From this stage it takes about 1 day for the reticulocyte to terminally differentiate into a 7 - 8 µm, donut-shaped, mature erythrocyte that lost its ribosomes and mitochondria. In mice, the pro-erythroblast measures about 12 µm in diameter, and finally reaches a diameter of 4 µm as a mature erythrocyte. The erythrocyte has an average life span of 120 days in humans and 55 days in mice. Worn-out erythrocytes are phagocytosed by macrophages in the liver and the spleen, whereas nuclei extruded during differentiation are phagocytosed by macrophages in the bone marrow.

3. The major factors that regulate erythropoiesis

3.1. Erythropoietin is required for erythroid expansion and differentiation

In the hematopoietic system, the receptor for erythropoietin (EpoR) is selectively expressed in the erythroid lineage resulting in a lineage specific Epo response. Epo and its receptor are both essential from the CFU_E stage of erythroid differentiation; mice lacking Epo (*Epo)* or the Epo-receptor (*EpoR*) die at day 12.5 of gestation from a lack of fetal erythrocytes (313). CFU_E can be isolated from

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Epo null fetal livers and do form mature erythrocytes when supplemented with exogenous Epo *in vitro* (152, 313). Erythropoietin (Epo) is produced in the kidney in response to low oxygen levels (hypoxic conditions), but also by BFU_E in an oxygen-independent way (251). Under normal physiological conditions Epo levels are suboptimal, leading to homeostasis of red cell production with about 20% of erythroid progenitors going in apoptosis (107, 240). This suggests that Epo functions as a survival factor (77, 100, 115, 139). This is further established in experiments showing that ectopic expression of the GM-CSF, G-CSF or prolactin receptor rescues erythroid colony formation in absence of Epo signaling (97, 244, 246, 302). However, it is of note that Epo has also been shown to be essential for induction of erythroid expansion in cooperation with SCF, as will be discussed later.

3.1.1. EpoR signaling

The EpoR is a type I cytokine receptor characterized by the presence of four conserved cysteines (C), a WSXWS motif, a group of aromatic residues in its extracellular domain, the absence of intrinsic tyrosine kinase activity and its constitutive association with Janus kinase-2 (Jak2; 141, 264, 307). When Epo binds to the EpoR homodimer, the receptor conformation changes resulting in activation of Jak2 and phosphorylation of the EpoR (220, 257, 297) on 8 tyrosine motifs (48, 307). Receptor phosphorylation results in the recruitment and activation of various proteins that bind direct or indirect to the receptor (Figure 1.2) (48, 235). I will give a brief overview on the main pathways activated.

Stat5 is the main Stat-family member involved in EpoR signaling, and is recruited to Y343 and Y403 (51, 293). After phosphorylation by Jak2, Stat5 dimerizes and translocates to the nucleus to regulate target gene transcription (51, 113). Several Stat5 target genes have been identified. Bcl-X_L is up-regulated in differentiation in a Stat5-dependent way, and substitutes for Epo-induced survival during differentiation (65). Activation of the Stat5 targets cytokine-inducible protein (Cis), suppressor of cytokine signaling (SOCS), and Pim-1 serves as a negative feedback loop for Epo receptor signaling via multiple mechanisms (165, 186, 320). SOCS1 binds Jak2 and inhibits its catalytic activity (71, 186), whereas Cis has been shown to bind directly to phosphorylated tyrosines on the receptor, thereby competing with Stat5 for binding (165). Pim-1 causes reduced tyrosine phosphorylation of the receptor, probably through stabilization of the SOCS proteins (204).

Activation of the Ras/Raf/Mitogen-activated protein kinase (MAP kinase) pathway by the Epo receptor occurs via several steps. Epo stimulation results in complex formation between growth factor receptor-bound protein 2 (Grb-2) and Shc, and recruitment to tyrosine 464 of the EpoR (49, 50), upon which the guanine nucleotide exchange factor Son of sevenless (Sos-1) is recruited to the complex. Sos-1 than converts the inactive Ras-GDP into the active Ras-GTP form, resulting in the activation of the downstream target molecules Raf/MAP kinase (67, 70, 262, 263).

The p85α subunit of Phosphoinositide-3-OH kinase (PI3K) is recruited to Y481 (51, 130). The associated catalytic subunit (p110) phosphorylates phospholipids in the membrane, producing phosphatidylinositol-3,4-phosphate

(PIP2) and PI-3,4,5-phosphate (PIP3) from PI-4-phosphate and PI-4,5-phosphate, respectively. These PI3K lipid products act as signaling-dependent anchors to recruit multiple signaling molecules that contain a pleckstrin homology domain. Examples are protein kinase B (PKB), Phosphoinositide-dependent kinase-1 (PDK1) and Bruton's tyrosine kinase (Btk), as well as docking molecules such as Adaptor protein-2 (AP-2) and Grb-2 associated binding protein-1 and 2 (Gab-1, Gab-2) (109, 146, 268, 305, 318). In turn, proteins like Gab-1 and 2 serve as docking molecules for many other signaling molecules like SH2-containing protein Shc, SH2-containing inositol phosphatase (SHIP), Grb2, Phospholipase C (PLC) and PI3K (156, 304). Since PIP3-dependent Gabs are able to recruit PI3K, they serve as a positive feedback-loop for PI3K activity (225).

Recently the tyrosine kinase RON was also implicated in EpoR signaling. Ron has been shown to associate with Gab-1 and to activate the PI3K/PKB and the MAP kinase pathways (1, 78, 191, 324), Ron is phosphorylated by Jak2 in response to Epo (279) and Epo-induced phosphorylation of Gab-1 appeared to be critically dependent on Ron kinase activity (279). Direct activation of Ron results in the activation of the MAP kinase and PI3K/PKB pathway, but not the Jak/Stat pathway (279).

Since many of the signaling molecules can dock to several sites in the signaling complex, signal transduction pathways may be initiated in various ways. Notably, transduction of erythroid progenitors derived from *EpoR* -/- fetal livers with distinct EpoR mutants suggested that only tyrosine 479 of the EpoR is essential erythroid colony formation (131, 312). However, mice expressing a truncated EpoR lacking all tyrosines exhibit a normal erythropoiesis (321).

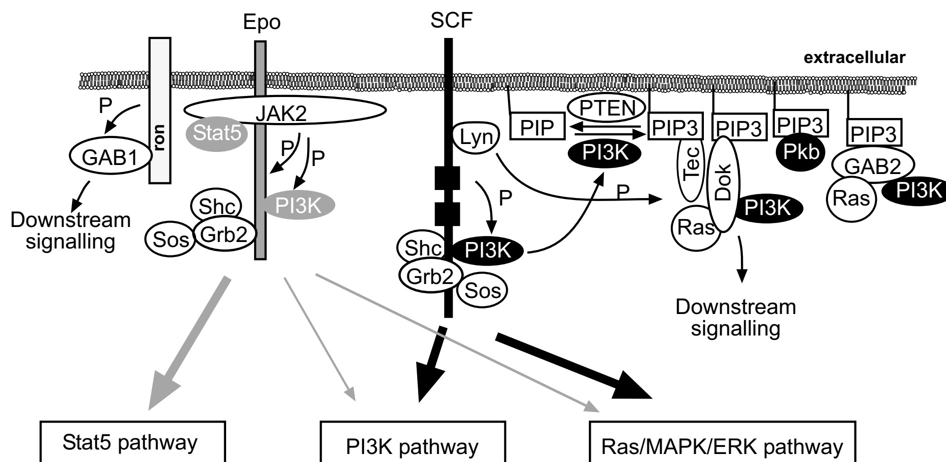

Figure 1.2. **Schematic representation of the signaling complexes and signal transduction pathways activated by Epo and SCF**. Ligand binding to the Epo- and c-Kit receptor triggers a cascade of downstream signaling events. Downstream signaling molecules are recruited to the receptors or to adaptor molecules (Gab-1, Gab-2, Dok, Grb2). The EpoR and c-Kit contribute differentially to activation of the three main downstream signal transduction pathways. Thick arrows indicate stronger activation of a certain pathway compared to thin arrows. The contribution of Epo to these different pathway is indicated in gray arrows, the contribution of SCF to the different pathways is indicated by black arrows. A more detailed representation of the PI3K pathway is shown on the right. In addition to PTEN, the phosphatase SHIP is also involved in dephosphorylation of the membrane associated phosphoinositol rings (PIP3).

3.2. Stem cell factor is required for erythroid expansion

The receptor c-Kit is a growth factor receptor with ligand-dependent tyrosine kinase activity, classified as a type III receptor kinase family member together with Fms-like tyrosine kinase-3 (Flt-3), the receptors for colony-stimulating factor-1 (CFS-1), and the platelet-derived growth factor receptor (PDGF). Common features of this family are the extracellular part with five immunoglobulin-like domains, and the cytoplasmic part with a kinase domain which is interrupted by a insert of variable length (10, 25). *c-Kit* was originally identified as the viral oncogene (*v-kit*) responsible for the transforming activity if the Hardy-Zuckerman IV feline sarcoma virus (18). The importance of c-Kit and its ligand SCF was long known, since two genetic mutants, mice with a homozygous *White* or *Steel* locus were white and severely anemic at birth. The *White* locus appeared to encode c-Kit, the *Steel* locus its ligand SCF. SCF turned out to be a growth factor with a wide biological range. In development, SCF is not only important for migration and proliferation of hematopietic stem cells, but also for migration and proliferation of melanoblasts and germ cells (10). In the hematopoietic system SCF acts on many different hematopoietic cells, starting from the pluripotent stem cell towards more mature stages (Figure 1.1; 25, 159). SCF is produced by various cell types (endothelial cells, fibroblasts, hematopoietic progenitor, and marrow stromal cells) as a membrane-bound and soluble factor (25). Mice lacking the membrane-bound form of SCF (Steel dicky mutation; Sld) suffer from severe anemia, indicating that soluble SCF alone is not enough for normal

erythropoiesis (79). *Sld* mice not only have strongly reduced numbers of BFU_E and CFU_E, but also have declined numbers of CFU-granulocyte-macrophage (CFU_{GM}) and CFU_S underlining the wide activity of SCF in the hematopoietic system (13). Also in *in vitro* cultures of erythroid progenitors, membrane bound SCF appeared to be more potent than soluble SCF to inhibit differentiation and promote expansion (122, 198). This could be due to the fact that membrane bound SCF induces prolonged c-Kit signaling (177). In addition, membrane bound SCF may play a role in the localization and/or the migration of the erythroid progenitors. For instance, the presence of membrane bound SCF on spleen cells is required for expansion of erythroid cells during recovery from hemolytic anemia in adult mice (26). In this process, BFU_E from the bone marrow travel to the spleen to support stress erythropoiesis (26, 99, 103, 190). This suggests that membrane bound SCF on the spleen is responsible for lodging and expansion of BFU_E in the spleen. Mice with mutated, non-functional c-Kit genes also suffer from severe anemia (28, 192, 306). Expression of c-Kit is found from the earliest erythroid progenitor up to the stage of basophilic erythroblast (277). This is supported by the finding that SCF is required for the *in vitro* expansion of erythroid progenitors as it retards differentiation of both human and mouse erythroid progenitor cells while stimulating proliferation (64, 184, 289, 290). Although c-Kit or SCF homozygous null mice may suffer from an overall decrease in blood cells, the stringent effect on the erythroid lineage indicates that this receptor is essential for the expansion of the erythroid lineage.

3.2.1. SCF signaling

Binding of SCF to the receptor results in activation of its intrinsic kinase activity and subsequent autophosphorylation, creating docking sites for several proteins. PI3K binds to tyrosine 721 in human c-Kit (239), and its activation creates docking sites for proteins containing a pleckstrin homology (PH)-domain (see 3.1.1). For example, SCF induces PI3K-dependent recruitment of a protein complex consisting of Lyn, Tec and Dok to its receptor (281). The Src family member Lyn associates with the juxtamembrane domain of c-Kit (Y568), and phosphorylates the cytoplasmic tyrosine kinase Tec and the docking molecule $p62^{Dok-1}$. $p62^{Dok-1}$ contains several protein-protein interaction domains, including tyrosine residues, to recruit proteins involved in the regulation of downstream events of c-Kit signaling. It has been suggested that $p62^{Dok-1}$ serves as a negative regulator of c-Kit signaling. In erythroid cells we have found that SOCS1 and 3, and SHIP are recruited by $p62^{Dok-1}$ (Thamar van Dijk, unpublished results), leading to receptor down modulation. A role for $p62^{Dok-1}$ in down modulation of receptor signaling has also been shown in other systems (255, 316). Like Epo, SCF also results in the PIP3-dependent recruitment of the docking molecule Gab-2 (279).

SCF-induced activation of the Ras/MAP kinase pathway requires activation of Lyn, which in turn recruits and phosphorylates Shc. Both Shc and phosphorylated residues in c-Kit recruit Grb2/Sos, which subsequently activates the Ras-Raf-MAP kinase cascade (24, 117, 147).

3.3. Cooperation between SCF and Epo signaling

The cooperation of Epo and SCF in erythroid expansion is shown by increased erythroid colony numbers in the presence of both factors (131). The cooperation is confirmed at the molecular level. We showed co-immunoprecipitation of the EpoR and cKit in murine primary fetal liver cells and in the erythroblasts cell line I/11 (278). Several domains have been shown to be important for this interaction. The tyrosine kinase receptor c-Kit interacts with the extended box-2 region in the cytoplasmic domain of the EpoR (313). In addition, we showed an interaction of the extracellular domain of both receptors in a yeast two hybrid assay (unpublished data). These data show that the Epo and SCF receptors can function in the same signaling complex.

Signal transduction pathways activated by Epo and SCF overlap to a large extent (Figure 1.2; and paragraph 3.1.1 and 3.2.1). In general, Epo and SCF stimulation lead to activation of the Ras/MAP kinase and PI3K pathway, and of downsteam targets such as the adapter molecule Gab-2 (279) and Bruton's tyrosine kinase (Btk; 237). However, there are also differences in downstream target activation. The most obvious difference relates to Epo-induced activation of the Jak/Stat pathway. Recruitment of adapter molecule Gab-1 by Ron, and its activation in response to Epo treatment (279), is another Epo-specific event. Other targets are phosphorylated in response to both Epo and SCF but appear to have stimulus-specific effects. Btk activation protects primary cells from Trail-induced apoptosis only when induced by SCF (237), whereas Epo-induced Btk phosphorylation acts as a positive regulator of EpoR signaling (237). Activation of Btk in response to SCF may affect SCF-induced gene expression, but it is equally possible that Btk mediates the cellular localization of specific proteins such as those involved in TRAIL-induced apoptosis. Since not all downstream pathways need to affect target gene expression, it will be revealing to profile SCF target genes e.g. in Btk-deficient cells. In addition, other differentially regulated targets of both receptors may be discovered in future experiments as well.

In conclusion, the partial overlap between Epo and SCF signaling explains why both receptors are required for erythroid expansion. Since the combined activation of common Epo/SCF signal transduction cascades results in a more enhanced and prolonged signal (135, 253), the strength of the Epo/SCF induced signal may also be essential for full support of erythroid expansion.

3.4. *In vitro* expansion of erythroid progenitors reflects stress-erythropoiesis

The cooperative effect of Epo and SCF on erythroid expansion has been shown in erythroid progenitors derived from different tissues and organisms. Erythroid progenitors derived from human neonatal cord blood and adult bone marrow, from mouse fetal liver and adult bone marrow, or from chicken bone marrow, can be expanded in the presence of Epo, SCF, and Dex (290, 291, 301, 303). Epo and SCF have also been reported to cooperate in expansion of human erythroid colony-forming cells (185), and in colony formation of erythroid progenitors isolated from murine fetal livers (112).

As mentioned above, the glucocorticoid receptor (GR) is also implicated in expansion of erythropoiesis. Dexamethasone, an artificial ligand of the GR,

does support erythroid colony formation of primary cells (19, 47, 275), but also supports erythroid expansion in *in vitro* cultures (219, 290, 291, 301, 303). Notably, mice expressing a GR mutant lacking transactivation activity (GRdim), are viable and show normal erythropoiesis. However these mice fail to induce stress erythropoiesis in response to hypoxia (14). This shows that the GR, and particularly its transactivation activity, is required for erythroid expansion under hypoxic stress (219). This and the fact that erythroid progenitors derived from $GR^{-/-}$ and $GR^{dim/dim}$ mice cannot be expanded *in vitro* (14, 43), suggests that *in vitro* erythropoiesis reflects stress erythropoiesis. The role of the GR in stress erythropoiesis is underscored by strongly increased release of glucocorticoids under anemic stress conditions (11, 271).

In addition to glucocorticoids, SCF also plays an important role in stress erythropoiesis. Mice in which c-Kit is mutated respond poorly to anemic stress (104). SCF levels are not affected under anemic conditions (145, 308), but SCF is responsible for the lodging of BFU_E from the marrow to the spleen, where murine stress-erythropoiesis takes place (26, 103, 199, 219). Treatment of hypoxic mice with a neutralizing antibody for c-Kit almost completely inhibited splenic erythropoiesis, and only modestly affected erythropoiesis in the bone marrow (26). This explains why the acute expansion of erythroid cells in response to hypoxia is at least in part due to migration of BFU_E from the bone marrow to the spleen, but also due to higher proliferation rates of CFU_E in the spleen (99, 103, 190). Finally, hypoxia immediately up-regulates Epo (228). Epo levels are known to be required for increased survival, proliferation and differentiation of erythroblasts. We showed that Epo-induced activation of PI3K-dependent signaling pathways as well as Epo-induced activation of the MAP kinase pathway is important for the expansion of erythroid progenitors in presence of SCF (279). These data suggest that the combined action of Epo, SCF and Dex is essential in stress-erythropoiesis.

Interestingly, we found that murine, fetal liver or bone marrow derived erythroid progenitors could be cultured indefinitely when derived from mice lacking p53 (237, 290). This resulted in e.g. the I/11 and R10 cell lines. These lines retain (1) the ability to differentiate synchronously to hemoglobinised, enucleated erythrocytes, (2) full factor dependence and (3) a normal diploid karyotype when maintained properly. Notably, development of murine erythroleukemia induced by spleen focus forming virus (SFFV) is critically dependent on loss of p53 (213), and p53 was shown to antagonize glucocorticoid-induced proliferation of erythroid progenitors (89).

4. The cyclic-AMP pathway

In addition to Epo, SCF and Dex, many additional factors modulate erythropoiesis. Among them TGF-β family members (322), Interleukin-3 (85), GM-CSF (74), or ligands for serpentine receptors coupled to trimeric G-proteins. One mechanism via which Serpentine receptor mediate erythropoiesis is via regulation of intracellular levels of cyclic-AMP (cAMP). This paragraph discusses the role of the cAMP pathway.

Hormones such as adrenaline (epinephrine), calcitonin, and prostaglandins induce an increase of the second messenger cAMP upon binding

to their receptor (Figure 1.3). The receptors are associated with guanine nucleotide-binding protein (G-protein), which consists of three subunbits, α (45 kD), β (35 kD), and γ (7 kD). Hormone binding to the receptor stimulates the exchange of GDP for GTP to the receptor bound Gα protein. The G-protein then falls apart into the three subunits. Gα subunits come in 3 functionally different subtypes: $G\alpha_q$, which activates phospholipase activity; $G\alpha_s$, which activates adenylate cyclase to generate cAMP; $G\alpha_i$, which inhibits adenylate cyclase. In addition, the heterodimer of Gβ,γ and or $G\alpha_i$ may activate kinases (e.g. Src-family members). The intrinsic GTPase activity of the $G\alpha_s$ hydrolyzes GTP to GDP and abrogates signaling (Figure 1.2; 23).

The cellular functions of cAMP are mediated through the serine/threonine protein kinase A (PKA). PKA consists of two different subunits, a 49 kD regulatory subunit, and a 38 kD catalytic subunit. In the absence of cAMP, the regulatory (R-unit) and catalytic (C-unit) subunits form an enzymatically inactive R_2C_2 complex. In this complex the C-unit binds a pseudo-substrate sequence in the R-unit that mimicks a PKA phosphorylation site (94, 133). Binding of cAMP to the R-unit results in an allosteric change of the R_2C_2 complex, which prevents binding of the C-unit to the pseudo-substrate, and release of the C-units. Although PKA also phosphorylates many cytoplasmic substrates, its nuclear localization and subsequent phosphorylation of cAMP response element binding proteins of the Creb/ATF family constitutes a major pathway (56, 233). In mammals at least three genes encode cAMP responsive transcription factors: Creb, Crem and Atf-1, of which various isoforms exist, and which are able to homo- or heterodimerize (179, 234). Serine phosphorylation by PKA on residue 133 in Creb, 117 in Crem, and 63 in Atf-1 results in association with Creb binding protein (Cbp)/p300, and subsequent recruitment of the basal transcription machinery (56, 233). Creb family members belong to the basic-domain-leucine-zipper class of transcription factors, and bind to the palindromic sequence TGACGTCA, first identified in the somatostatin promoter, and called the cyclic AMP responsive element (CRE; 179, 234).

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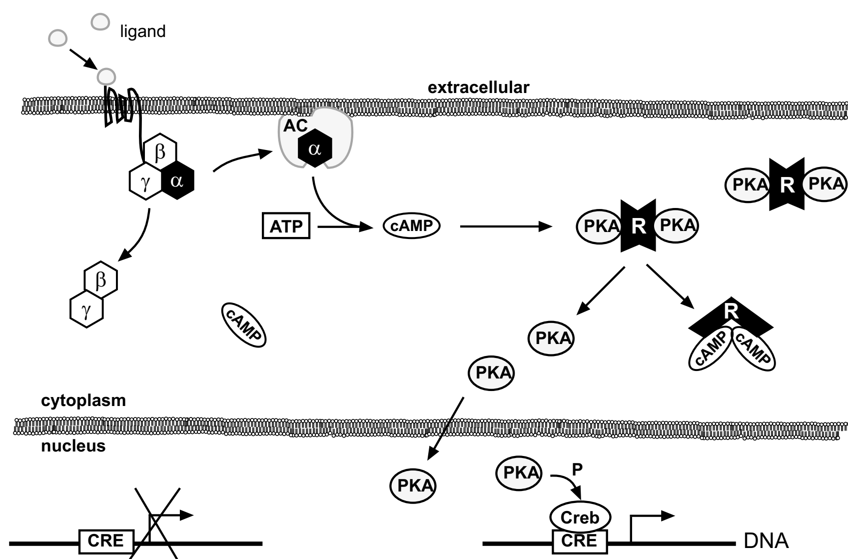

Figure 1.3. **Schematic representation of the cAMP signal transduction pathway**. A G-protein coupled receptor is activated upon ligand binding at the cell surface resulting in dissociation of the Gα unit from the Gβ and Gγ units. Gα binds adenylyl cyclase (AC) which triggers the conversion of adenosine tri-phosphate (ATP) into cyclic-AMP (cAMP). cAMP binds the regulatory subunit (R) of the protein kinase A (PKA) complex, inducing the release of the catalytic subunit of PKA. PKA is tranported to the nucleus where it is able to mediate transcription by phosphorylation of cyclic-AMP response element (CRE) binding proteins of the Creb family proteins (serine 133 for Creb).

4.1. The cAMP pathway and regulation of erythropoiesis

The cAMP pathway is involved in many physiological processes, such as memory and long term potentiation (242), circadian rhythms (82, 83), pituitary functions (252) spermatogenesis (233), and hematopoiesis. Activation of the cAMP pathway is associated with differentiation of many cell types, including hematopoietic cells (157, 164). For instance, increased cAMP levels inhibit growth and induce differentiation of leukemic blasts (101, 197, 231).

Some studies have pointed to a role for the cAMP pathway in regulation of erythropoiesis. Increased cAMP levels have been associated with growth suppression (105, 274), and cAMP was shown to promote chemically induced differentiation of mouse erythroleukemia cells, whereas inhibition of adenylyl cyclase suppresses differentiation (142). Furthermore, knock-down of the cAMP downstream target protein kinase A (PKA) impairs erythroid differentiation of mouse erythroleukemia cells (210) and reduces heme and hemoglobin synthesis (209, 254, 285). The latter was suggested to be caused by an impaired transcriptional activity of the erythroid specific transcription factor NF-E2 which is activated in differentiation (9, 91).

Physiologically, increased cAMP levels in erythroid cells are at least partially induced by prostaglandins. Stimulation of erythroid cells with prostaglandins promotes hemoglobin synthesis (52), which most likely is

regulated via PKA (209). Interestingly, the prostaglandin E2 receptor is more than 30-fold up-regulated during terminal erythroid differentiation (64), which may explain the more potent growth suppression of cAMP in differentiating cells (274). Interestingly, PGE2 has been shown to cooperate with the Epo-induced transcriptional activation Stat5 enhancing the expression of SOCS2, SOCS3 and Bcl-X_L (22).

The data show the involvement of the cAMP/PKA pathway in regulating gene expression in erythroid cells, and suppressing erythroid growth. However, the exact role of this pathway and its regulation in erythropoiesis is largely unknown.

5. Erythropoiesis and disease

Various erythroid malignancies have been reported. In polycythaemia vera an excess of erythrocytes is caused by hypersensitivity to Epo, or by EpoR mutations leading to hyper-responsiveness of the receptor (140). Erythroleukemia is a variant of acute myeloid leukemia with significant involvement of the erythroid lineage (226). Anemia is caused by a lack of functional erythrocytes. Anemia can occur when hemoglobin synthesis is disturbed (thalassemia and sickle cell disease; 114), or when reduced numbers of progenitors are present (e.g. Josephs-Diamond-Blackfan anemia and Fanconi Anemia; 295). If the defect is in the production, survival or differentiation of CFU-E, the anemia may be refractory to increased Epo levels. If mature erythrocytes are instable, increased Epo levels will increase the survival and proliferation of CFU-E, generating more erythrocytes. This results in the increased numbers of reticulocytes in the peripheral blood, a condition known as reticulocytosis.

6. The PI3K pathway

6.1. The PI3K pathway is required for erythroid expansion

As mentioned before, Phosphatidyl Inositol-3 kinase (PI3K) is activated both by the EpoR and c-Kit. PI3K is a heterodimeric complex consisting of 85- and 110 kD subunits (p85 and p110; 121). The p85 subunit contains two Src homology 2 (SH2) domains, which bind to phosphorylated tyrosines. The domain in between the two SH2 domains is required to bind to the catalytic p110 subunit (132). Active PI3K phosphorylates the 3'-OH group of the phosphatidylinositol (PI) ring present in the plasma membrane, resulting in phosphoinositol-3-phosphate PI(3)P1, PI(3,4)P2, or PI(3,4,5)P3. This results in recruitment and activation of pleckstrin-homology (PH)-domain containing proteins (283). Downstream effector molecules can have a preferential affinity for the distinct phosphoinositol rings. For instance, Btk and general receptor for phosphoinositides (Grp1) specifically bind PI(3,4,5)P3 (217). The pleckstrin homology (PH) domain of PKB was shown to have highest affinity to PI(3,4)P2, and somewhat lower for PI(3,4,5)P3 (84). Tapp1 and Tapp2 (tandem PH-domain-containing protein) only bind PI(3,4)P2 and not PI(3,4,5)P3, whereas phosphatidylinositol-three-phosphate-binding PH-domain protein-1 (Pepp-1), Pepp-2, Pepp-3, ATPH1, and the FYVE finger domain protein preferentially bind PI(3)P1 (66, 217). PI3K is implicated in regulation of survival, cell cycle progression, differentiation, and

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intracellular trafficing (81, 283, 284). Oncogenic transformation and growth factor stimulation result in increased levels of phospholipids in the membrane (35, 121, 249).

The PI3K pathway is essential for Epo and SCF induced erythroid expansion. Inhibition of the PI3K pathway by the inhibitor LY294002 (7.5 and 15 μM) strongly suppresses erythroid expansion in favor of differentiation (290, 291). This appears to be specific for the PI3K pathway because inhibition of the Ras/MAP kinase pathway also suppressed erythroid expansion but does not enhance differentiation (290, 291). The role of PI3K in erythroid expansion has also been shown *in vivo* using p85$\alpha^{-/-}$ mice. Deletion of the p85α subunit of PI3K in mice does not result in death (86). However, the colony forming ability of erythroid progenitors in E14,5 fetal livers of p85$\alpha^{-/-}$ embryo's was 2-3 fold decreased when the progenitors were cultured in the presence of Epo and SCF. Colony numbers were less drastically decreased when grown in the presence of Epo alone (112). This also suggests that PI3K is most important for expansion of early erythroid progenitors, but not for Epo-dependent survival and differentiation. Notably, p85$\alpha^{-/-}$ mice display a transient fetal anemia, a temporary shortage of red cells which becomes apparent when maximal expansion is required during fetal liver erythropoiesis (112).

In vivo studies using transgenic mice expressing mutant EpoR or c-Kit, lacking the PI3K docking sites do not show a requirement for PI3K in normal erythropoiesis. These transgenic mice are viable and have a normal definitive erythropoiesis (21, 321). However, stress erythropoiesis has not been examined in these mice. In addition, other PI3K docking sites present in the EpoR-/c-Kit signaling complex (e.g. on Gab-1, Gab-2 docking molecules) might substitute for PI3K activation (278).

6.2. Negative regulators of the PI3K pathway

The PI3K pathway is negatively regulated in several ways. The phosphatase protein PTEN was identified as a tumor suppressor (150, 260), and was shown to dephosphorylate the 3' position of phosphoinositol phosphates (160). PTEN-null embryonic fibroblasts have higher PIP3 levels, and constitutive PKB activity (248), which can be converted by re-expression of PTEN in PTEN deficient cells (149). Another down-regulator of PIP3 signaling is the SHIP family of phosphatases. These SH2-domain-containing inositol phosphatases (SHIP) have been shown to dephosphorylate PIP3 at the 5' position (227). To date, two SHIP proteins have been identified: SHIP1/SHIP and SHIP2. Whereas SHIP is predominantly restricted to the hematopoietic system, SHIP2 is more ubiquitously expressed (155, 206). Various studies have shown that SHIP and SHIP2 negatively downregulate the PKB activity (7, 154, 258). However, since PTEN dephosphorylates the 3' position, it is the direct and complete antagonist of PI3K. Therefore, activation of PKB, which preferentially binds PI(3,4)P2 above PI(3,4,5)P3, is probably most affected by PTEN, and to a lesser extent by SHIP, whereas FYVE-domain containing proteins which bind specifically to PI(3)P1 which can only be repressed by PTEN (92, 203).

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7. Protein Kinase B (PKB/Akt-1)

PKB was cloned independently by two groups (42, 119). Because the gene showed similarity to the kinase domains of protein kinase C and protein kinase A, it was named protein kinase B (16, 42, 119) Three PKB isoforms exist: PKBα/Akt-1, PKBβ/Akt-2 and PKBγ/Akt-3, sharing more than 80% homology on amino acid level. All three isoforms share an NH_2-terminal pleckstrin homology domain (about 100 amino acids (aa)), followed by a kinase domain (approximately 250 aa), and the COOH-terminal regulatory domain. PKB has two phosphorylation residues: threonine 308 (T308, situated in the kinase domain), and serine 473 (S473, present in the regulatory domain) which are important determinants for its activity.

The pleckstrin homology (PH) domain of PKB was shown to have highest affinity to PI(3,4)P2, and somewhat lower for PI(3,4,5)P3 (84). Once recruited to the plasma membrane, PKB is phosphorylated at two sites. Threonine 308 is phosphorylated by the serine/threonine kinase phosphoinositide-dependent-kinase-1 (PDK1), a protein that is also recruited to PI(3,4)P2 and PI(3,4,5)P3 via its PH-domain. T308 phosphorylation regulates substrate access to the catalytic site (5, 250), whereas subsequent phosphorylation of S473 is required for full activation (4). Phosphorylation of S473 is performed by the DNA-dependent protein kinase (DNA-PK; 327). Once activated, PKB translocates from the plasma membrane to the cytosol or to the nucleus where it phosphorylates its substrates.

PKB mediates a variety of cellular functions. It has been implicated in regulation of insulin uptake, glucose metabolism, protein synthesis and regulation of survival (41, 137, 283). PKB mediated survival and proliferation is regulated via multiple mechanisms. First, regulation of protein synthesis is established through phosphorylation of 4E-binding protein (4E-BP). It is thought that the signal is transduced from PKB, through mTOR to 4E-BP (17, 76). Phosphorylation of 4E-BP by mTOR results in its dissociation from eukaryotic initiation factor 4E (eIF4E), thereby initiating translation (33, 95). The role of PKB in stimulation of protein synthesis has clearly been shown in skeletal muscle, adipose tissue, and in erythroid cells (Blazquez-Domingo and Grech, in preparation; 126). In addition, overexpression of eIF4E in erythroblasts results in erythroid expansion (M. Blazquez-Domingo and G. Grech, in preparation).

PKB mediated inhibition of the apoptotic machinery is another way to support growth. This has been confirmed in a wide variety of cells (45, 75, 317). PKB up-regulates the anti-apoptotic factor Bcl-2, which bind and inhibits the pro-apoptotic factors Bcl-X_s, Bax, Bad and Bak. In addition, PKB inactivates the pro-apoptotic factor Bad directly via phosphorylation resulting in the association to 14-3-3 proteins (53, 58, 315). Furthermore, PKB stimulates cell cycle progression by direct phosphorylation of the cell cycle inhibitor $p27^{KIP}$. Phosphorylation of the nuclear localization motif of $p27^{KIP}$ impaired its nuclear import resulting in as release of cyclin dependent kinase-2 and stimulation of cell cycle progression (151, 287).

Studies in the hematopoietic system indeed have shown a proliferation-inducing role for PKB. In the myeloid cell line 32D, PKB is implicated in IL-3

induced proliferation. Both in T-cells and in the pre-B cell line Ba/F3, cytokines activate PKB, which results in inhibition of apoptosis and cell cycle progression through induction of Bcl-2 and c-Myc expression. In 1999, PKB was shown to regulate cell survival via regulation of a subfamily of forkhead transcription factors.

8. The Forkhead family of transcription factors

The first member of the forkhead family was identified in *Drosophila melanogaster* in 1989. Mutations in this gene resulted in homeotic transformation of gut structures into head-derived elements, therefore the gene was called fork head (299). About two years later, mammalian family members were found and described as a small family of hepatocyte enriched DNA-binding transcription factors, the hepatocyte nuclear factors, which showed homology to the Drosophila fork head gene (144, 298). But it was the characterization of the forkhead DNA binding domain that led to the identification of the forkhead family of transcription factors (143). The winged helix DNA-binding domain encompasses about 110 amino acids and is conserved in a wide range of species. Since than, more than 100 forkhead family members have been identified in organisms ranging from yeast to humans (123).

8.1. The Foxo subfamily of Forkhead transcription factors

The first member of the O subclass of forkhead family members (Foxo) was identified in *Caenorhabditis elegans* and was named Daf-16. Daf-16 was shown to regulate dauer formation. Dauer formation is a developmental stage in the 15 weeks life span of the worm, which ensures survival under nutrient poor conditions through closing its mouth and anus and by lowering its metabolism. This developmental arrest can be reversed upon improving conditions (261). After the identification of Daf-16 the mammalian homologues were investigated. Daf-16 showed a large degree of homology to the mammalian forkhead transcription factors AFX (Foxo4), FKHR (Foxo1), and FKHR-L1 (Foxo3a) and was negatively regulated by the insulin receptor (Daf-2) and PI3K (Age-1; 8, 127, 153, 182, 195). All Foxo family members contain three PKB phosphorylation sites (RXRXX(S/T)X) (Figure 1.4; 3, 294). PKB was shown to phosphorylate the Foxo family members on these three PKB motifs: a NH_2-terminal threonine (T32), a forkhead box serine (S253), and a COOH-terminal serine (S315; Figure 1.4). T32 and S253 are required for nuclear export and binding to 14-3-3 proteins (27, 30). Phosphorylation of S315 was shown to enhance nuclear export (Figure 1.5; 32, 222).

Other kinases regulate Foxo proteins as well. These are: Casein kinase 1 (CK1), dual specificity tyrosine phosphorylated and regulated group of kinase (Dyrk1a), and serum and glucocorticoid regulated kinase (SGK). CK1 recognizes serine or threonine phosphorylated protein motifs. PKB phosphorylation of the C-terminal PKB site is essential for CK1 phosphorylation of the two downstream serines (Figure 1.4; 222). CK1 can be activated upon DNA damage (232). Dyrk1a is activated by an as yet unknown kinase, and its cellular function is still unclear (311). One Dyrk1a phosphorylation site is present in the Foxo family members. This site (S325) is located adjacent to the two CK1 phosphorylation

sites (S318, S321) and is constitutively phosphorylated (222, 311). Mutation of the S325 Dyrk1a site decreased nuclear localization and transcriptional activity, suggesting that it is functional (311). SGK is regulated at the transcriptional level and on protein level by serum, glucocorticoids and stress factors (148). Activation of SGK is induced in a PI3K- and PDK1-dependent way (134, 201). Because PKB and SGK have identical substrates it is difficult to distinguish between their activity (134, 201). However, the fact that SGK is activated by a much broader range of stimuli, that it lacks a pleckstrin homology domain, and that it prefers a serine as a phospho-acceptor, probably determine their differential role *in vivo* (32, 134). Although both kinases phosphorylate the NH_2-terminal PKB site, SGK preferentially phosphorylates the COOH-terminal phosphorylation site whereas PKB preferres the serines in the forkhead box (32). Finally, Foxo3a was shown to interact with and to be phosphorylated by IκB kinase (Ikk), independent of PI3K activity (110). Serine 644, present in a Ikk target sites (DSX (hydrophobic amino acid)XX(S/T)) appeared to be the Ikk target site in Foxo3a. Phosphorylation of this site repressed transcriptional activity and induced nuclear exclusion of a PKB phosphorylation deficient Foxo3a mutant, indicating that Ikk phosporylation serves as an alternative pathway that regulates Foxo activity (110).

Taking all possible phosphorylation events into account, there seems to be a certain order in these phosphorylation events. Phosphorylation of the PKB site in the forkhead box (S253) is a prerequisite for phosphorylation of the N- (T32) and C-terminal (S315) PKB sites (221). Namely, mutation of S253 abolished insulin induced phosphorylation of Foxo1 whereas mutation of the NH_2- or COOH-terminal PKB sites did not (187). Since phosphorylation of the CK1 sites is again dependent on phosphorylation of the COOH-terminal S315 PKB site, phosphorylation of S253 by PKB site can be regarded as the triggering event leading to Foxo phosphorylation.

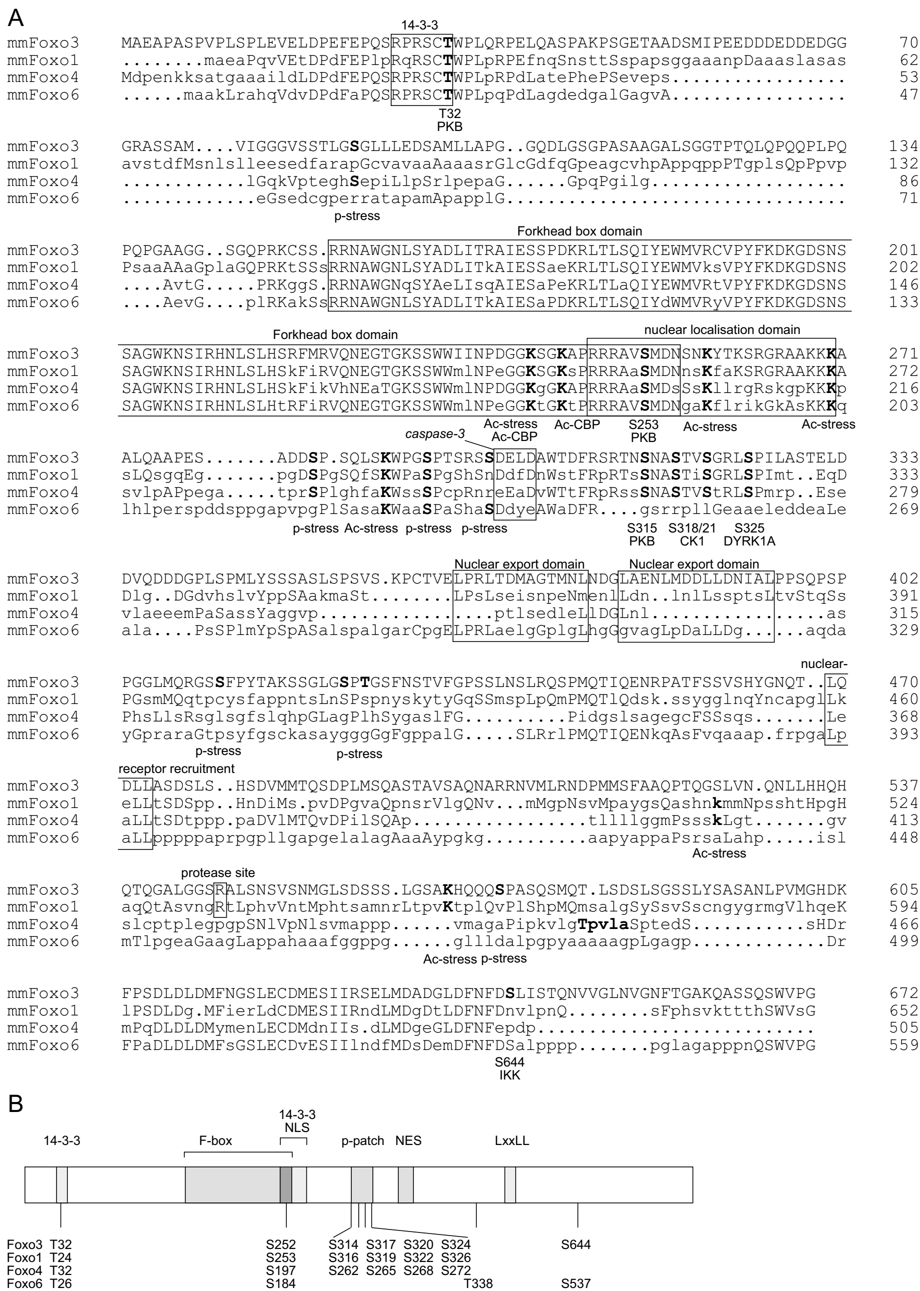

Figure 1.4. **Allignment of murine Foxo family members.** (**A**) Common elements (boxed) and known phosphorylation sites (S and T) are indicated. Stress induced Acetylation and phosphorylation sites are indicated as Ac-stress and p-stress respectively (Brunet et al., 2002). Two domains have been identified to bind 14-3-3 proteins: the indicated N-terminal site, and the Forkhead box PKB phosphorylation site. The positions of the phosphorylation sites are numbered according to the positions in Foxo3. Ac-CBP: Lysines known to be target of the acetylase CBP/P300. Caspase and protease cleavage sites are also indicated (see text). (**B**) Schematic drawing of Foxo proteins. Several domains are indicated, and positions of the phosphorylation sites in all family members are given for the murine Foxo proteins. Figures adapted from Birkenkamp *et al.* (2003) Biochem Soc Trans., 31, 292-297, and Van der Heide *et al.* (2004) Biochem J., 380, 297-309.

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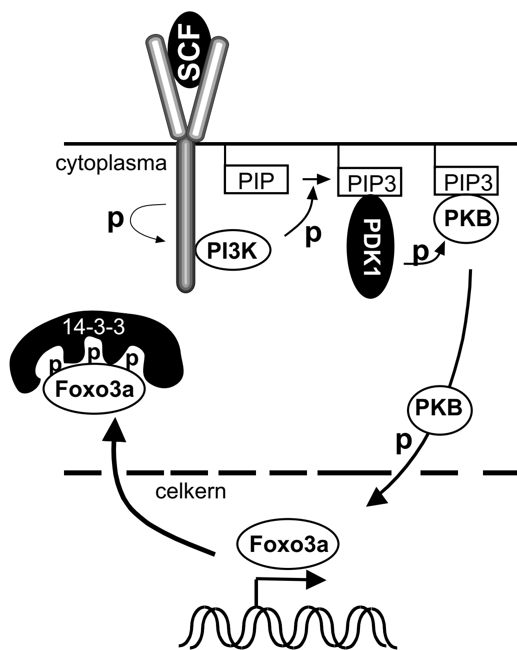

Figure 1.5. **The PI3K/PKB represses transcriptional activity of the Forkhead family of transcription factors subclass O (FoxO).** Binding of SCF to c-Kit induces the tyrosine kinase activity of c-Kit, which leads phosphorylation of the receptor and downstream targets such as PI3K. Active PI3K phosphorylates inotisol rings (at the 3-position) present in the plasmamembrane. Proteins containing a pleckstrin homology domain (PDK1, PKB) are recruited by these phospholipids (PI(3,4,5)P3). Once PKB is activated through phosphorylation by PDK1 and DNA-PK, it translocates to the nucleus where it phosphorylates Foxo transcription factors on three different PKB phosphorylation sites. Once phosphorylated, DNA binding of Foxo's is disrupted and Foxo nuclear export is enhanced. PI3K activation thus inhibits Foxo transcriptional activation of Foxo target such as the cell cycle inhibitor $p27^{KIP}$.

8.2. Regulation of Foxo transcriptional activity

Transcriptional activity of subfamily O of forkhead transcription factors is regulated at three levels: I) cellular translocation, II) DNA binding and III) protein degradation (34, 280), as will be discussed below (Figure 1.5).

(I) The nuclear membrane forms a physical barrier between the cytosol and the nucleus. Transport between these compartments takes place through the nuclear pore complex. Small molecules (<50 kD or < 9 nm) are able to diffuse freely through this complex, whereas larger complexes require active transport (136). Foxo's depend on active transport mechanisms. Foxo1, 3, and 4 contain a nonclassical nuclear localization signal (NLS) consisting of three arginines next to the forkhead box PKB site (S253) and three lysine residues located 19 residues downstream of the arginines (Figure 1.4; 27, 158). Whereas a basic domain is required for NLS function, phosphorylation of the PKB site (S253) and the adjacent CK1 sites establishes a negative charge inhibiting nuclear translocation (27). Recently, a second NLS was identified in Foxo1 (325). Nuclear import of Foxo3a requires binding to importin and shuttling from the cytosol to the nucleus in a Ran-dependent manner (136, 280, 319).

Essential factors in nuclear/cytoplasmic localization of Foxo proteins are the 14-3-3 proteins. 14-3-3 proteins exist in seven isoforms. They consist of acidic dimeric subunits of about 27 kD (296). The 14-3-3 dimer binds tightly to single molecules containing tandem repeats of phosphoserine motifs (315). 14-3-3 proteins are chaperone proteins able to mask or expose nuclear export or import domains, thereby influencing the cellular distribution (31, 183, 224). Foxo

proteins contain one optimal 14-3-3 binding site which overlaps with the NH_2-terminal PKB site (T32). Mutation of this site disrupts 14-3-3 binding, resulting in nuclear localization (30, 187). Since Foxo binding to conserved exportin 1 (Crm1) is independent of the Foxo phosphorylation status (280), it can be concluded that the nuclear localization of the T32 mutant is caused by the inability of Foxo proteins to bind the cytoplasmic anchoring protein 14-3-3 and not by impaired nuclear export. 14-3-3 proteins can also bind to degenerate binding sites (314). It was shown that both phosphorylation of residues in the NH_2-terminus (T32) and the Forkhead box (S253) are essential for optimal 14-3-3 binding, suggesting the presence of an additional 14-3-3 binding site in the Forkhead box (30, 325). Since S253 is a prerequisite for phosphorylation of the other sites (see 7.1), and its phosphorylation also masks the NLS, phosphorylation of S253 is a key event in the control of Foxo proteins.

Non-DNA bound Foxo is exported from the nucleus dependent on exportin 1 (Crm1) and Ran-GTP (27, 116, 222). Mutation of the COOH-terminal phosphorylation site (S315), which also inhibits phosphorylation of the CK1 sites, greatly increased nuclear localization (27, 222). In addition, insertion of the stretch of 4 COOH-terminal serines in Foxo6 restored nuclear export (Figure 1.4; 116). Because S315 is not important for 14-3-3 and nuclear export, its phosphorylation may disrupt DNA binding. This may indirectly promote its nuclear export and cytoplasmic retention.

(II) It was initially thought that Foxo nuclear export was the major determinant for inhibition of Foxo function. However, phosphorylation of Foxo's appeared to block Foxo transcriptional activity as well. Phosphorylation of S253 in the forkhead box by PKB inhibits Foxo1 transactivation without affecting its cellular distribution (167, 205, 221, 323). Furthermore it was shown that phosphorylation of S253 disrupts DNA binding (158). These data were confirmed after the identification of Foxo family member 6. Foxo6 lacks the C-terminal stretch of phosphorylation sites, and also one of the two nuclear export domains,(116) both crucial for nuclear export (Figure 1.4) (31). By consequence, growth factor stimulation only slightly induced nuclear export of Foxo6 but potently inhibited Foxo6 transactivation activity (116). These data show that transcriptional activity of Foxo proteins can be suppressed without nuclear exclusion.

(III) Foxo proteins are relatively stable proteins. The half-life of Foxo4 is more than 10 hours (34), However, insulin stimulation of a murine pro-B cell line decreased the Foxo1 and 3 protein levels by proteolytic degradation (211). Insulin stimulation induced Foxo1 ubiquitination and subsequent degradation by the proteosome system in a PI3K-dependent way (167), which required cytoplasmic localization. Another pathway involved in degradation of Foxo proteins is the IκB kinase (Ikk) regulated pathway. Ikk, is also activated upon growth factors stimulation (62), and induced Foxo3a nuclear localization and subsequent ubiquitination and protein degradation via the proteosome (110).

Finally, Foxo3a can be cleaved by caspase-3-like proteases, yielding an NH_2-terminal, and a COOH-terminal domain. The cleavage site was also present in Foxo1 and 4, but not in Foxo6 (38). Furthermore it is shown that Foxo1 is negatively regulated by androgens in a proteolytic-dependent way (111).

8.3. Transcriptional regulation by Foxo proteins

Two elements have been identified to which Foxo-members bind preferentially: the insulin response element (IRE), and the Daf-16 binding element (DBE). The DBE was defined as TTGTTTAC (87), which overlaps with the IRE: CACTAGCAAAACAAACTTATTTTGAACAC (256). These domains have in common the core forkhead binding sequence which has been identified in the promoter of a variety of forkhead targets: (G/A)(T/C)(C/A)AA(C/T)A (123, 230). However, this core sequence alone is not enough for DNA binding. Probably, flanking DNA sequences are important for target specificity (123, 207, 230). Forkhead transcription factors in general have been described to bind DNA as monomers, through helix 3 of the winged helix domain (40, 118), and Foxo family members were shown to bind DNA similarly (300). Mutation of an essential histidine to an arginine in helix 3 disrupted DNA binding of Foxo1 (HRAAA mutant). Importantly, this mutation blocked regulation of only part of the Foxo1 target genes and retained the ability to repress growth (216). This observation indicated that Foxo1 transcriptional regulation might in part be independent of DNA binding. DNA-binding independent control of gene expression may occur through the interaction of Foxo's with several co-regulators such as, CCAAT/enhancer binding protein β, p300 and Creb/binding protein (CBP), several steroid and non-steroid nuclear receptors, and Smads (20, 238, 280). The examples outlined above suggest diverse mechanisms via which Foxo's regulate transcription.

8.4. Functions of Foxo proteins

At present, Forkhead transcription factors of the subclass O have been implicated in regulation of a variety of cellular processes in response to different stimuli. Among the first identified Foxo targets were the proapoptotic gene *Fas ligand* (*Tnfsf6*; 30), *Insulin growth factor binding protein-1* (*Ifgbp-1*; 102), and the cell cycle inhibitor *Cdkn1b* (*p27^{KIP}*; 170), which indicated a role for Foxo family members in regulation of apoptosis, cell cycle progression and metabolism. Foxo proteins were shown to regulate the cell cycle through regulation of *p27^{KIP}* (137) and *Ccng2* (*Cyclin G2*; 163), by downregulation of D-type cyclins (216, 236), and via regulation of *cyclin B* and *polo-like kinase* (*Plk*) (6). Foxo-mediated induction of apoptosis involves transcription of pro-apoptotic factors such as Fas ligand (*Tnfsf6;* 30), *Bim* (63), and *Trail* (93, 178). And in response to oxidative stress and DNA damage, Foxo transcription factors regulate *Gadd45* (269), whereas *Mnsod* and *p66^{shc}* are up-regulated in response to oxidative stress only (138, 188). Foxo transcription factors also mediate cellular metabolism via upregulation of *Igfbp-1* (102), *glucose-6-phosphatase* (*G6pc*; 12), and *pyruvate dehydrokinase-4* (*Pck1*; 214).

In conclusion, Foxo family members control growth rate via diverse mechanisms. In *C. elegans*, nutrient depletion leads to a developmental arrest that increases life span, whereas in mammalian cells Foxo's are also activated in response to nutrient and growth factor depletion, but also in response to oxidative stress and DNA damage. Dependent on the stimuli Foxo’s induce growth arrest, senescence or apoptosis.

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9. The PI3K/Foxo pathway and neoplastic transformation

In this introduction we first introduced SCF as a growth factor able to delay differentiation and promote expansion of erythroid progenitors. We argued that activation of the PI3K/PKB pathway is important for this function of SCF and that inhibition of Foxo's is an important function of PKB. Accumulating evidence shows that the regulation of the PI3K/PKB/Foxo3a pathway is important to control the balance of expansion and differentiation in many cell types and that disrupted control is involved in leukemogenesis.

First, mutations in cKit that render the receptor constitutively active occur approximately in one third of all inv(16) and t(8;21) AML (15, 90). Expression of constitutively active cKit mutant in factor-dependent cell lines renders these cells factor independent (129), and exogenous expression of mutant cKit in mouse marrow cells caused leukemia in transplanted mice (129). Notably, PI3K activity is critical for the transforming ability of the D816V cKit mutant (39). Furthermore, autocrine loops of cKit activation may be important in many other types of leukemia (208).

Second, constitutive activation of PI3K is widespread in many types of tumors. This can be caused by the breakpoint-cluster-region-abelson (Bcr-Abl) fusion protein in CML, which is a kinase that strongly activates PI3K (243). The BCR/ABL kinase resulting from a chromosomal translocation plays an essential role in the pathogenesis of chronic myelogenous leukemia and Philadelphia positive acute myeloid leukemia. The importance of PI3K/PKB pathway for the transforming potential of BCR/ABL has been supported by the observation that a kinase-dead PKB mutant was able to inhibit BCR/ABL-induced bone marrow transformation. It also suppressed outgrowth of leukemia in severe combined-immunodeficiency (SCID) mice transduced with the BCR/ABL kinase. Conversely, addition of an active PKB mutant enhanced leukemia development in SCID mice infected with a defective BCR/ALB mutant (2). Because PKB has been shown to regulate apoptosis and cell cycle progression, the transforming capacity is thought to occur at least partially through deregulation of these processes. PI3K activity can also be increased due to PTEN inactivation, which was observed in a variety of tumors (36). PTEN heterozygous mice may develop a progressive lymphoproliferative disorder (61, 212), whereas PTEN heterozygosity contributed to the development of myelodysplasia in SHIP deficient mice (180).

Third, a general hallmark of cancer is loss of growth control. In many tumors this results from activation of a growth promoting gene (proto-oncogene), and loss of a growth-suppressing tumor suppressor gene. The importance of tumor suppressor genes in tumorigenesis is underlined by the fact that inactivation of one of the two copies of the gene already contributes to cancer progression (80, 272, 286). Foxo transcription factors may function as tumor suppressor genes. They suppress cell growth and are able to induce apoptosis or cellular senescence through transcriptional activation of genes such as the cell cycle inhibitor *p27^{KIP}* and the pro-apoptotic factor *Bim* (20, 34). Inactivation of Foxo function has been associated with tumorigenesis and poor survival in breast cancer (FOXO3a; 110). Inactivation may be the consequence of chromosomal translocation. In acute lymphoblastic leukemia a chromosomal

abnormality was identified that involved a fusion between the MLL gene on chromosome 11, and *FOXO4* on the X-chromosome (202). Due to X-inactivation in females and the single copy of the X-chromosome in males , *FOXO4* disruption results in the complete inactivation of Foxo4. Chromosomal fusion between *FOXO1* and *PAX3* or *PAX7* in translocations t(2,13) and t(1,13), respectively, has been demonstrated in pediatric alveolar rhabdomyosarcoma (54, 88). In this case the fusion products exhibit stronger transactivation activity compared with PAX3 and PAX7 alone (72). This results in a stronger inhibition of myogenic differentiation, thereby presumably contributing to the tumorigenic phenotype. Notably, the PAX3-FOXO1 fusion gene up-regulates two tyrosine kinase receptors: the platelet derived growth factor receptor (PDGFR) and MET, which are not regulated by FOXO1 or PAX3 alone (73, 96). Because these receptors are potent activators of the PI3K pathway, the intact FOXO1 protein might be repressed as well. This potent FOXO1 inactivation may contribute to tumorigenesis.

Growth advantage through Foxo inactivation is not the only mechanism contributing to tumorigenesis. Foxo proteins are also activated in responses to oxidative stress and DNA damage indicating that suppression of Foxo activity does not only contribute to cell proliferation, but also to a cell's resistance to DNA damage and oxidative stress. Therefore it will be important to understand the mechanism of Foxo function not only as regulators of cell growth, but also as regulators of the cellular response to chemotherapeutics.

10. Scope of the thesis

A proper balance between cell expansion and differentiation is a central feature of normal hematopoiesis and deregulation of this balance may lead to development of leukemia. In the studies presented in this thesis we set out to investigate mechanisms that are involved in regulation of the expansion/differentiation balance in erythropoiesis and concentrated on the PI3K/PKB pathway. It had become clear that the PI3K pathway plays a role in erythropoiesis, but the impact of the downstream events remained largely elusive. Because inhibition of the PI3K/PKB pathway induces erythroid differentiation without affecting apoptosis, we were interested to sort out PKB mediated cellular mechanisms different from apoptosis. In a series of experiments we addressed the role of the PI3K/PKB-dependent Foxo forkhead transcription factors in controlling erythropoiesis (Chapter 2). We explored to what extent Epo and SCF regulate Foxo proteins and how the different Foxo family members are regulated during terminal erythroid differentiation. To test whether Foxo3a controls the balance between erythroid expansion and differentiation, erythroblast clones, stably expressing a tamoxifen-inducible, PKB-phosphorylation deficient Foxo3a(A3):ER mutant were obtained and tested. In additional approaches to explore the mechanisms via which Foxo3a controls erythropoiesis, we performed mRNA profiling studies to identify erythroid specific Foxo3a targets. We concentrated on a novel Foxo3a target identified in these studies, *Btg1*, and examined whether and how Btg1 is involved in regulation of the balance between erythroid expansion and differentiation (Chapter 2). A pathway different from the PI3K pathway that is implicated in regulation of erythropoiesis is the cAMP pathway. The cAMP pathway was also found to regulate *Btg1* transcription. Therefore, the transcriptional cooperation of Foxo3a and the cAMP pathway on this target was investigated in expanding and differentiating erythroblasts (Chapter 3). To identify other Foxo3a target genes, erythroblast clones were used that stably express a tamoxifen inducible, PKB-phosphorylation deficient Foxo3a(A3):ER mutant, in combination with a general 17K EST cDNA array (Chapter 4). The profiling results were compared with profiling data obtained from the identical arrays on which Epo and SCF controlled transcription was analyzed, to sort out the overlap between Foxo3a and Epo/SCF targets. Because the PI3K pathway is most potently activated by the combined stimulation of Epo and SCF in expanding erythroblasts, and is downregulated during differentiation we wished to know whether expression of selected Foxo3a targets is dependent on PI3K and how these genes are regulated in expanding and differentiating erythroblasts (Chapter 4). Since a large number of Foxo3a target genes did not show the expected transcriptional regulation by Epo and SCF, we finally set out to investigate whether this may caused by cooperation with additional transcription factors (Chapter 4).

In Chapter 5 we present both a summary and a discussion of the experimental observations of this thesis with emphasis on their relevance for understanding the molecular mechanism regulating erythropoiesis, as well as their impact on future scientific research concerning the cellular functions and mechanisms of Foxo proteins, and on hematopoiesis in general.

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Foxo3a regulates erythroid differentiation and induces Btg1, an activator of Protein Arginine Methyl Transferase 1

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CHAPTER 2

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Abstract

Erythropoiesis requires tight control of expansion, maturation, and survival of erythroid progenitors. Since activation of phosphatidylinositol-3-kinase (PI3K) is required for erythropoietin/stem cell factor-induced expansion of erythroid progenitors, we examined the role of the PI3K-controlled Forkhead box, class O (Foxo) subfamily of Forkhead transcription factors. Foxo3a expression and nuclear accumulation increased during erythroid differentiation, whereas untimely induction of Foxo3a activity accelerated differentiation of erythroid progenitors to erythrocytes. We identified *B cell translocation gene 1 (Btg1)/antiproliferative protein 2* as a Foxo3a target gene in erythroid progenitors. Promoter studies indicated *Btg1* as a direct target of Foxo3a. Expression of Btg1 in primary mouse bone marrow cells blocked the outgrowth of erythroid colonies, which required a domain of Btg1 that binds Protein Arginine Methyl Transferase 1. During erythroid differentiation, increased arginine methylation coincided with *Btg1* expression. Concordantly, inhibition of methyl transferase activity blocked erythroid maturation without affecting expansion of progenitor cells. We propose Foxo3a-controlled expression of *Btg1* and subsequent regulation of protein arginine methyl transferase activity as a novel mechanism controlling erythroid expansion and differentiation.

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Introduction

Homeostasis of the hematopoietic system requires tight control of expansion, differentiation and survival of progenitor cells, which is exerted by numerous cytokines and growth factors acting on specific cell types, e.g. erythropoietin (Epo), or on multiple types of progenitors, e.g. stem cell factor (SCF). The role of SCF in hematopoiesis is mainly to stimulate expansion and delay differentiation of hematopoietic progenitors in cooperation with more cell type specific factors (reviewed by 7).

Erythroid progenitors can be expanded in presence of Epo, SCF and dexamethasone (Dex), whereas they differentiate into enucleated, hemoglobinized erythrocytes in presence of Epo alone (18, 55). Differentiation involves four differentiation specific cell divisions with altered cell cycle regulation, until a terminal G1-arrest is reached. SCF considerably delays differentiation, eventually yielding ~20-fold increased numbers of mature erythrocytes. Erythroid differentiation is thought to be an autonomously regulated cascade of events in which Epo-signaling is mainly required for survival and constitutive BCL-X_L expression is sufficient to allow erythroid differentiation in defined medium lacking any factors (19, 45). SCF, a potent activator of PI3K in erythroid progenitors, is unable to induce cell survival in absence of Epo (18, 55). Instead, SCF signaling is required to delay differentiation, which is abrogated by PI3K-inhibitor LY294002 (55). Both Epo and SCF activate PI3K and its target Protein Kinase B (PKB or c-AKT; 1). However, compared to Epo, SCF is much more potent in inducing PKB phosphorylation (55).

Phosphorylation and activation of the serine/threonine kinase PKB controls fundamental processes such as cell cycle progression, apoptosis and mRNA-translation (6, 9, 12, 14, 41). The Forkhead box, class O (FoxO) subfamily of Forkhead transcription factors is an important effector of PKB in regulating apoptosis and cell cycle progression. Members of this subfamily, Foxo4 (AFX), Foxo1 (FKHR) and Foxo3a (FKHR-L1), are directly phosphorylated by PKB, leading to cytoplasmic retention and inhibition of their transcriptional activity (8, 28, 47). In absence of phosphorylation, these transcription factors induce expression of genes encoding proteins that inhibit the cell cycle such as $p27^{KIP}$ (17, 35), $p130^{Rb2}$ (27), and *Cyclin G2* (40), or pro-apoptotic proteins such as *Bim* (15, 16) and the transcriptional repressor *BCL-6*, which inhibits expression of the anti-apoptotic factor *BCL-X_L* (49). Whether Foxo activation results in cell cycle arrest, induction of apoptosis, or other cell fates will depend on the cellular context.

We investigated the potential role of Foxo family members during expansion and differentiation of erythroid progenitors. Foxo3a expression increases during differentiation resulting in nuclear accumulation 48 h after induction of differentiation. Activation of a phosphorylation-insensitive Foxo3a mutant accelerated differentiation under conditions favoring renewal. DNA micro-array screens identified *B cell translocation gene 1/anti-proliferative protein 2* (*Btg1/Apro2*) as a novel Foxo3a target. Ectopic expression of Btg1 inhibited the expansion of mouse erythroid progenitor cells, which was dependent on the Protein Arginine Methyl Transferase 1 (Prmt1) binding domain of Btg. We present

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data that suggest that modulation of protein arginine methylation activity by Btg1 may present a new Foxo-dependent mechanism regulating erythroid differentiation.

Results

Foxo3a expression and activity increase during erythroid differentiation
In search of PI3K-dependent pathways involved in maintenance of erythroid progenitor renewal, we analyzed expression and function of the PI3K/PKB-regulated Forkhead family members Foxo4, Foxo1, and Foxo3a during expansion and differentiation of the p53-deficient I/11 erythroid cell line. After induction of differentiation, expression of Foxo1 and Foxo4 increased for 24 h, but then rapidly declined to levels lower than those observed under renewal conditions (Figure 2.1A). In contrast, Foxo3a expression sharply increased 12-24 h after differentiation induction (Figure 2.1B), reaching maximal expression at 48 h and remaining high until completion of differentiation at 72 h (Figure 2.1A, B). Essentially the same results were obtained with primary erythroid progenitors expanded from E12.5 fetal livers, except that these cells express maximal levels of Foxo3a at 24 h and complete differentiation in 48 h (unpublished data).

Foxo3a function is regulated by phosphorylation. In factor-deprived and re-stimulated erythroblasts, SCF induced a strong transient phosphorylation of Foxo3a serine-residue 253, whereas Epo only weakly induced phosphorylation of Foxo3a (Figure 2.1C). In Figure 2.1C, the unidentified protein X is phosphorylated in factor deprived cells and dephosphorylated following Epo or SCF stimulation. Phosphorylation of protein X is equally strong at time 0 in both panels, the much more pronounced signal in the Epo-stimulation experiment is due to an accordantly longer exposure of the blot. Upon induction of differentiation, phosphorylation of Foxo3a reached maximum levels after 36 h, and decreased thereafter, whereas total Foxo3a expression still increased (Figure 2.1D, the band detected at 72 h by the phospho-specific antibody may be the same protein X detected in Figure 2.1C). Foxo3a phosphorylation coincided with reduced expression and phosphorylation of PKB (Figure 2.1D, middle panels). By consequence, unphosphorylated Foxo3a, and thus Foxo3a mediated transcription increased from 36 h onwards as monitored by expression of the Foxo3a target gene *p27*^{KIP} (Figure 2.1D, third panel). This is accompanied by increased nuclear localization of Foxo3a (Figure 2.1E; 72 h after induction of differentiation the cells are enucleated, hence no nuclear localization can be detected). Together, Foxo3a is upregulated early in differentiation and is transcriptionally active ~36-48 h after induction of differentiation as indicated by its phosphorylation status and the expression of its target *p27*^{KIP}.

Physiological effects of Foxo3a in erythroid differentiation
Although Foxo1, Foxo3a and Foxo4 may control cell cycle progression and cell survival of renewing cells, the expression pattern of Foxo3a suggests an additional role in erythroid differentiation. To investigate the role of Foxo3a we

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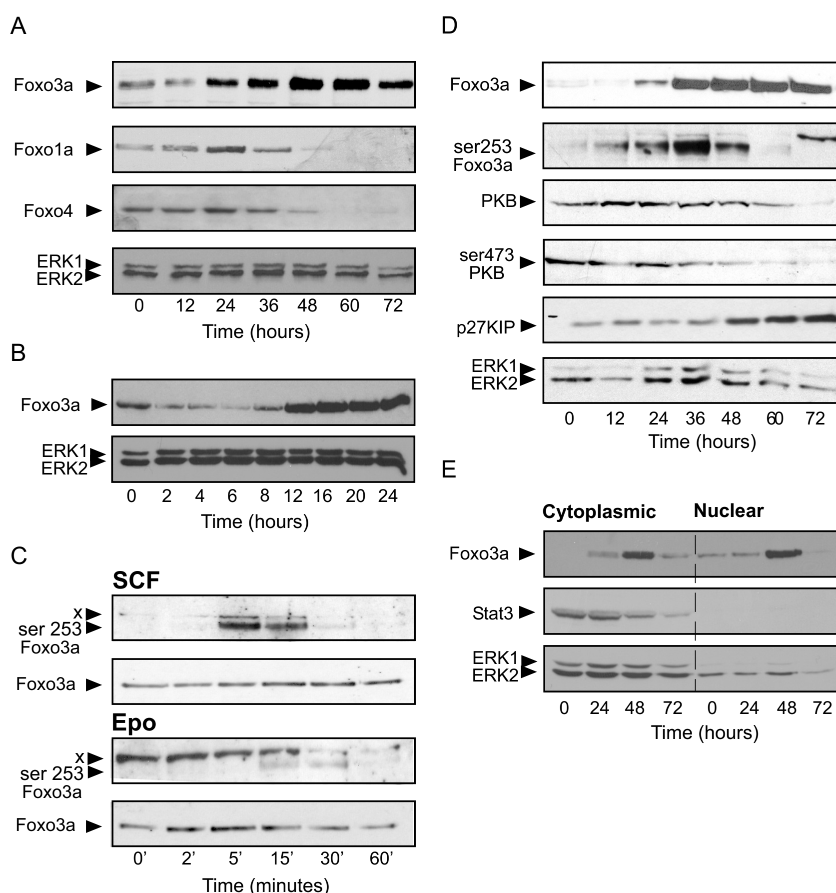

Figure 2.1. **Foxo3a expression and activity increases during erythroid differentiation**. (**A**) Western blots of differentiating I/11 cells (samples taken every 12 h) were analyzed with antibodies recognizing Foxo1, Foxo3a and Foxo4. ERK protein levels do not change during differentiation (loading control). (**B**) Western blot analysis of Foxo3a total protein levels during the first 24 h of differentiation (ERK expression serves as loading control). (**C**) I/11 cells were factor deprived and stimulated with Epo (5 U/ml) and SCF (1 μg/ml) for increasing time-periods as indicated. Blots were analyzed with phosphospecific Foxo3a antibodies (S253) and antibodies recognizing total Foxo3a. An unidentified, cross-hybridizing protein (X) is dephoshorylated in response to Epo and SCF. Its signal is comparable at t 0' in the Epo and SCF panel when equally exposed. (**D**) Western blots of differentiating I/11 cells (samples taken every 12 h) were analyzed with antibodies recognizing Foxo3a and S253-phosphorylated Foxo3a, PKB and S473-phosphorylated PKB, $p27^{KIP}$ and Erk. The protein recognized by Ser253-Foxo3a antibodies at t 72 h is unknown and may be the same background band X detected by this antibody in (**C**). (**E**) Cytoplasmic and nuclear protein extracts of differentiating I/11 cells were analyzed with antibodies recognizing Foxo3a, STAT3 and ERK. Under these conditions STAT3 is only present in the cytoplasm, indicating minimal contamination of nuclear extracts with cytoplasmic proteins. ERK expression serves as a loading control.

used an inducible, phosphorylation-insensitive Foxo3a(A3):ER fusion construct, in which three serine- phosphorylation sites are mutated to alanines. Fusion of this mutant to an estrogen receptor domain allows induction of nuclear translocation of Foxo3a(A3):ER by 4-hydroxytamoxifen (4OHT; 16). The Foxo3a(A3):ER protein was expressed in I/11 erythroid progenitors by retroviral transduction. Exposure of Foxo3a(A3):ER expressing cells to 50 nM 4OHT induced $p27^{KIP}$ protein levels in eight out of ten clones, which did not occur in empty vector transduced cells (unpublished data). To test whether activation of

Foxo3a(A3):ER could counteract SCF-induced delay of Epo-dependent differentiation, Foxo3a(A3):ER clones and empty vector control clones were cultured in Epo or in Epo plus SCF, both in presence or absence of 50 nM 4OHT. Cell numbers, cell size, hemoglobin levels and cell morphology were monitored daily. 4OHT did not affect proliferation or differentiation of control clones, whereas it slightly decreased proliferation and enhanced hemoglobinization of Foxo3a(A3):ER clones in presence of Epo (Figure 2.2A). In presence of Epo and SCF, proliferation was impeded which was accompanied with enhanced differentiation as analyzed by hemoglobinization (Figure 2.2B) and cell morphology (Figure 2.2C). 4OHT increased the number of partially mature and mature hemoglobinized cells, but cytospins showed no evidence for increased cell death (Figure 2.2C). We did not observe an increase in apoptotic cells upon Foxo3a(A3):ER activation, using a TUNEL assay (Figure 2.2D). This indicated that exogenous, active Foxo3a accelerates differentiation of erythroid progenitors. Also, in presence of Epo, SCF and Dex, activation of Foxo3a(A3):ER abrogated renewal and induced differentiation. However, the presence of Dex interferes with hemoglobin synthesis and precludes the use of this parameter to monitor differentiation accurately. Using siRNA, we examined whether underexpression of Foxo3a impaired or delayed erythroid differentiation. An siRNA that fully blocked Foxo3a in a transient assay (Figure 2.2E, FOXi2) resulted in a considerable, but not complete, reduction of Foxo3a expression upon stable expression in erythroid progenitors (Figure 2.2F). After induction of differentiation, reduced Foxo3a expression attenuated differentiation as measured by hemoglobinization (Figure 2.2G). Thus, activation of Foxo3a accelerated differentiation, and reduced levels of Fox3a suppress maturation during differentiation.

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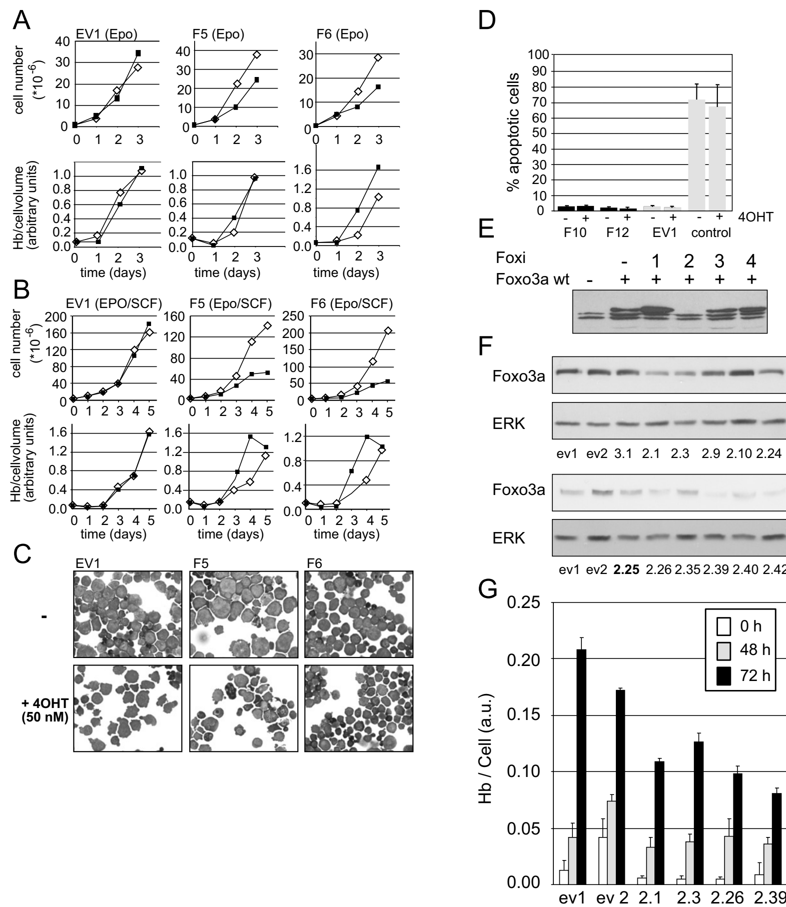

Figure 2.2. **Foxo3a induces erythroid differentiation.** All Foxo3a(A3):ER clones used had similar expression levels of the mutant Foxo3a protein and treatment of all clones induced rapid up-regulation of *p27*^{KIP} (not depicted). (**A** and **B**) A vector transduced clone (EV1) and Foxo3a(A3):ER expressing clones F5 and F6 were seeded in differentiation medium containing Epo (A) or Epo plus SCF (**B**), in absence (open diamonds) or presence (closed squares) of 50 nM 4OHT. Cumulative cell numbers (upper panel) and hemoglobin content per cell volume (lower panel) was determined at daily intervals. (**C**) At day 4 of the experiments, cell morphology and hemoglobin content was analyzed in cytospins. Hemoglobinized cells are smaller and darker (**D**). The vector control clone (EV1) and two Foxo3a(A3):ER clones (F10, F12) were cultured in presence of Epo, SCF, Dex in presence or absence of 4OHT. As a control, parental I/11 cells were seeded in medium lacking factors. After 24 h, the percentage of apoptotic cells was determined by a TUNEL assay. Values represent mean ± SD of apoptotic cells counted in five fields of a cytospin preparation (100 cells/field) in two independent experiments. (**E**) Phoenix E cells were transfected with Foxo3a wild-type alone or in combination with RNAi constructs FOXi1 to 4 (see material and methods). Transient expression of Foxo3a was determined by western blot (Erk serves as a loading control). (**F**) I/11 clones, transduced with pSuper-retro vector as a control (ev1 and ev2) or pSuper-retro FOXi2 (clone numbers indicated) were tested for Foxo3a on Western blot. (**G**) Two empty vector (ev1, ev2) and four FOXi2 clones (clone numbers indicated) were differentiated in the presence of Epo. The hemoglobin content of the cells was measured at 0, 48 and 72 hours in differentiation. Values represent mean ± SD of three experiments.

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Foxo3a target genes in erythroid cells

Although the Foxo3a target p27^{KIP} can induce cell cycle arrest and differentiation in some cell types, overexpression of p27^{KIP} induced apoptosis in erythroblasts, whereas erythroid differentiation was normal in *p27^{KIP}*-deficient cells (23). In addition to known targets that cause apoptosis or cell cycle arrest, Foxo3a could induce so far unknown targets that function in erythroid differentiation. To identify such target genes, cDNA derived from a Foxo3a(A3):ER expressing clone and a control clone exposed to 4OHT (50 nM) for 0, 2 or 6 h, were hybridized to custom-made 'hematopoietic' DNA microarrays containing ~9000 cDNAs derived from SSH libraries enriched for transcripts of expanding erythroblasts (I/11 cells) and mature T-cells (26). These microarrays contained multiple copies of abundant erythroid-specific cDNAs (up to a few hundred for ß-globin). The complete array results are available as supplementary data (Table S1, available at http://www.jcb.org/cgi/content/full/jcb.200307056/DC1). Upon 4OHT treatment of Foxo3a(A3):ER-expressing cells for 2 h, seven transcripts in the array were up-regulated >1.75 fold compared with no treatment, whereas no up-regulated genes were detected in similarly treated control cells. Five out of those seven transcripts represented *Btg1*, with an average up-regulation of 1.9 ± 0.14 (Figure 2.3A). Upon 6 h of 4OHT treatment, 98 transcripts indicated a >1.75 fold increase compared to no treatment. Eleven of these represented *Btg1*. Their average fold up-regulation was 2.9 ± 0.33 (Figure 2.3A,B). None of these transcripts was detected in the control experiment.

The same arrays were also screened for genes up- or down-regulated in I/11 cells that were factor depleted and subsequently restimulated with Epo and/or SCF (26). Among the Foxo3a targets up-regulated after 6 h, only the transcripts representing *Btg1* were down-regulated by Epo and SCF-signaling as expected for genes primarily regulated by Foxo3a (Figure 2.3C and unpublished data). Therefore, we concentrated on *Btg1* as a novel putative Foxo3a target. Two additional Foxo3a(A3):ER clones were treated for 2 h with 50 nM 4OHT, and *Btg1* transcript levels were determined by real-time PCR. In both clones, *Btg1* expression was two-fold up-regulated, whereas control clones showed no change in *Btg1* expression (Figure 2.3D). Cyclohexamide treatment of I/11 cells strongly up-regulated *Btg1*, which precluded its use to determine whether protein synthesis is required for *Btg1* up-regulation.

Together, we identified *Btg1* as a prominent Foxo3a target in this screen. We also show that mitogenic signaling suppresses *Btg1* expression, which is in accordance with the observed Epo- and SCF-induced phosphorylation of Foxo3a.

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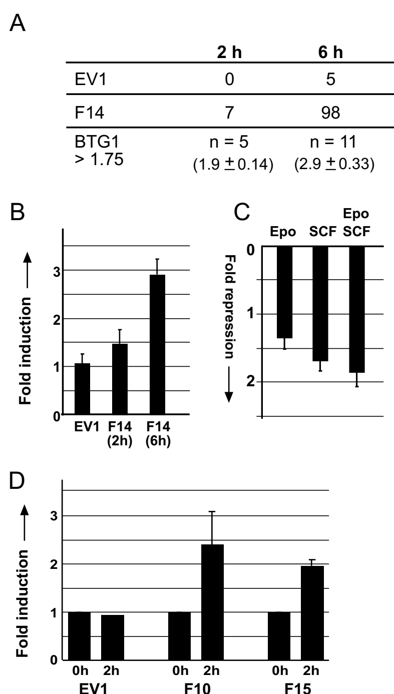
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	2 h	6 h
EV1	0	5
F14	7	98
BTG1 > 1.75	n = 5 (1.9 ± 0.14)	n = 11 (2.9 ± 0.33)

Figure 2.3. **Btg1, a Foxo3a target gene.** Labeled cDNA from Foxo3a(A3):ER clone F14 and vector clone EV1, exposed to 50 nM 4OHT for 0, 2 and 6 h in presence of Epo, SCF and Dex, was hybridized to a 9K cDNA micro-array enriched for hematopoietic transcripts. (**A**) The number of spots that detected a >1.75 fold increase in signal upon treatment with 4OHT compared to nontreated cells is indicated for both clones after 2 and 6 h of treatment. *Btg1* transcripts were represented at least 11 times on these arrays, 5 of these *Btg1*-spots showed >1.75 fold up-regulation after 2 h induction with 4OHT. (**B** and **C**) The average regulation on the 11 *Btg1* spots is calculated after 2 and 6 h 4OHT treatment (**B**) and after 2 h Epo-, SCF- or Epo plus SCF-induction of factor-deprived cells (**C**), 1 meaning no regulation. Error bars indicate SD. (**D**) Control clone EV1 and Foxo3a(A3):ER clones F10 and F15 were treated with 50 nM 4OHT for 2 h and relative *Btg1* expression was determined by real-time PCR (Taqman), using expression of RNAse-inhibitor to normalize the values. Values represent mean ± SD of three independent experiments.

Btg1 is a direct Foxo3a target

To examine whether *Btg1* is a direct target of Foxo3a, we analyzed the *Btg1* promoter region for putative Foxo-binding sites. Among the sequences submitted to the public database, mouse cDNA clone L16846 contained the longest 5'UTR sequence. A comparison with genomic sequences in the CELERA database showed that the start of cDNA L16846 (designated +1) is located 40 nt downstream of a conserved TATA-box sequence (Figure 2.4A). We found four potential forkhead binding sites (Daf-16 binding element (DBE)) at position -219, -454, -826 and -922 (Figure 2.4B), allowing a 1-bp mismatch compared with the consensus sequence TTGTTTAC (21). outside the TGTT core sequence. Only DBE1 (position -219) completely matched the consensus sequence, and an alignment between the mouse and human *Btg1* promoter sequence (BAC AC025164) revealed that only DBE1 was 100% identical between mouse and human (Figure 2.4A).

To determine which part of the *Btg1* promoter mediates Foxo3a-induced expression, genomic fragments containing all 4 DBEs (-1033/+82), DBE1 (-314/+82) or no DBE-sites (-67/+82) were tested for basal promoter activity in Ba/F3 (hematopoietic, pro-B cells), COS (monkey kidney cells) and NIH3T3 (fibroblasts; Figure 2.4B, C). The activity of the different reporter constructs was similar in all cell types. The promoter activity of the -1033/+82 and the -314/+82 fragments was equally high, whereas the promoter activity of the -67/+82 *Btg1* promoter fragment was almost reduced to background levels, indicating the presence of a crucial regulatory element between -314 and -67.

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To investigate the role of DBE1, the TGTT core was mutated to AAAT in the -1033/+82 and -314/+82 constructs. This mutation caused a significant loss of basal promoter activity in both fragments (Figure 2.4D), indicating that DBE1 is a critical element in the *Btg1* promoter. Cotransfection of Foxo3a(A3) with the wild-type and mutant -314/+82 promoter induced wild-type but not mutant 314/+82 *Btg1* promoter activity (Figure 2.4E). Together these data suggest that Foxo3a is able to activate transcription of *Btg1* via the DBE1 element in the *Btg1* promoter.

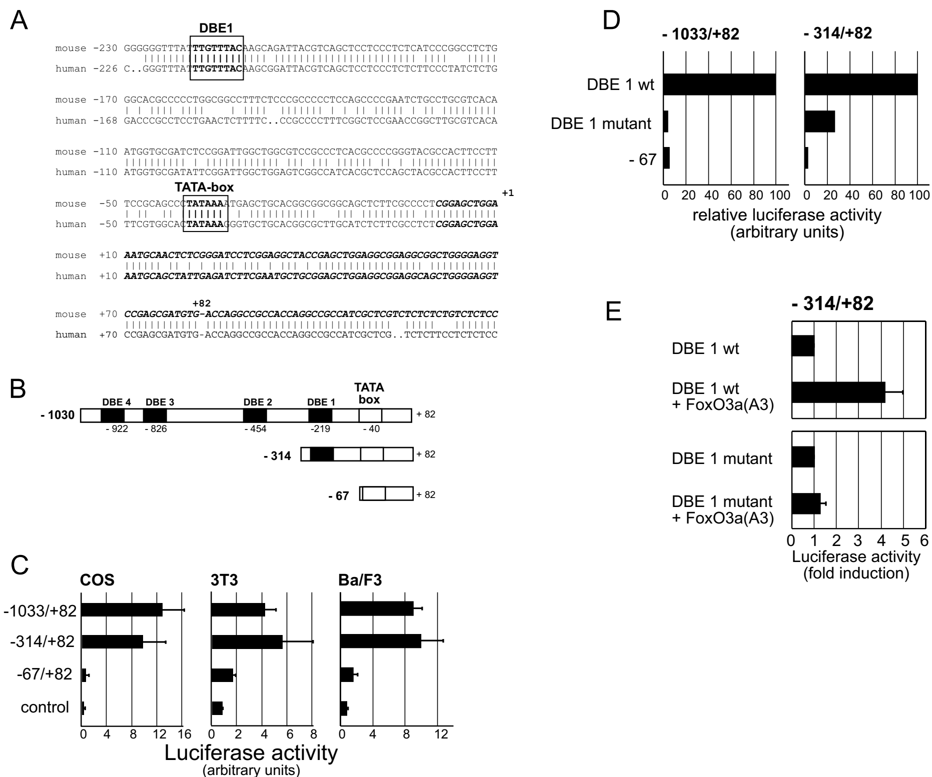

Figure 2.4. **Regulation of Btg1 promoter activity by Foxo3a.** (**A**) Sequence of the promoter region (plain) and part of the first exon (bold, italics) of *Btg1*. Top sequence is derived from the mouse CELERA database and cDNA clone L16846, the bottom sequence is derived from the human BAC AC025164. The start of mouse cDNA L16846 was assigned as position +1. A potential TATA-box and FoxO-binding site (DBE) are indicated. (**B**) Schematic drawing of the *Btg1* promoter fragments used in reporter assays. Four potential FoxO-binding sites, DBE1-4, were found in the -1033/+ 82 promoter fragment. (**C**) Basal *Btg1* promoter activity of these fragments was tested in COS, NIH3T3 and Ba/F3 cells and compared with a vector control (pGL3). Luciferase activity is represented as arbitrary units. Values represent mean ± SD of three measurements. (**D**) Both -1033/+82 and -314/+82 fragments with either a wild-type or a mutated DBE1 were tested for basal promoter activity in COS cells. The -67/+82 fragment, serves as a negative control. (**E**) COS cells were cotransfected with Foxo3a(A3) and the -314/+82 *Btg1* promoter with either a wt or a mutated DBE1. Luciferase activity presented as fold induction on the horizontal axis. Values represent mean ± SD of three measurements.

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Btg1 is up-regulated in erythroid differentiation

If *Btg1* is a Foxo3a target, its mRNA expression should follow Foxo3a activity during differentiation of erythroid progenitors. We determined *Btg1* transcript levels during erythroid differentiation by Northern blot and real-time PCR (Figure 2.5), using mRNA prepared from I/11 cells harvested at 12-hour intervals after differentiation induction. *Btg1* mRNA expression was low until 36 h after induction of differentiation when cells still proliferate. *Btg1* transcript levels strongly increased 48 h after differentiation induction when cells become postmitotic, and remained high until the final stages of erythroid differentiation (Figure 2.5). Thus, *Btg1* is expressed upon appearance of active Foxo3a during differentiation, suggesting a role of Btg1 in late erythroid differentiation.

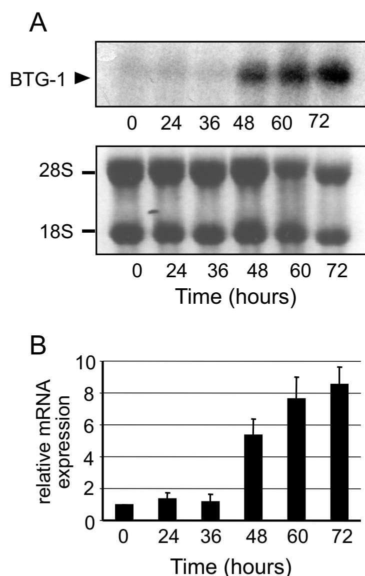

Figure 2.5. ***Btg1* is transcriptionally upregulated during erythroid differentiation.** (**A**) Total RNA was isolated from differentiating erythroid progenitors at 12-h intervals. *Btg1* transcript levels were detected using a Sma1/BamH1 211 bp fragment as a probe (top). Ethidium bromide staining (bottom) indicated equal loading. (**B**) Real-time PCR on the RNA samples confirmed the kinetics in a quantitative way, using SYBR green in Taqman-analysis and normalizing to the expression of the RNAse-inhibitor RI. Values represent mean ± SD of three measurements.

Btg1 may act via protein arginine methylation during differentiation

Btg1 is one of six family members, all sharing two highly homologous domains (BoxA and B; Figure 2.6A). It shares a third region of homology with Btg2 (BoxC), which associates with Prmt1 (2, 31). Overexpression of the Btg1 BoxC domain has been shown to inhibit differentiation of PC12 cells (2). We expressed Btg1 in the I/11 erythroid progenitors, but only obtained small, differentiated clones that could not be expanded (unpublished data). Btg1:ER fusion constructs as used for Foxo3a were not regulated tight enough. Because bone marrow is transduced with much higher efficiencies than I/11 cells, we next transduced murine primary bone marrow to examine proliferation and differentiation in suspension cultures and in colony assays, using retroviral expression vectors containing *Btg1* wild-type or a *Btg1* construct lacking boxC (*Btg1-ΔBoxC*). Colony formation of transduced cells was determined in serum-free semisolid medium supplemented

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with granulocyte-macrophage colony-stimulating factor (GM-CSF; myeloid colonies), or with a combination of Epo, SCF and Dex (producing exclusively erythroid colonies as Dex inhibits outgrowth of SCF-dependent, nonerythroid colonies; controlled by cytospins (unpublished data). Addition of puromycin selected for the outgrowth of transduced cells only. Bone marrow was infected with supernatants containing equal numbers of virus as controlled by dot-blot experiments (unpublished data; 20). Colonies were counted 7 days after plating. Ectopic expression of Btg1 drastically reduced both the size and the number of erythroid colonies (Figure 2.6B: smallest, average and largest colonies are shown), whereas it only slightly reduced the number of myeloid colonies (Figure 2.6C). In contrast, erythroid colonies expressing Btg1-ΔBoxC were normal in size and only slightly reduced in number compared with the vector-control colonies (Figure 2.6B,C). Though the total number of colonies obtained differed between experiments (between 95 and 266 colonies in the vector control), the relative number of colonies obtained upon transduction of the various constructs was constant. Therefore colonies are given as a percentage of control. Essentially the same results were obtained in suspension cultures (Figure 2.6D). This result indicates that Btg1 abrogates proliferation, and that this effect depends on the presence of the Prmt1 interaction domain in boxC.

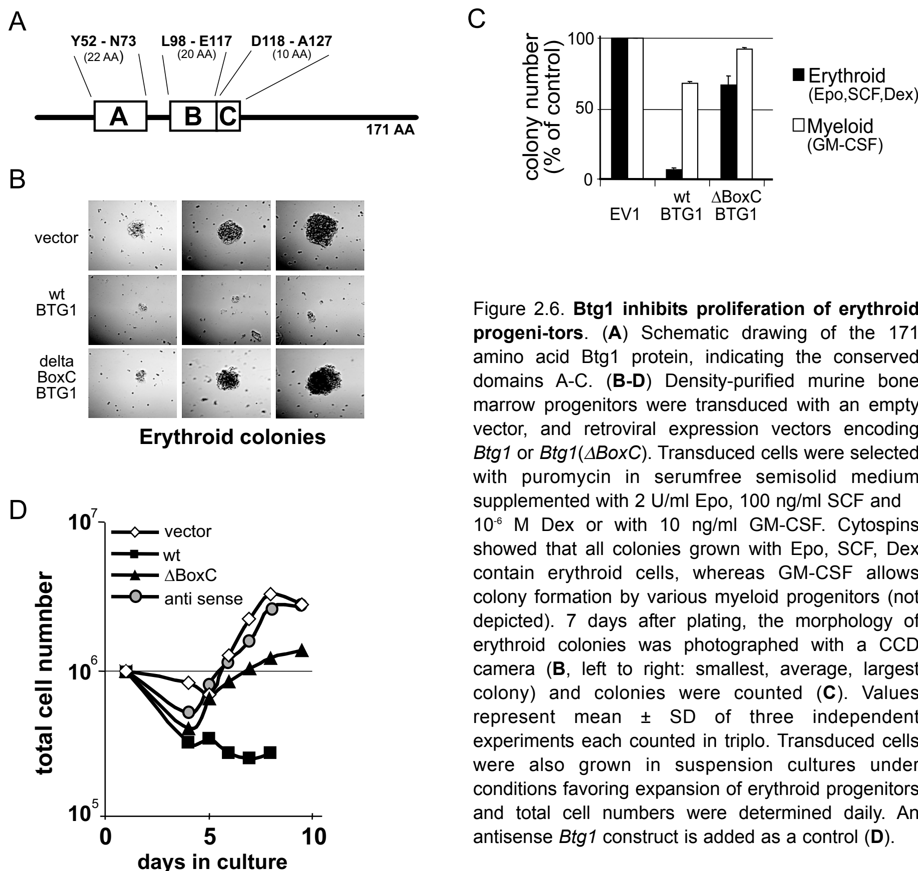

Figure 2.6. **Btg1 inhibits proliferation of erythroid progeni-tors**. (**A**) Schematic drawing of the 171 amino acid Btg1 protein, indicating the conserved domains A-C. (**B-D**) Density-purified murine bone marrow progenitors were transduced with an empty vector, and retroviral expression vectors encoding *Btg1* or *Btg1*(*ΔBoxC*). Transduced cells were selected with puromycin in serumfree semisolid medium supplemented with 2 U/ml Epo, 100 ng/ml SCF and 10^{-6} M Dex or with 10 ng/ml GM-CSF. Cytospins showed that all colonies grown with Epo, SCF, Dex contain erythroid cells, whereas GM-CSF allows colony formation by various myeloid progenitors (not depicted). 7 days after plating, the morphology of erythroid colonies was photographed with a CCD camera (**B**, left to right: smallest, average, largest colony) and colonies were counted (**C**). Values represent mean ± SD of three independent experiments each counted in triplo. Transduced cells were also grown in suspension cultures under conditions favoring expansion of erythroid progenitors and total cell numbers were determined daily. An antisense *Btg1* construct is added as a control (**D**).

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Because boxC of Btg1 interacts with Prmt1, we investigated whether protein arginine methylation is associated with Btg1 expression. Two different antibodies (7E6, recognizing mono- and dimethyl-arginine, and ASYM24, recognizing only asymmetrical dimethyl-arginine) were used to immunoprecipitate arginine-methylated proteins from lysates of cells at different stages of differentiation. Concurrent with the activation of Foxo3a and the up-regulation of *Btg1*, the 7E6 antibody detected a prominent protein of 100 kD (Figure 2.7A top panel). The ASYM24 antibody detected proteins of 45, 40 and 37 kD, which appeared after up-regulation of *Btg1* (Figure 2.7A, second panel). ASYM24 also detects abundant proteins that are methylated at all stages of differentiation. In contrast, the overall Prmt1 protein levels in the same lysates remained constant during differentiation (Figure 2.7A, lower panel).

To examine whether inhibition of methylation affects the balance between expansion and differentiation of erythroid progenitors, I/11 erythroblasts were seeded in differentiation medium containing Epo or Epo plus SCF to analyze differentiation. Aliquots were exposed to the methyl-transferase inhibitor 5'-deoxy-5'-methylthioadenosine (MTA; 10 and 25 mM) or its solvent. Cell numbers, cell volume, hemoglobinization and cell morphology were monitored daily. In presence of Epo, I/11 cells achieve full hemoglobinization in 3 days, but addition of MTA (both 10 and 25 mM) impairs hemoglobin accumulation (Figure 2.7B). In cytospins generated at day 3, the control cells are hemoglobinised and enucleated, whereas MTA treated cells maintained blast morphology (Figure 2.7C). In presence of Epo and SCF, erythroblasts reduced cell size and accumulated hemoglobin at day 8, whereas they retained blast morphology and continued to proliferate in presence of MTA. This showed that inhibition of methyl transferases impairs differentiation but fails to affect proliferation of erythroid progenitors. Although the inhibitor is not specific for Prmt1, these results support a potential role of Prmt1 in terminal erythroid differentiation.

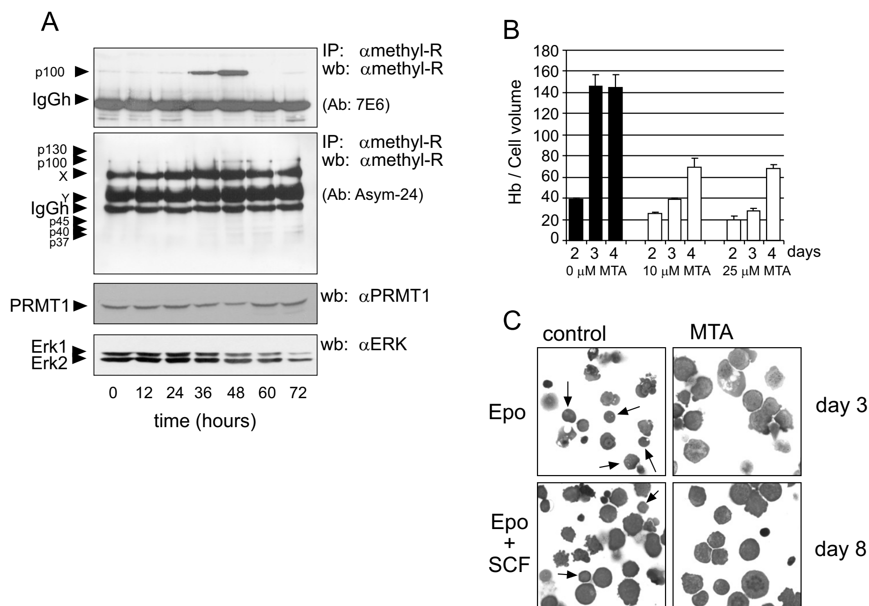

Figure 2.7. **Protein methylation is associated with differentiation not with renewal of erythroid progenitors**. (**A**) Arginine-methylated proteins were immunoprecipitated and stained with two anti-methyl-arginine antibodies (E76 and ASYM24) from cell lysates taken at 12 h intervals during differentiation of I/11 cells. The size of specific proteins is indicated in kD. X and Y represent uncharacterized proteins detected by ASYM24 at all stages of differentiation. The lower panels represent whole cell extract probed for total levels of Prmt1 and ERK (loading control). (**B**) During differentiation of I/11 cells in presence of Epo and in absence or presence of MTA (10 and 25 mM) hemoglobin accumulation was quantified at day 2 - 4. Hemoglobin per cell volume is measured in arbitrary units. Values represent mean ± SD of three measurements. (**C**) I/11 cells were seeded in differentiation medium in the presence of Epo (top) or Epo/SCF (bottom), in absence (left) or presence (right) of the methyl-transferase inhibitor MTA (25 mM). At day 3 (Epo) or at day 8 (Epo/SCF), cell morphology was analyzed in cytospins stained for hemoglobin (hemoglobinized cells stain orange/brown) and histological dyes. In presence of MTA cells retained a blast morphology and failed to accumulate hemoglobin.

Discussion

Foxo3a expression increases sharply during erythroid differentiation, followed by loss of phosphorylation and transcriptional activity. Inappropriate activation of Foxo3a accelerates differentiation of erythroid progenitors to erythrocytes. *Btg1* was identified as a novel target gene of Foxo3a, repressed by SCF and exerting a negative effect on erythroid progenitor renewal. Deletion of BoxC in Btg1, a domain known to bind protein arginine methyl transferase 1 (Prmt1), abrogated proliferation inhibition by Btg1, suggesting that protein arginine methylation might interfere with renewal. This is supported by the observation that inhibition of methyl transferase activity does not interfere with progenitor expansion, but blocks differentiation into mature erythrocytes.

PI3K and Foxo3a control the balance between renewal divisions and differentiation of erythroid progenitors

PI3K-dependent signaling is associated with expansion of erythroid progenitors

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(22, 25, 36, 46, 55). Both Epo and SCF promote erythroid proliferation by inhibition of FoxO's, whereas active Foxo3a was shown to cause cell cycle arrest and apoptosis in erythroblasts unless inhibited by phosphorylation through PKB (24, 33). We find Foxo1, Foxo3a and Foxo4 to be expressed in renewing erythroid progenitors. The disappearance of Foxo1 and Foxo4 in differentiation suggests that these FoxO-members may specifically have a role in the PI3K-dependent control of progenitor expansion, possibly via previously reported effector pathways (17, 27, 35, 40). Actually, the three FoxO-members may have overlapping functions in the control of progenitor expansion. However, since not only activation of Foxo3a, but also inhibition of PI3K activity induced differentiation rather than apoptosis, all three FoxO-members may be able to abrogate renewal and initiate the differentiation program. Once the differentiation program is started, only Foxo3a is upregulated and only Foxo3a may control gene expression in late differentiation, including *Btg1*. While activation of Foxo3a enhanced differentiation, RNAi suppressing Foxo3a impaired differentiation as monitored by reduced hemoglobinization. However, inhibition of Foxo3a did not abrogate differentiation. Possibly, the remaining expression of Foxo3a is sufficient to allow the differentiation process to proceed. Alternatively, the tight control of erythroid cell numbers, evidenced by the very rare occurrence of erythroid leukemia, has recruited several complementary mechanisms that guarantee proper differentiation of erythroid progenitors. The observation that *Foxo3a*-deficient mice develop a compensated anemia with reticulocytosis (10). illustrates the importance of Foxo3a in erythroid differentiation. The reticulocytosis suggests that the anemia is caused by instability of erythrocytes rather than by increased apoptosis of progenitors.

The role of Foxo3a in erythroid differentiation

After induction of differentiation, erythroid progenitors undergo 3 - 4 cell divisions whereas maturing to hemoglobinized, enucleated erythrocytes (18, 55). Activated Foxo3a induced expression of $p27^{KIP}$ and $p130^{Rb2}$ in erythroblasts as has been shown for other cell types (17, 29, 35; confirmed by quantitative-PCR, unpublished data). Forced activation of Foxo3a accelerated differentiation and reduced the number of cell divisions. Foxo3a-induced expression of $p27^{KIP}$ and $p130^{Rb2}$ most likely contributed to G1-arrest. However, cell cycle arrest caused by exogenous $\mathrm{p27^{KIP}}$ fails to induce terminal differentiation of erythroid progenitors, causing apoptosis instead (23). Furthermore, erythroid progenitors lacking $p27^{KIP}$ did not show any alterations in erythroid differentiation (unpublished data). Thus, erythroid differentiation requires more than a ($\mathrm{p27^{KIP}}$-mediated) cell cycle arrest. In contrast to other cells, erythroid differentiation requires 3 - 4 cell divisions without size control to mature into erythrocytes. We cannot conclude at present, whether the newly identified Foxo3a target *Btg1* contributes to differentiation related phenotypic changes like an altered cytoskeleton organization, cell cycle arrest, chromatin condensation and enucleation, or whether it directly contributes to the early control of the balance between renewal and differentiation. However, the fact that Btg1 controls protein methylation in erythroid differentiation and that this may contribute to control of renewal versus differentiation identifies a novel Foxo3a-dependent mechanism that may regulate many aspects of the

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differentiation process. The importance of Btg function is underscored by the fact that also the homologous family member Btg2 is similarly suppressed by SCF and induced during differentiation (26), although via a distinct mechanism. This complementary regulation underscores the biological importance of Btg activation but precludes conclusive experiments on the requirement for Btg1 by underexpression.

The role of Btg1 in protein methylation and differentiation

Screening of a cDNA array enriched for hematopoietic transcripts identified *Btg1* as a major Foxo3a target. Others also reported *Btg1* to be a potential forkhead target (40), but so far its role and regulation were not studied in detail.

Activation of Foxo3a in expanding erythroblasts induced a >2-fold up-regulation of *Btg1* within 2 h, indicating a rapid induction. A more pronounced up-regulation is detected during differentiation. However, Foxo3a(A3):ER protein can only be expressed at low levels in expanding cells, resulting in Foxo3a(A3):ER concentrations at best similar to endogenous levels. In contrast, Foxo3a levels rise considerably during differentiation. This difference on Foxo3a expression may explain the difference in *Btg1* expression in these experimental conditions. Expression of *Btg1* is not solely regulated by Foxo3a, we also reported *Btg1* to be regulated by glucocorticoids (26). and it was shown to be a putative vitaminD3 target (44). Moreover, preliminary data suggest that CREB and Foxo3a have to cooperate in *Btg1* induction (unpublished results). Thus, regulation of *Btg1* may be complex, suggesting an important role in cell fate determination.

The Btg1 protein lacks enzymatic activity but contains several protein interaction domains (Figure 2.6A), suggesting a function as an adapter molecule for enzymes and their targets or as a regulatory cofactor. Btg1 has been shown to interact with Prmt1 via its BoxC domain (2, 31), resulting in positive regulation of Prmt1 activity. Thus, Btg1 may direct the associated methyl transferase activity towards substrates binding to its NH_2-terminal domains (e.g. HOXB9 and the carbon catabolite repressor (CCR4)-associated factor-1, CAF1; 4, 38, 39, 43). Although expression of Btg1 in mouse bone marrow cells fully blocked expansion of erythroid progenitors, deletion of the Prmt1-associated BoxC domain largely abolished this negative effect, indicating that recruitment of Prmt1 is essential to the function of Btg1 in erythroid differentiation. Erythroid progenitors may be more sensitive to this function of Btg1, since Btg1 expression only marginally affected myeloid colony formation induced by GM-CSF. Erythroid differentiation seemed to require arginine methylation, since its inhibition by 5'-deoxy-5-methylthioadenosine (MTA) completely blocked terminal erythroid differentiation without affecting the proliferation of immature erythroblasts. Despite the fact that MTA inhibits other S-adenosyl-L-methionine-dependent methyl transferases, we can still conclude that methylation is required for terminal differentiation and not for renewal of erythroblasts. Similarly, global inhibitors of methylation also inhibited differentiation in the PC12 cell line (11) and PC12 cells loaded with a penetratin-Btg1/BoxC fusion peptide failed to differentiate, suggesting that neuronal differentiation involves Prmt1 regulation by Btg1 (2). Overexpression of Btg1 inhibited myoblast proliferation and induced differentiation (42). Btg2, the

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closest homologue of Btg1 was shown to be upregulated in neuronal differentiation (5, 13) and to play a role in germ cell and muscle cell differentiation (reviewed in (51)).

It has become increasingly clear that arginine methylation functions as a molecular switch, promoting or preventing specific protein-protein interactions (for a review see 34). Prmt1 contributes to 90% of the total cellular methyl transferase activity (37, 48) and mice lacking Prmt1 die at day 6.5 of development just before gastrulation occurs (37). We showed that increased *Btg1* expression following induction of differentiation is accompanied by arginine methylation of multiple proteins, ranging from 37 to 100 kD. The p100 detected by 7E6 is detectable at 36 h, maximal at 48 h and not detected at later time points, although expression of Btg1 and Prmt1 persist. Possibly its expression is only transient, or the Btg1/Prmt1 complex is directed to other substrates in the course of the differentiation program. Prmt1 was reported to induce methylation of Stat1, but Stat1, Stat3 or Stat5 antibodies did not recognize the 100 kD protein(s). So far we do not know the targets of Prmt1 in erythroid differentiation. Interestingly, Btg1 interacts with CAF-1 and the CCR4/CAF1 complex is involved in heterochromatin formation, gene silencing and negative regulation of mRNA stability (4, 32, 38, 43, 50, 52, 53). In erythroid cells both Prmt1 and CAF1 are expressed throughout differentiation at constant levels (this paper and unpublished results), leaving the possibility that Btg1 mediated activation of Prmt1 and CAF1 contributes to epigenetic gene regulation including condensation of the nucleus and enucleation late in erythroid differentiation.

Materials and Methods

Cells and reagents

COS, 3T3 and ecotropic Phoenix cells were cultured in DMEM (Life Technology) supplemented with 10% fetal calf serum (FCS; Life Technology), BA/F3 were cultured in RPMI 1640 supplemented with 10% FCS and 10 ng/ml murine IL-3. LY294002 was obtained from Alexis (Schwitzerland), 4-hydroxytamoxifen and 5'-deoxy-5'-methylthioadenosine (MTA) from Sigma-Aldrich.

Expansion and differentiation of erythroid progenitors

The erythroid cell line I/11 was cultured as described before (55). Cell numbers and cell size were determined using a CASY1 electronic cell counter (Schärfe-System). Cell morphology was analyzed in cytospins stained with histological dyes and neutral benzidine (3). using an Olympus Bx40 microscope (40x objective, NA 0.65), an OlympusDp50 CCD camera and Viewfinder Lite (1.0) acquisition software. Images were cropped using Adobe photoshop 6.0. Hemoglobin was measured as described previously (30). In short, 2 - 4 x10^4 cells were washed in PBS, lysed in 20 ml H_2O and frozen until all samples were collected. 100 ml reagent mix (0.5 mg/ml o-phenylenediamine (Sigma), 0.03% H_2O_2 in 0.1 M citrate/phosphate buffer pH 5.0) was added to thawn samples, the reaction was stopped after 3' with 20 ml 8 N H_2SO_4 and the extinction of the

reaction product was read on an Elisa photometer at 492 nM, using the extinction at 690 nM as a control. Extinction/cell number/cell volume was taken as a measure for hemoglobinisation. Apoptosis was determined using the TUNEL assay according to the manufacturer's protocol (Roche).

Generation of stable Foxo3(A3):ER or Foxo3-RNAi expressing I/11 clones
The Foxo3(A3):ER construct (15). was cloned in the retroviral expression vector pBABE-puro. Four RNAi constructs were cloned in pSuper-retro (kindly provided by Anton Berns, NKI, Amsterdam, The Netherlands):
FOXi1 AATGAAGGCACGGGCAAGAGCTCTT;
FOXi2 AACCAGACACTCCAAGACCTGCTT;
FOXi3 AGTGACTTGGACCTGGACATGTT
and FOXi4 AGCCAGCTCGGCCATGGTGAT. These were transiently expressed in Phoenix cells together with the Foxo3(A3):ER construct. Only the FOXi2 sequence suppressed Foxo3 expression and was used for stable expression in I/11 cells. To obtain stable expression, 0.5 x 10^6 ecotropic Phoenix cells, seeded in 60 mm dishes, were transfected with 16 mg plasmid DNA using calcium-phosphate coprecipitation. 40 h after transfection, cells were treated with 10 mg/ml mitomycinC (Kyowa Hakko Kogyo, Tokyo, Japan) for 1 hour and washed 3 times with PBS, twice with an interval of > 4 h. I/11 cells (0.5 x 10^6 / ml) were added and co-cultured for 24 h in StemPro-34™ plus factors. I/11 cells were removed and grown in puromycin containing (2 mg/ml, Sigma) semisolid medium (Methocel-containing StemPro-34™ (Invitrogen), supplemented with factors). After 7 days, well-separated colonies were picked, expanded and analyzed for Foxo3(A3):ER expression.

Western blotting and antibodies
I/11 cells were growth factor deprived for 4 h in plain IMDM (Life Technology) and stimulated at 37°C with SCF (100 ng/ml) or Epo (5 U/ml). Reactions were stopped by addition of ice-cold PBS. Cell lysis, immuneprecipitation, SDS-PAGE and Western blotting were performed as described previously (54). Antibodies used in this study were: α-HA (F-7, Santa Cruz), α-Foxo4a (N-19; Santa Cruz), α-Foxo1 (#9462, Cell Signaling Technology), α-Foxo3 (#06-951, Upstate Biotechnology), α-phospho-Foxo3 (S-253; #06-953, Upstate Biotechnology), α-p27KIP (#K25020, BD Transduction Laboratories), α-ERK1/2 (K-23, Santa Cruz), α-STAT3 (C-20, Santa Cruz), α-mono- and dimethylarginine (7E6; Abcam), α-dimethylarginine (ASYM24; Upstate) and α-Prmt1 (a generous gift from J. P. Rouault, Hôpital Edouard Herriot, Lyon, France).

RNA isolation and Real-time quantitative PCR
After 2 h stimulation (4OHT: 50 nM; Epo: 5 U/ml; SCF: 100 ng/ml), cells were lysed, nuclei were removed and RNA was isolated as described previously (26). 1 mg total RNA was used to synthesize cDNA, exactly as described previously (26). The cDNA was diluted 1:10 to 1:200 prior to PCR amplification. The primer-sequences used for the amplification of Btg1 were forward 5'-TGC AGG AGC TGC TGG CAG-3', reverse 5'-TGC TAC CTC CTG CTG GTG A-3'; murine ribonuclease inhibitor, forward 5'-TCC AGT GTG AGC AGC TGA G-3', reverse 5'-

TGC AGG CAC TGA AGC ACC A-3'. The real-time PCR assay involves TaqMan technology (PE Aplied Biosystems Model 7700 or 7900 sequence detector). The reactions were performed as described previously (26). The amplification program consisted of 1 cycle of 50ºC with 2' hold, 1 cycle of 95 ºC with 10' hold, followed by 40 cycles of denaturation at 95 ºC for 15'', annealing at 62 ºC for 30'' and extension at 62 ºC for 30''. The CT-values of Rnase Inhibitor were used to normalize the Btg1 values.

cDNA array hybridisations and analysis

Total RNA was used to hybridize a custom made 'Hematopoietic' micro-array containing approximately 9000 cDNAs, enriched for erythroid and T-cell specific cDNAs by subtracting cDNA of expanding I/11 cells and quiescent CD4+ T-cells from cDNAs prepared from 3T3 fibroblasts and EpH4 epithelial cells. A full description of the array and the array hybridization is available as supplementary data. The quality of RNA was determined with a bioanalyzer (2100 Bioanalyzer, Agilent), according to the manufacturers instructions. For a single hybridization, 30 µg total RNA was reverse transcribed into cDNA using Cy5-UTP (CyDye, Amersham Biosciences), whereas control total RNA was labeled with Cy3-UTP. The micro-arrays were hybridized and analyzed as described (26). The scanning was performed using a Genepix 400A (Axon Instruments) scanner, the analysis using the GenePix program.

Cloning of the Btg1 promoter and Luciferase Reporter assays

The Btg1 cDNA clone L16846 was aligned to the mouse *Btg1* genomic sequence (CELERA) and the human BAC clone AC025164. The -1033/+82 *Btg1* promoter fragment was cloned into the pGL3-Basic vector (Promega) after expansion by PCR using a 5' oligo (5'-GTG GTG TGT ATT GCA TCT GAT GAC C-3'), a 3' oligo (5'-CAC ATC GCT CGG ACC TCC CCA GCC-3') and the Expand High Fidelity PCR System (Roche). The -314/+82 and the -67/+82 promoter fragments were obtained using the internal NheI and SmaI sites, respectively. The DBE1) was mutated using the Quickchange Site Directed Mutagenesis kit (Stratagene) according to the manufacturers protocol with primers 5'-CGG GGG GTT TAT TTA AAT ACA AGC AGA TTA CG-3', and its complementary sequence. For reporter assays, COS cells were seeded at 2.5x105 cells/35mm well (Costar) and transfected with 4 mg of DNA by calcium-phosphate co-precipitation. After 24 h cells were washed with PBS and subsequently lysed in 25 mM Tris-phosphate pH 7.8, 15% glycerol, 1% Triton X-100, 1 mM DTT, 8 mM $MgCl_2$. Luciferase activity was measured using the Steady-Glo system (Promega). LacZ determination was used to correct for transfection efficiency.

Online supplemental material

Table S-I is an excel file that contains the data of the microarray analysis. A MIAME compliant description of the microarray, the samples and the hybridization and scanning procedures is given as a separate text file.

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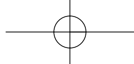
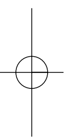
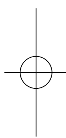
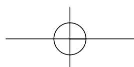

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The cAMP pathway cooperates with Foxo3a to induce *Btg1* expression in the final stage of erythroid differentiation

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CHAPTER 3

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Abstract

The transcriptional activity of forkhead transcription factor Foxo3a and expression of its target *B-cell translocation gene 1* (*Btg1*) are repressed by growth factor-induced phosphatidylinositol-3-kinase (PI3K) activity. Btg1 controls the activity of protein arginine methyl transferase 1 (Prmt1) and abrogates expansion of erythroid progenitors. Foxo3a expression increases and PI3K activity decreases during erythroid differentiation, resulting in increased expression of Btg1 late in erythropoiesis. We examined regulation of *Btg1* expression and found that a cAMP response element (CRE) present in the *Btg1* promoter close to the Foxo3a binding site was important for *Btg1* expression. This site appeared to bind the cAMP-dependent transcription factors CREB and ATF1, as well as ATF2 and cJun that are controlled by Jun-kinase (JNK). The level of cAMP and phosphorylation of CREB and ATF1 strongly increased late in differentiation, indicating that both Foxo3a and the cAMP pathway can induce *Btg1* in late erythroblasts. The prostaglandin E2 receptor, which has been shown to activate the cAMP pathway, is strongly induced during erythroid differentiation, and cooperates with Foxo3a on induction of erythroid differentiation.

Introduction

Erythropoietin (Epo) is essential for expansion, survival and differentiation of erythroid progenitors (30, 55). Stem cell factor (SCF) has an essential role in hematopoiesis (9), but in control of erythropoiesis it is mainly involved in expansion of erythroid progenitors under stress conditions (8, 35, 53) in cooperation with glucocorticoids (3). The ability of erythroid progenitors to undergo expansion *in vitro* in presence of Epo, SCF and glucocorticoids, whereas they differentiate to enucleated erythrocytes in presence of Epo, reflects stress erythropoiesis and offers a unique opportunity to study the regulation of expansion versus differentiation of erythroid progenitors (18, 53). An important signaling pathway in the control of this balance is the phosphatidylinositol-3-kinase (PI3K) / protein kinase B (PKB) pathway. Inhibition of PI3K decreased erythroid expansion and induced differentiation instead (53). Concordantly, mice lacking the p85 subunit of PI3K display a transient fetal anemia due to decreased progenitor expansion (24). PKB is a major effector of PI3K activity. Through direct phosphorylation, PKB controls multiple proteins with key functions in distinct cellular processes, among which class O forkhead transcription factors (Foxo's; (7, 10, 28) Phosphorylation of Foxo's results in reduced DNA-binding and nuclear exclusion (10, 28). Mutation of the 3 serine residues that are targeted by PKB into alanine residues, renders Foxo3a constitutively active (17). Expression of this Foxo(A3) mutant suppressed erythroid proliferation and induced erythroid differentiation instead (2). During differentiation, Foxo3a expression gradually increases, whereas PKB activity is abrogated 48h after differentiation induction, resulting in a sudden increase of Foxo3a activity concurrent with major changes in the differentiation program such as G_1 arrest of the cell cycle and chromatine condensation. By micro-array analysis we identified *B-cell translocation gene-1* (*Btg1*)/*anti-proliferative gene-2* (*Apro2*) as a direct Foxo3a target (2). Expression of *Btg1* sharply increases during erythroid differentiation concomitant with Foxo3a activity. Btg1 inhibits proliferation of erythroid progenitors, which is dependent on its ability to bind to arginine methyl-transferase-1 (Prmt1), suggesting that Foxo3a controls the balance between erythroid expansion and differention thought modulation of protein arginine methylation (2).

Btg1 and its close homologue *Btg2* are members of an anti-proliferative (apro) gene family, which suppresses growth in a wide variety of cells (32, 43, 44). Btg family members are small proteins without enzymatic activity. They all share two homologous domains (boxA and B), whereas Btg1 and Btg2 share a third homologous region (boxC), which interacts with Prmt1 (4). Their expression increases in the G_0/G_1 phases of the cell cycle and they induce differentiation in a variety of cells. For instance, Btg2 expression is increased at the onset of early neuronal differentiation (6, 48) and was suggested to mediate the growth arrest that serves as a prelude to differentiation (25). Concordantly, overexpression of boxC, which acts as a dominant negative peptide, blocked neuronal differentiation (4). Btg1 was shown to mediate cAMP-induced differentiation of myboblasts into myocytes (31, 42), suggesting a link between the cAMP pathway and Btg1 function.

The cAMP pathway, like Btg1 and Btg2, has been linked to inhibition of

cell growth and induction of terminal differentiation. For instance, both cAMP and Btg1 are involved in spermatid differentiation (40, 54) and cAMP was shown to impair proliferation and induce differentiation of leukemic blasts both *in vivo* and *in vitro* (21). In primary and transformed erythroid progenitors, cAMP impairs proliferation (22, 29), whereas knock down of the cAMP downstream target protein kinase A (PKA) impairs differentiation of mouse erythroleukemia cells (37). Expression of *Ala-S* (5-aminolevulinate synthase), the rate-limiting enzyme in heme-synthesis, and *NF-E2* (nuclear factor erythroid 2) appeared to be regulated by cAMP (20, 47). A major deficit of most studies concerning the role of cAMP in erythroid differentiation is that they address the role of cAMP in chemically induced differentiation of erythroleukemia cells, whereas little is known about the role of cAMP in normal erythropoiesis.

Here we demonstrate that cAMP levels as well as phosphorylation of the cAMP-responsive transcription factors CREB and ATF1 is increased during differentiation. CREB and ATF1 appeared to cooperate with Foxo3a to induce *Btg1* expression. One of the factors able to induce cAMP in maturing erythroid progenitors is prostaglandin E2, which enhanced Foxo3a induced erythroid differentiation. Thus, the cAMP pathway cooperates with Foxo3a to control terminal differentiation of erythroid progenitors and they cooperate directly in the regulation of Btg1.

Results

A CRE site in the *Btg1* promoter contributes to *Btg1* expression

Analysis of the *Btg1* promoter revealed the presence of cyclic-AMP responsive element (CRE) in a highly homologous promoter region (Figure 3.1A), i.e. at position -204, 15 basepairs downstream of the previously reported Daf-16 binding element (DBE), (Figure 3.1A) which is essential for induction of *Btg1* expression by Foxo3a (2). We previously reported Foxo3a-dependent promoter activity of both a -1030/+82 and a -314/+82 promoter fragment. To investigate the contribution of the CRE to control of *Btg1* expression, the CRE was mutated by a two basepairs substitution (TTACGTCA to TTTGGTCA), in both the wild-type promoter fragments and in conjunction with a mutation of the DBE (2). Promoter activity was assayed using luciferase activity as a reporter. Deletion of the CRE or the DBE repressed basal promoter activity to a similar extent, whereas the promoter activity was not further repressed in the absence of both sites (Figure 3.1B). Both the longer and smaller promoter fragments are similarly regulated confirming that the -314/+82 *Btg1* promoter fragment contains the essential elements for promoter regulation.

The close proximity of the CRE to the DBE and the observation that deletion of either site represses *Btg1* promoter activity raises the question whether both sites are essential for Foxo3a-induced *Btg1* transcription. Co-expression of Foxo3a and the various reporter constructs show that the DBE, but not the CRE site is essential for Foxo3a-induced *Btg1* expression (Figure 3.1C). It was also tested whether stimulation of the cAMP pathway resulted in increased

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Btg1 expression. To this end the different *Btg1* promoter constructs were transfected to Ba/F3 cells and cultured overnight in the absence or presence of 10 mM cAMP. Cyclic-AMP stimulation increased *Btg1* promoter activity of the wild type promoter, but not of the DBE and CRE mutants, suggesting that induction of *Btg1* by cAMP is dependent on Foxo activity (Figure 3.1D). Thus, both the DBE and the CRE site contribute to basal *Btg1* promoter activity, but the CRE-site is dispensable for Foxo3a-induced *Btg1* promoter activity, whereas the cAMP pathway has to cooperate with Foxo3a to induce *Btg1* promoter activity.

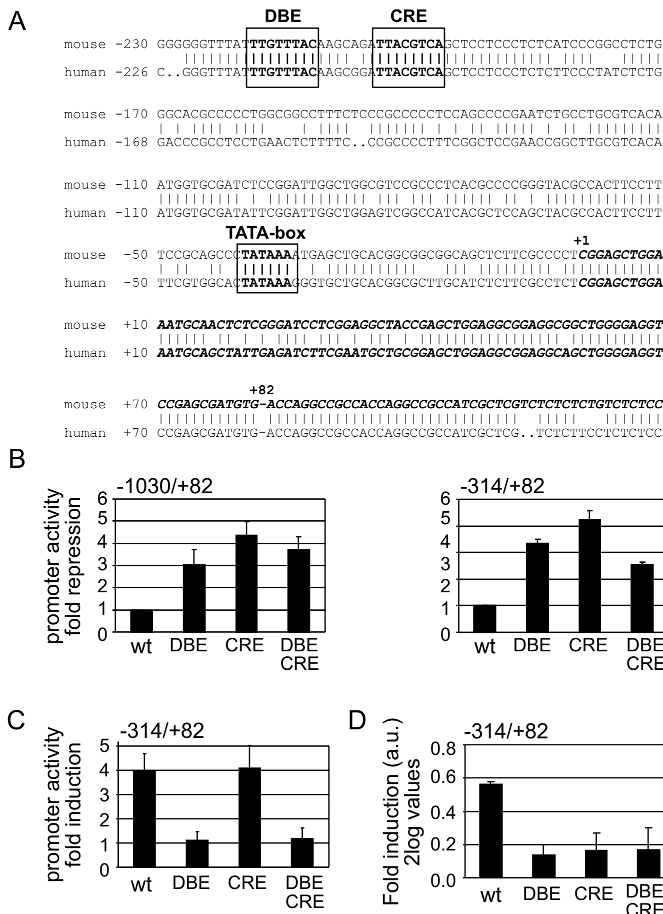

Figure 3.1. **Expression of *Btg1* is controlled by Foxo3a and by factors binding to a cAMP response element (CRE) close to the Foxo binding site (DBE).** (**A**) Alignment of the mouse and human *Btg1* promoter. The transcription startsite is indicated as +1, transcribed sequence in bold. At +82 the first intron is indicated by a small dash in both sequences. The TATA-box as well as the Foxo binding site (DBE) and cAMP responsive element (CRE) are boxed. (**B**, **C**) Long (-1030/+82) and Short (-314/+82) *Btg1* promoter fragments (wild-type (wt), with a mutated DBE (indicated as DBE), a mutated CRE (indicated as CRE) or both (indicated as DBE/CRE)) were cloned in a luciferase reporter construct, transfected in 295HEK cells and luciferase activity was measured 24 h after transfection. Values were normalized for transfection efficiency using β-galactosidase activity encoded by a co-transfected expression plasmid. (**B**) Promoter activity of mutated promoter fragments is given as fold repression compared to the wild-type promoter fragment. (**C**) Foxo3a expression plasmid or empty vector has been cotransfected with the reporter constructs and promoter activity in presence of Foxo3a is given as fold induction compared to promoter activity in absence of Foxo3a. Values represent mean and standard deviation of 3 experiments. (**D**) *Btg1* reporter constructs were transfected in Ba/F3 cells and were treated overnight with 20 mM db-cAMP, or left untreated. Promoter activity is presented as fold induction of db-cAMP over non-treated cells (in ^{2}log values). Values represent mean and standard deviation of 3 experiments

The cAMP pathway enhances *Btg1* expression in erythroid progenitors

To examine whether the cAMP pathway regulates *Btg1* expression in erythroid cells, I/11 cells were treated with a stable form of cAMP (dibutyryl-cAMP; db-cAMP, 10 μM), and ligands of the prostaglandin E2 receptor (PGE2, 10 μM) and the adrenergic receptor (norepinephrine; NE, 100 μM), two ligands that activate the cAMP pathway and play a role in erythropoiesis (39). Western blots containing cytoplasmic and nuclear extracts of stimulated cells were stained with an antibody recognizing phosphorylated serine 133 and 63 in CREB and ATF1 respectively. All three compounds induced phosphorylation of nuclear CREB and ATF1 (Figure 3.2A). Treatment with PGE2 induced a strong, transient response, whereas db-cAMP and NE resulted in a more prolonged activation of the cAMP pathway. All three compounds modestly enhanced *Btg1* expression (Figure 3.2B). NE and db-cAMP which both resulted in prolonged cAMP pathway activation, also resulted in a more pronounced activation of *Btg1* expression.

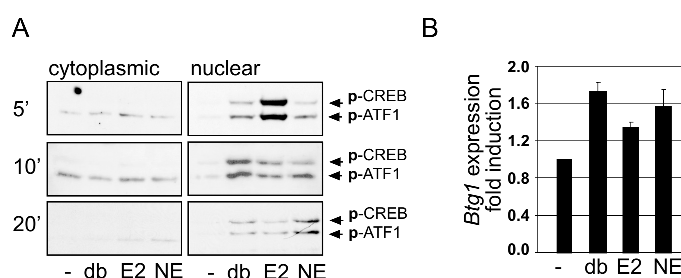

Figure 3.2. **Different inducers of cAMP cause phosphorylation of the transcription factors CREB and ATF1 and increase expression of *Btg1*.** (**A**) I/11 cells were factor deprived (4 h) and stimulated with dibutyryl cAMP (db; 10 μM), prostaglandin E2 (E2; 10 μM) or norepinephirine (NE; 100 μM) for 5, 10 or 20 minutes. Cytoplasmic and nuclear extracts were prepared and stained with an antibody recognizing phosphorylated serine 133 and 63 in CREB and ATF1 respectively. (**B**) I/11 cells were treated similarly for 2 h and Btg1 mRNA expression was quantified by realtime PCR. Expression induced by cAMP-activators is given as fold induction compared to no treatment. Values represent mean and standard deviation of 3 experiments.

cAMP pathway activity during erythroid differentiation

Foxo3a is strongly up-regulated during erythroid differentiation and by consequence also *Btg1* transcript levels are elevated between 48 and 72 hours (2), i.e. during the final stage of erythroid differentiation when the cells arrest in the G_1 phase of the cell cycle, enucleate and accumulate hemoglobin (53). The possible involvement of the cyclic-AMP pathway in *Btg1* regulation through the CRE prompted us to investigate activity of the cAMP pathway during erythroid differentiation. Cyclic-AMP levels increased during differentiation, reaching a maximum between 40 and 60 h after induction of differentiation (Figure 3.3A), concomitant with strongly increased *Btg1* levels from 40 hours in differentiation (2). Phosphorylation of CREB and ATF1 followed the levels of cAMP during differentiation. Whereas expression of CREB and ATF1 was constant during differentiation, phosphorylation increased from 48 hours after differentiation induction (Figure 3.3B). Phosphorylated CREB and ATF1 are located

predominantly in the nucleus (Figure 3.3C).

The observations that cAMP can induce *Btg1* expression and that the activity of the cAMP-pathway increases during the final stages of erythroid differentiation, strengthens the hypothesis that activation of the cAMP-pathway contributes to *Btg1* expression during erythroid differentiation.

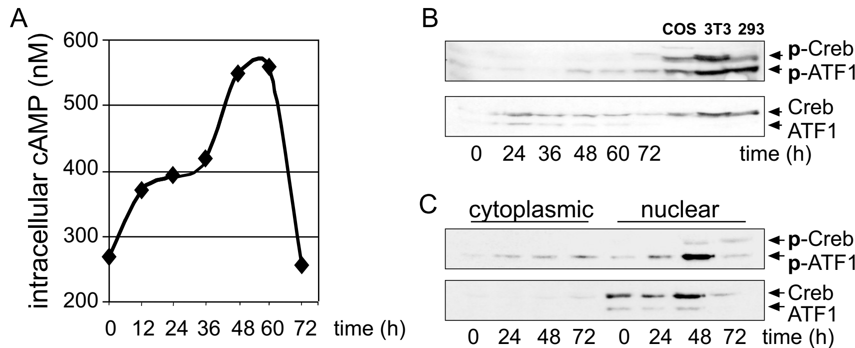

Figure 3.3. **cAMP and phosphorylation of CREB and ATF1 is increased late in erythroid differentiation. I/11 cells were induced to differentiate and samples were isolated at 12h intervals.** (**A**) Intracellular cAMP levels were determined using a cAMP enzyme-immunoassay. (**B**) Cell lysates were stained with an antibody recognizing phosphorylated serine 133 and 63 in CREB and ATF1 respectively. Cell lysates from COS, 3T3 and 293HEK cells were used as positive controls. (**C**) I/11 cells were induced to differentiate, cytoplasmic and nuclear extracts were generated at 24 h intervals and stained for phosphorylated CREB and ATF1 (see B).

CREB family members bind to the *Btg1* promoter

We next investigated binding of CREB/ATF1 to the CRE site in the *Btg1* promoter in expanding and differentiating erythroblasts. Nuclear extracts were incubated with a radioactively labeled oligonucleotide probe encompassing the CRE site and tested in an electrophoretic mobility shift assay (EMSA). Two protein complexes were found to bind the oliginucleotide probe, both in expanding erythroblasts and 48 h after differentiation induction. Competition with wild-type (wt), but not with a CRE mutated probe, inhibited binding of both complexes, indicating that they bind specifically to the CRE (Figure 3.4A,B). The CRE element in the *Btg1* promoter (TTA CG TCA) is not completely similar to the common palindromic CRE sequence identified for instance in the somatostatin promoter (TGA NN TCA) (33, 45). Instead, it resembles the binding site identified for the cJun/ATF2 complex (TTA CC TCA), through which the cJun/ATF2 complex regulates e.g. cJun expression (23, 50, 51). The CREB family members CREB, CREM and ATF1 are able to form heterodimers, but they do not heterodimerize with other bZIP classes such as AP1 family members like cJun and cFos (15, 46). To discriminate between CREB/ATF1 and c-Jun/ATF2 binding to the CRE-element in the *Btg1* promoter, probes with an optimal CREB (TGA CA TCA), and an optimal cJun/ATF2 binding site (TTA CC TCA) were used as competitors for the wild-type CRE *Btg1* probe. Interestingly, competition with a CREB/ATF1 probe competed efficiently with both complexes, whereas

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competition with the cJun/ATF2 probe only inhibited binding of the slower migrating complex (Figure 3.4A,B). Consistent with these findings, binding of the faster migrating complex but not the slower migrating complex, could be supershifted upon addition of the CREB/ATF1 antibody (Figure 3.4A,B).

In conclusion, two complexes are able to bind the CRE in the *Btg1* promoter. A CREB/ATF1 complex was identified as the faster migrating complex, whereas a cJun/ATF2 complex may be present in the slower migrating complex. Binding of these complexes did not differ between 0 and 48 hours, but the cAMP pathway may regulate the activity of the CREB/ATF1 complex during differentiation to contribute to up-regulation of *Btg1*.

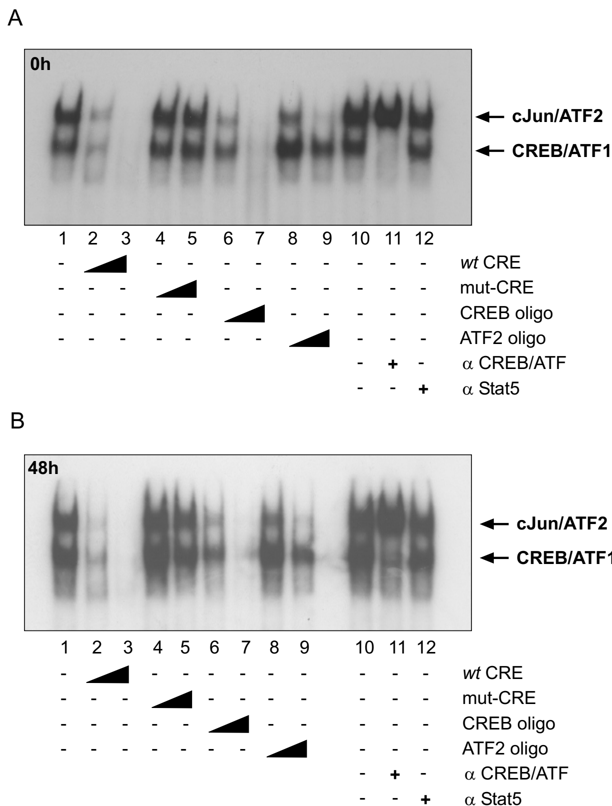

Figure 3.4. **The CRE in the *Btg1* promoter binds CREB/ATF1 as well as cJun/ATF2.** Nuclear extracts from expanding (**A**) and 48h differentiated (**B**) I/11 cells were incubated with a ^{32}P-labeled oligonucleotide probe derived from the *Btg1* promoter encompassing the CRE. Binding of protein complexes was assessed by EMSA. To verify specific binding we added an excess of oligonucleotide probe: wild-type (wild-type CRE; lanes 2,3) or with a mutated CRE (mut-CRE; lanes 4,5). To identify the protein complexes, excess of a CREB/ATF1 specific (CREB oligo; lanes 6,7) or a cJun/ATF2 specific (ATF2 oligo; lanes 8,9) oligonucleotide probe were added. In supershift experiments anti-CREB/ATF1 (lane 11) or anti-Stat5 (lane 12) were added. Arrows at the right side indicate the mobility of cJun/ATF2 and CREB/ATF1 complexes.

The cAMP pathway enhances Foxo3a induced erythroid differentiation

During differentiation, the activity of the PI3K/PKB pathway decreases whereas the activity of the cAMP pathway increases. As a result, the transcriptional activity of both Foxo3a and CREB/ATF1 increases. To test whether both pathways cooperate in induction of erythroid differentiation, I/11 cells were differentiated in the presence of Epo and SCF, supplemented with 10 μM dibutyryl cAMP and /or 15 μM of the PI3K inhibitor LY294002, as indicated (Figure 3.5). Addition of cAMP did not affect the growth of differentiating erythroblast, and LY294002 modestly reduced the number of cell divisions. However, the simultaneous

addition of both compounds strongly suppressed growth during differentiation (Figure 3.5A). In addition, both LY and cAMP alone did accelerate differentiation, shown by the increased hemoglobin synthesis upon addition of LY or cAMP alone, which was further enhanced in the presence of both compounds (Figure 3.5B), showing a cooperation between the PI3K/Foxo3a and the cAMP pathway in the control of erythroid differentiation.

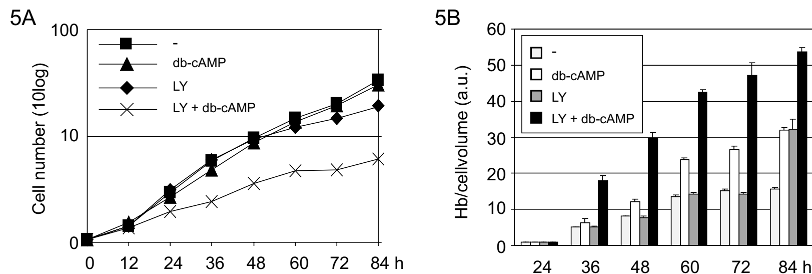

Figure 3.5. **Cyclic-AMP enhances erythroid differentiation induced by inhibition of the PI3K/PKB pathway.** Erythroblasts (I/11 cell line) were seeded in Epo/SCF containing medium, supplemented with the PI3K inhibitor LY294002 (15 μM), and/or 10 μM Cyclic-AMP as indicated. (**A**) Growth curve of the differentition course. Cell number presented in a logaritmic scale, time (hours) on the X-axis. (**B**) Hemoglobin (Hb) levels per cell volume (Y-axis, in arbitrary units (a.u.)) are shown for the timepoints 24 - 84 hours (X-axis).

To investigate the cooperation between the PI3K/Foxo3a and cAMP pathways in control of erythroid differentiation in more detail, it was investigated whether stimulation of the PGE2 receptor, which is up-regulated in differentiation about 40-fold (18), could enhance Foxo3a-induced differentiation. Therefore, erythroblast clones from the erythroid progenitor cell line I/11, stably expressing a 4OHT-inducible, active Foxo3a mutant (Foxo3a(A3):ER; 2), were treated with 4OHT and/or PGE2. Previously we have shown that Foxo3a induced differentiation is represented by decreased number of cell divisions and increased hemoglobin synthesis (2). Addition of 4OHT or PGE2 in control cells did not affect differentiation-associated proliferation, but PGE2 did enhance hemoglobin accumulation. As expected, addition of 4OHT strongly induced growth arrest and differentiation in clones with a high expression of Foxo(A3):ER. Under these conditions addition of PGE2 did not enhance differentiation (data not shown). However, in cells expressing moderate levels of Foxo(A3):ER, the differentiation induced by 4OHT was further accelerated by PGE2 as evidenced by decreased proliferation and increased hemoglobin accumulation (Figure 3.6). Addition of PGE2 alone again did not affect differentiation. Therefore we conclude that Foxo3a and the cAMP pathway cooperate on induction of erythroid differentiation.

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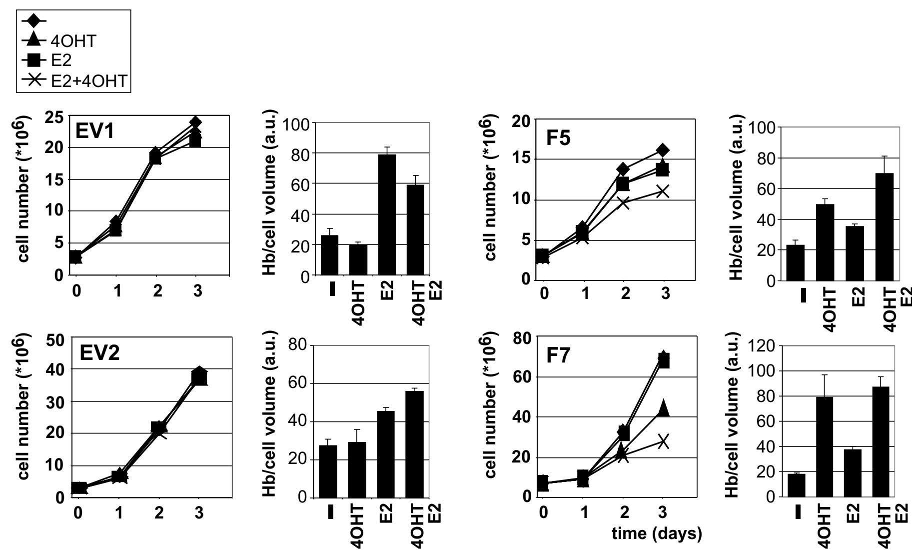

Figure 3.6. **Prostaglandin E2 and Foxo cooperate on induction of erythroid differentiation.** I/11 control clones (EV1 and EV2) and Foxo3a(A3):ER expressing clones (F5 and F7) were induced to differentiate in absence (diamants) or presence of 50 nM 4-hydroxy-tamoxifen (4OHT; triangles), 10 µM prostaglandin E2 (E2; squares) or both (crosses). Cells were counted at 24 h intervals to calculate cumulative cell numbers, and hemoglobin content per cell volume was determined in arbitrary units (a.u.).

Discussion

Btg1, encoding a small adaptor molecule controlling the activity of Prmt1, was identified as a Foxo3a target gene able to abrogate renewal divisions of erythroid progenitors. In this study we show that *Btg1* is also regulated by the cAMP responsive transcription factors CREB and ATF1. Both the Foxo-binding site (DBE) and the cAMP responsive element (CRE) are required for full activity of the *Btg1* promoter. The level of cAMP as well as phosphorylation of CREB and ATF1 increases late in differentiation, concomitant with *Btg1* expression. Thus, factors activation adenylate cyclase and increasing cAMP levels can enhance Foxo3a-mediated *Btg1* expression late in differentiation. As an example we demonstrated the cooperation of prostaglandin E2 and activation of Foxo3a in erythroid differentiation.

Cooperation of the cAMP pathway and Foxo3a during late erythroid differentiation

Differentiation of I/11 erythroblasts takes three days. At the onset of differentiation, which is induced by Epo, the cells undergo 3 - 4 "differentiation divisions", characterized by loss of size control, before they reach a terminal G_1-arrest and expel the nucleus. During this process the cells accumulate hemoglobin, which requires the balanced expression of the globin genes and

heme synthesis (53). Previously we have reported that Foxo3a accelerates the erythroid maturation process that is shown by an earlier onset of the G_1-arrest and hemoglobinization (2). Here we show that the cAMP pathway, upon activation of the prostaglandin E2 receptor, is able to accelerate the Foxo3a induced differentiation. Cyclic-AMP and PGE2 alone did not affect differentiation kinetics but increased hemoglobinisation.

When cells are induced to differentiate, cAMP levels rise late in differentiation. Erythroid cells express several $G\alpha_s$-coupled receptors that induce cAMP upon activation among which the adrenergic receptor and receptors for thrombin and PGE2 (39). The PGE2 receptor is up-regulated almost 40-fold late in differentiation (18). In addition, gene expression profiling showed that expression of NAD(+)-dependent 15-hydroxyprostaglandin dehydrogenase (*PGDH1*) is induced by dexamethasone in expanding erythroid progenitors, whereas it is rapidly down-regulated during differentiation (27; unpublished data). PGDH1 is the major enzyme that degrades prostaglandins and suppresses PGE2 activity. This tight regulation of PGE2 activity suggests that PGE2 has an important function in erythropoiesis and may also explain why PGE2 does not affect differentiation-associated proliferation of normal erythroid cells.

The observation that PGE2 induced differentiation in clones with an intermediate Foxo3a expression may indicate that cAMP/PKA-signaling specifically enhances Foxo3a function. Cooperation of Foxo's and the cAMP/PKA pathway has been observed in other cell systems as well. FoxO1-induced expression of prolactin, a major differentiation marker in human ES cells, was dependent on PKA, and cAMP was shown to enhance Foxo1 function in endometrial stroma (12).

The role of cAMP in erythropoiesis

The expression of PGDH1 in expanding erythroid progenitors and the up-regulation of the PGE2 receptor late in differentiation restrict the action of PGE2 to the final phase of erythroid differentiation. This is in agreement with the initial observation that activation of cAMP/PKA controls hemoglobin expression (14, 36). The cAMP/PKA-dependent transcription factor CREB appeared to control at least two key molecules in hemoglobin expression (20, 47): Ala-S, which is the rate-limiting enzyme in heme-synthesis (38) and NF-E2, a transcription factor binding the β-globin enhancer (1). This direct effect of CREB on heme synthesis and globin transcription may explain the effect of PGE2 on hemoglobin accumulation. However, the cooperation of CREB/ATF1 with Foxo3a implies that the effect of cAMP extends beyond the regulation of hemoglobin accumulation to the regulation of genes expressed in the final phase of erythroid differentiation. PGE2 has also been shown to cooperate with Epo-induced Stat5 transcriptional activation to regulate *SOCS2*, *SOCS3* and $Bcl\text{-}X_L$ (5). Similar to *Btg1*, $Bcl\text{-}X_L$ is also up-regulated late in differentiation (19).

Regulation of *Btg1* transcription

Gene expression profiling studies revealed *Btg1* as a Foxo1 (41) and as a Foxo3a target (2). A conserved DBE was identified in the human and mouse *Btg1* promoter, which appeared to be the target site via which Foxo3a induces *Btg1*

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expression (2). Here we show that CREB and ATF1 bind to a CRE present in the human and mouse *Btg1* promoter to regulate its expression. Foxo3a is able to induce *Btg1* expression in the absence of the CRE, suggesting that these pathways can act independently from each other. However, in absence of exogenously expressed Foxo3a, both the DBE and the CRE site were required for promoter activity and deletion of both sites did not further decrease promoter activity. Therefore, maximal expression in erythroid differentiation likely requires the activation of both transcription factors. This cooperation is furthermore underlined by the observation that cAMP induction of the *Btg1* promoter requires an intact DBE. Interestingly, both induction of cAMP levels and expression of *Btg1* have been associated with cell cycle arrest in several cell types. Since the mechanism of cAMP-induced cell cycle arrest has not been elucidated, induction of *Btg1* should be considered.

The function of Foxo proteins is to restrict cell growth by inducing cell cycle arrest, apoptosis, senescence or differentiation. Recently it became clear that Foxo proteins are not only activated upon down-regulation of the PI3K/PKB pathway, but also in response to DNA damage and oxidative stress (11, 34, 49). These different stress conditions appeared to induce different modifications of the Foxo protein, which affects the association with transcriptional coregulators (11, 34). As a result, DNA damage, oxidative stress and factor deprivation may activate different subsets of Foxo target genes. Interestingly, the CRE site in the *Btg1* promoter also recruits cJun and/or ATF2. The cJun/ATF2 complex is not regulated by the cAMP/PKA pathway, but through Jun-kinase (JNK), a member of the MAP kinase family. JNK is a stress-activated kinase, activated by e.g. DNA damage. As a result, cJun/ATF2 transcriptional activity is strongly enhanced in response to genotoxic stress (16, 26, 51). Interestingly, Btg1 expression is induced in response to DNA-damage (13). In case of Btg1 expression, the promoter apparently allows for potent activation of the gene under all conditions. The DBE binds all Foxo's, allowing a potent response to factor deprivation, when all Foxo's translocate to the nucleus (2). Binding of CREB/ATF1 to the CRE site in the *Btg1* promoter enforces Foxo3a mediated expression late in differentiation, whereas binding of cJun/ATF2 after activation of JNK enforces Foxo-mediated expression in response to oxidative or genotoxic stress.

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Materials and methods

Cells and reagents

BA/F3 were cultured in RPMI 1640 (Invitrogen) supplemented with 10% FCS (Hyclone, PerBio) and 10 ng/ml murine IL-3 (supernatant). The erythroid cell line I/11 was cultured in Stempro medium (Invitrogen) supplemented with 0.5 U/ml Epo (kind gift of Ortho-Biotech, Tilburg, The Netherlands), 100 ng/ml SCF (supernatant) and 1 mM dexamethasone (Sigma-Aldrich) (53). To activate Foxo3aa(A3):ER 50 nM 4-hydroxytamoxifen (4OHT; Sigma-Aldrich) was added to these expansion conditions. Stable FOXO3Aa(A3):ER expressing I/11 clones were generated using the retroviral expression vector pBabe as described previously (2). LY294002 was obtained from Alexis. Prostaglandin E2, dibutyryl cAMP and norepinephrine were obtained from Sigma.

Western blotting and antibodies

Cell lysis, SDS-polyacrylamide gel electrophoresis and Western blotting were performed as described previously (52). Nuclear and cytoplasmic extracts were prepared as described previously (1). Antibodies recognizing phosphorylated and total CREB/ATF1 were obtained from Santa Cruz (sc-7978, ser-133). Concentrated aliquots of the CREB/ATF1 (Santa Cruz, ATF-1 sc-270 X) and Stat5 antibodies (Santa Cruz sc-835 X) were used for EMSA.

Real-time quantitative PCR

cDNA synthesis and quantitative RT-PCR were performed using Taqman technology and Sybr-green detection (Applied Biosystems) of dsDNA as described (27). Murine primer sequences used:
Btg1: forward 5'-GCAGGAGCTGCTGGCAG-3',
reverse 5'-TGCTACCTC CTGCTGGTG A-3'.
Ribonuclease inhibitor: forward 5'-TCC AGT GTG AGC AGC TGA G-3',
reverse 5'-TGC AGG CAC TGA AGC ACC A-3'. The CT-values of Rnase Inhibitor were used for normalisation.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared, and EMSA was performed as described (1). The following oligonucleotide probes were used:
Btg1 CRE forward 5'-AGCTGAGCAGATTACGTCAGCTCCTA;
Btg1 CRE reversed 5'-AGCTGAGGAGCTGACGTAATCTGCTA;
Btg1 CRE-mutated forward 5'-AGCTGAGCAGATTTGGTCAGCTCCTC;
Btg1 CRE-mutated reversed 5'-AGCTGAGGAGCTGACCAAATCTGCTC;
cJun forward 5'-AGCTGAGCAGATTACCTCAGCTCCTC;
cJun reversed 5'-AGCTGAGGAGCTGAGGTAATCTGCTC;
Creb forward 5'-AGCTGAGCAGATGACATCAGCTCCTC;
Creb reversed 5'- AGCTGAGGAGCTGATGTCATCTGCTC;
β-casein forward 5'-AGCTAGATTTCTAGGAATTCAATCC;
β-casein reversed 5'-AGCTGGATTGAATTCCTAGAAATCT;

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Luciferase Reporter assays

The *Btg1* reporter constructs were reported previously (2). The TTACGTCA to TTTGGTCA mutation in the CRE was made using the Quickchange Site Directed Mutagenesis kit (Stratagene) according to the manufacturers protocol, using the primers: forward 5'-AGCAGATTTGGTCAGCTCCTC -3', and the opposite strand for the reverse primer. For reporter assays 293HEK were transfected by Ca-phosphate as described (2). Otherwise, $10x10^6$ Ba/F3 cells were electroporated (0.28 kV, capacitance 960 µFD) with maximum 20 mg of DNA. After recovery for several hours in normal media, cells were washed and grown overnight in the presence or absence of IL-3 and SCF, or cells were IL-3 deprived overnight and next day stimulated for 7 h with IL-3. Luciferase activity was measured using the Steady-Glo system (Promega). Transfection efficiency was determined by cotransfecting lacZ and analyzing b-galactosidase activity.

Differentiation of I/11 cells

To differentiate I/11 cells, cells were washed and reseeded in StemPro medium supplemented with 5 U/ml Epo and 0.5 mg/ml iron-loaded transferring. Cells and cell size were counted electronically (Casy counter, Schärfe-systems). Hemoglobin was measured by a calorimetric assay as described (2).

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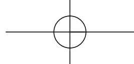

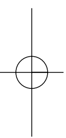

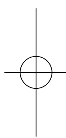

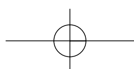

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Differential regulation of Foxo3a target genes in erythropoiesis

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CHAPTER 4

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Abstract

Stem cell factor (SCF) cooperates with erythropoietin (Epo) to sustain renewal divisions and delay differentiation of erythroid progenitors. Activation of Foxo3a accelerates differentiation of these progenitors. SCF-induced Protein kinase B (PKB) inactivates Foxo3a by phosphorylation-induced translocation to the cytoplasm. To examine how regulation of Foxo3a activity contributes to the role of SCF in erythropoiesis, we assessed whether Foxo3a target genes correspond to genes controlled by SCF stimulation. Expression profiling revealed a subset of Foxo3a target genes regulated by factor deprivation and SCF restimulation. Among these were genes involved in cell cycle control and erythroid differentiation. Foxo3a target genes not regulated by factor deprivation included genes involved in oxidative stress response, signal transduction and transcription factors such as Cited2. We show *Cited2* regulation by a Foxo3a/Stat5 complex, which is sustained in the presence of Epo but inhibited by SCF. Thus, Foxo3a is not only an effector of PKB, but integrates multiple signals to coregulate gene expression in erythropoiesis.

Introduction

Since the discovery of the forkhead DNA binding domain (36), about 100 members of the forkhead family have been identified and categorized in 15 subclasses (30). The subclass O of forkhead transcription factors (Foxo), encompassing Foxo1, 3, 4, and 6 (8, 29), is phosphorylated by Protein Kinase B (PKB; 13, 33, 54), which results in transcriptional inactivation through nuclear export and cytosolic retention by binding to 14-3-3 proteins (11, 13). Only Foxo6, which lacks the COOH-terminal PKB site important for nuclear export, shows a predominant nuclear localization independent of PKB activity (29).

Initial studies on Foxo proteins in hematopoiesis pointed to a role for Foxo's in the regulation of the cell cycle and apoptosis (9, 16). Foxo's were shown to induce the cell cycle inhibitor *p27^{KIP}* and the pro-apoptotic Bcl-2 family member *Bim* in Ba/F3 cells (18), and *Fas-ligand* in Jurkat T-cells (13). On the other hand, Foxo1 was suggested to induce survival and maturation in thymocytes (37) and we showed that activation of Foxo3a in erythroid progenitors induces differentiation instead of apoptosis (4). Together the data suggest that the role of Foxo proteins in hematopoiesis is diverse, and possibly cell type specific.

The induction of differentiation by Foxo3a is consistent with the role of the PI3K/PKB pathway in erythropoiesis. Major factors regulating erythropoiesis are erythropoietin (Epo) and stem cell factor (SCF) (12, 40, 58). Expansion of erythroid progenitors can be achieved *in vitro* using serum free medium supplemented with Epo, SCF and glucocorticoids (19, 56), which reflects *in vivo* expansion of erythroid progenitors under stress conditions (5, 10). Both Epo and SCF activate the PI3K/PKB pathway, but SCF activates PKB most potently (56). Inhibition of PI3K abrogates Epo/SCF-induced expansion of *in vitro* cultures, suggesting that pathways downstream of PI3K/PKB are required to expand erythroid progenitors (56). Notably, mice lacking the PI3K subunit p85 display a transient fetal liver anemia (28), caused by a reduced number of BFU_E and CFU_E. Lack of p85 did not increase the rate of apoptosis in erythroid progenitors and mast cells but decreased proliferation (24, 28, 42). Foxo3a$^{-/-}$ mice suffered from a mild compensated anemia associated with reticulocytosis, suggesting normal expansion but defects in erythrocyte maturation (17). Together these data indicate an important role for the PI3K/PKB/Foxo pathway in regulating the balance between erythroid expansion and differentiation.

Recently, we screened a hematopoietic cDNA array enriched for erythroid specific cDNA's for Foxo3a target genes. We identified *Btg1*, an activator of protein arginine methyl transferase 1 (Prmt1) as a direct Foxo3a target, and showed that Btg1 repressed expansion of erythroid progenitors (4). In this study we extended our search for Foxo3a targets using a 17K EST cDNA array, which we previously screened for Epo and SCF target genes (32). We identified several differentially regulated clusters of Foxo3a target genes and analyzed two clusters of Foxo3a upregulated genes that are differentially regulated by Epo and SCF in erythroblasts. One cluster encompasses target genes are repressed by SCF and upregulated in differentiation (*Cdkn1b*, *Btg1*, *Ccng2*, *Ulk1*), whereas the other cluster contains genes that are hardly affected

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by SCF or differentiation (*Dcn*, *Sesn1*, and *Cited2*). To investigate how Foxo3a targets escape negative control by SCF-induced PKB activation, we focused on the regulation of the transcriptional regulator *Cited2*. We present a novel mechanism of transcriptional control in which an alleged growth stimulatory transcription factor, Stat5, cooperates with an assumed growth inhibitory transcription factor, Foxo3a, to control expression of *Cited2*. Our data implicate that Foxo3a is not only an effector of PKB, but that it functions to integrate and transmit multiple signals which cooperate to regulate the gene expression program of erythroid progenitors.

Results

Identification of Foxo3a targets in the erythroblasts

To identify Foxo3a target genes activated in erythroid progenitors we used I/11 erythroblast clones stably expressing an inducible, active Foxo3a mutant (Foxo3a(A3):ER). This Foxo3a mutant was constructed by mutating the three inhibitory PKB phosphorylation sites to alanine residues, and by fusing it to the ligand binding domain of the estrogen receptor to render it inducible upon addition of 4-hydroxy-tamoxifen (4OHT; 4, 18). cDNAs derived from a Foxo3a(A3):ER-expressing erythroblast clone and an empty vector control clone induced by 4OHT under expansion conditions were hybridized to 17k EST cDNA arrays (32). Potential Foxo3a target genes were selected using an arbitrary threshold of >1.75 fold change (positive or negative) comparing the ratio plus/minus 4OHT in Foxo3a(A3):ER clones and <1.3 for the same genes in the vector control clone, to exclude nonspecific effects of 4OHT. This yielded 299 potential target genes. The ratio of 1.75 was set previously using the same arrays to obtain the 1% genes most strongly regulated by Epo and SCF (32). Regulation of the potential Foxo3a target genes was clustered with available data on the regulation of these genes by Epo-, SCF- and dexamethasone detected on the same arrays (32) (Figure 4.1 and supplementary data). Hierarchical clustering indicated that cluster A (22 genes) is the first outstanding cluster, containing genes upregulated by Foxo3a and downregulated by Epo and SCF. Cluster A contains all genes already known to be Foxo targets such as *p27^{KIP}*, *Btg1* and *Cyclin G2* (Table I). Remaining Foxo3a target genes were split in 4 additional clusters. Clusters B and C contain all Foxo3a downregulated genes, of which cluster B genes (191 genes) are not or hardly regulated by Epo/SCF signaling, whereas cluster C genes (38 genes) are upregulated by Epo/SCF signaling. Cluster D and E contain the remaining Foxo3a upregulated genes that are either not regulated by Epo/SCF signaling (cluster D, 42 genes) or also upregulated by Epo/SCF signaling (cluster E, 5 genes). Because phosphorylated Foxo3a is excluded from the nucleus, we expected Foxo3a target genes to be counter regulated by Epo/SCF signaling as seen in clusters A and C. However, these clusters appeared to contain only 60 (20%) of the 299 selected genes (Figure 4.1).

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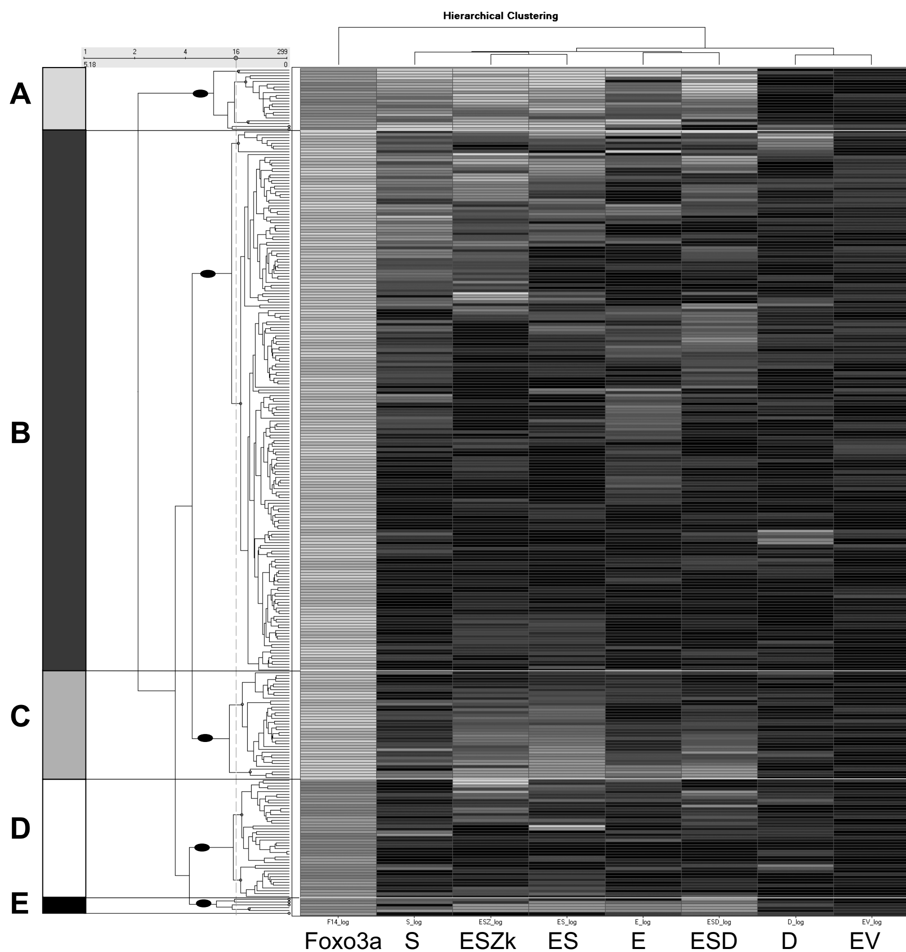

Figure 4.1. **Dendogram of Foxo3a target genes and their regulation by Epo, SCF and Dex.** Erythroid progenitors (I/11) expressing Foxo3a(A3):ER or a control vector were treated with or without 4OHT (50 nM) for 6h under expansion conditions (Epo/SCF/Dex). cDNAs were hybridized to 17k EST arrays in pairs using dual labeling with fluorochromes. Genes were selected when induced or repressed by 4OHT more than 1.75 fold in Foxo3a(A3):ER expressing cells (ratio +4OHT/-4OHT: 'Foxo3a') and less than 1.3 fold in control cells (ratio +4OHT/-4OHT 'EV'). These genes were clustered with data on the regulation of these genes by Epo (E), SCF (S), Epo/SCF (ES), Epo/SCF/Dex (ESD), Dex (D) and Epo/SCF/Zk (3 X 10^{-6} M glucocorticoid antagonist Zk1123.993 (ZK)) by Spotfire software using Euclidean distance and average linkage to assess distance. I/11 cells were factor deprived and induced for 2 h with the respective ligand (ratio +/- ligand). Five major clusters of target genes are indicated at the left side. Black ovals indicate the branches of the distinct clusters. Dark-grey bars (as shown in the Foxo3a column, cluster A) indicate upregulation, light-grey bars (as shown in the Foxo3a column, cluster B) indicate repression by 4OHT or growth factors. Black bars indicate non-regulated sequences.

Table I. Cluster A: Foxo3a upregulated, Epo/SCF downregulated genes.

accession number	Gene*	gene description	F14#	E§	S§	ES§	ESD§	ESZk§	D§	EV#
AI451894	**Cyclin G2**	Ccng2	1.30	-1.2	-1.04	-1.95	-1.31	-2.04	0.08	-0.05
AI846040	RIKEN cDNA 1500004A08 gene	EST	0.96	-0.84	-1.35	-2.02	-0.51	-1.44	0.51	-0.12
AI846647	carnitine palmitoyltransferase 1	Cpt1	1.05	-0.98	-0.89	-1.45	-1.24	-0.71	-0.11	-0.09
AI843786	**cyclin-dependent kinase inhibitor 1B (p27^{KIP})**	**Cdkn1b**	1.33	-0.45	-1.00	-1.48	-1.28	-1.37	0.11	-0.17
AI853707	cell cycle progression 1	Ccpg1	1.25	-0.08	-0.69	-1.35	-0.62	-0.95	0.01	0.09
AI451891	RIKEN cDNA 4833420G17 gene	EST	0.83	-0.49	-0.78	-1.31	-1.00	-0.86	0.02	0.02
AI596353	RIKEN cDNA 2410003M04 gene	Kbras2	0.94	-0.5	-0.82	-0.97	-0.98	-0.84	-0.13	0.14
AI850194	**Unc-51 like kinase 1**	**Ulk1**	0.83	-0.48	-0.67	-0.74	-1.05	-0.92	-0.16	0.03
AI429475	exportin 7	Xpo7	1.13	-0.61	-0.68	-0.74	-0.89	-0.66	0.19	0.02
AI843236	**retinoblastoma-like 2**	**p130**	0.89	-0.36	-0.61	-0.83	-0.77	-0.98	0.00	-0.28
AI845268	RIKEN cDNA E430026E19	EST	0.92	-0.41	-0.46	-0.74	-0.73	-0.82	0.05	-0.18
AI450702	ATPase, Na+/K+ transporting, beta 3 polypeptide	Atp1b3	0.82	-0.33	-0.73	-0.56	-0.53	-0.93	0.05	-0.10
AI449499	Glucocorticoid induced transcript 1	Glcci1	0.81	-0.66	-0.56	-0.71	-0.49	-0.83	0.05	0.18
AI450119	Acidic ribosomal phosphoprotein P0	EST	0.99	-0.48	-0.44	-0.75	-0.62	-0.64	0.04	0.14
AI847059	PT EN induced putative kinase 1	Pink1	0.92	-0.38	-0.61	-0.93	-0.39	-0.53	0.15	0.22
AI854419	RIKEN cDNA 2610318K02 gene	EST	0.90	-0.41	-0.66	-0.68	-0.44	-0.43	0.04	-0.09
AI845739	phosphoglucomutase 2-like 1	Pgm2l1	0.86	-0.32	-0.80	-0.50	-0.57	-0.44	0.23	-0.01
AI851307	RIKEN cDNA C630043F03	EST	0.95	-0.34	-0.50	-0.31	-0.66	-0.69	0.13	0.10
AI835817	thymus expressed acidic protein	Trp53inp1	1.28	- 0.93	-0.39	-0.64	-0.15	-0.80	-0.12	0.08
AI450899	adenosine monophosphate deaminase 2	Ampd2	1.90	-0.85	-0.76	-0.86	-0.58	-0.86	0.30	0.29
AI848411	**B-cell translocation gene 1**	**Btg1**	1.77	-0.49	-1.06	-1.14	0.09	-1.30	0.75	-0.37
AI448121	IMAGE:558102	EST	0.81	-0.18	-1.00	-0.7 3	0.09	-0.80	0.53	0.31

*) abbreviations according to the Unigene database (NCBI), genes in bold have been validated.

#) F14 is a Foxo3a(A3):ER expressing clone; EV de vector control. The expression ratio is plus/minus 4OHT (data in ^{2}log values).

§) Expression ratio plus/minus ligand: E, Epo; S, SCF; D, dexamethasone

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Differential regulation of Foxo3a targets by starvation/stimulation, corresponds with their differential regulation in differentiation

Because only 20% of the potential Foxo3a target genes were regulated as expected, we investigated the regulation of selected Foxo3a upregulated genes from cluster A (downregulated by Epo/SCF as expected) and cluster D (no regulation by Epo/SCF) to assess the reliability of the array hybridization and clustering. These included *Btg1* (*B-cell translocation gene 1*), *Ulk1* (*Unc-51-like kinase*) and *Ccng2* (*Cyclin G2)* to represent cluster A and the surface molecule *Dcn* (*Decorin)*, the anti-proliferative gene *Sesn1* (*Sestrin 1)*, and *Cited2* (*CBP/p300-interacting transactivator, with glu/asp-rich C-terminal domain*) to represent cluster D.

First, Foxo3a-dependent expression of the selected targets was assessed in two independent Foxo3a(A3):ER clones (F17 and F18) and a control clone using a short induction time (Figure 4.2A). All targets were induced between 2- and 4-fold within 2 h in both Foxo3a(A3):ER expressing clones but not in the control cells. Next, regulation of selected targets was examined in factor depleted and restimulated erythroid progenitors in the presence or absence of the PI3K inhibitor LY294002 (LY) to determine PI3K/PKB dependence. Cluster A targets *Btg1*, *Cyclin G2* and *Ulk1* were strongly repressed by Epo, SCF and Epo/SCF (Figure 2B, between 2-fold for *Ulk1* and 30-fold for *Cyclin G2*), which was abrogated upon inhibition of PI3K, indicating repression through PI3K/PKB-mediated phosphorylation of Foxo3a. In contrast, cluster D targets *Decorin* and *Sestrin* were less than 2-fold downregulated by Epo/SCF and inhibition of PI3K had no effect on the expression of these genes (Figure 4.2B). Notably, expression of *Cited2* was upregulated by Epo and by Epo plus SCF (2-fold). Inhibition of PI3K did not affect Epo-induced upregulation of *Cited2*, but further increased Epo/SCF-induced expression of *Cited2* (>4-fold).

Finally, we determined expression of the selected cluster A and D target genes during erythroid differentiation. Previously we showed that Foxo3a expression increases during erythroid differentiation, whereas PKB activity and Foxo3a phosphorylation decrease. Consequently, expression of *Btg1* and *p27*^{KIP} sharply increase from the moment unphosphorylated Foxo3a accumulates in differentiating erythroblasts (Figure 4.2C; (4). Transcription of the cluster A genes *Cyclin G2* and *Ulk1* similarly increased 48h after differentiation induction (between 5-and 10-fold as previously observed for *Btg1*). However, none of the cluster D genes was significantly upregulated during differentiation (Figure 4.2C lower panels).

In conclusion, transcription of *Btg1*, *Cyclin G2*, and *Ulk1* (and *p27*^{KIP}; 4) is clearly repressed by Epo and SCF via a PI3K-dependent pathway and is induced in differentiation. In contrast, the transcription of *Decorin*, *Sestrin*, and *Cited2* is not or hardly affected by the PI3K/PKB pathway in response to factor-deprivation and restimulation by Epo/SCF, and is also not stimulated during differentiation. Since Foxo's have been shown to cooperate with multiple transcription factors, these data suggest that Foxo3a may function within distinct transcriptional complexes to enhance expression of cluster A and D targets.

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Table II. Cluster D: Foxo3a upregulated genes not regulated by Epo/SCF.

accession number	Gene*	gene description	F14#	E§	S§	ES§	ESD§	ESZk§	D§	EV#
AI449437	ATPase, Cu++ transporting, alpha polypeptide	Atp7a	0.99	-0.48	-0.09	-0.27	0.09	-0.99	-0.15	-0.26
AI450792	RIKEN cDNA B230106I24 gene	EST	0.82	-0.21	0.05	-0.19	-0.08	-1	0.19	0.04
AI662267	homeodomain interacting protein kinase 1	Hipk1	0.88	-0.21	0.01	-0.48	0.01	-0.67	-0.07	-0.01
AI451316	RIKEN E430019P06 cDNA	EST	0.81	-0.23	-0.02	-0.39	0.28	-0.33	-0.17	0.16
AI843965	**Sestrin 1**	**Sesn1**	0.87	-0.1	-0.12	-0.31	-0.7	-0.79	-0.14	-0.15
AI325508	expressed sequence AW049829	EST	1.04	-0.28	-0.32	-0.21	-0.53	-0.44	-0.23	0.22
AI415470	Neuropathy target esterase	Nte	0.92	-0.22	-0.18	-0.23	-0.41	-0.47	-0.22	0.09
AI449513	glutamate receptor, ionotropic, AMPA3	Gria3	0.91	-0.37	-0.06	-0.48	-0.23	-0.18	-0.45	0.17
AI447150	insulin-like growth factor I receptor	Igf1-R	1.03	-0.1	-0.05	-0.16	-0.18	-0.21	-0.32	0.31
AI852445	DnaJ homolog, subfamily C, member 12	Dnajc12	0.84	0.15	-0.2	-0.2	-0.65	-0.26	0.06	0.09
AI845479	oxysterol binding protein-like 9	Osbpl9	0.88	-0.19	-0.23	-0.13	-0.19	-0.53	0.07	0.07
AI451237	protein geranylgeranyltransferase type I, beta	Pggt1b	0.87	-0.14	-0.17	-0.08	-0.24	-0.47	0.07	0.17
NM_008138	guanine nucleotide binding protein, alpha inhibiting 2	Gnai2	0.84	-0.11	-0.22	-0.11	-0.29	-0.17	0.26	0.22
AI842614	NAD(P)H:menadione oxidoreductase 1	Nmor1	1	-0.02	-0.01	-0.18	-0.4	-0.4	0.13	-0.21
AI448727	core promoter element binding protein	Copeb	1.28	-0.22	-0.19	-0.42	-0.23	-0.49	0.28	0.03
NM_007799	cathepsin E	Ctse	1.17	-0.09	-0.18	-0.24	-0.36	-0.22	0.24	0.06
AI414015	Expressed sequence AI852444	EST	1.05	0.05	-0.32	-0.93	0.35	0.04	-0.13	0.26
AI845199	selenoprotein P, plasma, 1	Sepp1	0.92	0.14	-0.1	-0.69	-0.11	0.02	0.36	-0.14
AI449375	MAD homolog 4 interacting transcription coactivator 1	Mitc1	1.17	-0.33	-0.54	0.03	-0.26	-0.02	-0.23	0.01
NM_007482	arginase 1	Arg1	0.81	-0.07	-0.7	0.26	-0.18	-0.06	0.34	-0.05
AI464367	EST AA536815	EST	0.81	-0.18	-0.14	0.28	0.38	-0.18	-0.14	-0.17
AI661009	IMAGE:719175	EST	0.95	-0.45	-0.12	-0.26	-0.12	-0.09	0.18	-0.05
AI449354	RIKEN cDNA 6330516O17 gene	EST	0.89	-0.02	-0.23	-0.05	0.01	-0.12	0.06	-0.03
AI452320	RIKEN cDNA D030028O16 gene	EST	0.83	0.06	-0.22	0.13	0.16	-0.09	0.13	-0.06
AI465319	thioredoxin domain containing 1	Txndc1	0.91	-0.01	-0.04	0.01	0.02	0.02	0.13	0.09
D44443	Endogenous mouse mammary tumor virus	Mtv1	0.86	0.1	-0.08	0.06	0.13	0.04	0.44	0.19
D44443	Endogenous mouse mammary tumor virus	Mtv1	0.86	0.1	-0.08	0.06	0.13	0.04	0.44	0.19
NM_021099	kit oncogene	cKit	1.3	0.04	0	-0.3	-0.03	-0.08	0.14	0.12
AI846778	**Decorin**	**Dcn**	1.38	0.28	0.04	-0.32	0.09	-0.14	0.11	0.07
NM_007781	colony stimulating factor 2 receptor, beta 2	Csf2rb	1.56	0.18	-0.08	0.11	-0.16	-0.08	0.31	0.08
AI430768	**Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2**	**Cited2**	0.9	0.23	-0.34	-0.06	-0.32	0	-0.61	0.28
AI838934	RIKEN cDNA 2010005O13	EST	0.88	0.56	-0.51	0.02	0.19	0.27	-0.38	-0.06
AI429552	expressed sequence AW146242	EST	0.91	0.21	0.07	0.38	-0.06	0.36	-0.22	0.01
AI323564	neurofibromatosis 2	Nf2	0.87	0.03	0.08	0.3	0.06	0.14	0.09	0.36

AA123949	complement receptor 2	Cr2	0.81	0.03	0.28	0.45	0.16	0.33	0.1	-0.02
AI385712	Vinculin	Vcl	0.92	-0.16	0.33	0.27	0.31	0.15	0.12	0.17
AI413346	N-acylsphingosine amidohydrolase 1	Asah1	1.04	0.04	0.17	0.49	0.27	0.19	0.05	0.18
AI452330	Hypothetical protein B930075F07	EST	1.09	0.13	0.15	0.35	0.33	0.13	0.34	0.18
AI844042	protein tyrosine phosphatase, non-receptor type substrate 1	Ptpns1	0.85	0.33	0.3	0.56	0.26	0.59	0.08	0.07
NM_007781	colony stimulating factor 2 receptor, beta 2	Csf2rb	1.45	0.1	-0.07	0.44	0.01	0.52	0	0.1
AI426361	cytoplasmic polyadenylation element binding protein 4	Cpeb4	1	0.4	-0.41	0.7	0.43	0.53	0	0.08

*) abbreviations according to the Unigene database (NCBI), genes in bold have been validated.

#) F14 is a Foxo3a(A3):ER expressing clone; EV de vector control. The expression ratio is plus/minus 4OHT (data in ^{2}log values).

§) Expression ratio plus/minus ligand: E, Epo; S, SCF; D, dexamethasone

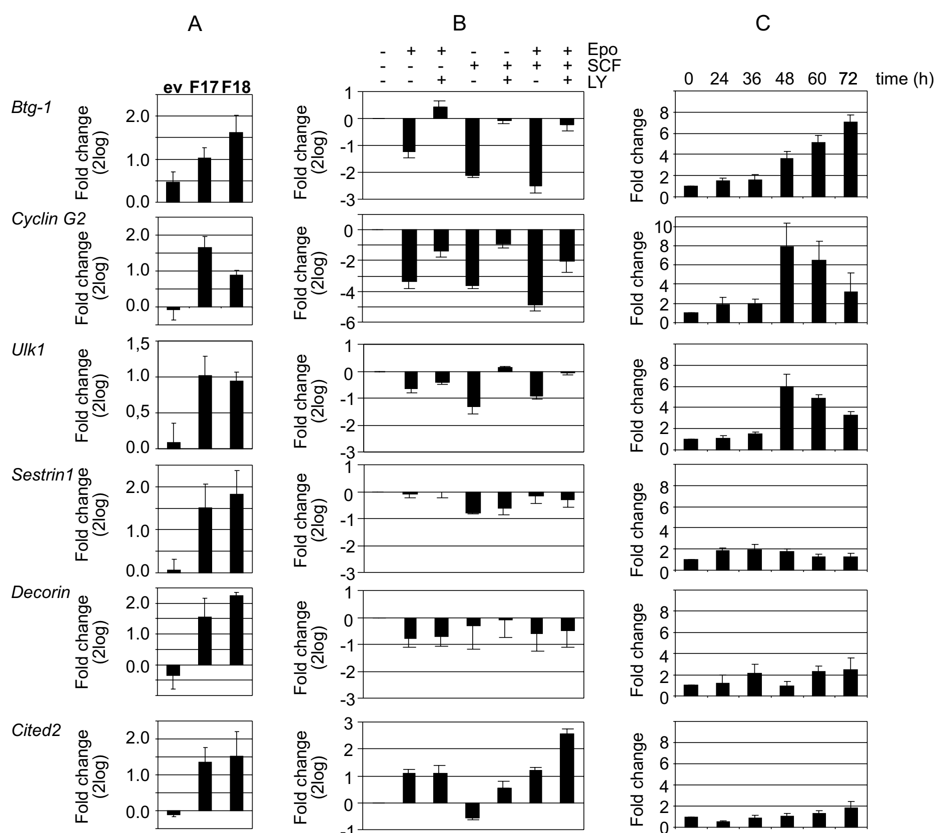

Figure 4.2. **Expression of FoxO3a target genes in response to growth factors and during differentiation of erythroid progenitors.** (**A**) Two independent Foxo3a(A3):ER overexpressing clones, and a control clone were treated with 50 nM 4OHT for 2h. Expression of selected target genes indicated at the left side of the panels was compared between the Foxo3a(A3):ER clones F17 and F18 and the control clone. (**B**) I/11 cells were factor deprived, and subsequently stimulated for 6h with Epo, SCF, or Epo plus SCF, in the presence or absence of the PI3K inhibitor LY294002 (15 μM). Expression of selected targets was compared between the various conditions and unstimulated cells. Ratio's are given as ^{2}log-values. (**C**) I/11 cells were differentiated and samples were taken every 12 h, until the end of erythroid differentiation (72 h). Transcript levels from the selected genes were compared between the start of the differentiation experiment and ensuing time points. In all experiments transcript levels were determined using poly-A mRNA and real-time PCR.

Epo induces Foxo3a/Stat5 complex formation

The association of Foxo3a with additional transcription factors may conceal the normal regulation of Foxo3a by phosphorylation. To examine this possibility we investigated Epo- and Foxo3a-induced expression of *Cited2* as an example. *Cited2* is known to be a cytokine- and growth factor inducible gene, that is transcriptionally controlled in a Jak/Stat dependent manner (51). In addition, *Cited2* was also found as a potential Foxo1 target (45). This suggests that *Cited2* expression may be regulated by the cooperate action of the Foxo and Jak/Stat pathways, similar to the reported association and cooperation of Foxo1 and Stat3, in response to IL-6 stimulation, on transcriptional activation of the $\alpha 2$-

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*macroglobuli*n promoter (34).

Because Stat5, but not Stat3, is robustly activated by Epo in erythroblasts, we examined a possible association between endogenous Foxo3a and Stat5 in response to Epo and SCF stimulation. I/11 cells were factor deprived and stimulated with Epo, SCF and Epo plus SCF (Figure 4.3A). In agreement with previous data (4) Epo stimulation resulted in modest Foxo phosphorylation, whereas SCF phosphorylated almost all Foxo3a. In contrast, Stat5 is phosphorylated exclusively by Epo, not by SCF (56). Thus, all combinations of predominantly phosphorylated and unphosphorylated Foxo3a and Stat5 are represented by these conditions. Phosphorylated Foxo3a was prominently present in Stat5 immunoprecipitates of unstimulated and Epo-stimulated cells, although most Foxo3a is unphosphorylated under these conditions (Figure 4.3A). This suggests that Stat5 associates more efficiently, but not exclusively, with phosphorylated Foxo3a. In addition, association of Foxo3a with Stat5 increased upon Epo-stimulation. However, in the presence of Epo plus SCF both Foxo3a and Stat5 are phosphorylated, but less Foxo3a was present in the Stat5 immunoprecipitates. This suggests that the phosphorylation status of Foxo3a and Stat5 is not be the major determinant of Foxo3a/Stat5 association. Compartmentalization may be a second determinant of Foxo3a/Stat5 association. Maximally phosphorylated Foxo3a is located in the cytoplasm, whereas phosphorylated Stat5 is located in the nucleus.

To investigate the role of compartmentalization, we analyzed Foxo3a/Stat5 association in cytoplasmic and nuclear cell fractions under various conditions. The colocalisation of phosphorylated Stat5 with unphosphorylated Foxo3a in the nucleus of Epo stimulated cells induced a much more prominent co-immunoprecipitation than the colocalisation of unphosphorylated Stat5 with phosphorylated Foxo3a in the cytoplasm induced by SCF (Figure 3B). The nuclear fractions were not contaminated with cytoplasmic proteins, since Stat3, which is always cytoplasmic in erythroid progenitors grown under serum-free conditions (4), was restricted to the cytoplasmic fraction (Figure 4.3C). Therefore the data indicate that colocalization of Stat5 and Foxo3a in the nucleus enhances the Foxo3a/Stat5 interaction. The interaction is not completely dependent on phosphorylation, which is demonstrated by the interaction of unphosphorylated, cytoplasmic Stat3 with unphosphorylated Foxo3a (Figure 4.3B lower panel).

To examine the role of compartmentalization in more detail, we assessed the cellular location of Foxo3a complexes in response to Epo and Epo plus SCF over time. In I/11 cells, both Epo and SCF induced transient translocation of Foxo3a from the nucleus to the cytoplasm. Epo induced partial phosphorylation of Foxo3a and by consequence also partial exclusion of Foxo3a from the nucleus. In the presence of Epo plus SCF, both phosphorylation and nuclear exclusion of Foxo3a were almost complete (Figure 4.3C). In cytoplasmic fractions of Epo-induced I/11 cells, Foxo3a always co-immunoprecipitated with Stat5 (Figure 4.3D), which is abundantly present in the cytoplasm of these cells. In contrast to its abundant presence in the cytoplasm, nuclear Stat5 was hardly detectable in factor-deprived cells and only induced in response to Epo. Nevertheless, low levels of a nuclear Foxo3a/Stat5 complex was precipitated from factor deprived cells. Upon Epo stimulation this complex first disappeared,

then reappeared as nuclear Stat5 increases (Figure 4.3D). The same pattern was observed in three independent experiments (it has to be noted that the exposure of the cytoplasmic and nuclear panels can not be compared). Apparently a nuclear Foxo3a/Stat5 complex can be formed when Foxo3a or Stat5 is abundantly present in the nucleus.

In conclusion, the assumed proliferation-inhibiting factor Foxo3a and proliferation-promoting Stat5 associate to regulate common target genes such as *Cited2*. The association of Foxo3a and Stat5 is not strictly dependent on phosphorylation of either Foxo3a or Stat5, but phosphorylation of Stat5 and nuclear co-localization of Stat5 and Foxo3a in presence of Epo enhances Foxo3a/Stat5 complex formation, whereas increased phosphorylation of both proteins in response to Epo plus SCF results in nuclear exclusion of Foxo3a and decreased Foxo3a/Stat5 complex formation. This suggests that association with Stat5 is not sufficient to retain phosphorylated Foxo3a in the nucleus.

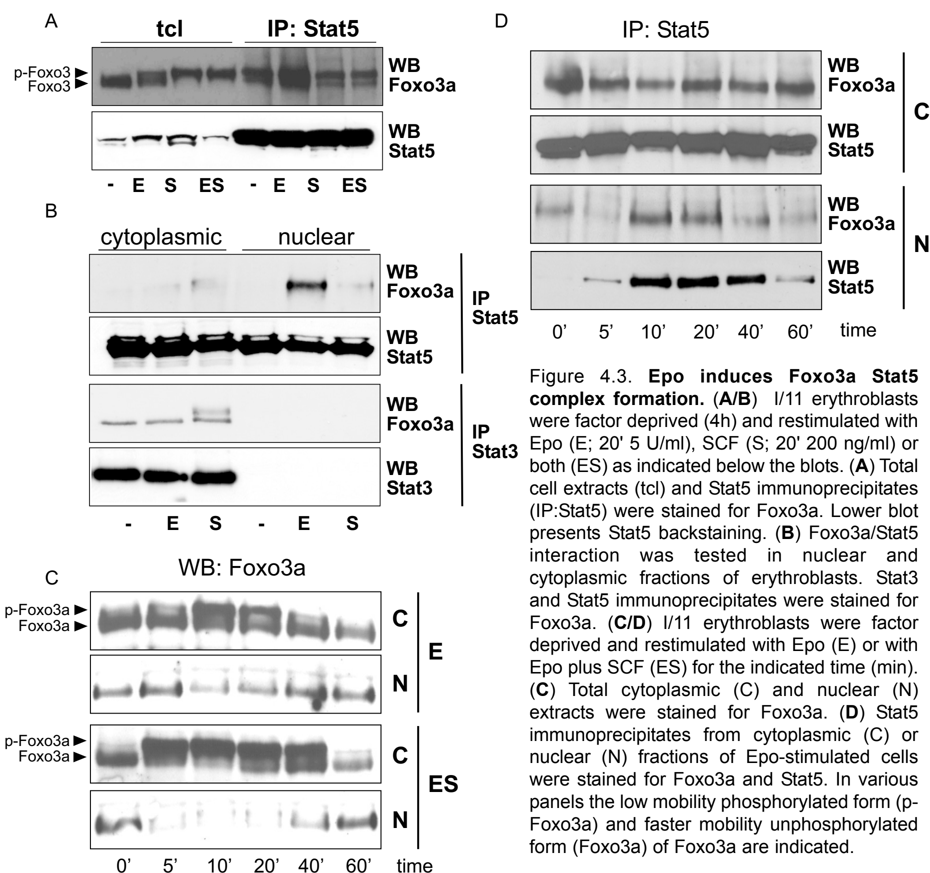

Figure 4.3. **Epo induces Foxo3a Stat5 complex formation.** (**A/B**) I/11 erythroblasts were factor deprived (4h) and restimulated with Epo (E; 20' 5 U/ml), SCF (S; 20' 200 ng/ml) or both (ES) as indicated below the blots. (**A**) Total cell extracts (tcl) and Stat5 immunoprecipitates (IP:Stat5) were stained for Foxo3a. Lower blot presents Stat5 backstaining. (**B**) Foxo3a/Stat5 interaction was tested in nuclear and cytoplasmic fractions of erythroblasts. Stat3 and Stat5 immunoprecipitates were stained for Foxo3a. (**C/D**) I/11 erythroblasts were factor deprived and restimulated with Epo (E) or with Epo plus SCF (ES) for the indicated time (min). (**C**) Total cytoplasmic (C) and nuclear (N) extracts were stained for Foxo3a. (**D**) Stat5 immunoprecipitates from cytoplasmic (C) or nuclear (N) fractions of Epo-stimulated cells were stained for Foxo3a and Stat5. In various panels the low mobility phosphorylated form (p-Foxo3a) and faster mobility unphosphorylated form (Foxo3a) of Foxo3a are indicated.

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Transcriptional activation of *Cited2* by the Foxo3a/Stat5 complex

We next examined how the Foxo3a/Stat5 complex regulates *Cited2* gene expression. The sequence of a genomic mouse fragment, encompassing the -1128/+238 promoter region identified previously (NT039491) (39), was aligned with the human *CITED2* promoter (AF129290). Although 22 putative monomeric Stat binding sites have been reported for the human promoter based on the 5'-TTNNNNNAA-3' sequence (39), alignment of the human and murine *Cited2* promoter did not reveal clear Stat5 binding sites. Two Foxo binding sites (DBE for Daf-16 binding element) were identified in the murine *Cited2* promoter, one of which was identical between mouse and human. This DBE is located in a highly conserved promoter region at position -872 (Figure 4.4A).

To analyze whether a Foxo3a/Stat5 complex binds to the DBE, nuclear extracts were incubated with wild-type or mutated DBE oligonucleotide probes in EMSA assays. One protein complex specifically associated with the wild-type *Cited2* oligo, suggesting its dependence on an intact DBE (Figure 4.4B). However, Epo induction did not enhance binding, and this complex could not be supershifted by a Stat5 antibody. Epo did induce Stat5 DNA-binding in these lysates, since an Epo-dependent complex was binding to a classical Stat5-probe (β-casein; Figure 4.4C) and this complex could be supershifted with anti-Stat5. These data suggest that Epo-induced induction of the *Cited2* promoter activity by the Foxo3a/Stat5 complex does not require the DBE at -872.

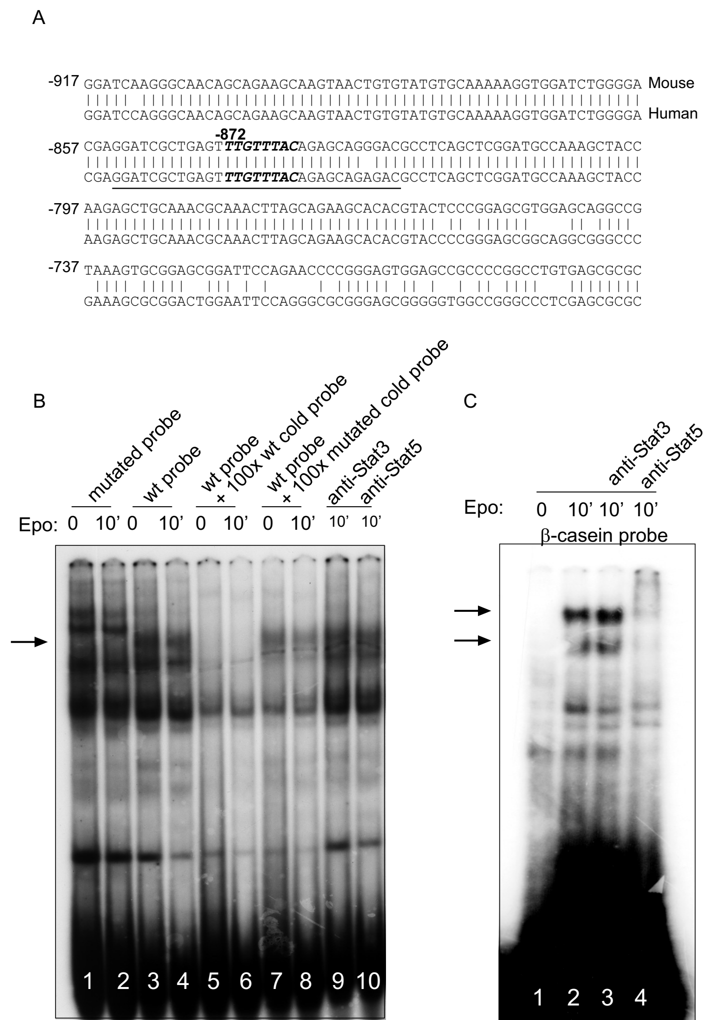

Figure 4.4. **Analysis of conserved DBE in *Cited2* promoter.** (**A**) Allignment of the mouse (upper line) and human (lower line) *Cited2* promoter. The Daf-16 binding element (DBE) is shown in bold. The position of the DBE, as indicated in the figure, is based on analysis of the human *CITED2* promoter (39). The line under the sequence containing the DBA indicates the oligonucleotide probe used in the EMSA assay. (**B**) Gel retardation assays using *Cited2* oligonucleotide probes comprising the DBE element. Probes with a wt or mutated DBE were incubated with nuclear extracts from I/11 erythroblasts that were factor deprived for 4 hours (0') and restimulated with Epo for 10'. The arrow indicates the complex specifically binding the DBE. Lane 5 - 8 contain competition experiments with 100-fold excess unlabeled probe (mutated and wt). In lane 9 - 10 anti-Stat3 and anti-Stat5 were added. (**C**) The same nuclear extracts were also incubated with a b-casein probe, to test for Stat5 activation. Anti-Stat3 (lane 3), and anti-Stat5 (lane 4) were added as a control for the experiment shown in (B). Epo induced Stat5 complexes are are indicated by arrows.

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To test the regulation of the entire -1128/+238 promoter fragment by Foxo3a and Stat5, the fragment was cloned into a luciferase reporter construct and tested in Ba/F3 cells. The *Cited2* promoter stimulated luciferase activity 40-fold (Figure 4.5A), indicating basic promoter activity. To express and control Foxo3a and Stat5 in Ba/F3 cells, Foxo3a transfected cells were cultured in presence of SCF, which prevents activation of wt Foxo3a, and subsequently cells were left untreated or treated with combinations of IL-3 and LY294002 (LY), to induce nuclear localization of Stat5 and Foxo3a respectively (Figure 5B). In the absence of active Foxo3a (no LY), IL-3 stimulation only modestly increased promoter activity. In contrast, active Foxo3a (plus LY) induced *Cited2* promoter activity 4-fold, which was enhanced to 9-fold upon stimulation with IL-3 (Figure 4.5B). To examine whether the observed IL-3 stimulation of Foxo3a was dependent on Stat5, Ba/F3 cells were transfected with the *Cited2* promoter together with wt Foxo3a and/or dominant negative Stat5. Reproducibly, IL-3 cooperated with Foxo3a to stimulate *Cited2* promoter activity, which was inhibited in the presence of dominant negative Stat5 (Figure 4.5C). Together these experiments indicate that Foxo3a-induced *Cited2* induction is Stat5-dependent.

To investigate whether a Foxo3a/Stat5 complex could act through the consensus DBE, the core sequence TGTT was mutated to AAAT (26). The wild-type and DBE mutated *Cited2* promoter had similar promoter activity in Ba/F3 cells (Figure 4.5D). The DBE-mutant and wt reporter construct were similarly responsive to Foxo3a and Stat5 activation (Figure 4.5E), which indicates that the Foxo3a/Stat5 complex does not bind a consensus DBE. This is in agreement with the failure to detect a Foxo3a/Stat5 complex bound to the DBE in a bandshift assay (Figure 4.4B) and suggests that the Foxo3a/Stat5 complex does not act through a classical DBE. This also predicts that Foxo3a/Stat5 complexes do not regulate classical Foxo target genes such as present in cluster A. To confirm this prediction, regulation of the *Cited2* promoter was compared with regulation of the *Btg1* promoter, cloned previously (4). Whereas IL-3 stimulation of Ba/F3 cells activated the *Cited2* promoter, it repressed *Btg1* promoter activity more than two fold (Figure 4.5F). In addition, inhibition of PI3K induced the *Btg1* promoter, but did not affect the activity of the *Cited2* promoter (Figure 4.5G). These data confirm the differential promoter regulation of the cluster A target *Btg1* and the cluster D target *Cited2* in response to factor stimulation and deprivation.

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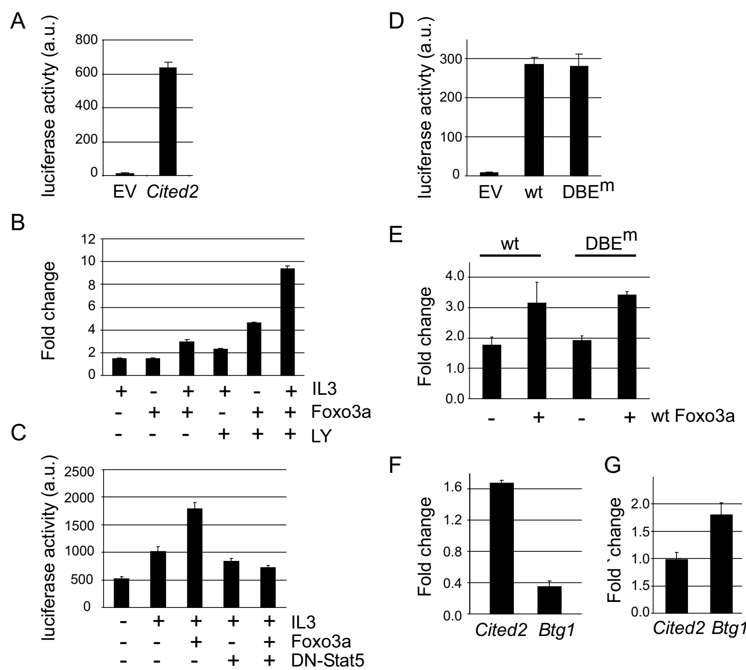

Figure 4.5. **Foxo3a and Stat5 cooperate to induce *Cited2* promoter activity.** For promoter studies, Ba/F3 cells were electroporated with *Cited2*-luciferase constructs (-1128/+238 of the *Cited2* promoter) and a β-galactosidase construct to correct for transfection efficiency. Luciferase activity is expressed as arbitrary units (a.u.) (**A**) The pGL3 vector without (EV) or with *Cited2* promoter sequence (*Cited2*) were examined for basal promoter activity in Ba/F3 cells. After electroporation cells were grown overnight in the presence of IL-3. (**B**) Ba/F3 were transfected with the *Cited2*-reporter construct together with wt Foxo3a, where indicated, were grown overnight in presence of SCF. Subsequently cells were treated (7 h) with combinations of IL-3 and the PI3K inhibitor LY (15 μM). Luciferase activity is presented as fold induction, calculated compared with *Cited2* promoter activity obtained after 24 h of culture in presence of SCF only. (**C**) Ba/F3 cells were transfected with combinations of the *Cited2* promoter, Foxo3a and dominant negative Stat5 (DN-Stat5). Cells were allowed to recover from electroporation for 2 hours, and subsequently incubated overnight in the presence or absence of IL-3. (**D**) Basal pomoter activity was compared between the wild-type (wt) and the DBE mutated (DBEm) *Cited2* promoter. Electroporated Ba/F3 cells were grown overnight in the presence of IL-3. (**E**) The wt and DBE-mutated (DBE^m) reporter constructs were transfected into Ba/F3 cells with or without Foxo3a. After 16 h cells were left untreated or were stimulated with IL-3 for 7 h. Fold induction indicates the luciferase ratio +/- IL-3. (**F/G**) The *Cited2* and *Btg1* reporter constructs were transfected into Ba/F3 cells and the ratio of luciferase activity was determined +/- IL-3 (**F**) or +/- LY294002 (15 μM)(**G**).

Discussion

In this study we identified Foxo3a target genes in erythroid progenitors and investigated their regulation by factor deprivation and Epo/SCF restimulation. Hierarchical clustering identified 5 major clusters of Foxo3a target genes. Of 69 putative Foxo3a-upregulated target genes, only 22 were downregulated by signaling as expected (cluster A), whereas 42 were not regulated (cluster D), and 5 were upregulated by signaling (cluster E). Of 229 putative target genes downregulated by Foxo3aa, only 38 were upregulated by signaling (cluster C), whereas the majority was not regulated by signaling (cluster B). The low response to factor deprivation and restimulation of Foxo3a upregulated target genes suggested that these targets may require cooperation of Foxo3a with additional transcription factors. Stat5 appeared to be one such a transcription factor binding to Foxo3a to alter the expected expression of the Foxo3a target gene *Cited2*. Foxo3a/Stat5 complex formation is not strictly dependent on phosphorylation of Stat5 and Foxo3a, but co-localization of Stat5 and Foxo3a in the nucleus enhanced *Cited2* expression. This co-localization was optimal in presence of Epo, which potently phosphorylates Stat5 resulting in nuclear translocation and which only partially phosphorylates Foxo3a, allowing Foxo3a to remain in the nucleus. Foxo3a-induced expression of *Cited2* depends on Stat5 and is independent of a classical Foxo-binding site. Thus, we identified a novel mechanism of Foxo-dependent gene regulation through the cooperation of an assumed growth inhibitory (Foxo3a) and growth promoting (Stat5) transcription factor.

Differential regulation of Foxo3a targets in response to stress conditions
Because phosphorylation of Foxo3a by PKB is supposed to diminish DNA binding and retain the protein in the cytoplasm (54), expression of Foxo3a target genes was expected to be dependent on SCF-induced PI3K activity. Moreover, we previously showed a strong upregulation of Foxo3a during erythroid differentiation (4) and expected a general upregulation of Foxo3a target genes during differentiation as observed for *Btg1*, *Cyclin G2*, *Ulk1* and other genes listed as cluster A. However, the majority of Foxo3a targets did not show this expected expression pattern (e.g. cluster D). Because we performed single hybridizations, we cannot be sure that all putative target genes are reliable. However, there is large consistency between the array data of Epo, SCF, Epo/SCF, Epo/SCF/Dex and Epo/SCF/ZK hybridizations (32). Moreover, we validated several selected target genes from cluster A and D in distinct Foxo3a(A3):ER expressing clones. Together the data suggest that cluster D targets are not involved in a cellular response to factor deprivation. Foxo's activate gene expression in response to various stress situations including oxidative stress, DNA damage, and growth factor depletion (16, 54). These various stress conditions all result in inhibition of cell growth and Foxo's are involved in these processes via regulation of distinct target genes; *p27*^{KIP} and *Bim* are upregulated in response to factor deprivation, whereas *Gadd45* is induced in response to DNA damage and oxidative stress (14, 18, 25, 33, 43, 53). Recently, it was shown that oxidative stress induces acetylation of Foxo on five different

lysine residues and phosphorylation on eight serine/threonine residues, concomitant with nuclear localization (14). Oxidative stress induced association of Foxo's with the acetylase p300/CBP-associated factor (PCAF), possibly responsible for stress-induced acetylation on five lysines. This subsequently induced the association with the deacetylase Sirt1, whereas it decreases interaction of Foxo with p300 (14, 54). Interestingly, interaction of Foxo with Sirt-1 was shown to repress Foxo-induced expression of *Bim*, *p27^{KIP}*, *PECK*, and *IGFBP1* (14, 46), but appeared to induce expression of *Gadd45* (14). As a result, Sirt-1 deacetylation of Foxo's enhanced cell cycle arrest and replicative senescence, and suppressed apoptosis (14). These data indicate how Foxo protein modifications in response to distinct stimuli may specify Foxo target gene activation. In addition, these data may also explain how forced activation of Foxo3a(A3):ER can activate Foxo targets that are normally induced in response to oxidative stress but fail to be activated by factor deprivation.

Role of target genes in cell fate determination

As the Foxo3a target genes can be divided into genes activated by factor deprivation or stress conditions, these target genes may be involved in distinct cellular processes. From the 14 genes with a known function that are up-regulated by factor deprivation, at least 8 have a function late in erythroid differentiation, or in mature erythrocytes. We previously showed p27^{KIP} and Btg1 to be transcriptionally activated late in differentiation. We now show the same for the antiproliferative Cyclin G2 , for the serine/threonine kinase Ulk1 and for p130^{Rb2}, which has a similar role in inhibition of the cell cycle as p27^{KIP} (16). Also phosphoglucomutases (Pgm2l1), adenosine monophosphate deaminase 2 (Ampd2) and carnitine palmitoyltransferase I (Cpt1a) are abundant proteins in mature erythrocytes (1, 3, 6), regulating glucose metabolism (Pgm2l1), the affinity of hemoglobin to bind oxygen (Ampd2) and membrane stability (Cpt1a; 3, 6). Failure to induce these target genes may contribute to the compensated anemia observed in Foxo3a-deficient mice (17). In contrast, from 24 genes with a known function that were hardly regulated by factor deprivation only the common IL3-receptor β-chain was found to be upregulated in differentiation (~30-fold). We found no genes with an obvious function in late differentiation in this group. Instead 6 putative target genes function in a response to oxidative stress: a thioredoxin-related membrane protein (*Txndc1*), selenoprotein P (*Sepp1*, 2x), arginase1 (*Arg1*; depletes cells of arginine to inhibit mRNA translation and induce senescence; 23), homeodomain-interacting protein kinase (*Hipk1*; upregulates the Daxx, Ask1, Jnk pathway in an oxidative stress response; 21, 50), Sestrin1 (*Ses1*; regenerates thioredoxin, 15) and NAD(P)H:menadione oxidoreductase 1 (*Nqo2*, deficient mice show a prominent bone marrow hyperplasia; 41). In addition, 9 putative target genes control signal transduction, including the IGF-I receptor and protein geranylgeranyltransferase Ib (*Pggt1b*; the enzyme transferring a membrane anchor to Ras). Enhanced IGF-I receptor signaling and sustained Ras/Mek/Erk activation are hallmarks of cell senescence. Finally it is noteworthy that cluster D contains at least two prominent tumor suppressor genes: *NF2*/*Merlin* (31) and *Copeb*/*Klf6* (22, 47). In conclusion, cluster D seems to contain genes that control the induction of senescence. Notably, mitotic

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hematopoietic progenitors cannot execute a senescence program similar to e.g. fibroblasts. Instead, activation of a senescence program including stabilization of p53 seems to enhance differentiation. These targets may, therefore, still contribute to the observed enhanced differentiation in response to Foxo3a activation (4).

Regulation of *Cited2* expression by Foxo3a and Stat5

Differential activation of target genes by Foxo's in response to different stimuli can be established through differential cooperation with other transcription factors. At present Foxo is known to mediate transcription via interaction with a large variety of transcription factors (9, 54). Complex formation with other transcription factors also explains how a mutant Foxo1, incapable of DNA binding, retains the ability to regulate expression of part of its target genes (49). This mutant lost the ability to induce apoptosis but was still able to cause a G_1-arrest (49), underscoring that different mechanisms of target gene activation are associated with distinct cellular responses.

We showed that an Epo induced Foxo3a/Stat5 complex regulates *Cited2* expression. Epo induced *Cited2* expression was not affected by the PI3K inhibitor LY294002 (Figure 2B). This is not surprising since Epo strongly induces Stat5 activation, whereas it only causes weak Foxo3a phosphorylation (Figure 4.3A, C). SCF repressed *Cited2* expression in a PI3K-dependent way, both in presence and absence of Epo, although less pronounced than the repression of genes in cluster A. The data show that a Foxo3a/Stat5 complex can be formed when either Foxo3a or Stat5 is abundantly present in the nucleus, i.e. upon factor deprivation and Epo/SCF stimulation respectively. However, induction of Stat5 in absence of Foxo3a phosphorylation favors Foxo3a/Stat5 interaction and *Cited2* expression.

The observation that a complex is binding specifically to the DBE of the *Cited2* promoter in an EMSA assay, although the DBE seems not to be required for Foxo3a-induced up-regulation of *Cited2*, raises a question as to the identity of the DBE-binding complex. Unfortunately, the Foxo3a antibodies are unable to supershift Foxo3a-containing complexes, not even on a proper DBE in e.g. the *Btg1* promoter. Therefore, we cannot be sure that the complex binding the DBE contains Foxo3a. Possibly, a different Foxo3a-containing complex binds to the DBE to promote *Cited2* expression in response to other signals. The observation that the Foxo3a/Stat5 complex activates *Cited2* independent of the DBE is not surprising, since the specific function of this complex is likely to require a specific DNA-binding site, otherwise all Foxo3a target genes would be subject to Stat5 regulation. This is very unlikely since genes in cluster A are predominantly repressed in response to Epo stimulation (Table I). The fact that Cited2 transcript levels are strongly and rapidly increased by growth factor or cytokine stimulation (51), and by Foxo3a activation supports the hypothesis of direct transcriptional control through the Foxo3a/Stat5 complex. Interestingly, the Il-6 induced cooperation of the Foxo1/Stat3 complex on the *α-2-macroglobuli*n promoter was suggested to act via two Stat3 elements present in the promoter (34). Also the glucocorticoid receptor induced Stat3 transcriptional activation of the *α-2-macroglobulin* promoter by enhanced recruitment other transcriptional

components, independent of DNA binding (38). Therefore it seems likely that Foxo3a enhances *Cited2* transcription via association with a Stat5 containing complex, possibly acting through an unidentified Stat5 promoter element. Another interesting observation is the cytoplasmic interaction between Stat3 or Stat5 and Foxo3a, which may be part of the reported statosome protein complexes (48). However, the function of the cytosolic Foxo/Stat interaction remains unclear.

Our data implicate that differential binding of Foxo's to cooperating transcription factors results in a distinct response to growth factors. Genes encoding Riken clone 2010005O13, EST AW146242 and *Cpeb4* (cytoplasmic polyadenylation element binding protein 4) are among the targets in cluster D that are regulated similarly to *Cited2*, being upregulated by Epo and downregulated by SCF (Table II). These genes are likely candidates to be regulated by the Foxo3a/Stat5 complex as well. Notably, neither Cited2 nor Cpeb4 proteins are associated with inhibition of growth, but rather with developmental processes. Cited2 is a chromodomain protein that interacts with the transcriptional co-activator p300/CBP to alter its interaction with transcription factors among which HIF1a (7). Among the genes positively regulated by Cited2 are the polycomb proteins Bmi-1 and Mel-18 (35). Cpeb binds to the cytoplasmic polyadenylation element (CPE) to regulate polyadenylation and translation in response to activation of PKA and CaMKII (44, 52).

Other Foxo target genes such as *Ptpns1* (protein tyrosine phosphatase, non-receptor type substrate 1) and *P2ry14* (purinergic receptor P2Y14) are upregulated by both Epo and SCF and may be regulated by yet another complex of transcription factors activated by mitogenic signals and Foxo's.

We did not observe a marked regulation of *Cited2* during differentiation. Notably, Stat5 is expressed early but not late in differentiation, whereas Foxo3a expression increases from the start of differentiation induction to reach maximal levels 48 h after differentiation induction when Stat5 expression becomes undetectable (4, 20). Both Foxo3a and Stat5 are required for terminal differentiation. The cooperation between the two factors apparently ensures ongoing *Cited2* transcription during differentiation, either through a Foxo3a/Stat5 complex early in differentiation or later by Foxo3a alone.

In conclusion, we identified putative Foxo3a target genes that were assigned to different clusters based on their regulation by Epo and SCF signal transduction. We analyzed some genes in more detail that belonged to cluster A, being upregulated by factor-deprivation and repressed by SCF-induced PI3K activity, or cluster D, not prominently controlled by factor deprivation and SCF restimulation. We do not propose that the latter group is regulated independent of PKB-mediated phosphorylation of Foxo transcription factors. Rather we propose that this group of genes requires further modification of Foxo proteins e.g. by acetylation and phosphorylation in response to a specific stimulus, or requires cooperation with other transcription factors. As an example we describe a novel mechanism for Foxo3a-mediated control of gene expression. A Foxo3a/Stat5 complex controls expression of genes such as *Cited2* independent from a classical Foxo-binding element (DBE). The data strengthen the notion that

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Foxo's function at the heart of signal dependent gene-regulation and in complexes that integrate multiple signals to decide between cell death or survival and senescence.

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Materials and Methods

Cells and reagents
BA/F3 were cultured in RPMI 1640 (Invitrogen) supplemented with 10% FCS (Hyclone, PerBio) and 10 ng/ml murine IL-3 (supernatant). The erythroid cell line I/11 was cultured in Stempro medium (Invitrogen) supplemented with 0.5 U/ml Epo (kind gift of Ortho-Biotech, Tilburg, The Netherlands), 100 ng/ml SCF (supernatant) and 1mM dexamethasone (Sigma-Aldrich) (56). To activate Foxo3aa(A3):ER 50 nM 4-hydroxytamoxifen (4OHT; Sigma-Aldrich) was added to these expansion conditions. For stimulation, I/11 cells were incubated for 4h in plain IMDM (Invitrogen) and stimulated at 37°C with 200 ng/ml SCF or 5 U/ml Epo. Reactions were stopped by addition of ice-cold PBS. Stable FOXO3a(A3):ER expressing I/11 clones were generated using the retroviral expression vector pBabe as described previously (4). LY294002 was obtained from Alexis (Schwitzerland).

cDNA array hybridisations and analysis
Total RNA was generated from cells treated with or without 4OHT. Dual labelling was used to hybridise the cDNAs pairwise to custom made EST micro-array containing 17.000 cDNAs (17K). For MIAME-compliant description of the procedures, see on-line supplement (32). Hierarchically clustering of data was performed and visualised with the Spotfire application, using Euclidean distance and average linkage to assess distance.

Real-time quantitative PCR
cDNA synthesis and quantitative RT-PCR were performed using Taqman technology and Sybr-green detection (Applied Biosystems) of dsDNA as described. (32) Murine primer sequences used:
Btg1: forward 5'-GCAGGAGCTGCTGGCAG-3',
reverse 5'-TGCTACCTC CTGCTGGTG A-3'.
Ccng2: forward 5'-TGAAACCGAAACACCTGTCC,
reversed 5'-TCGAGTTTATCGAGGCTGAGA.
Ulk-1: forward 5'-TACCAGAATGTTCTCAGTGG,
reverse 5'-TGCTCCATGAGGGTCTCC.

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Sesn1: forward 5'- TCTGATGTGACAAGGTGACA,
reverse 5'-TGTTACCGCCAACACGGTC.
Dcn: forward 5'-TGGGCGGCAACCCACTG,
reverse 5'-TCAGGCTGGGTGCATCAAC.
Cited2: forward 5'-TGAACCACGGGCGCTTCC,
reverse 5'-TGGCGTGCCTGATGCCGC.
Ribonuclease inhibitor: forward 5'-TCC AGT GTG AGC AGC TGA G-3',
reverse 5'-TGC AGG CAC TGA AGC ACC A-3'.
The CT-values of Rnase Inhibitor were used for normalisation.

Western blotting and antibodies

Cell lysis, immunoprecipitation, SDS-polyacrylamide gel electrophoresis and Western blotting were performed as described previously (55). Nuclear and cytoplasmic extracts were prepared as described previously (2). Lysate of 20x106 cells was used for one immunoprecipitation. Antibodies used in this study were: anti-FOXO3a (#06-951) from Upstate Biotechnology, anti-Stat3 (C-20), anti-Stat5 (N-20) from Santa Cruz. Concentrated aliquots of the Stat3 (C-20; Santa Cruz) and Stat5 (L-20; Santa Cruz) antibodies were used for EMSA.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared as described (2), Oligonucleotide probes were end-labelled with 32P and EMSA were performed as described (27). The following oligonucleotide probes were used:
wt-*Cited2* 5'-GCTGATCGCTGAGTTTGTTTACAGAGCAGGGAC,
DBE-mutated *Cited2* 5'-AGCTGATCGCTGAGTTTAAATACAGAGCAGGGAC-3'.
A standard ß-casein oligonucleotide (5'-AGATTTCTAGGAATTCAATCC) binding Stat5 and Stat1 (57) was used as a positive control.

Cloning of the *Cited2* promoter and Luciferase Reporter assays

The mouse *Cited2* promoter was cloned in pGL3-basic (Promega) using the following primers: forward 5'-CCTATTGCTCCACTGAACAAT-3', reverse 5'-CTCACCTTCCGTCTTTGCGATTTC-3', and the Expand High Fidelity PCR System (Roche). Promoter alignment between human (AF129290) and murine *Cited2* (NT039491) was performed using the DNAMAN program, version 5.2.9. The position of the identified Foxo promoter element was based on the cloned human *CITED2* promoter (39). The AAAT mutation in the DBE of the *Cited2* promoter was made using the Quickchange Site Directed Mutagenesis kit (Stratagene) according to the manufacturers protocol, using the primers: forward 5'-GATCGCTGAGTTTAAATACAGAGCAGGGAC-3', and the opposite strand for the reverse primer. For reporter assays, 10 x 10^6 Ba/F3 cells were electroporated (0.28 kV, capacitance 960 µFD) with maximum 20 mg of DNA. After recovery for several hours in normal media, cells were washed and grown overnight in the presence or absence of IL-3 and SCF, or cells were IL-3 deprived overnight and next day stimulated for 7 h with IL-3. Luciferase activity was measured using the Steady-Glo system (Promega). Transfection efficiency was determined by cotransfecting lacZ and analyzing β-galactosidase activity.

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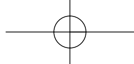
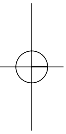
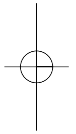
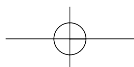


Overview and General Discussion

CHAPTER 5



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General Discussion

1. Overview

This thesis describes the role of Forkhead transcription factor Foxo3a in erythropoiesis. We observed a strong increase in Foxo3a activity during erythroid differentiation through up-regulation of Foxo3a with concomitant loss of PKB activity, suggesting a role for Foxo3a in the control of gene expression in terminal differentiation. This was confirmed by the observation that activation of Foxo3a accelerated terminal erythroid differentiation (Chapter 2). Expression profiling studies performed on erythroid specific and general cDNA arrays enabled the identification of Foxo3a regulated genes. A subset of the Foxo3a target genes was indeed up-regulated during late differentiation (Chapter 2 and 4). Among these targets we could identify genes that play a role in red cell function and cell cycle arrest, but also novel genes for which a function in erythropoiesis was unknown. Of the latter targets, the role of the anti-proliferative gene *Btg1* was investigated. Expression of *Btg1* is regulated both by Foxo3a binding to the DBE (Chapter 2), and by the cAMP pathway via a CRE in the *Btg1* promoter (Chapter 3). Both sites are required for optimal expression of *Btg1*. Increased intracellulair cAMP, and phosphorylated CREB/ATF1 levels at terminal stages of differentiation indicate involvement of the cAMP pathway in erythroid maturation. Stimulation of the cAMP enhanced Foxo3a-induced differentiation (Chapter 3), showing cooperation of these pathways in regulating erythroid differentiation. One of the functions of Btg1 is regulation of the protein arginine methyl transferase-1 (Prmt1). We found that Btg1 repressed erythroid colony growth dependent on the domain interacting with Prmt1. This indicated protein arginine methylation as a novel mechanism regulating the balance between erythroid expansion and differentiation (Chapter 2).

Because PKB regulates the function of Foxo proteins, we expected the identified Foxo3a targets to be regulated by factor deprivation and Epo/SCF re-stimulation. Although this was true for the genes regulated during differentiation, it was not the case for about 70% of the Foxo3a regulated genes. From this we concluded that a minority of the Foxo3a regulated sequences is controlled upon down-regulation of the PI3K pathway, whereas the other sequences may be regulated in response to stimuli such as DNA damage and oxidative stress. Notably, one subset of Foxo3a up-regulated genes encodes proteins able to protect against oxidative stress, a cellular mechanism regulated by Foxo proteins (Chapter 4). Interestingly, The CRE site in the *Btg1* promoter bound both cAMP responsive transcription factors CREB/ATF1, which are activated during differentiation, and a cJun/ATF2 complex, which is activated by JNK in response to DNA damage and oxidative stress. Thus Foxo-induced expression of *Btg1* is enforced via the CRE both in differentiation and possibly also under stress conditions (Chapter 3).

To examine how Foxo target genes can escape control by factor deprivation and restimulation, we studied expression regulation of the transcriptional co-regulator Cited2. This demonstrated that Foxo3a associates with Stat5, an association that is enhanced upon stimulation with Epo, which is consistent with Epo induced up-regulation of *Cited2*. The Foxo3a/Stat5 complex

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does not act via a regular Foxo3a or Stat5 binding sites, but may act via a specific, so far unidentified binding site in the *Cited2* promoter. The transcriptional regulation of *Cited2* exemplifies how Foxo's are able to integrate multiple signals to the transcriptional machinery.

2. The erythroblast cell line I/11 - A relevant model to study erythropoiesis

Normal hematopoiesis requires a tight balance between progenitor expansion and differentiation, which is regulated by the concerted action of extra cellular factors regulating the signaling network and transcription factors (99). The analysis of SCF-induced signaling pathways that regulate expansion and differentiation of erythroid progenitors, requires the use of a proper cell model. Although erythroid progenitors can be expanded to large numbers from umbilical cord blood (114), bone marrow (114), and day E12-E14 murine fetal livers (113) in the presence of Epo, SCF and Dex (in serum free media), these progenitors have a limited life-span due to spontaneous differentiation. However, immortal cultures can be established reproducibly from fetal livers and bone marrow of p53-deficient mice. These cultures retain their dependence on Epo, SCF and Dex, as well as their ability to differentiate synchronously to enucleated erythrocytes in the presence of Epo. This permits factor-dependent expansion to numbers that allow large scale molecular and biochemical analysis of mechanisms regulating erythroid expansion versus differentiation. Unlike the *in vivo* situation, other erythroid-leukemic cell lines (HCD57 (52), AS-E2 (103), K562 (34), UT-7 (45, 59)) depend only on Epo for expansion and display an incomplete differentiation, which is in some cases artificially induced upon addition of DMSO or factor deprivation. Therefore, a clonal cell line derived from a $p53^{-/-}$ fetal liver cells (I/11 cells) is used for most studies in this thesis, because it most faithfully mimics the expansion/differentiation aspects of *in vivo* erythropoiesis.

3. The role of the PI3K/PKB/Foxo3a pathway in erythropoiesis

3.1. The PI3K/PKB pathway is not restricted to regulation of apoptosis

In previous studies it was shown that the PI3K and not the MAPK pathway controls erythroid expansion versus differentiation (113). Although these experiments were performed using the I/11 model, they have been consolidated in primary cells. Treatment of human primary erythroblasts with 7.5-15 mM LY294002 resulted in an acceleration of erythroid differentiation (Figure 5.1), but did not affect the survival of these cells (determined by cell morphology, Figure 5.1, and TUNEL assay, not shown). In addition, 24 h activation of the active Foxo3a in the erythroblasts clones also did not increase the apoptotic index (Chapter 2). Contradictory results come from several reports that did link the PI3K pathway primarily to regulation survival and apoptosis of erythroblast. However, these studies use high concentrations of PI3K inhibitors (> 50 μM LY294002; > 1 μM wortmannin; 44, 73, 92, 93, 105) resulting in inhibition of additional pathways (92, 107, 112). For instance, we have reported that a concentration of 30 μM LY294002 in erythroblasts significantly inhibits tyrosine phosphorylation of the EpoR, Jak2, Gab-1, PKC, and Erk, whereas a LY294002

concentration of 7,5 μM completely blocks PKB activation but hardly affects the phosphorylation of these proteins (107, 112) Furthermore, 0.5 μM wortmannin not only blocked PI3K activity but also Erk activation (92). Other studies are performed in differentiation-incompetent cell lines and cells that attempt to differentiate may enter a conflict situation resulting in apoptosis (52, 103). Therefore these and our data are not in conflict but we reach different conclusions. In granulopoiesis, inhibition of the PI3K/PKB pathway also does not result in cell death. Umbilical cord blood derived CD34+ cells that were differentiated towards the eosinophilic lineage did not show increased apoptosis upon PKB or mTOR inhibition (16). Only inhibition of PI3K activity using 20 μM LY294002, a concentration that affects other pathways as well, did increase the apoptotic rate during eosinophilic differentiation (16).

These data indicate that inactivation of PI3K/PKB pathway in hematopoietic cells does not automatically result in apoptosis and may regulate other cellular endpoints as well.

3.2. The PI3K/PKB pathway controls the balance between erythroid expansion and differentiation

The PI3K family contains a large number of highly related lipid kinases. The role of each of these kinases in regulation of hematopoietic development is only beginning to be understood. Mice lacking the P110γ catalytic subunit of PI3K display impaired T-cell development (88), p110δ appeared to be essential for allergic reactions initiated by mast cells (3), and mice lacking p85α, the regulatory subunit of class 1 PI3K show a block in B-cell development at the pro-B cell stage (35). Mice lacking PKBα only show an increased apoptosis in the thymus (104), whereas mice lacking both PKBα and PKBß have defects in multiple tissues (78). Although erythropoiesis has not been studied in detail in most of these models, mice lacking P110γ have reduced numbers of BFU_E and CFU_E. The various models show that signaling through PI3K/PKB defines cell survival in some cells and in some stages of development, whereas it is involved in control of proliferation and differentiation in other cells. This has recently been confirmed for the eosinophilic lineage. CD34+ cells derived from umbilical cord blood that were differentiated to the eosinophilic lineage showed dramatically enhanced differentiation upon PKB or mTOR inhibition, whereas activation of PKB in these cells resulted in inhibition of eosinophilic differentiation (16). Strikingly, activation of PKB in these CD34+ cells enhanced neutrophilic differentiation, showing an important role for PKB in myeloid lineage determination (16). In addition to hematopoietic cells, PKB promotes neuronal differentiation(111), and PKBβ but not PKBα, enhanced myoblast differentiation (55, 109). To test the role of PKB in erythropoiesis we tried to overexpress PKB and PKB mutants in the erythroid cell line I/11, but stable clones could not be obtained suggesting tight regulation of PKB in erythropoiesis. However, inhibition of PI3K using low LY294002 concentrations, did not result apoptosis but induced differentiation (Figure 5.1; 113). This shows that PKB is implicated in regulation of erythoid proliferation and differentiation instead of apoptosis.

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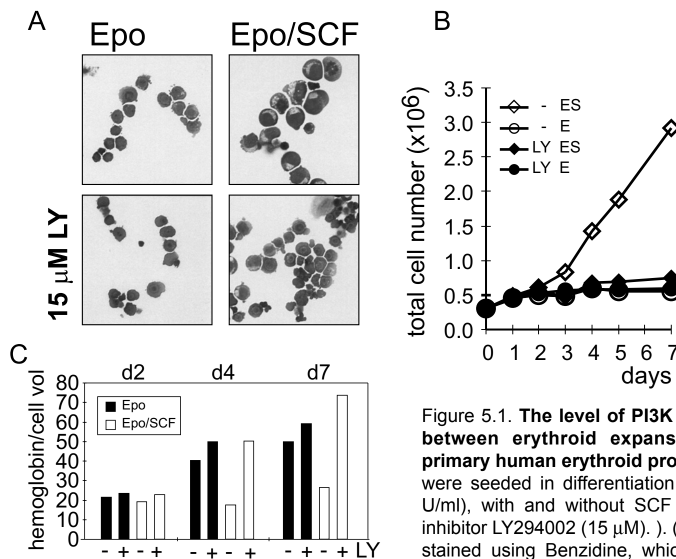

Figure 5.1. **The level of PI3K activity controls the balance between erythroid expansion and differen-tiation of primary human erythroid progenitors.** Erythroid progenitors were seeded in differentiation medium containing Epo (E; 2 U/ml), with and without SCF (S; 100 ng/ml) and the PI3K inhibitor LY294002 (15 μM).). (**A**) After 4 days, cytospins were stained using Benzidine, which stains hemoglobinized cells orange-brown (here shown as small, dark cells). Addition of SCF delays Epo induced differentiation, which can be reversed upon inhibition of PI3K activity. (**B**) Growth curve of the cultures. In the presence of Epo alone, cells divide 3-4 times and than terminally differentiate. SCF significantly enhances the expansion capacity of these cells. This effect was abrogated when PI3K was inhibited, showing the importance of PI3K for expansion. (**C**) Hemoglobin levels were determined during the assay as a measure for differentiation. Primary erythroid cells cultured in the presence of Epo plus SCF showed a significantly delayed hemoglobinization compared with Epo induced differentiation. However, addition of the PI3K inhibitor LY294002 to Epo/SCF cultures fully restored hemoglobinization. Notably, addition of the MEK inhibitor PD98059 only slightly reduced the proliferation but did not alter the balance between expansion and differentiation (not shown).

4. The role of Foxo3a in erythropoiesis

4.1. Foxo3a and regulation of hematopoietic differentiation

Initially the role of Foxo proteins in the hematopoietic system was thought to be regulation of blood cell turnover (17) because overexpression in hematopoietic cell lines (Jurkat cells, 14; Ba/F3 cells, 29) induced apoptosis. However, these studies often suffered from the same deficits as studies on the role of PI3K in hematopoiesis; they were performed in differentiation incompetent cells. Therefore, a potential Foxo-induced cell cycle arrest may not lead to differentiation but results in a conflict situation leading to apoptosis. We showed that Foxo3a is markedly increased late in erythroid differentiation. Activation of an active Foxo3a mutant at the onset of differentiation accelerated the differentiation process but did not induce apoptosis (Chapter 2 and 3). To confirm the control of erythopoiesis by the PI3K/Foxo3a pathway in primary cells, murine primary cells were differentiated in the presence of Epo, and expression kinetics of PKB, Foxo3a and $p27^{KIP}$ were examined by western blot (Figure 5.2). These kinetics were similar as observed in differentiating erythroblasts from the I/11 cell line (Chapter 2), suggesting an identical role for the PI3K/Foxo3a pathway in the control of erythropoiesis in primary cells. A role for Foxo3a in regulation of

erythropoiesis is furthermore supported by the fact that Foxo3a null mice suffer, among other abnormalities, from a mild compensated anemia with reticulocytosis showing that Foxo3a is required for the generation of stable erythrocytes (18).

Foxo proteins regulate differentiation in other cell types as well. In thymocytes, endogenous Foxo1 is expressed in parallel with differentiation markers, and ectopic expression of a dominant negative Foxo1 mutant reduced cell numbers, without affecting the apoptotic rate of these cells (62). The commitment of myeloid progenitors to either eosinophilic or neutrophilic granulocytes which is controlled by PKB (previous paragraph) is at least in part mediated by Foxo's through transcriptional repression of the helix-loop-helix transcription factor Id1 (16). Furthermore, Foxo1 is involved in differentiation of myoblasts. Although Foxo1 activation induced differentiation of primary myoblasts (11), Foxo1 had to be inactivated to induce differentiation of the myoblast cell line C2C12 (47). These seemingly contradictory results may point to cell type specific differences (2).

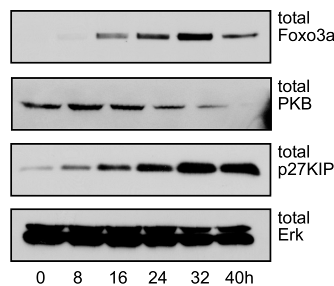

Figure 5.2. **Regulation of PKB, Foxo3a, and p27^{KIP} in murine primary erythroblast differentiation.** Erythroid progenitors derived from murine fetal livers were differentiated in the presence of Epo (20 U/ml). Cell lysates were harvested every 8 hours and proteins were separated performing SDS-PAGE. Blots were stained for total levels of Foxo3a, PKB, and p27^{KIP} as indicated. Total Erk was used as a loading control.

4.2. Overlap between Foxo3a and Epo/SCF regulated targets

To identify the mechanisms via which Foxo3a mediated accelerated erythroid differentiation, mRNA expression profiling studies were performed to screen for Foxo3a targets, using erythroid clones expressing an inducible, active Foxo3a mutant. Because the Foxo forkhead transcription factors are defined as PI3K/PKB targets, we assumed that Foxo up-regulate genes would be repressed by SCF-induced PI3K activity. *Vice versa* we expected Foxo's to mediate SCF-induced repression of target genes. Therefore, we assessed how Foxo3a regulated sequences were controlled by Epo/SCF. Clustering of array-data revealed Foxo3a up- and down-regulated clusters, which were differentially regulated by Epo/SCF signaling (Chapter 4). Surprisingly, about 70% of the Foxo3a regulated sequences, are not regulated in a starvation/stimulation experiment (Figure 5.3 and Chapter 4). When selected for Foxo3a and Epo/SCF regulated sequences with a threshold of a factor 1.75, only 13% of the Foxo3a regulated sequences were regulated by Epo/SCF stimulation. Similarly, 588 out of 11585 sequences were regulated by Epo/SCF of which 80% is not regulated by Foxo3a. Notably, these data clearly show that only a minor subset of Foxo3a targets are regulated upon factor deprivation. and suggest that a majority of the Foxo3a targets is controlled via alternative mechanisms (see paragraph 4.4).

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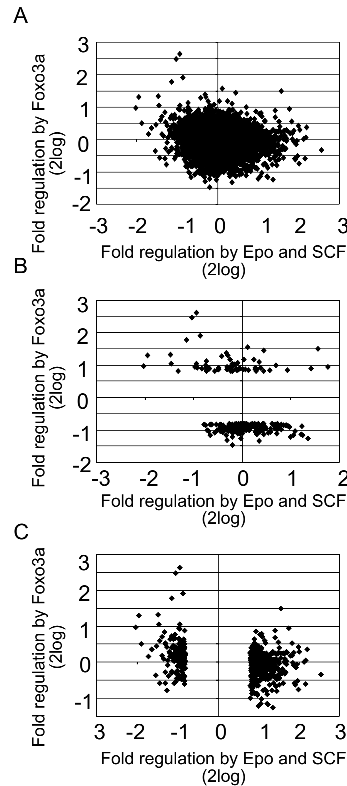

Figure 5.3. **Analysis of target gene regulation by Foxo3a and Epo/SCF (E/S) stimulation as detected using the 17K EST cDNA array.** For the analysis presented in this figure, we discarded all sequences that showed a 'bad' signal in the Foxo3a or Epo/SCF hybridization and all sequences that were regulated by 4OHT in the control clone (ev) more than 1.4 fold (^{2}log >0.49 or < -0.49), This yielded a selection of 11585 sequences which are presented in (**A**). The X-coordinate of each gene presents the fold regulation by Epo/SCF stimulation over starvation, and the Y-coordinate presents the fold regulation by Foxo3a (4-hydroxy-tamoxifen stimulated over non-stimulated Foxo3a clones). Fold regulation for both axis are presented as 2log ratio's. (**B**) Out of 11585 sequences, 238 were regulated by Foxo3a with a 2log ratio of >0.81 or <-0.81 (factor 1.75 up- or down-regulated). The fold Foxo3a regulation is plotted against their fold regulation by E/S stimulation. Using MS-access, we found 74% of the Foxo3a regulated sequences not to be regulated by E/S stimulation (non-regulation is defined as less than 1.4 fold. (**C**) The reversed analysis as shown in B: 588 sequences are regulated by E/S (factor 1.75 up- or down-regulated), of which 80% is not regulated by Foxo3a (factor regulation < 1.4). These data show that the majority of the Foxo3a sequences are not regulated by factor depletion and re-stimulation experiments, and that about 80% of the Epo/SCF regulates sequences are not regulated by Foxo3a.

4.3. Foxo3a targets and their role in differentiation

In addition to the mRNA profiling studies described in this thesis, we have also performed profiling analysis on differentiating erythroblast cells using affymetrix cDNA arrays (manuscript in preparation). Combining the results of both studies allows the characterization of Foxo3a controlled gene expression in differentiation. The differentiation studies were performed using the p53-deficient erythroblast cell lines I/11 and R10 (the R10 cell line is derived from $p53^{-/-}$ mice with a different genetic background). Because the 17K EST array and the affymetrix array have a different background, only ~50% of the Foxo3a controlled genes could be traced back on the affymetrix arrays. These overlapping Foxo3a targets (130) were clustered with the differentiation data (Figure 5.4). Because Foxo3a activity increases in differentiation, Foxo3a-regulated genes identified on the 17K array were expected to be regulated in differentiation. The Foxo3a regulated sequences showing this consistent regulation are show in Table 5.1 and some of them are discussed below.

This procedure confirmed the up-regulation of some Foxo3a induced genes already identified in Chapter 4 such as *Btg1*, *Cdkn1b* (*p27^{KIP}*) and *Ccng2* (*Cyclin G2*), and identified additional targets up-regulated during differentiation. Core promoter element binding protein (*Copeb*), also known as Krüppel like factor-6 (KLF6) is a transcription factor that functions as a tumor suppressor. A Copeb mutant was cloned from a B-cell chronic lymphocytic leukemia patient and

exhibited transforming capacity (31). Furthermore, *Copeb* is frequently mutated human prostate cancer (74). Its late up-regulation in differentiation probably contributes to terminal G1-arrest since *Cdkn1a* (*p21*^{CIP1}) was found as a Copeb target (74). Another differentiation upregulated Foxo3 target is Selenoprotein P plasma 1 (*Sepp1*). Selenium has been shown to play a role in oxidant defense (122). Sepp1 functions as a heparin-binding protein associated with endothelial cells and has been implicated as an oxidant defense in the extracellular space (123). Exportin 7 is a Ran-binding protein involved in nuclear transport, and was identified from the t(5;14) translocation that is found in acute lymphoblastic leukaemia suggesting loss of growth suppression through inactivation (57).

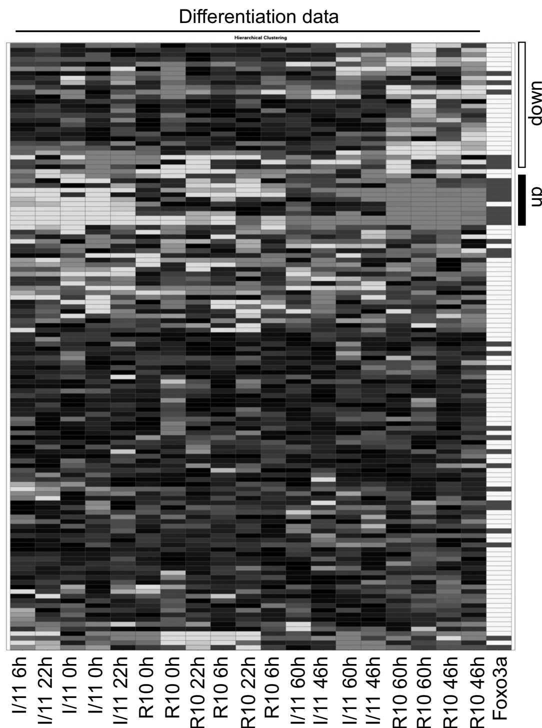

Figure 5.4. **Regulation of Foxo3a targets in differentia-tion.** Clustering of Foxo3a up-regulated targets (right column, grey bars) and down-regulated genes (right column, white bars) in terminal erythroid differentiation (non-regulated sequences are shown in black). This analysis was performed by linking Foxo3a regulated genes identified on the general 17K EST cDNA array with differentiation profiling studies performed on affymetrix gene chips, using the erythroblast cell lines I/11 and R10. From the 299 Foxo3a regulated genes identified with the 17K EST chip (fold regulation > 1.75), 130 genes could be traced back in the affymetrix hybridization and are presented in this graph. Differentia-tion expression profiling studies were performed for the following timepoints: 0 h, 6 h, 22 h, 46 h, and 60 h. Duplicate hybridizations were performed for each cell line at each timepoint (except for 6 h I/11: one hyb.).

The list of Foxo3a-induced genes outlined above underlines the growth-suppressing role of Foxo3a in differentiation (Chapter 2). Transcriptional induction of cell the cycle inhibitors *Cdkn1b*, *Ccng2*, *Btg1*, and *Rbl2* (p130^{Rb}; Chapter 2 and 4) is probably part of the mechanism via which Foxo3a controls differentiation. This is underlined by the fact that Foxo3a activity is induced after completion of the 3 - 4 differentiation-divisions when cells terminally arrest in G_1 (113). The importance of a terminal cell cycle arrest in erythroid differentiation is suggested by Rb null mice which display a differentiation defect,(51) and by a block of erythroid differentiation resulting from GATA1 overexpression which is also thought to occur through deregulation of the cell cycle (118). Furthermore, cell cycle inhibition is believed to be a general mechanism involved in regulation of the terminal differentiation of hematopoietic cells (36).

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Tabel 5.1. Foxo3a regulated genes with consistent regulation in erythroid differentiation

Foxo3a upregulated genes, upregulated in differentiation

unigene #	gene name	gene function
Mm .333738	acidic ribosomal phosphoprotein P0	unknown
Mm .254297	ATPase, Cu++ transporting, alpha polypeptide (*Atp7a*)	transport
Mm .152987	Exportin 7 (*Xpo7*)	transport
Mm .3527	Cyclin G2 (*Ccng2*)	nuclear transport
Mm .24642	hypothetical protein	cell cycle regulation
Mm .275036	Core promoter element binding protein (*Copeb*)	transcription
Mm .1940	Colony stimulating factor 2 receptor, beta 2 chain (*Csf2rb*)	Signalling
Mm .22699	Selenoprotein P, plasma, 1 (*Sepp1*)	oxidant defense
Mm .2958	p27KIP (*Cdkn1b*)	cell cyle regulation
Mm .305438	B-cell translocation gene 1 (*Btg1*)	arginine methylation

Foxo3a downregulated genes downregulated in differentiation

unigene #	gene name	gene function
Mm .44219	DEAD/H BOX 18; (Ddx18)	proliferation
Mm .26392	THUMP domain containing 1 (*Thumpd1*)	unknown
Mm .321440	2010309E21Rik	unknown
Rn .11074	Chondroitin sulfate proteoglycan 6 (*Cspg6*)	induces proliferation
Mm .38193	zinc finger protein 292 (*Zfp292*)	transcription
Mm.35089	Renela cell carcinoma, papillary (*Prcc*)	unknown
Mm .41555	member RAS oncogene family (*Rab28*)	embryogenesis
Mm.24117	DNA segment, Chr 8, ERATO Doi 319, expressed	unknown
Mm .2756	non-histone chromosomal protein HMG-14	unknown
Mm .28124	growth factor, erv1 (*Gfer*)	proliferation
Mm .12459	ankyrin repeat domain 10	unknown
Mm .27131	Ribonuclease P 21 subunit (human) (*Rpp21*)	mRNA regulation
Mm .512	WW domain binding protein 5 (*Wbp5*)	unknown
Mm .2478	splicing factor, arginine/serine-rich 4 (*Sfrs4*)	pre-mRNA splicing
Mm .343230	Similar to protein translocation complex beta	protein transport
Mm .24643	centrin 2 (*Cetn2*)	calcium binding protein
Mm .28392	Deformed epidermal autoregulatory factor 1 (*Deaf1*)	transcription, growht suppression
Mm .52583	putative protein	unknown

Foxo3a targets were accepted as regulated when 4 or more out of 8 hybridizations from the later timepoints (46, 60h) showed regulation. Fold regulation Foxo3 clone > 1.75 (ev < 1.3).

Complementary to the induction of growth inhibitory genes, Foxo3a may also accelerate differentiation through down-regulation of target genes (Figure 5.3; table 5.1). Other profiling studies aimed to identify Foxo3a target genes also revealed clusters of down-regulated genes (31, 81). A subset of genes was down-regulated both by Foxo3a and in differentiation (Table). In general, these targets can be linked to expansion: DEAD/H Box 18 (*Ddx18*), is a putative ATP-dependent RNA helicase. *Ddx18* expression is induced by the Myc-Max transcriptional complex, after proliferative stimulation of primary human fibroblasts and B cells, and is down-regulated during terminal differentiation of HL60 leukemia cells and primary keratinocytes consistent with the disappearance of c-Myc RNA (41). Chondroitin sulfate proteoglycan (*Cspg6*), causes transformation when overexpressed, and is frequently found to be induced in human and murine tumors (39). The zinc finger protein 292 (*Zfp292*) is involved in transcriptional activation of growth hormone (108), and the Renela cell carcinoma, papillary (*Prcc*) gene was identified from a translocation occurring in papillary renal cell carcinoma (117). Finally, the growth factor Erv1 (*Gfer*) is essential for cell cycle progression in yeast (65) and plays a role in liver regeneration (33).

The fact that these genes all support proliferation suggest that their suppression by Foxo3a may present a complementary mechanism via which Foxo3a controls erythropoiesis. Such a mechanisms has recently been proposed for eosinophilic differentiation. It was suggested that Foxo3a transcriptional repression of the helix-loop-helix protein Id1 is required for execution of the differentiation program (16). Whether Foxo3a regulates *Id1* in erythroid cells is not known. However, *Id1* expression is potently induced upon cytokine stimulation myeloid cells (24), and ectopic expression of Id1 in erythroid cells blocks erythroid differentiation (66, 67).

4.4. Foxo3a may play diverse functions in erythroid cells

Foxo proteins are not only activated upon down-regulation of the PI3K pathway, but also in response to genotoxic stress, oxidative stress, and stimulation of the cAMP pathway (15, 21, 71, 102). Oxidative stress induces Foxo3a acetylation on multiple lysine residues as well as phosphorylation of serine/threonine residues (15), causing differential co-regulator binding (15), and target gene activation (15, 71), which resulted in cell cycle arrest and replicative senescence rather than apoptosis (15). From the studies described in this thesis it can be hypothesized that Foxo3a plays diverse function in erythroid cells as well. This is suggested by the fact that some targets regulate levels of reactive oxygen species (Chapter 4). Whereas genotoxic stress is able to induce Foxo3a activity, other transcription factors induced by genotoxic stress may cooperate with Foxo3a to control a specific set of target genes as demonstrated by the Foxo3a target gene *Btg1*. Genotoxic stress induces *Btg1* expression (25) and we describe it as a Foxo3a target gene. Interestingly, Foxo3a seems to cooperate with the DNA damage inducible cJun/ATF2 complex, which was found to associate with the CRE present close to the DBE in the *Btg1* promoter (Chapter 3). However, further investigation is required to establish the function of Btg1 in response to DNA damage.

It becomes increasingly clear that both factor deprivation and stress factors (DNA damage, oxidative stress) induce Foxo's and that the cellular response to Foxo activation may vary from differentiation to cell cycle arrest, senescence or apoptosis, depending on the stress factor but probably also on the strength of the stress-induced signal (15). Modification and regulation of Foxo's seem to be crucial in the regulation of Foxo-function. Not only PKB, also Casein kinase 1 (CK1), SGK1, IκB Kinase (IKK) and Dyrk1 phosphorylate Foxo's. CK1 and SGK1 induce Foxo phosphorylation in response to genotoxic stress, whereas IKK and Dyrk1a phosphorylate Foxo's independent from the PI3K pathway (28, 43, 87, 119). Furthermore, Foxo3a is also acetylated on multiple lysine residues in response to oxidative stress (15).

In conclusion, these data suggest that Foxo's function at the heart of signaling controlled gene expression and exert multiple functions. Stimulus-specific Foxo protein modification and (subsequent) co-factor binding are fundamental in their selective activation of target genes, determining cell fate. Future investigation is necessary to establish the different roles for Foxo transcription factors in erythroid cells.

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4.5. Cooperation of Foxo3a with co-regulators in regulation of erythropoiesis As indicated above, these different Foxo functions may be mediated by differential activation of gene expression resulting from protein modifications and subsequent distinct co-regulator binding. The importance of Foxo co-regulators is exemplified by a Foxo1 mutant, which lost its DNA-binding capacity. This mutant is still able to mediate gene expression and inhibit cell cycle progression, whereas its ability to induce apoptosis was diminished (81). Nowadays, numerous co-regulators have been identified (9, 106). The ATF1/CREB and ATF2/cJun complexes binding to the CRE in the *Btg1* promoter may comprise novel Foxo co-regulators, suggested by the close proximity of only 7 basepairs between the DBE and CRE (Chapter 3). However, an association between Foxo3a and CREB (or CREM) could not be detected in overexpression studies (not shown). However, an interaction with the CREB family member ATF1 was not tested. The binding of both Foxo3a and CREB/ATF1 to the *Btg1* promoter may enhance the recruitment of other transcriptional components and lead to the cooperative induction of *Btg1* transcription as suggested in Chapter 3. A similar cooperation between Foxo1 proteins and the cAMP pathway was shown on transcriptional activation of the prolactin in human ES cells (21).

The interaction of Foxo transcription factors with nuclear hormone receptors is also of interest in erythropoiesis. Expansion of erythroid progenitors is dependent on glucocorticoids, whereas retinoic acid and thyroid hormone induce differentiation (61). Transient transfection of FKHR into mammalian cells represses transcription mediated by the glucocorticoid and progesterone receptors and stimulates retinoic acid and thyroid hormone receptor-mediated transactivation (121). Thus, activation of Foxo's could suppress GR function and thereby suppress erythroid expansion. However, we observed cooperation on gene transcription between Foxo3a and the GR. The Foxo3a target gene *Btg1* has also been identified as a GR induced gene (58). In addition, other genes such as *Glcci1* and *Zfp36* also showed induction both by Foxo3a and the GR (Figure 4.1). Since glucocorticoids have a growth suppressive function in e.g. macrophages and lymphocytes, cooperation of Foxo's and the GR in the abrogation of cell proliferation in general seems plausible. However, the role of glucocorticoids may be different in erythropoiesis and in granulopoiesis.

The importance of Foxo transcriptional co-regulators was clearly shown by the interaction between Foxo3a and Stat5 (Chapter 4). This interaction was Epo-induced and could be abrogated by co-stimulation with SCF, resulting in a decreased binding of these proteins because of subcellular separation: SCF induced an almost exclusive cytoplasmic localization of Foxo3a, and Epo induced predominantly nuclear localized Stat5. In absence of SCF, Epo stimulation only modestly affects Foxo3a nuclear export and resulted in an enhanced nuclear interaction between Foxo3a and Stat5. A similar mechanism has been observed for Foxo1 and Stat3 in liver cells. Stimulation of HepG2 liver cells with IL-6 induced interaction between Stat3 and Foxo1, which could be reversed by co-stimulation with insulin (60), the main activator of the PI3K pathway in liver cells. Interestingly, Foxo1 and Stat5 did not interact in these cells, suggesting that there may be cell specific interactions between Foxo and Stat proteins. It is most likely that the Foxo3a/Stat5 complex directly binds the *Cited2*

promoter resulting in *Cited2* expression. This is suggested by the findings that *Cited2* transcript levels are strongly increased after 1 hour of growth factor/cytokine stimulation in a variety of cells (95). Notably, this induction depended on activation of the Jak/Stat pathway (95). We observed increased *Cited2* transcript levels after 2 hours of Foxo3a activation in the presence of Epo (Chapter 4). Although we identified a conserved DBE in the *Cited2* promoter, deletion of this site did not alter the cytokine response of the promoter suggesting that another regulatory element is required for activation of the promoter by the Foxo3a/Stat5 complex. This is plausible since Epo does not induce all other identified Foxo targets. This hypothesis is supported by the cytokine induced cooperation between Foxo1/Stat3 on induction of α-2-macroglobulin, an inhibitor of many proteases, including trypsin, thrombin and collagenase (7).Two Stat3 binding sites are essential for the Stat3 response (63). Increasing amounts of Foxo1 did enhance the promoter activity only in the presence of activated Stat3 suggesting that this occurs via Stat3 binding sites (60). Furthermore, optimal transcriptional activation of the α-2-macroglobulin is dependent on the cooperation between Stat3 and the GR. Interestingly, in this cooperation the GR does not bind directly to the DNA whereas Stat3 does (63). The cooperative activation of the promoter was caused by a more efficient recruitment of a large transcriptional complex called the enhanceosome (63). Similarly, Stat5 may bind to the *Cited2* promoter to a yet unidentified element, whereas Foxo3a may act in a DNA-binding independent manner. This may result in the more efficient recruitment of other transcriptional complexes like the enhanceosome. Since Foxo1 did not enhance the transcriptional activity of Stat3 itself, and also did not affect cellular distribution of Stat3 (60), recruitment of other transcriptional components to the promoter is probably the mechanisms via which Foxo and Stat proteins cooperate on transcriptional activation of genes. In support of this hypothesis it will be necessary to identify a Stat5 promoter element in the *Cited2* promoter.

We also noticed a cytoplasmic interaction between Foxo3a and Stat3 or Stat5. Since Foxo1 cellular transport did not alter Stat3 cellular localization in liver cells, it was suggested that this interaction does not affect the transcriptional activity of both proteins and may serve a different function. Notably, large cytosolic protein complexes of 200 - 400 kD and 1 - 2 mD were described in which both non-tyrosine phosphorylated and tyrosine phosphorylated Stat proteins are present (75). The functions of these Statosomes are yet unknown. However our data suggests that Foxo proteins could be part of the Statosome complexes. Possibly, these protein complexes may function as large cytosolic anchoring sites for both Stat and Foxo proteins.

5. Regulation of protein arginine methylation as a novel cellular mechanism that controls erythropoiesis From the data described in this thesis it can be hypothesized that Foxo3a controls erythropoiesis by multiple mechanisms. As described in Chapter 2 and 4, Foxo3a induces genes that stimulate growth arrest and maturation of these cells, and down-regulate growth promoting genes, as outlined in 4.3. Another mechanism via which Foxo3a may control erythropoiesis is via regulation of protein arginine methylation. This is mediated via up-

regulation of *Btg1*. Btg1 is a member of the antiproliferative (Apro) gene family. The Apro family consists of six members that are labile and share an Apro-homology domain consisting of two highly conserved boxes (A and B) separated by a non-conserved spacer region (69). Family members function as regulators of cell growth, differentiation and DNA-repair (69, 101). In addition, Btg1 and its closest homologue Btg2 share an additional conserved domain termed box C (8), suggesting a different function for these proteins. Btg1 and 2 are both expressed in quiescent cells and during the G_1 phase of the cell cycle (83, 85). Overexpression of Btg1 suppresses growth and is negatively correlated with proliferation in a variety of cell types, such as T-lymphocytes, fibroblasts, macrophages, and during testis development (80, 85, 94). However, the mechanisms involved in the antiproliferative effect of this gene family are largely unsolved (69, 101), and can only be implied from their interaction partners.

Btg1 and Btg2 have been shown to interact with the transcription factor carbon catabolite repressor protein (CCR4)-associated factor 1 (Caf-1; 10, 49, 84, 30). This interaction occurs via box-B (85) suggesting that it is a common binding partner among the family members. Caf-1 is part of a transcriptional regulatory complex and is also part of the major cytoplasmic mRNA deadenylase complex in yeast (104). Caf-1 is involved in transcriptional regulation of a variety of targets that regulates chromatin structure, and interacts with the cell cycle regulated protein Dbf2 in yeast (68). Caf-1 also regulates expression of *Rad51*, a gene involved in DNA repair (89). These data suggest an interesting link between the Btg1/CCR4 complex, cell cycle regulation and DNA damage response. Interestingly, both Btg2 (42) and Foxo1 (81) suppress transcription of D-type cyclins. This suggests that Foxo induced growth suppression may in part be performed via Btg1 mediated transcriptional downmodulation of D-type cyclins.

Another Btg1 interacting protein is protein arginine N-methyltransferase (Prmt1). Since the box-C domain is responsible for this interaction (8), Prmt1 only associates to Btg1 and Btg2. Type I arginine N-methyltransferase produces ω-monomethylarginine and asymetric ω-NG, NG-dimethylarginine. Interestingly, Prmt1 null mice die during development (77), and arginine methylation by type I arginine N-methyltransferase is a common post-translational protein modification that seems to be catalyzed predominantly by Prmt1 (38, 77, 96, 97). Btg1 and Btg2 have both been shown to stimulate arginine methylation activity of Prmt1 (64). Although the function of type I protein arginine methylation is unknown, it has been suggested to mediate transcriptional regulation (19), protein shuttling (90), and RNA processing (38). Interestingly, Prmt1 has been shown to associate to the Ifnαr-1 chain of the interferon type 1 receptor and contributes to the growth arrest induced by this receptor via methylation of Stat1 (1, 72). Other targets of Prmt1 are Interleukin-enhanced-binding factor 3 (97), poly-A-binding protein II (5), hnRNP A2 (76), Fibroblast growth factor-2 (56), and histones (70).

We showed that Btg1 suppressed colony formation of murine bone marrow cells, which could be reversed upon deletion of box-C. Therefore, association of Btg1 with Prmt1 seems to be responsible for the Btg1 growth suppression. Prmt1 is expressed throughout the differentiation process, but the expression of Btg1 late in differentiation may activate Prmt1 during terminal

differentiation or recruit it to a different set of target proteins. The observations that (1) arginine methylation of a number of unknown proteins was increased during differentiation and (2) a global inhibitor of methylation blocked erythroid differentiation (Chapter 2), supports a role for Prmt1 during terminal erythroid differentiation. Interestingly, overexpression of the box-C peptide, which inhibits Prmt1, blocked neuronal differentiation in PC12 cells (8), and NGF-induced PC12 differentiation could be inhibited by an global inhibitor of methylation (22). Therefore, Btg1 mediated arginine methylation could serve as a general mechanism that regulates differentiation. This is supported by the finding that Btg1 and Btg2 regulate not only erythroid differentiation but also neuronal (12) and myoblast differentiation (82).

6. Foxo activation *versus* other mechanisms

Inactivation of Foxo3a by PKB presents one mechanism via which the PI3K/PKB pathway controls erythropoiesis. Another mechanism important for the expansion of erythroid progenitors is the activation of the translation machinery. PKB phosphorylates and activates the mTOR kinase, which results in phosphorylation of the translation elongation and initiation factor 4E (eIF4E) binding protein (4EBP) leading to its dissociation from eIF4E (40). The level of free eIF4E is particularly important for the translation of structured mRNAs. Interestingly, inactivation mutations in the C. elegans homologue of mTOR (CeTOR) result in dauer formation (53) similar to what was observed for Daf-16 activation. Activation of the PKB/eIF4E pathway leads to cell growth. This is clearly shown by the role of this pathway in tumorigenesis in various tissues (86, 110, 116). To investigate the role of the PKB mediated translation in erythroid cells, we constructed eIF4E overexpressing I/11 clones. Notably, constitutive eIF4E expression induced erythroid expansion and delayed erythroid differentiaton (M. Blázquez-Domingo and G. Grech, manuscript in preparation). Currently, Epo/SCF-induced translationally regulated targets are under investigation (G. Grech). Interestingly, Drosophila dFOXO induces expression of the translation inhibitor d4EBP (79). However, in erythroid cells, activation of Foxo3a does not affect 4EBP transcription (unpublished observations).

We observed that overexpression of eIF4E abrogates the requirement for PI3K activation during expansion of erythroid progenitors, whereas experiments presented in this thesis argue that PI3K activity is required to suppress activation of Foxo3a. Although one may argue that it should be one or the other mechanism that is important, the data are complementary (Figure 5.5). If PI3K is activated, mRNA translation is promoted and Foxo activity is suppressed. When PI3K activity is abrogated, translation of structured mRNA ceases and the differentiation program is actively induced by enhanced Foxo activity.

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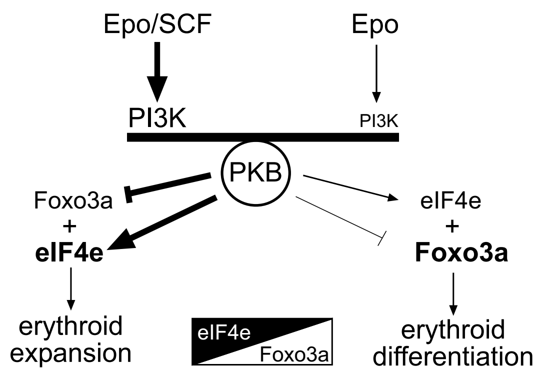

Figure 5.5. **PKB regulates the balance between erythroid expansion and differentiation by control of Foxo3a and eIF4e activity.** Potent activation of PI3K/PKB by Epo/SCF results in an complete Foxo3a inactivation and a strong activation of eukaryotic translation initiation factor 4E (eIF4e). These events support erythroid expansion. Epo induced activation of the PI3K/PKB pathway only results in minor inactivation of Foxo3a (thin arrow) and low activation of eIF4e. The majority of the Foxo3a proteins are activated and accelerate differentiation. The activity of the PI3K/PKB pathway thus controls erythropoiesis. This is obtained via complementary mechanisms of PKB induced eIF4e activation and inhibition of Foxo3a.

7. Future directions

7.1. Foxo transcription factors and cancer

It has been suggested that Foxo proteins may function as tumor suppressor genes, however, up to date, this remains to be established. Despite some interesting observations concerning the identification of *FOXO* genes from translocations involved in tumorigenesis (27, 37) and the association of tumorigenesis and poor breast cancer survival with *FOXO* inactivation (48), their role as tumor suppressor genes is still unclear. So far, *Foxo* knock-out mice have not shown any evidence for increased tumor risk (18, 46). However, this may be caused by complementary functions for the three most homologous Foxo proteins (Foxo1, Foxo3a, Foxo4). Studies on Foxo target genes have revealed the regulation of a common set of targets, suggesting overlap in function of the Foxo members. This could explain why *Foxo4* null mice do not shown any abnormalities (46). The observation that only *Foxo3a* null mice display disturbed erythropoiesis (18) may be due to the fact that Foxo3a, but not Foxo4 or Foxo1, plays a role in terminal erythroid differentiation. Therefore, the contribution of Foxo proteins to cancer has to be tested in double or triple knock out mice, or via simultaneous knock down of their mRNA's using siRNAs. Until then their role in tumorigenesis remains to be established.

7.2. Regulation of Foxo function

Foxo proteins can be activated in response to DNA damage, oxidative stress, growth factor depletion and stimulation of the cAMP pathway (15, 21, 71, 102). So far, several stimulus specific target genes have been identified. Further identification of these stimuli specific targets will help to understand the different cellular functions of Foxo proteins. One way via which Foxo stimuli specific targets may be identified is by performing mRNA profiling experiments to detect the changes in gene expression induced by sublethal DNA damage or oxidative stress, in the absence or presence of the described Foxo3a specific siRNA (Chapter 2). A detailed analysis of these Foxo mediated mechanisms may give

important clues with respect to the relation between oxidative stress, DNA repair and human cancer, as well as other degenerative diseases of aging (4).

Another important question concerning Foxo proteins is what triggers Foxo activation in response to the different Foxo activating stimuli, and which Foxo modifiers may be involved in regulation of target specificity. A powerful screening method that I plan to use in the near future to isolate Foxo modifier genes is the genetic *Drosophila* P-element screen. In this method, transgenic *Drosophila* lines are used in which overexpression of dFOXO is specifically targeted to the eye, which prevents intervention with early development (13), and results in a 'small eye phenotype' (54). Crossing of these flies with an library of 1600 mutant fly lines in which single genes are disrupted by P-element integration (91) will allow the identification of genes that alter the degenerate eye phenotype imposed by dFOXO. Using this method, putative modifiers of dFOXO can be identified and potential Foxo modiers can be rapidly tested that either positively or negatively change Foxo3a function. The Foxo3a transcriptional co-regulator Stat5 may present a co-regulator that alters Foxo function because it may tip Foxo-induced growth arrest towards growth stimulation via transcriptional up-regulation of *Cited2*.

7.3. Foxo transcription factors and regulation of hematopoietic quiescence

A role for Foxo transcription factors in regulation of cellular quiescence was initially suggested from studies in *C. elegans* (see 8.1 of Chapter 1). In these worms, activation of the Foxo transcription Daf16 induces a temporal developmental arrest that results in an increased life span. This is at least partially obtained via lowering of the metabolism (100), and possibly through increased protection against oxidative stress (98). The role of Foxo proteins in regulation of cellular quiescence and protection from oxidative stress has been confirmed in mammalian cells (17) and is underscored by several *in vivo* observations. Foxo3a knock-out mice exhibited ovarian follicle activation that may result from loss of quiescence (18). In addition, *in vivo* overexpression of a dominant negative Foxo1 mutant in the T-cell lineage resulted in increased thymocyte proliferation (62), suggesting a role for Foxo1 in maintaining a quiescent state in resting T-cells (23, 120).

Because quiescence is a characteristic of hematopoietic stem cells, Foxo proteins may be implicated in regulation of stem cell quiescence. For example, the cell cycle inhibitor $p21^{CIP}$ regulates the quiescent state of hematopoietic stem cells. Mice deficient for *$p21^{CIP}$* show increased cycling and exhaustion of hematopoietic stem cells (20). In addition, $p27^{KIP}$ also stimulates the quiescence of hematopoietic stem cells (26, 32). Foxo's may thus contribute to hematopoietic stem cell quiescence by regulating the cell cycle. Strikingly, protection against oxidative stress is also important for hematopoietic stem cell maintenance (50), and may present another mechanism via which Foxo's regulate quiescence of hematopoietic stem cells. To decipher the role of Foxo proteins in hematopoietic stem cell maintenance, bone marrow cells from *Foxo3a*-deficient mice could be used in transplantation experiments.

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7.4. Btg and arginine methylation

Protein arginine methylation is nowadays believed to be a common protein modification that regulates a variety of cellular processes such as signaling, RNA processing and chromatin remodeling and transcription (6, 70). The recent cloning of peptidylarginine deiminase 4 (Pad4), a protein which exhibits arginine-demethylation activity (115), underscores the dynamic character of protein arginine methylation. Since we proposed protein arginine methylation as a novel cellular mechanism regulating the balance between erythroid expansion and differentiation, as also shown in neuronal cells, the identification of arginine methylated proteins will be required to unravel the underlying mechanisms. This may be performed by analysis of arginine methylated proteins, using the antibodies described in this Chapter 2, and comparing conditions with or without Btg1 overexpression. Differentially methylated proteins may be identified using mass spectrometry. Because *Prmt1* deficient mice are lethal (22, 77), erythroid specific *Prmt1* null mice should be constructed to determine whether protein arginine methylation is essential for erythropoiesis.

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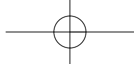

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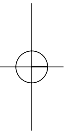
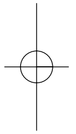
Inleiding en Samenvatting





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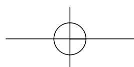

Inleiding op, en overzicht van de behaalde resultaten

Een mens is opgebouwd uit miljarden cellen. Al deze cellen zijn onder te verdelen in verschillende typen cellen. Zo zijn er o.a. spiercellen, hersencellen, huidcellen, botcellen, bloedcellen, en zenuwcellen. Deze verschillende cellen ontstaan uit slechts één enkele embryonale 'stamcel' via een complex proces dat differentiatie wordt genoemd. Een soort gelijk differentiatie proces vindt ook plaats bij het aanmaken van bloedcellen. Vanuit de 'bloedstamcel' kunnen zich rode bloedcellen, bloedplaatjes of witte bloedcellen (de overige bloedcellen) ontwikkelen (Figuur 1). Witte bloedcellen beschermen ons lichaam tegen indringers zoals virussen en bacteriën. Bloedplaatjes zijn noodzakelijk voor het stollen van bloed wanneer er een bloeding optreedt. Rode bloedcellen transporteren zuurstof (O_2) van de longen naar de weefsels, en koolstofdioxide (CO_2) vanuit de weefsels naar de longen. Een andere onderverdeling van bloedcellen kan worden gemaakt op basis van de plaats waar de bloedcel gemaakt wordt. Bloedcellen die uitrijpen in het beenmerg behoren tot de myeloïde reeks, terwijl bloedcellen die uitrijpen in de lymfklieren behoren tot de lymfoïde reeks.

Het proces waarbij de verschillende bloedcellen ontstaan vanuit de bloed-stamcel wordt hemopoïese genoemd. De differentiatie van een stamcel naar de functionele bloedcellen is een proces dat verloopt via zogenaamde voorlopercellen. Naarmate voorlopercellen verder gedifferentieerd zijn kunnen ze minder rijpe bloedcellen produceren. Zo kan een vroege voorloper-rode bloedcel (BFU; Figuur 1) zo'n $5*10^3$ rode bloedcellen produceren, terwijl een meer gedifferentieerde voorloper-rode bloedcel (CFU) nog maar enkele tientallen rode bloedcellen kan produceren. Differentiatie wordt voor een belangrijk deel geregeld door groeifactoren. Sommigen groeifactoren kunnen er voor zorgen dat een voorlopercel zich gaat delen in plaats van te differentiëren. Hierdoor zal het aantal rijpe bloedcellen dat de voorlopercel kan voortbrengen toenemen. Dit proces wordt expansie genoemd. Andere groeifactoren ondersteunen juist alleen de differentiatie van een bepaalde voorlopercel. Op een zelfde manier kan de aanwezigheid van een bepaalde 'groeifactormix' er voor zorgen dat er op een bepaald moment meer rode bloedcellen gemaakt worden, of juist een bepaald type witte bloedcel.

Het doel van dit proefschrift was te onderzoeken of en op welke manier bepaalde eiwitten een rol spelen in de vorming van rode bloedcellen (erythropoïese). Eiwitten spelen een cruciale rol in het functioneren van een cel en kunnen gezien worden als de robotten, of robotonderdelen van de cel. Voor een aantal eiwitten is onderzocht of en hoe zij de balans tussen erythroïde expansie en differentiatie bepalen. Een beter begrip van erythropoïese mogelijk leiden tot een betere behandeling van bepaalde typen anemie (te kort aan rode bloedcellen) en bloedkanker. Voordat ik de resultaten van mijn studie beschrijf wil ik eerst kort uiteenzetten hoe een cel 'opgebouwd' is, hoe eiwitten gemaakt worden en op welke wijze eiwitten de deling van een cel kunnen reguleren.

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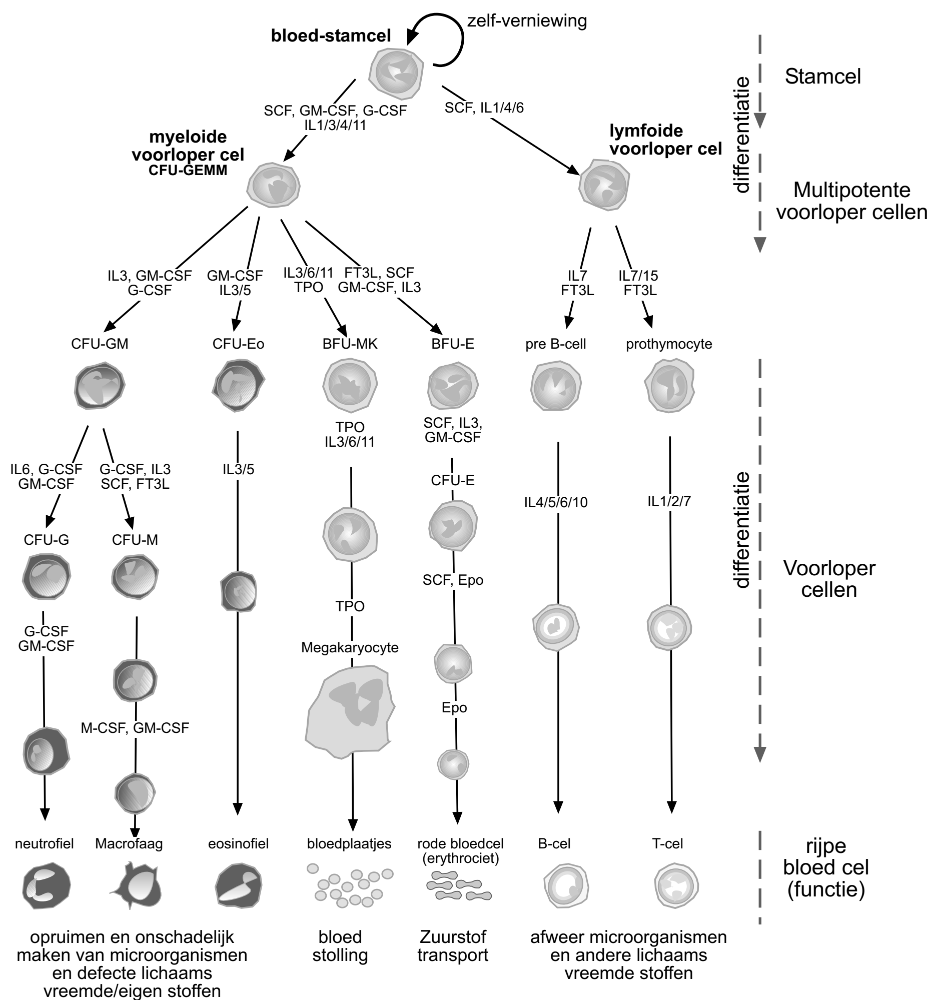
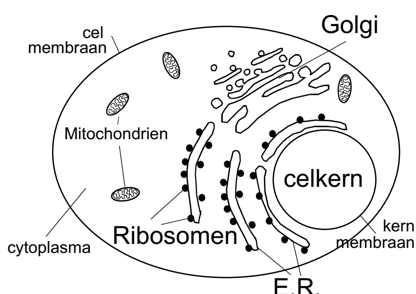


Figuur 1. **Schematische weergaven van de bloedcel vorming (hematopoïese).** Stamcellen zijn zeldzaam en kunnen door deling van de cel of zichzelf dupliceren/vernieuwen, of differentiëren in een myeloïde or lymfoïde voorloper cel. Differentiatie van voorloper cellen kan alleen plaats vinden in de aangegeven richting van de pijlen. Des te verder een voorloper cel in het differentiatieproces gevorderd is, des te minder bloedcellen de voorloper cel kan produceren. Uit een stamcel kunnen zo verschillende typen bloedcellen ontstaan met ieder hun eigen functie. De groei en differentiatie van de hematopoietische voorloper cellen vereist de aanwezigheid van bepaalde groeifactoren zoals aangegeven bij de pijlen. De volledige namen van deze groeifactoren zijn: SCF (Stem cell factor), IL3 (Interleukin 3), FLT3L (FMS-like tyrosine kinase 3 ligand), Epo (erythropoietin), GM-CSF (granulocyte/macrophage-colony stimulating factor), G-CSF (granulocyte -colony stimulating factor), TPO (thrombopoietin). De volledige naam van weergegeven voorloper cellen is: CFU-GEMM (colony forming unit-granulocyte-erythroid-monocyte-megakaryocyte), CFU-Eo(colony forming unit-eosinophilic-granulocyte), BFU-MK (burst forming unit-megakaryocyte), BFU-E (burst forming unit-erythroid). CFU-GM (colony forming unit-granulocyte-macrophage).

Moleculaire mechanismen betrokken bij de regulatie van celgroei

Een cel wordt begrensd door een celmembraan (Figuur 2). In deze membraan bevinden zich kanalen die nodig zijn voor uitwisseling van zouten en water met het medium buiten de cel. Ook bevinden zich in de celmembraan receptoren voor groeifactoren en hormonen die het functioneren van de cel reguleren. In de cel bevinden zich onder andere mitochondriën, dit zijn de energiefabriekjes van de cel. Ribosomen zijn verantwoordelijk voor de productie van eiwitten. Het endoplasmatisch reticulum en Golgi systeem modificeren en sorteren de eiwitten voor gebruik in of buiten de cel. Endozomen transporteren eiwitten van en naar de celmembraan, en in de lysozomen worden eiwitten afgebroken. In de celkern bevindt zich het erfelijk materiaal (DNA) .



Figuur 2. **Schematische weergave van de cel.** De celmembraan vormt een fysieke barrière tussen de omgeving en de cel-inhoud. In het cytoplasma bevinden zich de volgende cel-compartimenten: de celkern (bevat het erfelijk materiaal (DNA)), het endoplasmatisch reticulum met de ribosomen (waar een groot deel van de eiwit synthese plaatsvindt), het Golgi-apparaat (waar de in het ER gesynthetiseerde eiwitten modificaties ondergaan wat gevolg heeft voor hun functie en transport, en de mitochondriën (energie produktie).

Omdat eiwitten slijten, of alleen tijdelijk nodig zijn, moet de productie van eiwitten goed gereguleerd zijn. Eiwitten worden gecodeerd door het DNA (**d**eoxy-**n**ucleïne zuur (**a**cid)) dat zich in de celkern bevindt en ook wel de 'blauwdruk van het leven' wordt genoemd (Figuur 3). DNA bestaat uit twee lange aan elkaar gekoppelde strengen. Deze strengen zijn opgebouwd uit 4 verschillende moleculen die basen worden genoemd: adenine (A), guanine (G), cytosine (C), en thymine (T). Hierbij is een A (of T) uit de ene streng altijd gekoppeld aan een T (of A) uit de andere streng. Hetzelfde geldt voor G en C. Omdat de twee strengen van het DNA een spiraal-vorm hebben wordt de ruimtelijke structuur van het DNA ook wel vergeleken met een wenteltrap waarbij de verbindingen tussen de basen A-T en G-C de treden vormen. Iedere cel van het menselijk lichaam bevat 23 paar chromosomen die opgebouwd zijn uit dit DNA. Een gen is een stuk DNA dat alle informatie bevat voor het maken van een specifiek eiwit. In 2001 werd een eerste ruwe versie van de totale basenvolgorde van het menselijke DNA bekend (alle chromosomen = het genoom), en op dit moment wordt het totale aantal genen van de mens geschat op zo'n 20.000 - 25.000.

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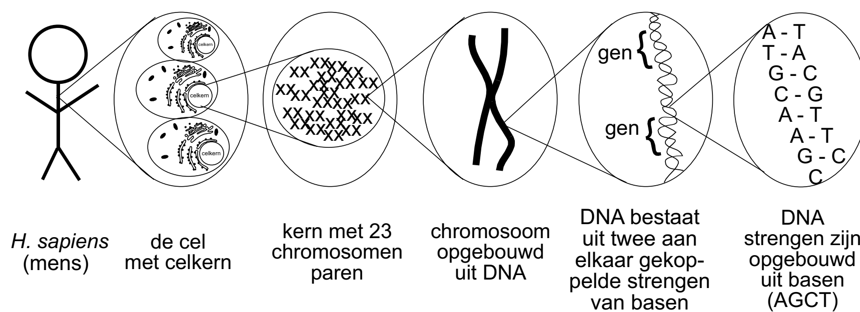
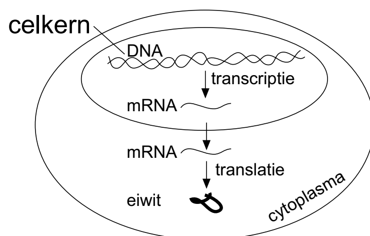


Figuur 3. **Weergave van het DNA**. In iedere cel van het menselijk lichaam bevindt zich in de celkern een kopie van het menselijk genoom (het totaal van alle 46 chromosomen). DNA is opgebouwd uit twee complementaire strengen van aan elkaar geschakelde basen adenine (A), thymine (T), guanine (G), en cytosine (C). Hierbij kunnen uitsluitend de basen A en T een verbinding aangaan (hetzelfde geldt voor G en C). De ruimtelijke structuur van het DNA is een wenteltrap, waarbij de verbindingen tussen de basen (A met T, en G met C) de treden vormen.

Een volgende belangrijke vraag is hoe een gen nu 'vertaald' kan worden in een eiwit. Allereerst wordt er een kopie (mRNA) van het gen gemaakt (Figuur 4). Dit proces heet transcriptie en wordt uitgevoerd door eiwitten genaamd transcriptie factoren. Een complex van transcriptie factoren bindt aan een specifieke bindingsplaats in het DNA die naast het gen ligt (promoter DNA). Het transcriptie complex leest het gen af en maakt een kopie (mRNA) van het gen. Alle gedeelten in het mRNA die geen informatie bevatten worden verwijderd, en het resterende mRNA wordt omgezet in een eiwit. Dit proces heet translatie. Translatie wordt uitgevoerd door de in het cytoplasma gelegen ribosomen. In het translatie-proces codeert iedere set van drie basen (codon) voor een bepaald aminozuur. Zo codeert de basenvolgorde ATG-AAA-TCC- voor een keten van de aminozuren methionine-lysine-serine. In totaal kunnen de vier verschillende basen 64 verschillende codons vormen die coderen voor de 20 verschillende aminozuren. Op deze manier kan de grote verscheidenheid aan genen vertaald worden in een minstens zo grote verscheidenheid aan eiwitten.



Figuur 4. **Schematische weergave van het vertalen van een gen in een eiwit**. Transcriptie factoren maken een kopie van het gen (mRNA) door specifiek te binding aan het promoter DNA van een gen (stuk DNA gelegen vóór het coderende DNA van een gen dat nodig is voor de activativering van transcriptie). Vervolgens wordt het DNA afgelezen waarbij gelijktijdig het mRNA gemaakt wordt (transcriptie). Na synthese wordt het mRNA de kern uit getransporteerd naar het cytoplasma waar het als blauwdruk dient voor de synthese van het eiwit. Dit proces wordt translatie genoemd en wordt uitgevoerd door ribosomen.

Specifieke eiwitsynthese

Zoals gezegd heeft de cel niet alle eiwitten tegelijkertijd nodig. Sommige eiwitten bevorderen de celdeling terwijl andere deze juist remmen. Tijdens de celdeling moeten de eiwitten van de laatste categorie dus niet gemaakt worden. Daarnaast zijn er eiwitten die juist wel of juist niet in een bepaald celtype voorkomen. Zo wordt het zuurstof bindende eiwit hemoglobine uitsluitend in rode bloedcellen

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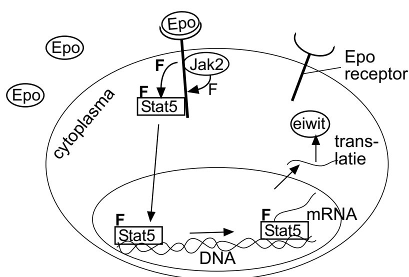
gemaakt. Iedere situatie in ieder celtype vereist zo een specifieke set van eiwitten. Een belangrijke vraag binnen de moleculaire biologie is hoe deze specificiteit in eiwitsynthese gerealiseerd wordt.

Eiwit-synthese is gekoppeld aan de signalen van buiten de cel. Een cel is namelijk niet autonoom maar werkt samen met andere cellen in het uitoefenen van een bepaalde functie in een organisme. Signalen zoals bijvoorbeeld groeifactoren, kunnen binden aan receptoren in de celmembraan. Vanaf de celmembraan wordt het signaal door eiwitten doorgegeven naar de celkern, waar transcriptie factoren de mRNA's maken van de eiwitten die nodig zijn voor dit specifieke signaal. Een cel heeft verschillende signaal routes, waardoor specifieke signalen van buiten de cel kunnen leiden tot de aanmaak van een specifieke set mRNA's.

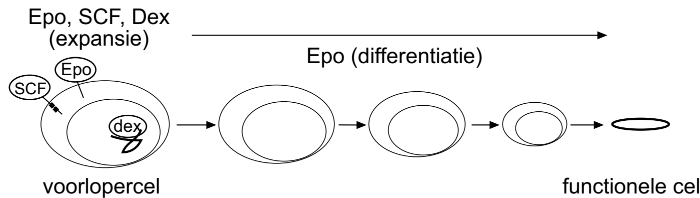
Een voorbeeld van een signaal route is de Epo signaal route. Epo is een groeifactor voor rode bloedcellen (Epo) die verhoogd wordt uitgescheiden door de nieren bij een tekort aan zuurstof in het lichaam. Epo stimuleert de groei van voorloper-rode bloedcellen door aan de in de celmembraan aanwezige Epo-receptor te binden (Figuur 5). Dit resulteert in de 'activatie' van het aan de Epo-receptor gekoppelde kinase-eiwit Jak2, een eiwit dat fosfaatgroepen koppelt aan de receptor (fosforylatie). Aan deze fosfaatgroepen kunnen vervolgens weer allerlei andere eiwitten binden die op hun beurt ook door Jak2 gefosforyleerd worden. Stat5 is een transcriptie factor die op de wijze door Jak2 geactiveerd wordt. Hierdoor wordt Stat5 naar de kern getransporteerd waar het de transcriptie van Stat5 target genen stimuleert.



Figuur 5. **Voorbeeld van signaaltransductie in voorloper-rode bloedcellen (erythroblasten)**. De groeifactor Epo bindt aan zijn receptor, waarna de kinase Jak2 geactiveert wordt om fosfaatgroepen te koppelen (fosforylering) aan de receptor. Hierdoor kunnen er verschillende eiwitten aan de receptor binden waaronder de transcriptie factor Stat5. Stat5 wordt vervolgens ook gefosforyleerd door Jak2. Fosforylering van stat5 leidt tot activering van Stat5 waardoor Stat5 naar de kern getransporteerd wordt en het betrokken is bij transcriptie van specifieke genen (genen die een Stat5-bindingsplaats in de promoter hebben). Op deze manier kunnen signalen van buiten de cel de activiteit en synthese van eiwitten reguleren.

Model en Vraagstelling

Regulatie van rode bloedcel productie (erythropoïese) is afhankelijk van Epo, stamcel factor (SCF) en corticosteroïden (een bepaald type hormoon). SCF bindt en activeert zijn specifieke receptor (c-Kit) zoals Epo bindt aan de Epo-receptor. Corticosteroïden binden een receptor die zich in de cel bevindt en niet aan de membraan. De activering van alledrie de signaal routes (Epo, SCF, Dex) is vereist om erythroblasten (voorloper-rode bloedcellen) tot expansie te aan te zetten (Figuur 6). In de aanwezigheid van alleen Epo rijpen de erythroblasten uit in functionele rode bloedcellen. Ondanks dat de factoren die erythropoïese reguleren bekend zijn, is het grotendeels onduidelijk welke moleculaire mechanismen hierbij betrokken zijn. Het in dit proefschrift beschreven onderzoek heeft tot doel deze mechanismen te identificeren.



Figuur 6. **Regulatie van rode bloedcel vorming**. De expansie van voorloper-rode bloedcellen wordt gestimuleerd door de samenwerking van de groeifactoren: Erythropoietin (Epo), Stamcel factor (SCF), en corticosteroïden (waarvan Dexamethasone (Dex) een synthetische variant is). Als de voorlopercellen in de aanwezigheid van alleen Epo worden gekweekt, differentiëren ze uit tot functionele rode bloedcellen.

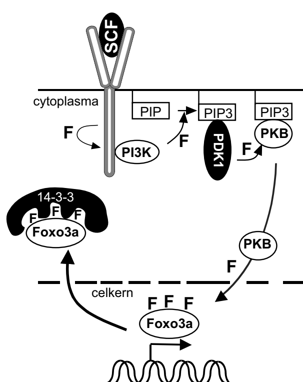
Resultaten

De PI3K signaal route voorkomt erythroïde differentiatie

Twee bekende signaal routes die een rol spelen in erythropoïese zijn de Ras/Raf/MEK/Erk, en de PI3K/PKB signaal routes. Beide worden zowel door Epo als door SCF geactiveerd. De activatie van deze signaal routes door SCF is echter het sterkst. Om de bijdrage van deze signaal routes aan erythropoïese te analyseren is er gebruik gemaakt van moleculen die specifiek de activiteit van de MEK of de PI3K signaal route blokkeren. Toevoeging van deze remmers aan erythroblasten die gekweekt worden in de aanwezigheid van Epo en SCF resulteert voor de Erk remmer in een onderdruking van celdeling, terwijl remming van PI3K niet alleen resulteert in een geremde expansie maar ook in een stimulatie van de differentiatie. De PI3K signaal route is dus een belangrijke route die de blans tussen expansie en differentiatie van rode bloedcellen regelt.

Foxo transcriptie factoren worden door de PI3K/PKB signaal route gereguleerd. In de afwezigheid van Epo en SCF zijn Foxo's actief en bevorderen ze de aanmaak van eiwitten die het delen van een cel remmen. Activatie van de PI3K/PKB signaal route door Epo en SCF resulteert in inactivering van de Foxo transcriptie factoren zoals aangegeven in Figuur 7. Om te bepalen of Foxo's mogelijk een rol spelen in het door PI3K/PKB gereguleerde proces van erythropoïese is de hoeveelheid eiwit van Foxo1, Foxo3a en Foxo4 bepaald in expansie en differentiatie van voorloper cellen. Terwijl de eiwit hoeveelheid van Foxo1 en Foxo4 snel afneemt in differentiatie, neemt het van Foxo3a juist toe (Hoofdstuk 2). Dit suggereert dat de versnelde differentiatie door remming van PI3K mogelijk ten dele wordt veroorzaakt door activatie van Foxo3a. Om te testen of Foxo3a inderdaad de differentiatie van rode bloedcellen kan stimuleren werd er in voorloper-rode bloedcellen een gen ingebracht dat codeert voor een actieve mutant van Foxo3a. Deze mutant kan niet meer door PKB geremd worden. Ook is deze mutant gefuseerd met een gedeelte van de estrogeen receptor (ER) waardoor de Foxo3a mutant (Foxo3a(A3):ER) specifiek te activeren is door toevoeging van 4-hydroxy-tamoxifen (4OHT) aan de cellen. Toevoeging van 4OHT resulteert inderdaad in een door Foxo3a versnelde differentiatie, en een reductie van het aantal celdelingen tijdens de differentiatie (Hoofdstuk 2). Concluderend: inactivatie van Foxo3a door de PI3K/PKB signaal

route is een mechanisme waarmee SCF de differentiatie van erythroblasten uitstelt en daardoor de expansie mogelijk maakt.



Figuur 7. **Activatie van de PI3K signaal route inactiveert de activiteit van Foxo transcriptie factoren**. Binding van Stamcel factor (SCF) aan zijn receptor-kinase (c-Kit) resulteert in de fosforylatie van (en door) de receptor (weergegeven door een 'F'). PI3K bindt aan zo'n receptor-gekoppelde fosfaat groep en wordt vervolgens ook door de receptor gefosforyleerd. Actief PI3K fosforyleert vervolgens moleculen aan de membraan (PIP: fosfo-Inositol-fosfaat moleculen). Aan deze fosfo-moleculen binden o.a. Pdk1 en PKB. PKB wordt vervolgens geactiveerd (gefosforyleerd) door Pdk1 waarna transport naar de celkern plaatsvindt. In de kern fosforyleert PKB de Foxo transcriptie factoren. Deze fosforylering resulteert in de inactivatie van Foxo's door disruptie van DNA binding, transport uit de kern gevolgt door cytoplasmatische verankering aan 14-3-3 eiwitten.

Identificatie van Foxo3a target genen

Vervolgens willen we weten via regulatie van welke genen Foxo3a de rode bloedcel differentiaie stimuleert. Hiervoor hebben we de zogenaamde DNA-chip technologie gebruikt. Op deze chips bevinden zich DNA fragmenten van zo'n 10.000 verschillende genen. We hebben mRNA geïsoleerd uit cellen waarin Foxo3a(A3):ER aanwezig is en waarin deze Foxo3a mutant wel of niet geactiveerd is met 4OHT. Het mRNA uit cellen waarin Foxo3a(A3):ER gedurende 6 uur geactiveerd is, is met een rood fluorescerend molecuul gemarkeerd, het mRNA uit cellen die niet met 4OHT zijn behandeld met een groen fluorescerend molecuul. Het enkelstrengs mRNA bindt op de DNA-chip heel specifiek alleen aan het (enkelstrengs) DNA van het gen waarvan het een kopie is. Door te registreren welke spots op de DNA chip rood, groen of geel fluoresceren, weten we welke mRNA synthese wel of niet door Foxo3a wordt gereguleerd. Op een vergelijkbare manier is ook bepaald van welke genen Epo en SCF de mRNA synthese reguleren. Omdat Epo en SCF de PI3K signaal route activeren, en dus Foxo's inactiveren (Figuur 7), zouden genen waarvan Foxo3a de mRNA synthese activeert 'uitgezet' moeten worden na behandeling van de cellen met Epo en SCF (en *vice versa*).

Btg1

Er werd inderdaad een serie genen gevonden waarvan Foxo3a de transcriptie stimuleert en Epo en SCF de transcriptie remmen. Tot deze groep behoren *Btg1*, *p27^{KIP}*, *Ulk-1*, en *Cyclin-G2*). De hoeveelheid mRNA's van deze genen neemt sterk toe tijdens differentiatie van rode bloedcellen, overeenkomend met de verhoogde Foxo3a activiteit tijdens differentiatie. Van een aantal van deze eiwitten is bekend dat ze de celdeling remmen. Remming van celdeling is dus waarschijnlijk een belangrijke mechanisme waarmee Foxo3a de differentiatie van rode bloedcellen stimuleert. Van deze geïdentificeerde Foxo3a target genen is *Btg1* is uitgekozen voor verdere analyse.

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Allereerst is onderzocht of Foxo3a direct aan het promoter-DNA van Btg1 bindt. Het is bekend dat Foxo3a specifiek bindt aan de basenvolgorde TTGTTTAC. Deze basenvolgorde was ook aanwezig in *Btg1* promoter. Deletie van deze bindingsplaats is voldoende om transcriptie van *Btg1* door Foxo3a te voorkomen (Hoofdstuk 2). Behalve een Foxo3a bindingsplaats, bevat de *Btg1* promoter ook een bindingsplaats voor transcriptie factoren van de CREB familie. Inderdaad kon binding van de transcriptie factoren CREB/ATF1 aan de *Btg1* promoter worden aangetoond (Hoofdstuk 3). Transcriptie factoren van de CREB familie worden geactiveerd via de cyclisch-AMP (cAMP) signaal route: binding van hormonen aan zogenaamde G-gekoppelde receptoren zorgt voor toename van de stof cAMP in de cel. Deze toename van cAMP activeert de kinase PKA, die naar de kern getransporteerd wordt en door fosforylatie het CREB/ATF1 transcriptie complex activeert. Om te onderzoeken of cAMP bijdraagt aan transcriptie van *Btg1* in differentiatie is eerst de activiteit van de cAMP route in differentiatie getest. Dit is gedaan door de hoeveelheid cAMP en gefosforyleerd CREB/ATF1 te meten. Beide namen toe in differentiatie, gelijktijdig met de toename van het Btg1 mRNA (Hoofdstuk 3). Stimulatie van erythroblasten met cAMP bleek inderdaad de transcriptie van *Btg1* te stimuleren. Mogelijk leidt stimulatie van de cAMP signaal route tot een versnelde differentiatie via transcriptie van Btg1, zoals voor Foxo3a en Btg1 wordt verondersteld. Omdat verondersteld wordt dat Foxo3a de differentiatie versneld via transcriptie van *Btg1*, is hetzelfde te verwachten voor de cAMP signaal route. Echter, stimulatie van alleen de cAMP signaal route resulteerde in een nauwelijks toegenomen differentiatie, terwijl activatie van de cAMP route wel de Foxo3a versnelde differentiatie stimuleerde (Hoofdstuk 3). Foxo3a en de cAMP signaal route werken dus samen in het stimuleren van rode bloedcel differentiatie, waarschijnlijk via gezamenlijke stimulatie *Btg1* mRNA synthese.

Btg1 is ook wel bekend als anti-proliferatief eiwit 2 (Apro2). Om het effect van Btg1 op rode bloedcel-vorming te testen is het Btg1 gen ingebracht in muize-beenmergcellen. De ontwikkeling van deze cellen is vervolgens getest in een zogenaamde kolonie-assay waarmee de groei van voorlopercellen te bestuderen is. Terwijl zich in een controle experiment kolonies van rode bloedcellen ontwikkelen, is deze kolonie-vorming sterk geremd in de aanwezigheid van Btg1 (Hoofdstuk 2). Van Btg1 is verder bekend dat het arginine-methylering van eiwitten stimuleert (het koppelen van methyl groepen aan het amonizuur arginine in eiwitten). Dit gebeurt door binding van Btg1 aan, en stimulatie van de arginine-methylase Prmt1. Een Btg1 mutant die niet instaat is om Prmt1 te binden vertoont de reductie in kolonie-vorming van rode bloedcellen niet (Hoofdstuk 2). Regulatie van eiwit methylering door Btg1 speelt dus een belangrijke rol in de differentiatie van rode bloedcellen. Omdat de totale Btg1 activiteit sterk verhoogd is tijdens de differentiatie, is er met een antilichaam dat arginine-gemethyleerde eiwitten herkent gekeken of er een toename is in de methylering van eiwtten. Een aantal eiwitten waarvan de identiteit nog onbekend is vertoonen inderdaad een toenemende arginine-methylatie in differentiatie (Hoofdstuk 2). Toekomstig onderzoek moet uitwijzen welke eiwitten dit zijn.

Cited2

In tegenstelling tot Btg1 behoren *Cited2*, *Decorin* en *Sestrin* tot een groep genen waarvan de mRNA synthese wel door Foxo3a gestimuleerd wordt, maar die niet of nauwelijks geremd wordt door Epo en SCF (Hoofdstuk 4). Blijkbaar activeert Foxo3a deze genen in respons op een andere stress-factor. Uit ander onderzoek bleek inderdaad dat Foxo's niet alleen geactiveerd worden in de afwezigheid van groeifactoren, maar ook door het optreden van DNA schade en oxidatieve stress (schade aan cellen door bij-effecten van zuurstof). Inderdaad bevat de Cited2 groep van Foxo3a targets een aantal genen waarvan bekend is dat ze een rol spelen in de bescherming tegen oxidatieve stress (Hoofdstuk 4). De regulatie van deze genen door Foxo3a in respons op deze stress-factor zal echter nog bewezen moeten worden.

Een andere opmerkelijke observatie was dat de mRNA synthese van weer een ander aantal andere genen binnen de Cited2 groep gestimuleerd wordt door Epo. Deze observatie is bovendien opmerkelijk omdat Epo Foxo's remt door activatie van PI3K/PKB signaal route. Echter, we hebben aangetoond dat deze remming niet totaal is zoals dit bij SCF wel het geval is. Dit heeft tot gevolg dat een gedeelte van de Foxo3a eiwitten nog steeds actief is in de aanwezigheid van Epo (Hoofdstuk 4). Van *Cited2* was verder bekend dat de transcriptie door verschillende hemopoïetische groeifactoren gestimuleerd wordt via activatie van Stat transcriptie factoren. Foxo3a en Stat5, de Stat transcriptie factor die door Epo gestimuleerd wordt in erythroïde cellen (Figuur 5), werken dus mogelijk samen in de transcriptie van *Cited2*. We konden inderdaad aantonen dat Foxo3a en Stat5 een gezamenlijk transcriptioneel complex vormen na behandeling van de cellen met Epo (Hoofdstuk 4). Ook werd aangetoond dat gelijktijdige activatie van Stat5 en Foxo3a een maximale transcriptie geeft van het *Cited2* gen (Hoofdstuk 4). Zowel Epo behandeling van voorloper-rode bloedcellen als activatie van Foxo3a, laten binnen korte tijd (2 uur) een verhoogde mRNA synthese van *Cited2* zien. Daarom denken we dat het Stat5/Foxo3a complex de mRNA synthese van *Cited2* stimuleert door direct aan de *Cited2* promoter te binden. Verder onderzoek moet uitwijzen of deze hypothese juist is en welk DNA element in de *Cited2* promoter hierbij betrokken is.

Conclusies

De PI3K/PKB/Foxo3a signaal route speelt een belangrijke rol in de regulatie van de balans tussen expansie en differentiatie van voorloper-rode bloedcellen. Tijdens expansie wordt Foxo3a geïnactiveerd door maximale activatie van de PI3K/PKB signaal route door de aanwezigheid van Epo en SCF. Tijdens differentiatie neemt de activiteit van de PI3K/PKB route af doordat alleen Epo aanwezig is. Dit heeft tot gevolg dat Foxo3a geactiveerd wordt. Foxo3a versnelt vervolgens de differentiatie door stimulatie van eiwit methylering via transcriptie van *Btg1*, maar ook door het remmen van de celdeling via transcriptie van *$p27^{KIP}$* en *Cyclin G2*. Ook is er bewijs gevonden dat Foxo3a transcriptie van een aantal genen stimuleert in samenwerking met Stat5. Dit gebeurt in de aanwezigheid van Epo en is aangetoond voor het Foxo3a/Sta5 target gen *Cited2*. De functionele rol van de transcriptionele cooperatie van Foxo3a en Stat5 is echter nog onbekend.

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Verklarende woordenlijst

Hemopoïese	ontstaan van alle bloedcellen uit de bloed stamcel
Erythropoïese	ontstaan van rode bloedcellen uit de voorloper-rode bloedcel
Erythroblast	voorloper-rode bloedcel
Differentiëren	uitrijpen van een voorlopercel (uiteindelijk in een functionele bloedcel)
Erythroïde expansie	vermeerdering van voorloper-rode bloedcellen
Epo (Erythropoïetin)	groeifactor specifiek voor de rode bloedcellen
SCF (Stamcel factor)	algemene groeifactor, ook voor rode bloedcellen
Corticosteroïden	bepaald type hormoon waarvan bekend is dat het de expansie van erythroblasten stimuleren
Ribosomen	eiwitcomplex dat zorgt voor de synthese van eiwitten
Cytoplasma	cel-inhoud (zonder de celkern)
Genoom	al het DNA in de celkern
DNA	erfelijk materiaal, aanwezig in de celkern
Basen	Bouwstenen van het DNA (ATGC)
Gen	DNA dat informatie bevat voor een eiwit
promoter DNA	DNA gelegen vóór het coderende DNA van een gen (nodig voor de activativering van transcriptie)
mRNA	kopie van gen dat nodig is voor de synthese van het eiwit
Transcriptie	kopiëren van gen in mRNA
Transcriptiefactor	eiwit betrokken bij de transcriptie van een gen
Translatie	Synthetiseren van een specifiek eiwit aan de hand van een specifiek mRNA
Kinase	eiwit dat fosfaatgroepen koppelt aan andere eiwitten
Fosforylatie	het proces waarbij een kinase een fosfaatgroep koppelt aan een ander eiwit
Receptor	eiwit dat door de celmembraan heen, contact heeft met de omgeving buiten de cel
Signaal route	het omzetten van een signaal afkomstig van buiten de cel, in een cellulaire respons (signaal wordt doorgegeven m.b.v. eiwitten).
Btg1 (cursief)	hiermee wordt het *Btg1 gen* bedoeld
Btg1 (niet-cursief)	hiermee wordt het Btg1 eiwit bedoeld

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Abbreviations

1 h	1 hour
1'	one minute
1"	1 second
4OHT	4-hydroxy-tamoxifen
aa	amino acids
Apro2	Antiproliferative protein 2
ATF1	Activating transcription factor 1
BFU_E	Burst forming unit-erythroid
bp	base pair
Btg1	B-cell translocation gene 1
cAMP	Cyclic-adenosine-monophosphate
CBP	CREB-binding protein
CFU_E	Colony forming unit-erythroid
CFU_S	Colony forming Unit-spleen assay
Cited2	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2
CREB	Cyclic AMP response element binding protein
Dex	Dexamethasone
DNA	Deoxyribonucleic acid
eIF4E	Eukaryotic initiation factor 4E
Epo	Erythropoietin
EpoR	Epo-receptor
Foxo	Forkhead transcription factors subclass O
Gab-1	Grb-2 associated binding protein-1
Jak	Janus kinase
kD	kilo dalton
MTA	5'-deoxy-5'-methylthioadenosine
PDK1	Phosphoinositide-dependent kinase-1
PH	pleckstrin homology
PI3K	phosphatidylinositol-3-kinase
PIP3	PI-3,4,5-phosphate
PKA	Protein kinase A
PKB	Protein kinase B
PTEN	Phosphatase and tensin homolog
SCF	Stem cell factor
SD	standard deviation
SH2	Src homology 2
SHIP	SH2-domain-containing inositol phosphatases
SOCS	Suppressor of cytokine signaling
Stat	Signal transducer and activator of transcription
wt	wild-type

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Curriculum vitae

The author of this thesis was born on the 4th of February 1976 in Delft, The Netherlands. In 1993 he graduated from high school (HAVO) at the "Gereformeerde Scholengemeenschap" in Rotterdam. The same year he started his training as a technician in biotechnology at the Hoger Laboratorium Onderwijs in Delft. He received his degree in 1998 after completing a graduation project on biochemical analysis of cross talk between the Rac and Rho pathways. This research was performed under the supervision of Dr. Frank van Leeuwen in the group of John Collard at the Netherlands Cancer Institute. In 1997 he joined the post-HBO program for Biology at the University of Leiden were he specialized on Molecular/Cellular Biology. During this period, he constructed and analyzed a heme-deficient yeast strain with the aim to screen for anaerobic regulated genes in the yeast *Saccharomyces cerevisiae* under supervision of Drs. José ter Linde in the lab of Dr.ir. Yde Steensma at the Institute of Molecular Plant Sciences, University of Leiden. Another research study involved the isolation of Manganese oxidizing proteins, a project supervised by Drs. Geert-Jan Brouwers in the lab of Prof.dr. Peter Westbroek at the Gorlaeus Laboratory, section of Geobiochemistry, at the University of Leiden. After obtaining his master of science degree in Molecular/Cellular Biology at the University of Leiden, he continued to work at the Geobiochemistry section were he participated in a project funded by industry to perform large scale isolation of iron-oxidizing protein complexes from the fresh-water bacterium *Leptothrix discophora*. This project was performed under supervision of Dr. Elizabeth de Vrind-de Jong. In arpil 2000 he started the research described in this thesis. This research was performed at the department of Hematology of the Erasmus Medical Center in the lab of Prof.dr. Bob Löwenberg and supervised by Dr. Marieke von Lindern.

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Bakker WJ, Blazquez-Domingo M, Kolbus A, Besooyen J, Steinlein P, Beug H, Coffer PJ, Lowenberg B, Von Lindern M, Van Dijk TB. Foxo3a regulates erythroid differentiation and induces BTG1, an activator of protein arginine methyl transferase 1. J Cell Biol. 2004;164:175-184

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