Dynamic regulation of GATA transcription factors in hematopoiesis

Dynamische regulatie van GATA transcriptie factoren in hematopoïese

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 besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op
woensdag 27 oktober 2004 om 15.45 uur

door

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geboren te Coimbra (Portugal)

Promotiecommissie

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Cover: GATA (which means"female cat" in Portuguese and other languages) chasing a laboratory mouse on a DNA strand.

Cover design: Lucas Spensberger.

The work presented in this thesis was performed at the Department of Cell Biology at the Erasmus University Medical Center Rotterdam. This department is part of the Medisch Genetich Centrum Zuid-West Nederland.
The research was partially supported by the Nederland Organisatie voor Weterschappelijk Onderzoek (NWO) and the Nederlandse Kankerbestrijding Koningin Wilhermina Fonds (KWF).

Ao meu pai.

Contents

Scope of this thesis

Hematopoiesis, the process of generation of all mature blood lineages from a single hematopoietic stem cell, occurs by a succession of lineage-commitment steps. Each lineage-commitment step involves a restriction of differentiation potential and the establishment of lineage-specific expression profiles. These expression profiles rely on lineage-specific transcription factors to modulate the expression of their target genes. Therefore, hematopoiesis is an excellent model system to investigate how particular transcription factors influence the establishment of lineage-specific expression profiles and how their activity is regulated.

This thesis is focused on the study of transcription factors GATA1, GATA2 and GATA3, which are very similar to each other but have distinct expression profiles during hematopoiesis. Making use of transgenic mouse models where the expression of these genes was altered, the studies presented in this thesis were aimed to understand the importance of the correct spatio-temporal regulation of these genes for the differentiation of different hematopoietic lineages.

Chapter 1 gives an overview of the current knowledge about the general transcriptional control of hematopoiesis and what is known about the regulation and function of the transcription factors GATA1, GATA2 and GATA3.

In Chapter 2, evidence is shown that GATA1 expression has to be downregulated in the final stages of erythroid development for terminal differentiation to occur. When that fails to occur, erythropoiesis is impaired. Consequently, overexpression of GATA1 in transgenic mice leads to severe anemia resulting in embryonic lethality.

In Chapter 3 cDNA microarray technology was employed to analyse the expression profile of the GATA1 overexpressing mice (described in Chapter 2) in order to identify putative GATA1 target genes.

Hematopoietic GATA proteins are very similar and have partially overlapping expression patterns, suggesting that they may have redundant functions. In Chapter 4 we have used the rescue of a Gata1 *null* mutation by GATA1, GATA2 and GATA3 transgenes under the control of different promoters to demonstrate the importance of timing of GATA factor expression during erythroid development. GATA1, GATA2 and GATA3 expressed under the control of GATA1 regulatory sequences are able to rescue the Gata1 *null* phenotype but fail to do so when expressed under the control of the β-globin regulatory sequences.

Chapters 5 and 6 show the consequences of forced expression of GATA3 throughout T-cell differentiation. GATA3 overexpression in transgenic mice is shown to inhibit the maturation of CD8 single-positive cells and Th1 differentiation as well as induce thymic lymphomas.

In the last Chapter, the results presented in this thesis are briefly discussed, as well as the future prospect of the research on the regulation of GATA transcription factors in hematopoiesis.

Chapter 1

Introduction

Parts of this chapter have been submitted for publication

1. Hematopoiesis

The blood is one of the most fascinating tissues not only by its unique structure but also by its variety of cells. Each cell type has a distinct appearance and performs specific and essential biological functions[6,24,99].

The most abundant cells in the blood are the erythrocytes, occupying about 45% of its volume. Erythrocytes are enucleated biconcave discoid cells, filled with hemoglobin, responsible for the transport of oxygen and carbon dioxide throughout the body. The other hematopoietic cell types are less abundant, occupying about 1% of the blood volume, but performing equally important biological functions. Platelets are rod-shaped cell fragments, derived from large cells denominated megakaryocytes, with an essential role in coagulation. Lymphocytes, subdivided in B- and T-lymphocytes, play a crucial role in the specific immune response against microorganisms and foreign macromolecules. T-lymphocytes (or T-cells) act as direct killers of infected cells (cytotoxic T-cells) or by activating other cells to eliminate infected cells (T-helper cells). B-lymphocytes (or B-cells) produce soluble antibodies that can neutralise and promote the destruction of microorganisms or toxins. Granulocytes, subdivided in neutrophils, eosinophils and basophils are part of the innate immune system and involved in the inflammatory response and phagocytosis. Despite their hematopoietic origin, other cells such as macrophages and mast cells, which are also involved in the inflammatory response and phagocytosis, are not found in the circulation but within tissues. Macrophages arise from precursors denominated monocytes, which are circulating in the blood before they migrate to the tissues where they terminally differentiate. Mast cells originate from as yet unidentified progenitors in the bone marrow that migrate through the blood into mucosal tissues where they differentiate into mature mast cells.

Remarkably, all these different cells originate from a common cell type, the hematopoietic stem cell (HSC), by a highly complex process termed hematopoiesis (Fig. 1).

HSCs are characterised by the ability to duplicate themselves (self-renewal) and to give rise to all the different hematopoietic cell types (pluripotency)[124]. These cells are extremely rare, with an incidence of 1 to 10 HSCs per 100.000 cells in the murine bone marrow[3]. This small pool of HSCs is maintained because these cells can self-renew. In contrast, most of the descendant cells will lose this ability and commit to differentiation, becoming multilineage precursors (MLP).

The differentiation process occurs through a series of commitment steps, each leading to further restriction to a certain hematopoietic cell lineage. The nature of the first lineage commitment step remains a controversial issue since currently two models are proposed[106,195]. The most wildly accepted model claims that the first commitment step separates lymphoid from myeloid potential and is supported by the identification of common lymphoid progenitors (CLPs) and common myeloid precursor (CMPs). CLPs are restricted to the lymphoid lineage and can give rise exclusively to B, T-cells and natural killer (NK) cells[110] while CMP can give rise to granulocytes, erythrocytes, megakaryocytes and macrophages in *in vitro* colony assays[4,102]. These CMPs will undergo further lineage restriction when the granulocyte/monocyte potential is separated from the erythroid/megakaryocyte potential[4]. The existence of CLP is contested by the identification of myeloid/T-cell (MTP) and Myeloid/B-cell (MBP) bipotent precursors by Kawamoto and colleagues[107]. These suggested an alternative model in which myeloid (granulocyte/macrophage) potential is maintained in the early commitment stages of

all hematopoietic lineages. According to this model MLPs can give rise to CMPs or to common myeloid lymphoid precursors (CMLPs). CMLPs further commit into either MBPs or MTPs. Later on the myeloid potential is separated from the megakaryocytic/erythroid and the B-cell and T-cell potential (reviewed by Katsura[106]).

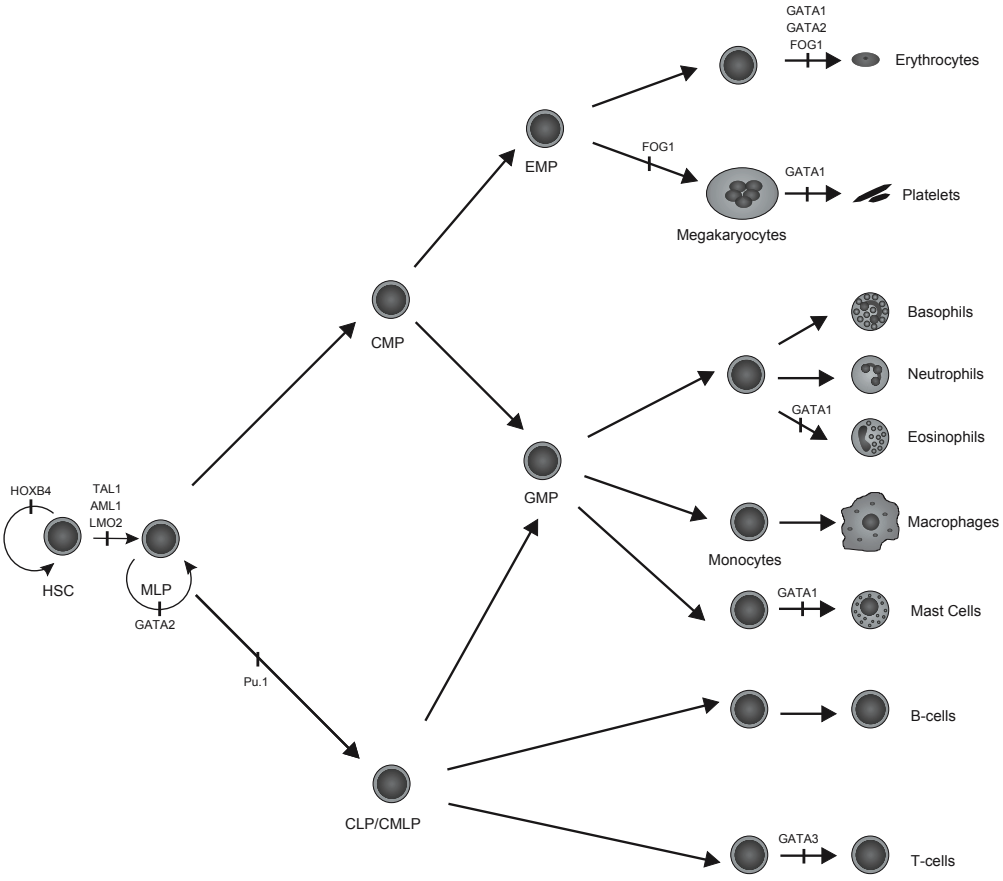

Figure 1 - Schematic representation of the main lineage commitment steps in hematopoiesis. The hematopoietic stem cell (HSC) is the basis of the hematopoietic hierarchy and give rise to multilineage progenitors (MLP), which can differentiate into all the hematopoietic lineages. MLP become lineage restricted to the lymphoid and myeloid lineage in the common lymphoid progenitor (CLP) and common myeloid progenitor (CMP) respectively. Common lymphoid progenitor can give rise exclusively to B and T-cells while CMP can give rise to megakaryocytes/erythroid progenitors (EMP) and granulocyte/monocyte progenitors (GMP). Alternatively, it is also believed that the first lineage-commitment separates myeloid and erythroid potential, in the CMP, from myeloid lymphoid potential, in common myeloid lymphoid progenitor (CMLP). CMLP can that further differentiate in B-cells, T-cells and GMP (dashed line). MEP can differentiate into erythrocytes and platelets while GMP can differentiate into granulocytes and macrophages. Hematopoietic transcription factors relevant for the development of particular hematopoietic lineages are indicated.

CMPs can further commit into either granulocyte/macrophage lineage-restricted progenitors (GMPs), also denominated CFU-GM, that give rise exclusively to granulocytes and macrophages or megakaryocyte/erythrocyte lineage-restricted progenitors (MEPs), also named CFU-MeE, which are restricted to the production of megakaryocytes and erythrocytes[4]. Each of these steps also contributes to the amplification of cell numbers since cell proliferation occurs at each stage of commitment[24]. In this way, a single HSC can be the source of a large number of mature hematopoietic cells.

During the last few years, however, the idea of this rigid hierarchy between the HSC and the different hematopoietic cells has been questioned. An increasing number of reports suggests the occurrence of transdifferentiation between different hematopoietic precursors (reviewed by Graf[66]).

How the decision to commit to a particular cell lineage is achieved is still a matter of speculation. Two contrasting models have been put forward to explain why and how a commitment step occurs at a given time. The instructive model states that a specific or combination of specific exogenous signals determines a commitment step. These signals are most likely ligands that interact with specific receptors in the cell, activating pathways that lead to the expression of genes specific for the subsequent commitment stage. This model reinforces the importance of the stroma in differentiation. The probabilistic model proposes that the critical gene expression changes leading to differentiation occur in a stochastic manner and the role of the stroma is only to provide a selective environment for the growth of the committed progenitor cells[39,52].

Recent reports favour the second model. In one report[5], oligonucleotide microarray technology was employed for gene expression profiling of HSCs, multipotent percursors and the more restricted CLPs and CMPs. This study revealed that both non-hematopoietic and hematopoietic lineage-restricted genes are expressed in HSCs but their numbers decrease throughout lineage commitment since CMPs and CLPs selectively express myeloid and lymphoid-specific genes, respectively. Another report[43] shows evidence for a stochastic activation of both α and β globin clusters. Upon stochastic activation the expression patterns are fixed and clonally maintained in differentiated cells.

Several different tissues are responsible for the production of hematopoietic cells during embryonic development (reviewed by Dzierzak and colleagues[49]).

The yolk sac is the first hematopoietic tissue in mammalian ontogeny and it is responsible for what is known as primitive hematopoiesis. This first wave of hematopoiesis consists mainly, but not exclusively, of the production of large, nucleated, primitive erythrocytes that synthesize embryonic globins. Primitive hematopoiesis starts between embryonic day 7.0 (E7.0) and E7.5 in mice, or day 15 and 18 in humans, in the blood islands that emerge from the mesoderm in the visceral yolk sac. Blood islands are masses of cells that gradually differentiate into primitive erythroblasts surrounded by visceral endoderm and mesoderm cells. These primitive erythroblasts enter the vascular system of the embryo proper where they continue to divide for several days, eventually reaching terminal differentiation characterised by G1 arrest. Hematopoiesis in the yolk sac continues until approximately E13 but primitive erythrocytes can be observed in the blood stream up to E16 (reviewed by and colleagues[182]).

Definitive hematopoiesis relies on the definitive HSC and is responsible for the production of all hematopoietic lineages present in the adult organism including the definitive

erythrocytes, characterised by the absence of nucleus and expression of adult globins. The embryonic origin of definitive hematopoiesis is a controversial issue since currently two very distinct models are proposed.

The first model proposes that definitive hematopoiesis is established by HSCs that have migrated from the yolk sac and colonise the Aorta-Gonads-Mesonephros (AGM) region and the fetal liver[153,181] (reviewed by and colleagues[182]). The second and more recent model proposes that definitive hematopoiesis relies on HSCs originated within the AGM region of the embryo, around E9.0, that later colonise the fetal liver [141,142,157] (Palis and Yoder[50]). In the past, studies using avian[46] and amphibian[232] embryos have identified an intra-embryonic source of definitive HSCs. This intra-embryonic hematopoietic source was mapped to the region containing the dorsal aorta but it was considered to be a feature of lower vertebrates, lost in mammals. Only years later the same intra-embryonic source of definitive HSCs was identified in the AGM region of the murine embryo[142].

Regardless of the controversy around its origins, it is generally accepted that definitive HSCs colonise the fetal liver which, by E11.5, becomes the principal hematopoietic tissue of the embryo[51,157]. Later in gestation the thymus and spleen are formed and colonised by hematopoietic progenitors involved in the production of differentiated lymphoid cells in fetal and adult stages. A recent report[10] suggests that the placenta is another major hematopoietic organ during embryonic development. Around birth, the HSCs migrate from the fetal liver to the bone marrow, which becomes the principal hematopoietic tissue throughout adult life. In humans, the bone marrow is the exclusive site of postnatal hematopoiesis, whereas in mice hematopoiesis also occurs in the spleen[24].

1.1 Erythropoiesis

The generation of mature erythrocytes is an important, and therefore tightly controlled process, since the amount of these cells in the body must remain fairly constant (approximately 45% of the blood volume) to guarantee the supply of oxygen to the body tissues. The average lifespan of an erythrocyte is 60 days in mice and 120 days in humans, which implies that new cells must be constantly produced.

Like all other cells in the hematopoietic system, erythrocytes descend from the HSC. This process, denominated erythropoiesis, involves a great variety of cells at different stages of differentiation between the HSC and the mature erythrocyte (Fig. 2).

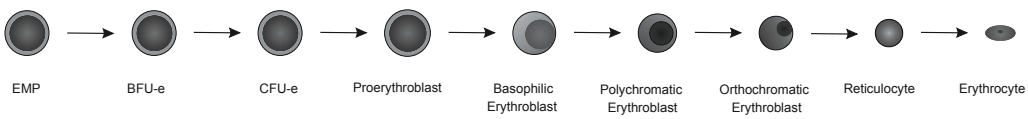

Figure 2 - Schematic representation of the different stages in erythroid differentiation. Erythroid/megakaryocyte progenitors (MEP) can give rise to both erythrocytes and megakaryocytes. Burst forming units- erythroid (BFU-e) and colony forming units-erythroid (CFU-e) are erythroid restricted progenitors that can only be identified via functional assays. Terminal erythroid differentiation starts with the proerythroblast, the first morphologically identifiable progenitor, and terminates upon enucleation.

As mentioned above, commitment to the erythroid lineage involves several steps. The first step consists of the differentiation of the HSC into a MLP, also called CFU-S, which is a pluripotent cell but without the capacity to self-renew. CMPs are derived from this MLP and can give rise to MEPs, which are fully restricted to the megakaryocytic and erythroid lineages. The Burst Forming Unit-Erythroid (BFU-E) is the most primitive erythroid-restricted progenitor, identifiable exclusively by functional assays[256]. In the presence of erythropoietin (Epo), interleukin 3 (IL3), granulocyte-macrophage colony-stimulating factor (GM-CSF), thrombopoietin and stem cell factor (SCF), this cell can give rise to large colonies containing more than 500 hemoglobinized erythroblasts after 5 to 7 or 14 to 16 days of culture of murine and human cells respectively. The BFU-E further differentiates into the Colony Forming Unit-Erythroid (CFU-E), a more mature erythroid progenitor closely related to the proerythroblast. In the presence of low concentrations of Epo, CFU-Es give rise to small colonies, containing 8 to 64 hemoglobinized erythroblasts[215,256] after 2 to 4 or 5 to 8 days of culture of murine and human cells, respectively.

The proerythroblast, derived from the CFU-E, is the earliest morphologically identifiable erythroid progenitor. The proerythroblast is a large cell (14-19μm and 11-13μm in diameter in human and mice respectively) characterised by a large nucleus (occupying about 80% of the cell), with visible nucleoli, surrounded by a basophilic cytoplasm. Basophilic erythroblasts are slightly smaller cells (12-17μm and 10-11μm in diameter, in human and mice respectively) where condensation of chromatin begins to occur, giving the nucleus a granular appearance. The cytoplasm is very basophilic due to the presence of large numbers of ribosomes. Hemoglobin can be detected for the first time by cytological staining methods at the polychromatophilic erythroblast stage. These cells are smaller (12-15μm and 9-10μm in diameter, in human and mice respectively) as are their nuclei. Full hemoglobinization occurs at the orthochromatic erythroblast stage. These cells are the smallest nucleated erythrocyte precursors (8-12μm and 7-8μm in diameter in humans and mice respectively) and their nuclei undergo pycnotic degeneration, that is, the chromatin becomes very condensed and the nuclei shrink. Finally the nucleus is extruded from the cell that is now denominated reticulocyte. Reticulocytes are larger than fully mature erythrocytes (7-8 μm and 3.5-4.5μm in diameter, in human and mice respectively) have irregular shapes but still contain certain cytoplasmic organelles[7,24].

Terminal erythroid differentiation, from the proerythroblast to reticulocyte, takes 48 to 72 hours. Reticulocyte maturation continues for another 48 hours until all unnecessary organelles are eliminated and the cell acquires the discoid shape of the fully mature erythrocyte[20].

Terminal erythroid differentiation occurs in an identifiable anatomic unit both in vivo[105] and in liquid culture[7], that provides a unique environment for maturing erythroid precursors: the erythroblastic island (Fig. 3). This is a sponge-like structure, consisting of a central macrophage surrounded by erythroid precursors at different stages of maturation. The more immature precursors are located closer to the centre but as they mature they move away from the body of the macrophage. Erythroid precursors keep in contact with the cytoplasmatic extensions of the macrophage during all stages of maturation until occurrence of enucleation[20]. Upon enucleation the reticulocyte detaches from the macrophage leaving its nucleus behind to be phagocytosed by the macrophage[7,8].

One obvious function of the macrophage, within the erythroblastic island, is phagocytosis but it is unlikely to be its only function. It is known that the presence of the macrophage is not essential for the amplification or maturation of the erythoblasts in different culture systems but

these do not represent the *in vivo* environment. It is probable that the macrophage influences the local concentration of nutrients and/or growth factors, especially considering the proximity of the erythroblasts and the presence of structures resembling gap junctions between the macrophage and the erythroblasts[7]. Furthermore, the macrophage is responsible for the destruction of the nucleus expelled by the erythroid precursors during differentiation.

A recent report[108] demonstrated that macrophages are indispensable for definitive erythropoiesis. Macrophages from Dnase II knockout mice are unable to degrade the DNA from the nucleus expelled by the erythroid precursors. The DNA from the engulfed nucleus therefore remains in cytoplasmatic inclusions and these macrophages are no longer able to perform their supportive functions. This leads to defective erythropoiesis and consequent embryonic lethality.

Amplification of the erythroid precursors also occurs during terminal differentiation. In the 48 to 72 hours that the erythoblast remains associated with the macrophage in the erythroblastic island, 4 to 5 cell divisions take place[8,20]. Extra evidence for this was obtained from the observation of the erythroblastic islands formed in bone-marrow liquid cultures[7]. Proerythroblasts occur singly while basophilic and polychromatophilic erythroblasts form groups of 4, 8 or 16 and orthochromatic erythroblast and reticulocytes are found in groups of up to 32 cells. These numbers correlate well with the notion that 4 to 5 divisions occur during erythroid terminal differentiation.

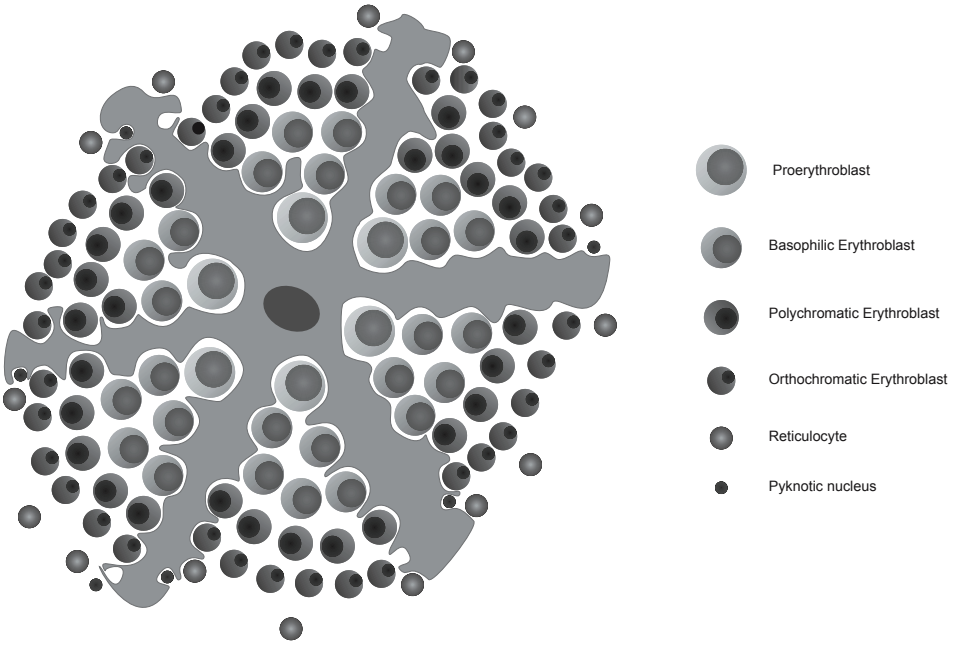

Figure 3 - Simplified model of the erythroblastic island. The central macrophage (in grey) is surrounded by erythroid precursors at different stages of maturation, the more immature cells located closer to the centre and the more mature cells further away from the body of the macrophage. Adapted from reference 252.

1.2 T-cell differentiation

The immune system is responsible for the defence of an organism against infection. Granulocytes and macrophages constitute the first line of defence by directly recognising and killing pathogens. Lymphocytes are responsible for a more sophisticated type of immunity, called adaptive immunity, since they can specifically recognise and eliminate extracellular and intracellular pathogens, and generate memory lymphocytes that can rapidly and effectively respond to re-infection. B-lymphocytes eliminate pathogens by producing specific antibodies that circulate in the plasma and can recognise pathogens, thus triggering phagocytosis by macrophages. T-lymphocytes eliminate pathogens either by direct interaction with infected cells (cytotoxic T-cells) or by activating other cells that will eliminate the infected cells (T-helper cells).

The remainder of this chapter will be focussed on T-lymphocytes, in particular on their development in the thymus and lineage commitment into the different subsets of mature T-cells.

T-lymphocytes recognise infected cells via the T-cell receptor (TCR) complex[99]. The TCR complex is composed of the TCR, a heterodimer composed of two transmembrane glycoproteins (usually TCRα and TCRβ)[18,60], and four other proteins, collectively called CD3[133]. Diversity of T-cell receptors, essential for the recognition of the different antigens, is achieved by the recombination of the genes encoding for the receptor α and β chains. During recognition of the antigen, CD4 or CD8 co-receptors, associated with the T-cell receptor complex, aggregate in the membrane bringing several TCRs in close proximity, which triggers the signalling cascade via the TCR[100].

Recognition of the infected cells occurs because these cells present specific peptides from the pathogen at their surface that can be recognised by the TCR complex. These antigens are displayed by specific membrane glycoproteins, the major histocompatibility complex (MHC) proteins (reviewed by Germain[62]). There are two classes of MHC molecules. MHC class I molecules display peptides from proteins synthesised in the cytoplasm, mainly viral antigens. MHC class II molecules display peptides from proteins in intracellular vesicles, derived from pathogens such as bacteria and eukaryotic parasites, internalised by macrophages. MHC class I molecules are expressed in the majority of nucleated cells while MHC class II expression is restricted to particular types of cells, such as dendritic cells, macrophages and B-cells, collectively denominated antigen presenting cells (APCs)[99].

Three distinct lineages of mature T-lymphocytes are identifiable based on the way they recognise the antigens and eliminate the infected cells. Cytotoxic T-cells recognise antigens presented by MHC class II molecules and directly kill the infected cell. These cells are characterised by the expression of the MHC class II co-receptor CD8. CD4 T-cells recognise antigens presented by MHC class I molecules and are characterised by the expression of the MHC class I co-receptor CD4. Depending in their way of elimination of the infected cells CD4 T-cells can be further classified into T-helper cell type 1 (Th1) or Th2. Th1 cells activate the infected macrophage to destroy the pathogen while Th2 cells induce B-cells to produce antibodies (reviewed by Abbas and colleagues[1]).

However, there are other types of T-cells that act in an antigen unspecific manner and are important for the regulation of T-cell activation (reviewed by Suciu-Focaand colleagues[217]).

Such T-cells, also produced in the thymus, possess suppressor/regulatory function over mature CD8 and CD4 T-cells mediated by cytokines, T-cell-T-cell or T-cell-APC interactions. CD4 regulatory T-cells (T_R) express high levels of CD25 (IL2 receptor α-chain) while CD8 suppressor T-cells (T_S) are characterized by the absence of CD28 expression.

Another type of T-cell has been named natural killer T-cell (NKT-cell) (reviewed by Godfrey and colleagues[65]). Such cells share some characteristics with NK cells like the expression of NK1.1 and the production of high levels of IL4 and interferon γ (INF-γ). $CD4^-$ $CD8^-$ double negative (DN), $CD4^+$ and $CD8^+$ subsets of NKT-cells have been identified but their specific functions remain unclear despite suggestion that they may participate in Th2 induction and Th1 inhibition.

1.2.1 Thymic T-cell differentiation

T-lymphocytes, like all hematopoietic cells, derive from the HSCs. In adults, the HSCs are confined to the bone marrow but T-cell progenitors require specific microenvironments to mature so they leave the bone marrow to colonise the thymus (thymus-dependent (T)-lymphocytes).

The thymus is a lymphoid organ composed of numerous lobules, each comprising two distinct regions: the cortex and the medulla. The cortex contains the T-cell precursors embedded in the thymic stroma, which provides the unique microenvironment necessary for maturation[236]. The more mature T-cells, together with dendritic cells and macrophages, are found in the medulla. The thymus rudiment starts to arise early in embryonic development (E9.0) and when it is fully formed (E11.5) it attracts bone marrow-derived progenitors that will become committed to the T-cell and dendritic cell lineages. Later on the thymus is also colonised by macrophages[119].

The identification of the bone marrow-derived lymphoid progenitor that migrates to the thymus and gives rise to the lymphoid lineage is still a matter of debate. Such progenitors are present in the thymus in very low numbers making their identification extremely difficult. Consequently, several research groups have identified distinct cells as the earliest T-cell progenitor in the thymus. They are generally characterised by the absence of both CD4 and CD8 co-receptors expression and therefore are generally called double negative (DN) thymocytes. Based on the expression of phagocyte glycoprotein-1 (CD44) and CD25, the DN T-cells can be subdivided into four different populations, reflecting differentiation stages[64]. The earliest T-cell progenitors in the thymus are characterized by a $CD44^+CD25^-$ phenotype (DN1). However, this classification is not very precise since cells other than T-cells express CD44 and do not express CD25. More detailed analysis of the DN1 cell population in the thymus revealed the existence of different cell types. Allman and colleagues[9] identified as an early T-cell progenitor the population of DN1 cells expressing CD117 (c-kit) but not CD127 (IL7Rα). This finding contradicts the idea that both B and T-cells originate from a CLP characterized by the expression of CD127 but not CD117 and suggests the existence of another bone-marrow progenitor for the T-cell lineage. However, Martin and colleagues[136] identified a $B220^+CD19^-CD117^+$ bone marrow cell type capable of generating mature T-cells, and suggest that these cells derive from the CLP and represent the most differentiated population of cell before commitment to the B-cell lineage, still possessing T-cell potential. Using a variety of cell markers Porritt and colleagues[194] identified five independent cell types within the DN1 population (DN1a-e) showing that the DN population may be composed by several distinct T-cell progenitors. DN1a and DN1b appear to be

classical T-cell progenitors since they have proliferation potential and exhibit characteristics of a differentiation intermediate between bone-marrow progenitors and committed T-cell precursors. Furthermore, DN1a seems to give rise to DN1b in culture suggesting that DN1a is the earliest intrathymic progenitor. DN1c, DN1d and DN1e are not conventional T-cell progenitors since they do not have proliferation potential but they do give rise to mature T-cells. DN1c can also give rise to B-cells, in agreement with the progenitors described by Martin and colleagues[136].

Commitment to the T-cell lineage, at the pro-T-cell or DN2 stage, is achieved by the induction of CD25 expression. At this stage cell proliferation is dependent on the presence of interleukin-7 (IL-7) and rearrangements of the T-cell receptor γ (TCRγ) locus are detected [31]. Loss of CD44 expression and proliferation capacity indicates the transition to the next developmental stage, the early pre-T-cell or DN3 stage, where the T-cell receptor β chain (TCRβ) locus starts to be rearranged. When the rearrangement is complete the TCRβ is presented at the surface of the cell in a complex with pre TCRα chain. Those cells that produce a functional TCRβ will proceed through the next step of differentiation, late pre-T-cell, by downregulation of CD25 expression after which they can proliferate (reviewed by Fehling and von Boehmer[54]).

Cells that have successfully undergone β-selection are ready to start expressing the CD4 and CD8 antigens and therefore enter the double positive (DP) stage of differentiation. At this stage, the TCRα locus is rearranged followed by selection of the TCRαβ molecules presented at the cell surface. T-cells are positively selected based on their capacity to recognise MHC molecules (reviewed by von Boehmer[238]). Negative selection consists on the removal of all the cells that can recognise self peptide bound to MHC molecules (reviewed by Nossal[168]).

T-cells expressing TCRαβ are able to recognise exclusively foreign peptides presented by MHC molecules and can differentiate further into either $CD4^+$ or $CD8^+$ single positive T-cells (Fig. 4).

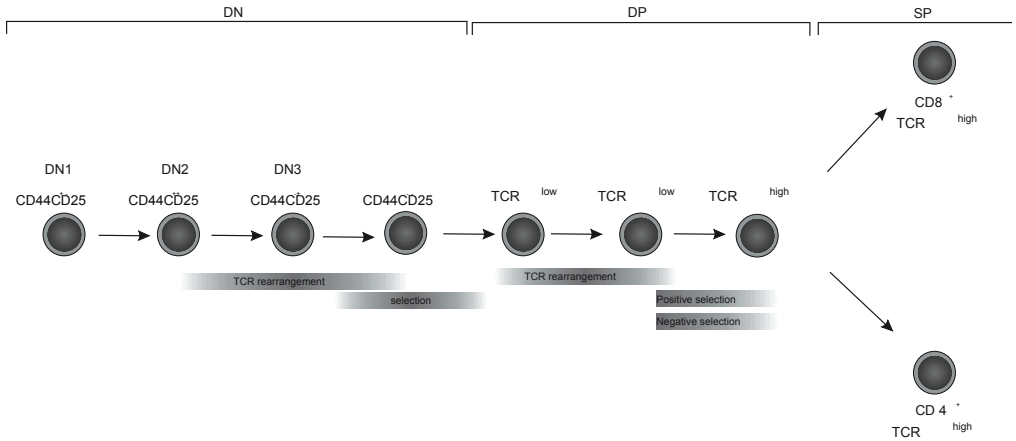

Figure 4 - Schematic representation of the different stages of T-cell differentiation in the thymus. The thymus is colonized by bone marrow-derived progenitors that constitute the CD4 CD8 double negative (DN) 1 stage; their identity is still a matter of debate. Precursors become restricted to the T-cell lineage at the DN2 stage. By the DN3 stage, the T-cell receptor β chain (TCRβ) locus starts to be rearranged. Cells that have successfully undergone β-selection start expressing the CD4 and CD8 antigens, thus entering the double positive (DP) stage. At this stage, the TCRα locus is rearranged followed by positive and negative selection of TCRαβ presented at the cell surface. Selected cells differentiate further into either $CD4^+$ or $CD8^+$ single positive T-cells.

1.2.2 Peripheral T-cell differentiation

Once they have completed their development in the thymus, $CD8^+$ and $CD4^+$ T-cells leave this organ and migrate through the blood into the peripheral lymphoid organs (lymph nodes and spleen). These cells are called naive T-cells because they never encountered their specific antigen and they will remain in circulation in the blood and peripheral lymphoid organs until such encounter takes place. Naive T-cells are arrested at the G0 stage of the cell cycle and can circulate many years without dividing. They have condensed chromatin and therefore synthesise little RNA and protein.

When these naive T-cells recognise their specific antigen, presented by MHC molecules on the surface of APCs, they stop to circulate, proliferate and finally differentiate into effector T-cells. Recognition of the antigen alone is not sufficient to activate a naive T-cell. In the absence of co-stimulatory signals from the APCs, T-cells express an IL2 receptor composed of β- and γ- chains with low affinity for IL2. In the presence of co-stimulatory signals from the APCs T-cells start to express the IL2 receptor α- chain, that confers higher affinity of the receptor for IL2, and IL2 itself. Binding of IL2 to the high affinity IL2 receptor triggers the cells to enter the G1 phase of the cell cycle, thus allowing proliferation.

After 4 to 5 days of proliferation, the naive T-cells differentiate into effector T-cells. Naive $CD8^+$ T-cells differentiate only into cytotoxic $CD8^+$ T-cells while naive $CD4^+$ T-cells can differentiate into either Th1 or Th2 cells (reviewed by Abbas and colleagues[1]; Fig. 5). Th1 cells are characterised by the expression of INF-γ, tumor necrosis factor-β (TNF-β) and IL2 and act by activating infected macrophages to destroy the pathogen[154]. In contrast, Th2 cells activate B-cells that produce antibodies against those pathogens and are characterised by the production of IL4, IL5, IL6 IL10 and IL13[154] (Fig. 5).

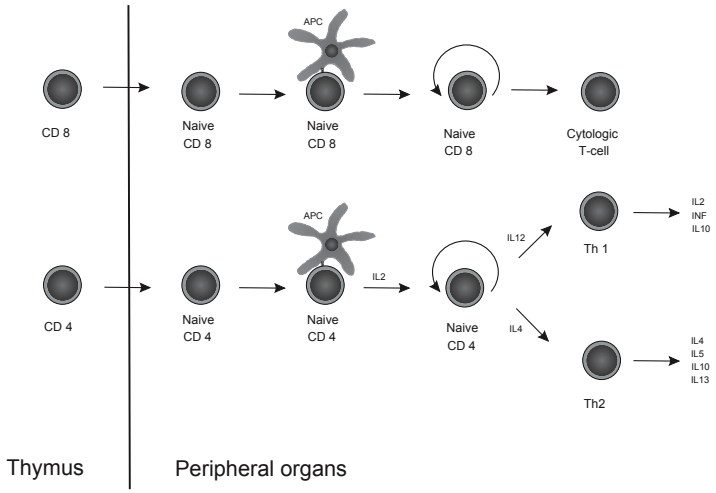

Figure 5 - Schematic representation of the stages of T-cell differentiation in the periphery. Upon antigen stimulation , naive $CD8^+$ T-cells, differentiate into cytotoxic T-cell. Naive $CD4^+$ T-cells differentiate into T-helper cells type 1 (Th1) or Th2 depending, at least partially, on the cytokines produced by the antigen presenting cell (APC). In the presence of IL12, cells become Th1 and start producing IL2, INFγ and IL10. In the presence of IL4, the cells become Th2 characterised by the production of IL4, IL5, IL10 and IL13. Adapted from reference 170.

The mechanism by which naive $CD4^+$ T-cells differentiate into either Th1 or Th2 is poorly understood (reviewed by O'Garra and Arai[170]). It appears to involve several steps, passing through an intermediate stage, Th0, at which both Th1 and Th2-specific cytokines are expressed followed by a restriction to expression of either Th1 or Th2-specific cytokines[2,104,161]. The choice of differentiation pathway appears to depend on environmental cues. The presence of IL12 and IL4 is known to influence Th1 or Th2 differentiation, respectively[111,206,207]. IL12 signalling, through the IL2 receptor, activates the transcription factor STAT4, which leads to the upregulation of INFγ[14,98]. IL18 was shown to synergize with IL12 in the upregulation of INFγ. The INFγ gene can also be transactivated by the Th1-restricted transcription factor T-bet. Ectopic expression of T-bet can initiate the Th1 genetic programme and repress the Th2 programme. IL4 directs differentiation into Th2 cells by similar mechanisms. IL4 signalling, through the IL4 receptor, activates Stat6 leading to the upregulation of IL4, IL5 and IL10 and repression of INFγ[121]. Stat6 is also responsible for the expression of Th2-specific transcription factors c-maf and GATA3, but GATA3 can also be regulated in a STAT6-independent way[178]. Both c-maf and GATA3 are involved in the transactivation of Th2-specific cytokines[88]. GATA3, like T-bet in Th1 differentiation, is crucial for Th2 differentiation since its ectopic expression can initiate the Th2 genetic programme and repress the Th1 programme (reviewed later in this thesis). A recent report[11] shows that Notch signalling is also involved in the regulation of $CD4^+$ T-cells differentiation into either Th1 or Th2. Different Notch ligands, expressed by the APCs, trigger different responses by the naive $CD4^+$ T-cells. While expression of Delta ligands promote Th1 differentiation, Jagged expression leads to Th2 differentiation by inducing IL4 expression and promoting GATA3 induction and T-bet repression. These may explain the observed STAT6-independent expression of GATA3 in Th2 cells.

2. Transcriptional control of hematopoiesis

The development of mature blood cells of distinct lineages, from the HSCs, involves a progressive restriction of differentiation potential and the establishment of lineage-specific gene expression profiles. The establishment of these expression profiles relies on lineage-specific transcription factors to modulate the expression of their target genes. In this Chapter a general introduction of transcription control of gene expression will be given followed by an overview of lineage-specific transcription factors involved in hematopoiesis.

2.1 Transcriptional control of gene expression

The synthesis of a functional protein involves several steps starting with transcription, proceeding through RNA processing, transport and translation and finally activation of protein activity. Consequently, gene expression control can, in principle, be exerted at each and every one of these steps.

The first and foremost important level of gene expression control is at the step of transcription. Transcription is the process through which an RNA copy of the DNA sequences encoding a gene is made. This RNA copy is processed and usually used as a template for the translation into protein. Controlling gene expression at this level presents the most benefits to the cell by preventing the synthesis of mRNA.

Transcription involves three independent stages: initiation, elongation and termination. During transcription initiation RNA polymerase, the enzyme responsible for RNA synthesis, binds to target DNA sequences allowing transcription to start. Eukaryotes posses three RNA polymerases responsible for the transcription of different types of genes: RNA polymerase I and III transcribe genes encoding transfer RNA, ribosomal RNA and several small RNAs while RNA polymerase II transcribes the vast majority of protein-encoding genes. RNA polymerase II can only initiate transcription with the help of several general transcription factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH). Through the elongation stage, the RNA polymerase, with the assistance of elongation factors, can then move along the DNA template making the RNA copy. A process involving the dissociation of the RNA polymerase from the DNA finally terminates transcription. Transcription is mainly regulated at the initiation stage, which will be the focus of this Chapter.

Chromatin

The first level of transcriptional control is chromatin structure. In order to fit in the nucleus DNA is compacted into chromatin, in which most of the DNA sequences are relatively inaccessible. The structural unit of chromatin is the nucleosome that consists of 146bp DNA stretches wrapped twice around an octameric core of histone proteins, containing two molecules each of histones H2A, H2B, H3 and H4. A region of linker DNA, of variable length, separates each nucleosome from the next giving rise to a structure resembling “beads on a string”, the 10nm fibre (reviewed by Kornberg and Lorch[112]). A second level of compaction, the 30nm fibre, is obtained by the binding of histone H1 to the linker DNA[221]. This level of compaction is still not sufficient to fit the DNA in the nucleus. A third level of compaction is therefore characterised by folding of the 30nm fibre into chromatin loops, reaching maximum compaction in mitotic chromosomes (reviewed by Belmont and colleagues[17]).

Two types of chromatin can be distinguished in the interphase nucleus: heterochromatin,

a more condensed form usually located in the periphery of the nucleus and euchromatin, less dense and spread throughout the nucleus. Heterochromatin is generally considered to be transcriptionally inactive while euchromatin is transcriptionally active. In fact, only about 10% of euchromatin is transcriptionally active at a given time. The remaining 90% of transcriptionally inactive euchromatin is in a reversibly closed chromatin configuration not allowing transcription (reviewed by Dillon and Festenstein[47]).

For transcription to occur, closed chromatin must be remodelled into a more relaxed form, which allows access of the transcription machinery to DNA. Modification of key amino acids at the N-terminal tails of histones is the best-studied mechanism of chromatin remodelling (reviewed by Berger[19]). For example, acetylation of particular lysine residues at the histone tails is associated with transcription activity since the presence of an acetyl group neutralises the positive charge of the lysine, therefore decreasing the affinity of the nucleosome for the DNA. Histone acetytransferases (HATs), several of which have been identified and extensively studied, mediate histone acetylation. The acetylated status is reversible and different histone deacetylases (HDACs) have also been identified (reviewed by Struhl[216]). Methylation of lysine and arginine residues can be associated with either transcriptional silencing or activation (reviewed by Kouzarides[113]). It is now believed that the combination of several modifications, rather than one single modification, dictates the transcriptional status of chromatin (reviewed by Iizuka and Smith[91] and Jenuwein and Allis[101]).

The enzymes involved in chromatin remodelling are often components of large protein complexes denominated chromatin-remodelling complexes. Several complexes have been identified, first in yeast and later in mammals (reviewed in Feng and Zhang[55], Kornberg and Lorch[112] and Martens and Winston[135]).

After access to the DNA is obtained, general transcription factors and RNA polymerase can, in principle, bind the promoter of the target gene. Promoters are specific regulatory sequences located immediately upstream of the RNA transcription initiation site. Eukaryotic promoters are constituted of about 100bp of sequence containing several conserved DNA motifs necessary for the recognition by general transcription factors and RNA polymerase. Several of these DNA motifs, such as the TATA box and upstream promoter elements such as CCAAT and CACC boxes, are well characterised. Promoters contain all the elements necessary for initiation of transcription but other *cis*-regulatory elements, including enhancers and locus control regions (LCRs), interact with the promoter in order to ensure the correct spatio-temporal levels of expression. Enhancers were originally identified[15] as cis-acting DNA sequences that increase transcription in a manner that is independent of their orientation and distance relative the RNA start site (reviewed by Blackwood and Kodanaga[22]). These regulatory elements have variable length and are composed of arrays of DNA motifs that can be specifically bound by transcription factors and can activate transcription independently of their orientation.

LCRs share many of the properties of enhancers but in addition posses the ability to open chromatin and prevent heterochromatinisation (reviewed by Grosveld[72]). The first LCR was identified in the human β-globin locus but several others have been identified since suggesting an important role in the regulation of transcription. LCRs are generally defined by their ability to drive high-level expression of linked genes in a tissue-specific, copy number-dependent and position-independent manner[71] .

Transcription factors

Despite the fact that all cells in an organism have identical genetic information, each

cell type expresses a restricted number of genes. The establishment of these expression profiles is orchestrated by specific transcription factors. These proteins, in response to environmental or physiological cues, can recognise specific target sequences located at the promoter, enhancer or LCR of a specific gene and activate or repress its transcription.

Transcription factors recognise their target sequences through specific DNA-binding domains, comprised of 60 to 100 amino acids (reviewed by Mitchell and Tjian[151]). Different types of DNA-binding domains can be found in transcription factors, including helix-turn-helix motifs, zinc-fingers, leucine zippers and helix-loop-helix motifs (reviewed by Harrison[80]). Amino acids at the surface of these domains recognise and bind the DNA target sequence, implying that even small differences in the amino acid sequence in this region can alter the recognised DNA sequence. Transcription factors are classified into families based on their DNA-binding specificity.

Binding of the transcription factor to DNA is necessary but not sufficient for transcription activation. Transcription factors interact with other proteins that influence the outcome of DNA binding by the transcription factor, *i.e.*, transcription activation or repression. Therefore, other types of domains can be identified in transcription factors such as transcription activation/repression domains and protein-binding domains. These domains, commonly regions of 30 to 100 amino acids, are usually separated from the DNA binding domain. Through these domains transcription factors can interact with the basic transcription machinery and recruit co-factors, transcriptional activator/repressor complexes or chromatin remodelling complexes (reviewed by Roberts[200]).

Transcription factors, like any protein, can also be subject to post-translational modifications. Post-translational modifications involve either the covalent binding of small molecules to specific amino acids or cleavage of parts of the protein after translation, which makes the final protein different from precursor. These alterations in proteins have consequences for their activity and therefore are extremely important for their biological function. Many types of post-translational modifications have been described including acetylation, phosphorylation, glycosylation, and ubiquitilation (reviewed by Han and Martinage[75]). Recently an increasing number of post-translational modifications in transcription factors have been reported. Despite the fact that in most of the cases the functional significance of such modification is not clear, in some cases it has been shown that modifications such as acetylation[16,94], phosphorylation[250], ubiquitilation[45] and sumoylation[63] are of the utmost importance for the normal function of transcription factors. A good example of the importance of post-transcriptional modification in the function of transcription factors is the tumour supressor gene p53 (reviewed by Xu[258]).

2.2 Hematopoietic transcription factors

Hematopoiesis is not only essential for the formation of blood, and therefore for the life of an organism, but it also provides a very interesting model system for the study of cell differentiation. Understanding how a pluripotent stem cell undergoes successive lineage restrictions and finally differentiates into a mature blood cell has been a challenge to many scientists over the years. Restriction to a particular cell lineage is associated with a lineage-specific expression profile, established by lineage-specific transcription factors. Therefore, understanding how these transcription factors function is essential to comprehend cell differentiation.

In the last two decades a wide variety of transcription factors were found to be involved

in the establishment of hematopoietic cell lineages (reviewed by Cantor and Orkin[29,30], Perry and Soreq[186] and Shivdasani and Orkin[211]) (Fig. 1). These findings relied on the study of genetically modified mice, either knockouts, where the gene is functionally deleted, or transgenics, where the gene is ectopically expressed. In this section the functions of lineage-specific transcription factors involved in hematopoiesis will be discussed. Since the number of transcription factors with identifiable functions during hematopoiesis is too vast to allow a thorough discussion of every one of them, the discussion will be focussed on those that appear to play the most important roles in particular stages of hematopoiesis. This does not mean that other transcription factors do not play equally important roles during hematopoiesis but only that they show more subtle phenotypes in studies using genetically modified mice and therefore their function is more difficult to be identified.

Several transcription factors were shown to be essential at very early stages of hematopoiesis, for the differentiation of MLPs from the HSCs (reviewed by Shivdasani and Orkin[211] and Zhu and Emerson[270]). TAL1, a basic helix-loop-helix transcription factor, and LMO2, a LIM-domain transcription factor, are critical for the onset of hematopoiesis since *null* embryos show complete absence of primitive and definitive hematopoiesis[193,199,210,244]. The zinc-finger transcription factor GATA2 is also essential for the early stages of hematopoiesis. Both primitive and definitive hematopoiesis are abrogated when the GATA2 gene is deleted, but it appears to play a role in the proliferation of the early precursors rather than in their differentiation (reviewed later in this thesis). AML1 is crucial for the early stages of definitive hematopoiesis. In AML1 *null* embryos yolk sac hematopoiesis is normal but fetal liver hematopoiesis is absent[171]. HOXB4, a member of the large HOX family of transcription factors, was shown to be important for the proliferation of HSCs. Overexpression of Hoxb4 in murine bone marrow[204] and embryonic stem (ES) cells[83] enhanced the proliferation capacity of the HSCs and definitive erythroid progenitors, respectively, without perturbing the differentiation into the different hematopoietic lineages. In agreement, mice deficient in both HOXB3 and HOXB4[21] or HOXB4 alone[27] show reduced numbers of hematopoietic progenitors and diminished cellularity of the hematopoietic organs but lineage commitment is not perturbed.

Once MLPs arise from the HSC and proliferate, the process of lineage restriction starts. A number of transcription factors that are preferentially expressed in cells destined to a particular lineage have been identified but few have been extensively studied. PU.1, a member of the *ets* family of transcription factors, is the best-studied transcription factor known to be involved in early lineage restriction. PU.1's effect on lineage commitment is dependent on its expression level: a high level of expression leads to commitment to the myeloid lineage while lower levels of expression lead to commitment to the lymphoid lineage. This is supported by two observations. First, PU.1 *null* mice die around birth and show absence of B-lymphocytes, granulocytes and monocytes[205]. Secondly, overexpression of PU.1 leads to commitment to the myeloid lineage, more specifically, to the granulocytic/monocytic lineage[165]. These findings are in agreement with the lineage commitment model in which a CMLP gives rise to MBPs and MTPs (reviewed By Katsura[106]).

More is known about transcription factors involved in specific hematopoietic lineages. Some examples of such transcription factors are GATA1, FOG1 and GATA3. The zinc-finger transcription factor GATA1 is essential for the development of the erythroid, megakaryocytic and eosinophilic lineages. FOG1, another zinc-finger transcription factor and co-factor of GATA1, is also crucial for the erythroid and megakaryocytic lineages. GATA3 plays an essential role in T-cell development. The functions of these factors will be discussed later in this thesis.

3. The GATA family of transcription factors

The GATA family consists of six transcription factors, GATA1 to GATA-6. These transcription factors are categorised as a family due to the fact that they all bind to the DNA consensus sequence (A/T)GATA(A/G) by two characteristic C_4 (Cys-X_2-Cys-X_{17}-Cys-X_2-Cys) zinc-finger motifs[109,137,143,253,260], commonly designated by GATA fingers.

The zinc-finger motifs are the regions with highest homology between the GATA family members. Outside these regions, the conservation between GATA factors is low[174,260]. Furthermore, the overall homology of an individual member is higher between species than between different members of the same species[260,272].

The GATA family is subdivided into two subfamilies based on the expression profiles of the individual transcription factors. GATA1, GATA2 and GATA3 belong to the hematopoietic subfamily since they are expressed mainly in the hematopoietic system[248] (Fig. 6). The non-hematopoietic subfamily is composed by GATA4, GATA5, and GATA6, which are expressed in several tissues including intestine, lung and heart but not in the hematopoietic system[152].

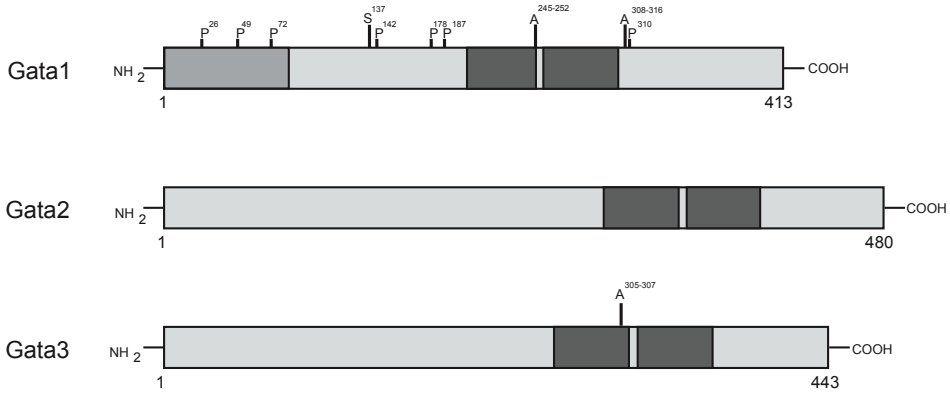

Figure 6 - Schematic representation of the murine hematopoietic GATA proteins. The zinc-finger domains (in black), N-terminus domain (dark grey) and the know sites modified by acetylation (A), phosphorylation (P) and SUMOylation (S) are shown; the numbers indicate the modified amino acid.

3.1 GATA1

GATA1, also known as NF-E1, NF-1, Ery-1 and GF-1, is the founder member of the GATA family of transcription factors. It was first identified as a protein with binding specificity to the β-globin 3' enhancer[53,243] and cloned from a murine erythroleukemia (MEL) cell line cDNA library[228]. The human homologue was soon after cloned and its localization assigned to the X-chromosome at position Xp21-11[271]. The murine GATA1 gene is also located on the X chromosome[271].

GATA1 is expressed in primitive and definitive erythrocytes[59,125], megakaryocytes[138,202], eosinophils[274], mast cells[138], and in the Sertoli cells of the testis [95,262]. Several gene targeting studies were performed to attempt to elucidate the importance of GATA1 function in these cells.

These studies have shown that GATA1 is essential for normal erythropoiesis. GATA1-

deficient embryonic stem (ES) cells are able to contribute to all different tissues in chimeric mice, with the exception of the mature red blood cells[188]. More detailed analysis of erythropoiesis in these chimeric mice revealed that GATA1 *null* erythroid cells fail to mature beyond the proerythroblast stage[189]. *In vitro* differentiation of GATA1-deficient ES cells confirmed this arrest of both primitive and definitive erythropoiesis at the proerythroblast stage[246] and showed that the arrested precursors die by apoptosis[247]. Not surprisingly, GATA1 *null* murine embryos die from severe anaemia between E10.5 and E11.5[59]. GATA1 knock-down embryos (GATA1.05), which express only approximately 5% of the wild-type GATA1 levels, also show an arrest of the primitive erythropoiesis and die between E11.5 and E12.5[219]. Other GATA1 knock-down mice (GATA1 low)[140], which express about 20% of the wild-type GATA1 levels, show a somewhat milder phenotype. Despite the fact that the majority of GATA1 low mice die between E13.5 and E14.5, due to ineffective primitive and definitive erythroid differentiation, some are born alive (2% of the expected 25%) and a small number survive to adulthood. These mice are anaemic at birth but they recover from the anaemia and show a normal life span. From the analysis of these different mouse models a direct relationship between the expression levels of GATA1 and the severity of the phenotype is evident.

The analysis of a megakaryocyte-specific knockout of GATA1 has revealed a critical role for this factor in megakaryocytic development[212]. Absence of GATA1 in megakaryocytes leads to an increased proliferation and deficient maturation of megakaryocytic progenitors as well as reduced number of circulating platelets. The platelets produced are not fully functional and show an abnormal morphology[239].

GATA1 also plays an essential role in eosinophil development. The first evidence for a role of GATA1 in eosinophil development comes from the observation that forced GATA1 expression in Myb-Ets-transformed chicken myeloblasts induced a reprogramming of these myeloblasts into cells resembling either transformed eosinophils or thromboblasts[114]. In a later report, forced expression of both GATA1 and GATA2 was also shown to instruct CD34-positive bone marrow cells to develop into eosinophils[86]. In the same report, it was also shown that GATA1 deficient fetal liver precursor cells are unable to generate eosinophilic colonies in culture[86]. Furthermore, the deletion of a double GATA site present in the GATA1 promoter was found to cause the selective loss of the eosinophilic lineage[263].

Mast cells are somewhat different from the majority of the hematopoietic cells. They originate from the HSC in the bone marrow but the precursor cells migrate, through the blood, to connective or mucosal tissues, where they proliferate and differentiate into mature mast cells. GATA1 is abundant in the more mature mast cells but it is almost undetectable in the bone marrow progenitors suggesting a possible role in the terminal differentiation of mast cells[79]. Also, it was noticed that GATA1.05 heterozygous female mice, expressing 5% of the wild-type GATA1 levels in approximately 50% of the cells owing to the process of X-inactivation, certain mast cells show a defective maturation[79]. Final proof of the importance of GATA1 in mast cell maturation arose from the analysis of GATA1 low mice[146]. These mice express about 20% of the wild-type GATA1 levels but in contrast to the GATA1.05 mice, some reach adulthood allowing a more detailed analysis of the mast cell phenotype. Indeed, connective tissues from GATA1 low mice contain large numbers of mast cell precursors but normal numbers of mature mast cells, with abnormal morphology. Many of these precursors die by apoptosis, which explains the normal numbers of mature cells. The defect observed is GATA1-specific, since forced expression of GATA1 rescues the maturation potential of these cells.

Although GATA1 is expressed in the Sertoli cells of the testis at critical stages of

spermatogenesis, testis-specific deletion of GATA1 does not result in an apparent phenotype[127].

Despite all the knowledge about the consequences of GATA1 absence in different hematopoietic lineages we are far from knowing the specific functions performed by this transcription factor in those cells.

Based on the observation that GATA1 *null* erythroid cells undergo apoptosis, it as been suggested that GATA1 is directly involved in cell survival. Several lines of evidence support this theory: GATA1 activates transcription of the Epo receptor (EpoR)[34] and Epo signalling is known to be important for erythroid progenitor survival[115]. Furthermore, one of the known target genes of GATA1 is Bcl-$_{XL}$, a gene encoding an anti-apoptotic protein[70].

Another possible GATA1 function is the regulation of G1/S cell cycle progression. Cell cycle control is of the utmost importance in hematopoietic differentiation since progenitors must be able to proliferate in order to proceed through hematopoietic development, but for terminal differentiation to occur cells must exit the cell cycle[254]. This idea is reinforced by the identification of a variety of GATA1 target genes involved in cell cycle regulation or with known functions in proliferation of differentiation processes[203].

GATA1 has also been implicated in the reprogramming of hematopoietic precursors. Forced expression of GATA1 was shown to reprogram myeloblasts and CD34$^+$ bone marrow cells to develop into eosinophils[86,114]. Furthermore, forced expression of GATA1 was also shown to reprogram GMPs to give rise to erythroid, eosinophilic and basophilic-like cells[85]. By clone tracking the authors demonstrated that the GATA1 effect is at the cell commitment level and not due to effects on clone selection. Another recent report[96] shows that ectopic GATA1 expression guides hematopoietic precursors to commitment to the erythrocyte-megakaryocytic lineage. It is not clear from these reports if the GATA1-induced lineage reprogramming is a GATA1–specific characteristic, or whether it is a general effect of the ectopic expression of lineage-specific transcription factors. In Chapter 4 of this thesis, the importance of spatial-temporal expression of the GATA transcription factors during erythroid development will be addressed.

At least three functional domains have been identified within the GATA1 protein: An N-terminal activation domain, the N-terminal zinc finger (N-finger) and the C-terminal zinc finger (C-finger). The C-finger is essential for GATA1 function since it is responsible for the recognition of the GATA consensus sequence and consequent binding to DNA[137,261]. The importance of the N-finger to GATA1 function has been more difficult to define. Although early studies, in non-erythroid cells, indicated that the N-finger was not essential for GATA1-mediated transcriptional activation[137], it was later shown that this zinc finger plays a crucial role in GATA1's ability to induce terminal erythroid differentiation[249]. The N-finger contributes to the stabilisation and specificity of DNA binding[137,253]. More recently, it was shown that this finger mediates the formation of complexes with cofactors. These interactions can involve only the N-finger, as is the case with FOG1[57], or as a collaboration between N and C-finger, for example with Sp1 and EKLF[69,144], and GATA1 itself[132]. Early studies, using reporter assays in non-erythroid cells, show that the most N-terminal 80 aminoacids of the GATA1 protein are essential for its transcriptional activation activity[137]. Surprisingly, in another report[249] this transactivation domain was shown to be dispensable for GATA1-mediated terminal erythroid differentiation. This discrepancy may be related with the expression levels of the GATA1 mutants (see below).

To examine the function of each of the three GATA1 domains in a more reliable way, Shimizu and colleagues[208] made use of transgenic rescue of GATA1.05 knock down mice. By

analysing the offspring resulting from the crossing between GATA1.05 mice and transgenic mice expressing different GATA1 mutants, the requirements for the different functional domains were unravelled. In agreement with the previous reports, the C-finger was shown to be indispensable for GATA1 function in both primitive and definitive erythropoiesis but the N-finger was shown to be necessary only for definitive erythropoiesis. Like the N-finger, the transactivation domain appeared to have different functions in primitive and definitive erythropoiesis. When expressed at levels higher than the endogenous GATA1, the transactivation domain mutant can sustain both primitive and definitive erythropoiesis but when expressed at lower levels, definitive erythropoiesis is impaired. From these data it can be concluded that all the GATA1 domains are required for definitive erythropoiesis while only the C-finger is absolutely essential for primitive erythropoiesis. This demonstrates that the primitive and definitive erythroid lineages have different requirements for GATA1.

3.1.1 Regulation of GATA1 activity

GATA1 activity *in vivo* is tightly regulated. Increasing GATA1 activity can lead to phenotypes as strong as embryonic lethality[251] (Chapter 2 of this thesis). The activity of proteins can be regulated by different mechanisms. The mechanisms thought to be involved in the regulation of GATA1 activity will be reviewed in this section.

Transcriptional regulation

The GATA1 transcription unit is composed of two alternative non-coding first exons, IT and IE[95], and five coding exons, II to VI[229]. Exon IT is primarily used in Sertoli cells of the testis while exon IE is used in hematopoietic cells[95]. The proteins expressed in hematopoietic and Sertoli cells are identical since exon II harbours the translation start site. The two zinc finger motifs are encoded separately in exon IV and V.

The testis promoter and exon IT are located 8kb upstream of exon IE. Disruption of the erythoid promoter leads to an arrest in primitive erythropoiesis without affecting the expression from the testis promoter[219]. Both testis- and erythroid-specific promoters contain GATA sites that are required for the proper functioning of the promoter[95,173,240], suggesting a positive feedback loop[229]. Interestingly, both GATA1 promoters lack a TATA box[76,229].
DNase I hypersensitivity analysis of the GATA1 locus, in erythoid cells, identified three main hypersensitive (HS) regions. HSI is located between 3.9 and 2.6kb upstream of IE, HSII corresponds to the region surrounding the IE promoter and HS III corresponds to intron I[139].

Transcription of the GATA1 gene in different hematopoietic lineages has different regulatory sequence requirements. HSI, also called GATA1 upstream activating element, can drive reporter gene transcription exclusively in primitive erythrocytes while together with intron I this element can drive expression of the reporter gene in both primitive and definitive erythroid cells[172]. Furthermore, GATA1 transcription in erythroid and megakaryocytic cells has different sequence requirements within HSI[212,240]. Expression in both lineages is dependent of the presence of an intact GATA site, but megakaryocytic expression requires the 3' end of HSI, which is dispensable for erythroid expression.

Translational regulation

GATA1 possesses an alternative translation initiation site located at methionine 84[28]. Translation from this alternative initiation site gives rise to a 40kD protein, GATA1s, which

lacks 83 amino acids at the N-terminal region, i.e. the N-terminal transactivation domain. GATA1s can be detected in MEL and K562 cells as well as in mouse tissues but its expression level is much lower that the full length GATA1 forms. This protein shows normal DNA binding activity but a reduced transactivation potential which is in agreement with the reported role of the N-terminus as a transactivation domain[137].

Exclusive translation of GATA1s has been implicated in myeloid leukemogenesis (reviewed by Gurbuxani and colleagues[73]) . Mutations in exon II of the GATA1 gene, introducing premature stop codons in the region encoding the N-terminal transactivation domain, have been identified in Down syndrome patients suffering from acute megakaryoblastic leukemia (AMKL)[245] and transient myeloproliferative disorder (TMD)[87,159]. The mechanism by which GATA1 contributes to the leukemogenic process is not known but it most likely involves the blockage of megakaryocytic differentiation since it is known that reduced levels of GATA1 lead to excessive proliferation and impaired differentiation of megakaryocytes. Despite the fact that GATA1 mutants lacking the N-termimal transactivating domain can rescue the GATA1.05 knock-down phenotype[208], when expressed at high levels, it can be hypothesized that GATA1s is not fully functional and therefore unable to drive terminal megakaryocytic differentiation.

Post-translational regulation

Acetylation

GATA1 can be acetylated both *in vitro* and *in vivo* by the ubiquitously expressed acetyltransferases P300 [25] and CREB-Binding Protein (CBP)[90]. Murine GATA1 is acetylated at two conserved lysine-rich motifs (aa245-252 and aa308-316) localised just C-terminal from each zinc finger. These motifs are conserved among members of the GATA-family and between different species.

The functional importance of GATA1 acetylation is not clear. The interaction between GATA1 and P300/CBP, and consequent acetylation of the transcription factor, seems to stimulate its transcription activity[23,25]. Boyes *et al*. also reported that in chicken GATA1 acetylation increased DNA-binding activity, but Hung and colleagues[25] did not see this effect with murine GATA1. It has also been suggested that acetylation plays an important role in targeting GATA1 for degradation by ubiquitination (J. Boyes, personal communication). Acetylation of GATA1 appears to be required for the *in vitro* differentiation of the GATA1 *null* cell line G1E[23].

Phosphorylation

GATA1 can be phosphorylated at seven serine residues[40] (Fig. 6). Six of these residues (S26, S49, S72, S142, S178 and S187), situated at the N-terminus of the protein, are phosphorylated in uninduced MEL cells. The seventh serine (S310), which is located near the DNA-binding domain, only becomes phosphorylated upon DMSO induction of the MEL cells. This suggests a possible role forphosphorylation in both DNA binding and transcriptional activity of the protein. Surprisingly, substitution of the serines for alanines did not have any consequence in GATA1 DNA binding or transcriptional activity[40].

Another somewhat contradictory report shows that the phosphorylation of GATA1, in induced K562 cells, increases DNA binding[184]. The same report confirms that such increase in DNA binding does not occur in induced MEL cells and suggests that GATA1 is already phosphorylated in uninduced MEL cells while in K562 cells GATA1 becomes phosphorylated upon induction, which leads to an increased DNA binding of the protein. A recent report[225]

shows that GATA1 phosphorylation at S26 and S178 is dependent of cytokine signalling via the MAPK pathway. A mutation of these serine residues interferes with the ability of GATA1 to interact with LMO2, a known interaction partner of GATA1 (reviewed later in this thesis).

SUMOylation

It was recently reported that GATA1 is modified by covalenty binding of SUMO1, both in vitro and in vivo[37]. Although SUMOylation is commonly assocated with transcriptional repression, no apparent effect on GATA1 transcriptional activity was observed by the authors in a number of assays. GATA1 SUMOylation also does not interfere with the cellular localization of the transcription factor therefore the specific function of GATA1 SUMOylation remains unknown.

Protein degradation

Another possible regulatory mechanism for protein activity in general is degradation. Our current hypothesis is that GATA1 activity must be high at early stages of erythroid differentiation, but must be downregulated for terminal erythroid differentiation to occur (see Chapter 2 and 4 of this thesis). This implies protein degradation as a potentially important regulatory mechanism for GATA1 function.

GATA1 degradation via caspase-mediate cleavage has been reported[44]. This report shows that activation of caspases, via death receptors, leads to an arrest in terminal erythroid differentiation. The authors attributed the differentiation arrest to a decrease of GATA1 protein levels due to caspase-mediated cleavage, since expression of a caspase-resistant GATA1 mutant, but not a wild-type GATA1, restored terminal erythroid differentiation.

From the observation that the Fas death receptor is expressed throughout erythroid differentiation but its ligand, FasL, is only expressed in the more mature erythroblasts a model emerges in which mature erythroblasts participate in a negative-feedback loop to attenuate differentiation of earlier erythroid progenitors.

In contrast, our group has shown that overexpression of GATA1 in erythroid cells, both *in vitro*[254] and *in vivo*[251], inhibits erythroid differentiation. Based on this fact a somewhat contrasting model can be envisaged: GATA1 degradation by caspases leads to a reduction of GATA1 levels at late stages of erythroid differentiation thereby allowing terminal differentiation. In the presence of high levels of GATA1 erythroid cells fail to differentiate but surprisingly if wild-type erythroid cells are present, the overexpressing cells can differentiate normally[251]. Further analysis[74] showed that differentiating erythroid cells can signal to GATA1 overexpressing erythroid cells, which are normally blocked in differentiation, to terminally differentiate. This might involve activation of death receptors present in erythroid precursors by ligands produced by differentiated erythroid cells, promoting caspase-mediated cleavage of GATA1.

3.1.2 Protein-protein interactions

GATA1 is now known to interact with a variety of proteins, either co-factors or transcription factors. These interactions play important roles in hematopoiesis since they lead to transcription activation or repression of GATA1 target genes. The most important interactions between GATA1 and other proteins known to date will be reviewed in this section.

GATA1

It has been shown that GATA1 can self-associate *in vitro*[42], both in solution and when bound to DNA. Both the C-finger and the N-finger can independently associate with full-length GATA1 protein since the interaction is mediated by N-finger-C-finger contacts[132]. GATA1 dimerization may play an important role in the regulation of promoters containing multiple GATA sites since mutation of particular residues in the finger regions reduces the GATA1 transactivation potential in reporter assays[132]. Furthermore, GATA1 dimerization was shown to be important for the positive regulation of the GATA1 promoter in zebrafish[167].

Other potential functions for GATA1 dimerization may be to establish contact between promoters and enhancers, and to recruit chromatin-remodelling complexes.

FOG1

Friend of GATA1 (FOG1), a protein containing nine widely-spaced zinc fingers, was identified in a yeast two-hybrid screen as a GATA1 co-factor[230]. It binds to the N-terminal zinc finger of GATA1 mainly via its zinc finger 6[57] although fingers 1, 5 and 9 also contribute to the binding[58]. FOG1 is co-expressed with GATA1 in the erythroid and megakaryocytic lineages and co-operates with GATA1 during erythroid and megakaryocytic differentiation[230].

The phenotype of FOG1 null mice closely resembles the GATA1 null phenotype. Both mutant mice die during midgestation from severe anemia caused by a defect in primitive and definitive erythropoiesis, suggesting that FOG1 is essential for GATA1 function. In contrast to GATA1 deficiency, however, loss of FOG1 leads to a complete ablation of the megakaryocytic lineage, revealing a GATA1-independent role of FOG1 in megakaryopoiesis[231]. Definitive proof that the FOG1/GATA1 interaction is essential for GATA1 function during erythroid differentiation was obtained by the analysis of GATA1 mutants defective in FOG1 binding and subsequent identification of compensatory mutations in the FOG1[38]. Erythroid precursors expressing GATA1 mutants unable to bind FOG1 fail to differentiate but this phenotype is rescued by the expression of the FOG1 mutants that can bind these GATA1 mutant proteins.

Although it has no apparent DNA-binding activity, FOG1 is known to differently modulate GATA1 activity depending on the promoter context. It synergizes with GATA1 in the activation of certain promoters[230] while repressing GATA1-mediated activation of other promoters[58].

One can speculate about possible mechanisms of action for FOG1. FOG1 may be a bridging factor coupling DNA-bound GATA1 to the general transcription machinery and repressor/activator complexes. Recent work in our laboratory shows that FOG1 links GATA1 to the MeCP1 transcriptional repressor complex[201].

RB

The tumor suppressor protein retinoblastoma (RB) plays important roles in many stages of the differentiation process, including regulation of progenitor proliferation, terminal cell cycle exit, induction of tissue-specific gene expression and protection from apoptosis[129]. The phosphorylation state of RB determines the progression of cell cycle from G1 to S-phase. In G1, RB exists in an active, hypophosphorylated, state and is in complex with E2F. The E2F family of transcription factors is the best characterized target of RB, regulating the timing and expression levels of many genes involved in cell cycle progression[77]. RB-E2F complexes stop cell cycle progression by preventing transcription of E2F target genes, either by sequestering E2F or by recruiting repression complexes such as SWI/SNF and histone deacetylases to promoters.

At the G1-S transition, CyclinD-cdk4/6 and CyclinE-cdk2 complexes phosphorylate RB. Hyperphosphorylation of RB results in the dissociation of transcription repression complexes and the release of E2F. Free E2F is now able to activate transcription of genes necessary for entrance S-phase and cell cycle progression[78].

Mice deficient for RB are embryonic lethal and show neuronal and hematopoietic defects[97,122]. Recently, the cause of death was attributed to placental malformation[257]. RB *null* embryos supplied with a wild-type placenta are viable, showing no signs of neuronal abnormalities. A mild defect in erythroid differentiation is still observed indicating that this defect is a direct consequence of RB insufficiency. However, RB null ES cells contribute to all tissues in chimaeric mice and these mice show no apparent defect in the erythroid lineage[131,255]. RB *null* fetal liver cells are also able to repopulate blood lineages of lethally irradiated recipient mice but, surprisingly, erythropoiesis is impaired[89]. RB *null* erythroid precursors show increased proliferation and a blockage of late stages of terminal differentiation leading to the presence of nucleated erythrocytes in the peripheral blood and anemia. This indicates that RB function is cell non-autonomous, that is, in the presence of wild-type cells RB *null* cells show a normal phenotype, suggesting that the RB requirement of an erythroid cell can be bypassed by signals supplied by another erythroid cell[252]. This is remarkably similar to the phenotype of the GATA1 overexpressing mice[251] (see Chapter 2 of this thesis), which is also cell non-autonomous.

Furthermore, GATA1 has been shown to bind RB in erythroid cells and erythroid-specific GATA1 overexpression leads to RB hyperphosphorylation via an as yet unknown mechanism[254].

Krüppel-like factors

GATA1 has been shown to physically interact and functionally synergize with Sp1 and erythroid Krüppel-like factor (EKLF or KLF1)[69,144], two transcription factors belonging to the wide Sp/XKLF family of transcription factors (reviewed by Philipsen and Suske[191]).

Sp1 is a ubiquitously expressed transcription factor essential for early embryonic development. Sp1 *null* embryos die around E9.5 and show a broad range of abnormalities, but transcription of embryonic globin genes is activated[134]. In contrast, EKLF is an erythroid-specific transcription factor[147]. EKLF *null* mice are embryonic lethal due to a defect in definitive erythropoiesis[169,185]. These embryos succumb to fatal anemia due to a defect in hemoglobin accumulation, explained by the EKLF requirement for β-globin expression[169,185].

The fact that Sp1 and EKLF can recognize GC and/or CACC motifs, which are found in close proximity of GATA motifs in several promoters, enhancers and LCRs, suggested a functional cooperation between these proteins. GATA1 was shown to bind the zinc-finger domain of Sp1 and EKLF mainly via its C-finger and reporter assays demonstrated that GATA1 transcriptional activity can be synergistically increased by these interactions[69,144]. The interaction between GATA1 and either Sp1 or EKLF was shown to be dependent on the promoter[69], suggesting discrete roles for these two factors in the regulation of erythroid-specific genes.

Interaction between GATA1 and Sp1 or EKLF may play a crucial role in, for example, bringing regulatory elements such as enhancers and LCRs in close proximity of promoters by promoting the formation of DNA loops. Recently, the formation of a complex containing the β-globin LCR and the promoters of the actively transcribing β-globin gene, the Active Chromatin Hub (ACH), has been demonstrated[32,223]. Furthermore, the presence of the transcription factor EKLF was shown to be crucial for the formation of the ACH[48].

LMO2

GATA1 had been reported to be in a complex together with LMO2, LDB1, TAL1 and E2A that can activate transcription from promoters containing an E-box (CANNTG consensus sequence) and a GATA binding site separated by 9 nucleotides[242]. The GATA-E-box motif has been reported in promoters of several genes[12,36,240] suggesting an important role for this motif.

GATA1 interacts directly with LMO2, but not with TAL1, in erythroid cells[176,177]. These authors also showed that GATA1 and TAL1, in the presence of LMO2, synergistically activate transcription of reporter genes[177]. From these data, a model was proposed in which GATA1 and the TAL1-E2A complex[233,241] bind DNA and LMO2, together with its partner LDB1. LMO2 makes the bridge between the transcription factors, either as single a molecule or by homodimerization.

The role of such a complex in hematopoiesis is not known but considering the important functions of the intervening proteins (reviewed elsewhere in this thesis) it is likely that this complex is of extreme importance. This complex was found to bind the GATA-E-box motif in the upstream regulatory sequence (HSI) of the GATA1 gene, and the integrity of the GATA binding site is crucial for binding. However, its functional importance remains unknown[240]. Furthermore, disruption of the GATA-E-box-GATA motif present in the EKLF promoter abrogates the expression of the gene. This suggests that the formation of GATA1/LMO2/TAL1 complex is required for transcription activation[12,36].

PU.1

PU.1 is a member of the *ets* family of transcription factor required for the development of the lymphoid and granulocytic/monocytic lineages (reviewed elsewhere in this thesis). Expression of PU.1 and GATA1 seems to be mutually exclusive suggesting an antagonistic effect of these two transcription factors.

Several lines of evidence indicate that GATA1 and PU.1 functionally antagonise each other via direct physical interaction[166,197,266]. PU.1 and GATA1 interaction takes place via the DNA-binding domains of both proteins[197,266] but the mechanisms by which these transcription factors antagonise each other are quite distinct. GATA1 inhibits PU.1 by preventing it to interact with its cofactor c-Jun[266] while PU.1 inhibits GATA1 by preventing its binding to DNA[267].

P300/CBP

P300 and CBP are ubiquitously expressed proteins with histone acetyltransferase (HAT) properties, known to interact with a large number of transcription factors. The mechanism by which P300 and CBP intervene in transcription regulation is not clear and several models have been proposed. Binding of P300/CBP to transcription factors can be a way to recruit histone acetyltransferase to the vicinity of nucleosomes and induce an open chromatin configuration, thus stimulating transcription. CBP and P300 can also serve as a bridging molecule between components of the general transcription machinery and enhanceosome complexes. Furthermore, P300 and CBP are known to be responsible for the acetylation of transcription factors which seems to have a direct effect on their function (reviewed by Vo and Goodman[237]).

GATA1 has been shown to bind P300[25] and CBP[90] both *in vitro* and *in vivo*. As mentioned before, P300 and CBP acetylate GATA1[25,90] but it is unlikely that this is the only function of these proteins. The involvement of P300/CBP in the recruitment of other transcription factors, co-factors or chromatin-remodelling complexes to regulatory sequences bound by GATA1 may be envisaged.

PIASy

It has been recently reported that GATA1 can be SUMOylated and such modification is promoted by the SUMO ligase PIASy[37]. Interestingly, SUMOylation does not seem to be required for GATA1 transcriptional activity but the interaction of GATA1 with PIASy, independent of SUMOylation, results in a repression of the GATA1 transactivation potential. PYASy is a member of the PIAS (Protein Inhibitor of Activated STAT) protein family original identified has negative regulators of STAT transcription factors but later on classified as E3-like SUMO ligases. E3-like PIAS proteins concentrate in nuclear foci that partially localize with PML bodies, nuclear bodies of as yet undefined biological function but that contain numerous transcription factors and transcription coregulators, most of them known to be SUMOylated (reviewed by Muller and colleagues[158]). This suggests an important role in the regulation of transcription factors.

3.1.3 GATA1 target genes

GATA sequences are quite abundant in the genome and GATA consensus sequences can be found in the promoters of many genes by searching the appropriate databases. An increasing number of GATA1 target genes are being identified using functional assays and more recently using high-throughput gene expression analysis afforded by DNA microarrays.

In this Chapter, a short overview of the most relevant of the growing number of known GATA1 target genes will be given. In Chapter 3 of this thesis data is presented from the gene expression analysis, using the cDNA microarray technology, of both *in vivo* and *in vitro* erythroid-specific GATA1 overexpression model systems.

α and β-globins

GATA1 was first identified by its interaction with the β-globin gene enhancer and was soon shown to bind to multiple regulatory regions in both the α- and β-globin locus[243,53,137]. Despite the absence of GATA1, GATA1 *null* erythroid cells are still able to produce hemoglobin suggesting that GATA1 may not play a critical role in the transcription of the globin genes[246]. Another possibility is that GATA1 is replaced by GATA2, which is known to be upregulated in GATA1 null cells, since GATA binding sites are essential for expression of β-LCR driven transgenes[190].

Heme biosynthesis enzymes

Hemoglobin, present in large quantities in erythrocytes, is the protein responsible for the transport of oxygen and carbon dioxide throughout the body. Hemoglobin is a tetrameric protein composed of two α-like and two β-like globin chains. Each globin chain carries a heme group, a ring shaped molecule containing a central iron atom, which can reversibly bind oxygen. Heme is synthesised from glycin and succinyl CoA via a series of steps involving the action of 8 enzymes (reviewed by Ponka[192]). Several of the genes encoding for enzymes involved in the heme biosynthesis (ALAS2, ALAD, HMBS) are known GATA1 target genes[174,203] emphasising once more the important and broad function of GATA1 in erythroid cells.

ABCme

ABCme (ABC mitochondrial erythroid) was identified as a GATA1 target gene[209] upon analysis of genes induced during GATA1-dependent differentiation of the G1E GATA1 *null*

erythroid progenitors[209]. ABCme belongs to the ABC (ATP-binding cassette) protein family, which members are involved in the transport of compounds through cellular membranes. ABCme is a mitochondrial inner membrane protein transporter, expressed in several tissues but predominantly in erythroid cells. ABCme overexpression in MEL cells enhances hemoglobin production suggesting a possible role in heme biosynthesis.

Erythropoietin (Epo) and Epo receptor (EpoR)

Erythropoietin (Epo) is the major growth factor for erythroid cells. Epo interacts with the Epo receptor (EpoR), a cell surface receptor expressed in erythroid, megakaryocytic and mast cells, triggering signalling cascades leading to the proliferation, differentiation and survival of erythroid progenitors (reviewed by Lacombe and Mayeux[115]).

GATA1 was first reported to be involved in the regulation of the EpoR by Zon and colleagues[273]. These authors showed that GATA1 could specifically bind and transactivate the EpoR promoter. A second report[34] also shows that GATA1 is expressed prior to the EpoR but its expression is strongly enhanced by EpoR-mediated signals. Surprisingly, Weiss and collegues[246] showed that EpoR is normally transcribed in GATA1 null erythroblasts, strongly suggesting a role for GATA2 in EpoR expression in early erythroid precursors.

In contrast to their function as Epo receptor activators, GATA proteins were shown to negatively regulate apoxia-induced Epo expression by binding to GATA sites in its promoter[92,93]. The reason for the contrasting functions of GATA proteins in the regulation of the EpoR and Epo genes remains unclear.

Bcl-$_{XL}$

It has been hypothesized that GATA1, in collaboration with Epo, can act as a survival factor during erythroid differentiation. The mechanism by which that is accomplished is not known but Bcl-$_X$, a member of the Bcl2 gene family, is a good candidate to mediate survival during erythroid development. Due to alternative splicing the Bcl-$_X$ gene can originate two distinct mRNAs: Bcl-$_{XL}$, a larger mRNA that codes for a protein with anti-apoptotic properties and Bcl-$_{XS}$, a smaller mRNA that, surprisingly, encodes for a pro-apoptotic protein. Bcl-$_{XL}$ expression increases in late stages of erythroid differentiation and appears to be dependent on Epo[155,213,68].

Based on these observations, Gregory and colleagues[70] analyzed the expression of Bcl-$_{XL}$ during erythroid differentiation in G1E GATA1 *null* erythroid progenitors rescued by the expression of GATA1. These data show that Bcl-$_{XL}$, is upregulated in a GATA1-dependent manner during erythroid differentiation. This suggests that GATA1, together with Epo, prevents apoptosis in differentiating erythroid cells by promoting the expression of anti-apoptotic proteins such as Bcl-$_{XL}$. A direct interaction between GATA1 and the BCL-$_{XL}$ promoter has yet to be demonstrated.

GATA2

GATA2 is an important regulator of hematopoiesis, its downregulation being crucial for hematopoietic differentiation (reviewed later in this thesis).

The first clue about a possible regulation of the GATA2 gene by GATA1 was the observation that GATA2 is upregulated in the absence of GATA1[246]. Further analysis of GATA1-regulated genes consistently identified GATA2 as being repressed by GATA1[203,209] through a mechanism involving a GATA1/FOG1/MeCP1 complex[201].

In a recent report[67], GATA1 was reported to bind to a region 2.4kb upstream of the GATA2 1G promoter. The same report also showed that GATA2, together with CBP, can bind to the same regions, as GATA1 in its absence and that displacement of GATA2 by GATA1 is the cause of repression. This data suggests a mechanism by which GATA1 directly represses GATA2: GATA2, when bound to the –2.8kb site of its own locus, recruits CBP to this region leading to histone acetylation and consequently activation of transcription. GATA2 displacement by GATA1 leads to a loss of CBP and the establishment of a closed chromatin configuration through the histone deacytylase activity of MeCP1[201].

EKLF

EKLF can bind to and synergistically function with GATA1[69,144] but it is also a GATA1 target gene. Three GATA binding sites, capable of binding GATA1, were identified in the EKLF promoter and one of them was shown to be crucial for initiation of transcription[41]. Moreover, forced expression of GATA1 can activate the EKLF promoter in non-erythroid cells and EKLF expression is downregulated in the absence of GATA1[246] but it is restored upon its reintroduction[203]. GATA1 appears to induce EKLF expression as a GATA1/LDB1/TAL1 complex[201].

TAL1

In the hematopoietic system TAL1 expression, driven by promoter Ia, is restricted to erythroid, megakaryocytic and mast cell lineages[156]. The similarities between TAL1 and GATA1 expression patterns and the presence of GATA consensus sequences in the TAL1 Ia promoter suggested regulation of TAL1 expression by GATA1. More detailed analysis of the promoter requirements for TAL1 expression confirmed that GATA1 could bind the GATA consensus sequences in the TAL1 promoter Ia and transactivate its transcription[120].

Cell cycle core components and proliferation-related genes

A function in G1/S cell cycle progression has been assigned to GATA1 during erythroid differentiation (reviewed elsewhere in this thesis). Recently, Rylski and collegues[203] have reported the identification of core cell cycle components as target genes of GATA1. Making use of a model system in which a proliferating GATA1 *null* cell line (G1E) can be induced to differentiate by reintroduction of GATA1[249], the authors analysed the influence of GATA1 in the transcription of genes involved in activation and inhibition of cell cycle progression. GATA1 appeared to be able to repress expression of core cell cycle protein such as Cyclin D2 and Cdk6, involved in cell cycle progression, and activate transcription of cell cycle progression inhibitors such as $p18^{INK4c}$ and $p27^{Kip1}$. Furthermore, it seemed to induce expression of growth inhibitors, including Btg2, Hipk2, JunB and Creg, and downregulate the expression of genes with mitogenic properties such as Myc, Myb and Nab2.

Unfortunately, the experiment described above was not able to distinguish between direct GATA1 target gene and differentially expressed genes due to secondary effects. In an attempt to clarify this issue, the authors performed a more detailed analysis of the interaction between Myc and GATA1 and showed that GATA1 represses transcription of the Myc gene by direct binding to its promoter. GATA1 binds the Myc promoter as a GATA/Gfi-1b complex[201]. Surprisingly, forced expression of Myc was shown to inhibit GATA1-induced cell cycle arrest but not erythroid maturation. This suggests that Myc may be responsible for the transcriptional control of cell cycle genes such as Cyclin D2 and $p18^{INK4c}$ and $p27^{Kip1}$ but does not interfere in the control of genes involved in erythroid differentiation.

3.2 GATA2

Yamamoto and colleges identified GATA2, the second member of the GATA family, in chicken, by homology to GATA1[260].

The expression pattern of GATA2 in the hematopoietic lineages substantially overlaps that of GATA1. It is expressed in CD34 positive multilineage progenitors, early erythroid progenitors, megakaryocytes and mast cells[160]. GATA2 expression is particularly high in multipotential progenitors but its expression is downregulated upon differentiation into more committed progenitors[125,175]. Furthermore, GATA2 expression was detected in the para-aortic splanchnopleura at E9.5 in the AGM region at E10.5, fetal liver and bone marrow[149].

GATA2 plays an important role in the regulation of proliferation and differentiation of stem cell/multipotential precursors. GATA2 *null* mice die between E10 and E11 due to severe anemia[226]. The primitive erythroid cells in these embryos are apparently normal, but the total number of cells is very low, causing the lethal anemia. The analysis of GATA2 *null* chimaeric mice showed the inability of GATA2 *null* ES cells to contribute to all definitive hematopoietic lineages[226]. *In vitro* differentiation of GATA2 *null* ES cells suggests an involvement in proliferation and survival of hematopoietic precursors, and possibly in the determination of their life span[227].

GATA2 overexpression studies reinforced this hypothesis. Enforced expression of GATA2 in adult bone marrow cells blocked normal hematopoiesis by inhibiting proliferation and differentiation of hematopoietic stem cells[187]. Erythroid differentiation is also blocked by erythroid-specific overexpression of GATA2[26] (see Chapter 4 of this thesis).

A recent report[150] suggests a role for GATA2 in the formation of HSCs. Using transgenic mice in which GFP expression is driven by the GATA2 promoter, the authors showed that GATA2 is highly expressed in endothelial cells, the potential candidates for HSCs precursors. Its expression decreases upon the start of differentiation, and is upregulated again after the cells have acquired HSC characteristics. A model is proposed in which GATA2 is expressed in hematogenic cells, inhibiting their differentiation and thereby preventing the unregulated differentiation of the precursor cells and preserving the pool of immature cells. The later upregulation of GATA2 in HSCs is in agreement with the previous described function of GATA2 in the expansion of HSCs. Furthermore, recent data shows a GATA2 dose dependent effect in the production and expansion of HSCs in the AGM region and in their proliferation in adult bone marrow[128].

Since GATA2 *null* mutants are embryonic lethal and hematopoiesis is severely impaired, it becomes very difficult to evaluate the importance of this transcription factor in particular hematopoietic lineages. In spite of that, there are some indications that GATA2 plays a crucial role in mast cell formation. GATA2 is abundantly expressed in mast cell precursors but is almost undetectable in more mature mast cells[79] and in *in vitro* cultures of GATA2 *null* yolk sac cells, formation of mast cells is completely ablated[227]

Outside the hematopoietic system GATA2 is expressed in placenta[130,162] , in the central nervous system, kidney and other tissues where, once more, it performs important functions. GATA2, together with GATA3, was shown to regulate the synthesis of placental hormones [130] and GATA2 *null* embryos display neurological defects consistent with impaired proliferation and/or survival of early ventral neuronal precursors[162].

Unexpectedly, when the hematopoietic phenotype of the GATA2 null mice was rescued by the presence of a YAC containing the GATA2 gene, an important role for GATA2 in urogenital

development was revealed[269]. Rescued GATA2 null mice were viable but died soon after birth due to hydroureternephrosis. Numerous other urogenital abnormalities were identified upon a more detailed analysis of the embryonic urogenital development.

3.2.1 Regulation of GATA2 activity

Transcriptional regulation

The GATA2 transcription unit, like GATA1, is composed of two alternative non-coding first exons, IS and IG, and five coding exons, II to V[148]. The common second exon contains the translation initiation site and the zinc fingers are coded separately in exons IV and V, respectively. Exon VI codes for the basic amino acid tail region, the 3' untranslated region and two polyadenylation signals, responsible for the expression of two mRNAs with different size[160].

The IS promoter is specific for hematopoietic progenitors while the IG promoter is generally used in the tissues and cell lines that express GATA2, including hematopoietic cells. Further analysis of the IS promoter using transgenic mice revealed that it can independently drive GATA2 expression in definitive HSCs and/or early hematopoietic precursors in the AGM and adult bone marrow[149]. Both GATA2 promoters contain putative DNA binding sites for a variety of transcription factors, including GATA binding sites.

Post-translational regulation

Acetylation

It has been reported recently that GATA2 exists as an acetylated protein in immature precursor cells[82]. GATA2 can be acetylated by P300 and GCN5 and its acetylation was shown to increase DNA-binding activity. Like GATA1, GATA2 displayed a transcriptional synergism with p300 that was impaired by mutation of acetylation sites. Acetylation appears to be important for the normal function of GATA2 since mutations in the acetylation sites abolished its growth inhibitory effect.

Phosphorylation

GATA2 was shown to exist in hematopoietic progenitors in a phosphorylated form[224]. Stimulation of hematopoietic precursors with IL-3 results in an increased phosphorylation of the GATA2 protein, possibly mediated by mitogen-activated protein kinases. The function of phosphorylated GATA2 is not clear, it does not influence its transactivation potencial but it is conceivable that it is involved in the GATA2-dependent proliferation of hematopoietic precursors.

SUMOylation

It has also been demonstrated that GATA2 can by modified by SUMO in endothelial cells[35]. SUMO modification of transcription factors is often associated with reduced transcriptional activity. However, the effect of SUMOylation in GATA2 function is not known.

3.3 GATA3

GATA3 was identified, together with GATA2, based on its homology to GATA1[260]. In mice, GATA3 starts to be expressed around E8 in the ectodermal cone and trophoblastic cells surrounding the embryonic cavity. At E10 its expression is detected within the central and peripheral nervous systems. The expression within the CNS and PNS remains throughout development but becomes more and more restricted to certain structures. By E14.5, GATA3 expression is also detected in the thymic rudiment, kidney and adrenal medulla[61].

The importance of GATA3 expression in the central and peripheral nervous systems was confirmed upon analysis of GATA3 *null* mice. Homozygous mice for the GATA3 *null* mutation die between E11 and E12 and display marked growth retardation and varying degrees of abnormalities in the brain and spinal cord[183]. The cause of death was later attributed to a noradrenaline deficiency[126] since feeding of pregnant mutant females with a synthetic catecholamine intermediate allowed survival of GATA3 null embryos up to E16.5.

GATA3 haplo-insufficiency in humans is associated with the hypoparathyroidism, deafness and renal anomaly (HDR) syndrome[235]. Mice heterozygous for GATA3 do not show any obvious renal abnormalities or parathyroidism, however, heterozygous GATA3 ko mice suffer from hearing loss[234]. GATA3 expression is detected in kidney during development, indicating a possible function for GATA3 in this organ. GATA3 is also know to be involved, together with GATA2, in the regulation of expression of placental hormones[130].

Within the hematopoietic system, the GATA3 expression pattern differs from the other hematopoietic GATA factors since it is expressed exclusively in T-lymphocytes[260]. The expression of GATA3 in early thymic rudiment and in $CD4^-$ $CD8^-$ double negative (DN) T-cells[61] suggested an important role in T-cell development. This was confirmed by analysis of GATA3 KO chimeric mice, in which GATA3 ES cells contributed to all the hematopoietic lineages with the exception of thymocytes[222,84].

A detailed analysis of GATA3 expression in T cells[84] demonstrated that GATA3 is expressed throughout T-cell differentiation in the thymus. Making use of a *lacZ* reporter insertion in the GATA3 locus, the authors showed that GATA3 is expressed at low levels during the DN stage of T-cell progenitors. Surprisingly, the lowest level of expression is observed during TCR rearrangement when the cells are in cell cycle arrest in G1. In cells where TCRβ rearrangement occurred successfully GATA3 expression is upregulated. GATA3 expression is maintained in $CD4^+$ SP T-cells but its expression is gradually downregulated in $CD8^+$ SP T-cells.

Several lines of evidence indicate that GATA3 is essential for the earlier stages of T-cell development in the thymus, in particular for the expansion of the T-cell progenitors. The first evidence for the requirement of GATA3 at the earliest stage of T-cell differentiation came from the observation that antisense GATA3 oligonucleotides inhibit T-cell differentiation of fetal liver lymphoid precursors but not of more committed fetal thymic precursors[81]. This was confirmed by RAG-2 KO complementation experiments showing that differentiation of GATA3 *null* T-cells is blocked at the $CD4^-CD8^-$ DN stage[222]. Another report also shows that GATA-3 *null* ES cells do not contribute to the earliest T cell precursors (DN1) in the thymus of chimeric mice[84]. A more detailed analysis of the precise function of GATA3 during the DN stage[179], using conditionally deficient mice, revealed its importance not only in regulating the expression of the TCRβ but also during β selection.

The observation that GATA3 is differentially expressed in $CD8^+$ and $CD4^+$ SP T-cells[84] suggests a functional role for GATA3 in these lineages. In Chapter 5 of this thesis[164] it is shown

that GATA3 indeed plays a role in the commitment to $CD4^+$ SP lineage, since forced expression of GATA3 during T-cell development in the thymus inhibits the maturation of $CD8^+$ SP T-cells and induces thymic lymphomas. Analysis of GATA3 conditional knockout mice[179] confirmed that GATA3 is necessary for the generation of $CD4^+$ thymocytes.

In activated $CD4^+$ T-cells, GATA3 is selectively expressed in the Th2 subset[264,268]. Two independent reports[264,268] showed that GATA3 is expressed in peripheral naive $CD4^+$ T-cells. This expression is maintained or even increased in cells induced to differentiate to the Th2 subset, but it is drastically downregulated in the Th1 subset. Differentiation of naive $CD4^+$ T-cells into either Th1 or Th2 T-cells is largely dependent on signals from the APC. IL12 promotes Th1 development by activation of the INFγ via a STAT4 mediated signalling pathway while IL4 promotes Th2 development by upregulation of IL4 production mediated by a STAT6 signalling pathway. Cytokines induction of Th1 and Th2 differentiation is also related with differential expression of the transcription factor T-bet (T-box expressed in T-cells) in Th1 and GATA3 Th2 cells (reviewed by O'Garra and Arai[170,198] and Rengarajan and colleagues[170,198]). T-bet expression is sufficient to convert Th2 cells into Th1 cells by inducing the expression of INFγ and repression of IL4 and IL5[218]. GATA3 also seems to control Th1/Th2 priming by controlling the expression of specific Th1 and Th2 cytokines. GATA3 influence the upregulation of IL4 in Th2 cells and represses Th12 mediated signalling by repressing the IL12 receptor β2-chain expression[178,196].

In Chapter 6 of this thesis[163], we show that forced expression of GATA3 during T-cell development inhibits the differentiation of $CD4^+$ naive T-cells into the Th1 lineage. This supports the hypothesis of the necessity for GATA3 in Th2 cells, but until recently the requirement for endogenous GATA3 in Th2 cells was not proven. Using a conditional-knock out approach, Pai and colleagues[180] have now demonstrated that endogenous GATA3 is essential for the maintenance of the Th2 phenotype. GATA3-deficient Th2 cells from GATA3 conditional knockout animals showed reduced IL4, IL5, IL-13 and IL-10 production and, conversely, IFN-γ production was increased.

The presence of GATA binding sites in the promoter regions of all Th2 cytokines suggests an important role in the establishment of the Th2 cytokine profile. GATA3 was shown to directly interact with the IL5 promoter[123,268] and indirectly with the IL4 promoter[196]. GATA3 is involved in chromatin remodelling of the IL4/IL13 locus in Th1-committed cells, thereby indirectly allowing its expression[123,220]. Furthermore, GATA3 plays a role in the downregulation of the Th1-specific cytokines, since forced expression of GATA3 in Th1-commited cells leads to a downregulation of INF-γ[56,178].

3.3.1 Regulation of GATA3 activity

Transcriptional regulation

The GATA3 transcription unit is composed of six exons. The first exon consists entirely of 5' untranslated sequence since the translation start site is present in exon II. The two zinc finger motifs are coded separately in exon IV and V and the polyadenylation signal and 3' untranslated region are located in exon VI. GATA3 transcription initiation site was mapped to 189 nucleotides upstream intron I and cis-regulatory elements present in a segment of the GATA3 locus from –2052 to +1004 were shown to be necessary but not sufficient to fully recapitulate GATA3 expression during embryonic development[61].

Extensive analysis of the GATA3 locus identified specific regulatory elements required

for the correct expression in CNS, urogenital and endocardial tissues[116,117].

Asnagli and colleagues[13] recently identified an alternative promoter for GATA3 (Ia) located approximately 10Kb upstream of the previously known promoter (Ib). Analysis of the promoter usage in different tissues and cell lines revealed that promoter Ib is preferentially used in T-cells while promoter Ia is used in the CNS and in later stages of Th2 development.

Post-translational regulation

Acetylation

Aiming to obtain a GATA3 dominant negative protein, Smith and colleagues[214] performed site-directed mutagenesis in the putative transactivation domain of GATA3 present between the two zinc-fingers. A mutant, where amino acids 305 to 307 (KRR) were mutated to alanine (AAA), was identified that behaved like a dominant negative when co-transfected with wild-type GATA3 and GATA1. This mutant was shown to bind DNA similarly to wild type but fail to activate transcription. This mutant was later shown to be hypoacetylated[259] due to the mutation in the KRR motif. The same report shows that the GATA3 acetylation level directly correlates with transcription activation. An increase in transcription activity by wild-type GATA3, but not by the KRR-mutant, is observed in the presence of P300 suggesting that GATA3 can be acetylated by P300.

Phosphorylation

GATA3 was shown to be phosphorylated in Th2 cells by mitogen-activated protein kinase p38[33]. The p38-kinase-induced phosphorylation of GATA3 seems to be important for the IL5 promoter activation but the exact function of acetylated GATA3 is unknown. It may be important for the interaction with other regulatory proteins or may be directly influence GATA3 activity.

3.3.2 Protein-protein interactions

ROG

Repressor of GATA (ROG) has been identified as a GATA3 interacting protein via an yeast two-hybrid screen of a Th2 cDNA library[145]. ROG is a member of the PDZ protein family, since it contain a BTB and three zinc finger domains. The interaction between ROG and GATA3 takes place via the zinc-fingers of both proteins, being the C-finger of GATA3 crucial for the interaction. ROG is specifically expressed in lymphoid cells and its expression is upregulated upon T-cells stimulation. ROG is a potent repressor of GATA3 induced transactivation of Th2 cytokines by preventing its binding to DNA. Surprisingly, ROG can also regulate the expression of Th1 cytokines most likely by a GATA3 independent mechanism.

3.3.3 GATA3 target genes

TCR

T-cell receptor δ chain was the first target gene of GATA3 to be identified. In fact, a fragment of the human TCR δ enhancer, containing two GATA consensus sequences, was used to identify and clone the human GATA3 gene[103]. GATA binding sites can also be found in the TCRα and TCRβ enhancers suggesting that these genes are also GATA3 target genes.

Th2 cytokines

As mentioned above GATA3 is differentially expressed in Th1 and Th2 subsets of activated $CD4^+$ T-cells. It is therefore probable that GATA3 is involved in the regulation of Th2-specific cytokines.

A direct effect of GATA3 in the expression of IL5 has been shown and forced expression of this transcription factor in Th1 committed cells promotes IL5 expression[123,268]. Interestingly, a direct role for GATA3 in IL4 promoter activation has not been shown[265] but GATA3 appears to play a permissive role in enhancing IL4 expression. This suggests that other factor may be required, together with GATA3, for the activation of this promoter[196]. Finally, Lavenu-Bombled and colleagues[118] reported a direct effect of GATA3 in the regulation of IL-13 expression.

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Chapter 2

An intrinsic but cell-nonautonomous defect in GATA-1-overexpressing mouse erythroid cells.

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Nature (2000) 406: 519-524

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GATA-1 is a tissue-specific transcription factor essential for the production of red blood cells[19,25]. Here we demonstrate that overexpression of GATA-1 in erythroid cells inhibits their differentiation, leading to a lethal anaemia. Using chromosome-X-inactivation of a GATA-1 transgene and chimaeric animals, we show that this defect is intrinsic to erythroid cells , but nevertheless cell nonautonomous. Usually, cell nonautonomy is thought to reflect aberrant gene function in cells other than those that exhibit the phenotype[20]. On the basis of our data, we propose an alternative mechanism in which a signal originating from wild-type erythroid cells restores normal differentiation to GATA-1 overexpressing cells *in vivo*. The existence of such a signalling mechanism indicates that previous interpretations of cell-nonautonomous defects may be erroneous in some cases and may in fact assign gene function to incorrect cell types.

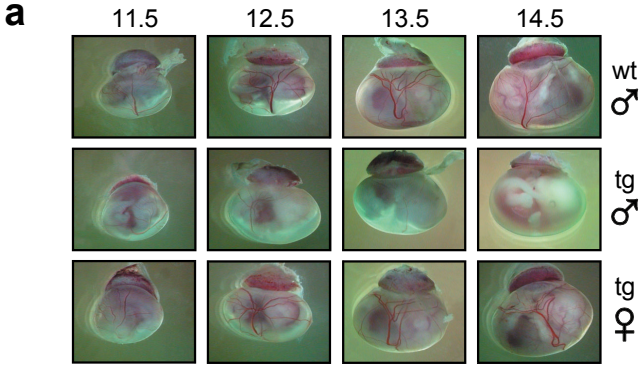
GATA-1 expression is required at the relatively immature proerythroblast stage[19,25], since GATA-1 null proerythroblasts undergo apoptosis[26] and reduced GATA-1 levels inhibit proerythroblast differentiation[23]. However, the role of GATA-1 later in erythroid differentiation remains obscure. Murine erythroleukemia (MEL) cells overexpressing GATA-1 under the control of the erythroid-specific human β-globin locus control region linked to the β-globin promoter (construct PEV3-GATA-1) fail to activate the expression of differentiation markers in response to the chemical inducer dimethyl sulphoxide (DMSO) and do not undergo differentiation-associated proliferative arrest[27]. Embryonic stem (ES) cell clones overexpressing GATA-1 also generate erythroid colonies that are inhibited in terminal differentiation[27]. Furthermore, overexpression of an inducible GATA-1 fusion protein (GATA-1-LBD, containing the tamoxifen-inducible ligand-binding domain of the oestrogen receptor) also inhibits erythroid differentiation (R.F. and D.W., unpublished data). These results may explain our failure to produce a transgenic line of mice by conventional microinjection of PEV3-GATA-1, since loss of erythroid differentiation would be lethal *in vivo*.

We therefore exploited the process of X-inactivation[13], as X-linked GATA-1 transgene should be transcriptionally active in 50% of female erythroid precursors. In males, X-inactivation does not occur and all erythroid cells should overexpress GATA-1. Mice can survive to term when 50% of erythroid precursors fail to differentiate normally, for example in females with one disrupted GATA-1 allele (which is X-linked); however, roughly 30% of GATA-1 null heterozygous females die from severe anaemia by15.5 days post coitus (dpc). In survivors, anaemia is transient and recovery is thought to be due to *in vivo* selection of normal progenitors[10,24].

DNA fluorecence-in-situ-hybridization (FISH) screening of male ES cells stably transfected with PEV3-GATA-1 showed localization of the transgene to the X chromosome in clone G4. The expected ratio in male to female progeny (38:34) was observed from 100% germline transmitting male chimeras generated from this clone, F1 females carrying the transgene and F1 males (except one) being non-transgenic. The phenotypically male transgenic animal contained two X chromosomes (one carrying the transgene) and one Y chromosome (data not shown). Chimeras generated from four other ES cell clones containing autosomal transgene integrations failed to give germline transmission.

We mated transgenic females and examined their progeny. Transgenic females were indistinguishable from wild-type littermates. Male transgenics were anaemic from 12.5 dpc and dead by 14.5 dpc (Fig. 1a and 1b). Back-crossing GATA-1 overexpressing females to FVB males for eight generations resulted in no viable transgenic males (excluding the XXY male). At 13.5 dpc, the number of enucleated erythrocytes relative to nucleated erythrocytes is reduced more than three-fold in transgenic males compared to controls, demonstrating that definitive erythropoiesis is inhibited (Fig. 1c). The fetal liver of transgenic male embryos, the site of definitive erythropoiesis at 13.5 dpc, was normal in size and cell number and contained similar numbers of apoptotic cells (terminal deoxyribonucleotide transferase (TdT)-mediated dUTP nick end labelling(TUNEL) assay; data not shown) compared to controls; however, transgenic male fetal livers contain significantly more early basophilic erythroid precursors and fewer late benzidine-positive cells (Fig. 1c), confirming that erythropoiesis is defective in fetal liver. Unexpectedly, female transgenics display no alteration in the morphology of cells in the fetal liver and have normal numbers of enucleated erythrocytes at this stage (Fig. 1c). Expression of putative GATA-1 target genes such as α-globin is unchanged in female transgenics, as RNA-FISH detection of α-globin nascent transcripts on 13.5 dpc fetal liver cells showed a normal number of cells transcribing α-globin (72% compared to 73% in controls). Furthermore, co-staining showed that half of these cells were positive for transgene-derived GATA-1 transcripts (data not shown), suggesting that the transgene is sensitive to X-inactivation.

Consistent with this interpretation, western blot analysis gave a higher level of transgene-derived GATA-1 protein in male versus female transgenic embryos (Fig. 2a). RNA-FISH using a GATA-1 probe demonstrates that at 12.5 dpc, 66% of transgenic male fetal liver cells were positive for transgene-derived GATA-1 nascent transcripts (appearing as a bright nuclear dot), while 32% of transgenic female cells were GATA-1 positive (Fig. 2b). This method did not detect endogenous GATA-1 transcripts. Co-staining for *Xist* RNA showed a single punctate signal in male fetal liver cells, corresponding to transcription from the *Xist* allele on the active X chromosome. Female cells also contained a larger area of accumulated *Xist* RNA on the inactive X chromosome (or Barr body)[18,22]. Apparent co-localisation of Barr body and GATA-1 signals occured in less than 5% of double positive cells (Figure 2b and data not shown), confirming that male transgenics express the transgene pancellularly and females heterocellularly in response to X-inactivation. Survival of transgenic mice was not dependent on being female *per se*, as a transgenic XXY male (where X-inactivation occurs[13]) was viable. One might argue that the observed phenotype arises from the X chromosomal integration event itself; however, overexpression of the tamoxifen-inducible GATA-1-LBD in mice inhibits erythroid differentiation independent of the integration site. GATA-1-LBD accumulated to lower levels than PEV3-GATA-1 derived protein and consequently induced a milder phenotype (15% reduction as compared to a 60% reduction in the proportion of differentiated erythroid cells in a CFU-E assay, data not shown).



b

	day post coitus 11.5	12.5	13.5	14.5	adult
wt male	2	14	18	2	56
wt female	2	21	13	1	52
GATA-1 tg. male	2	24 23 anaemic 1 dead	13 9 anaemic 4 dead	4 4 dead	0
GATA-1 tg. female	3	22	12	3	47

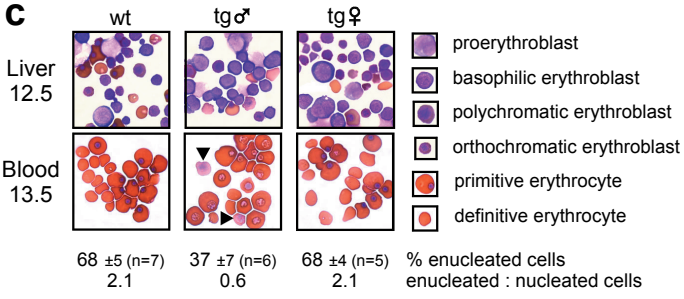
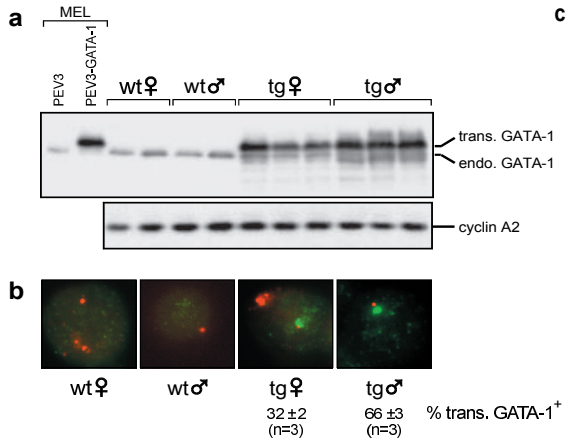


Figure 1 - GATA-1 overexpression *in vivo*. (**a***)* Representative wild-type (wt) males and transgenics (tg) of either sex, 11.5 dpc to 14.5 dpc, yolk sacs intact. **(b)** Genotype and phenotype of embryos and adults, excluding F1. Death was defined by lack of heartbeat, pallor was scored by anaemia. **(c)** Disaggregated 12.5 dpc fetal liver and 13.5 dpc blood. Indicated by key is morphology of differentiating erythroid precusors and erythrocytes. Arrowheads indicate erythroid precusors in male transgenic blood (<3% of all cells). Percentage of enucleated erythrocytes (>300 events/embryo), number (n) of independent embryos and standard deviations and ratio of enucleated to nucleated erythroid cells are shown below.

To examine the relative contribution of GATA-1-overexpressing cells to the erythroid lineage, we bred transgenic females with males containing a *lac*Z gene insertion in the X-inactivation sensitive Bruton's tyrosine kinase (Btk^{lacZ}) locus[11]. We found the same number of *lac*Z positive erythroid cells in the fetal livers of 13.5 dpc $Btk^{lacZ/+}$ and compound Btk^{lacZ+}/GATA-1 transgenic females (Fig. 2c). Adult Btk^{lacZ} males or females express *lac*Z in 50% or 25% of large

erythroid precursors in the bone marrow respectively. Compound Btk^{lacZ+}/GATA-1 transgenic females also express *lac*Z in 25% of large erythroid precursors (Fig. 2d). Thus, there is a normal representation of cells with an active lacZ gene in the erythroid compartment of GATA-1-overexpressing females throughout development. We conclude there is no selection for or against GATA-1-overexpressing cells in females. As these females generate normal numbers of definitive erythrocytes, the GATA-1 overexpressing cells must be differentiating normally *in vivo*. To confirm that GATA-1 overexpressing cells contribute to the adult erythrocyte pool, chimaeric animals were generated with clone G4 cells (which are male and express the GATA-1 transgene pancellularly). These cells contributed up to 50% of the erythrocytes, as assayed by globin chain isoform analysis (Fig. 3a) and confirmed by glucose phosphate isomerase analysis (data not shown). These cells were morphologically normal (Fig. 3b). Furthermore, analysis of the blood of female transgenic mice showed no effect on other blood cell parameters, including red cell number, cell volume and haemoglobin content (data not shown).



c

	%erythroid	% lacZ+ (of erythroid)
wildtype male (n=5)	85 ±2	0.5 ±0.2
Btk$^{lacZ/+}$ female (n=7)	84 ±3	9.0 ±3.3
GATA-1 tg. male (n=7)	79 ±3	1.3 ±0.3
GATA-1 tg. Btk$^{lacZ/+}$ female (n=9)	85 ±2	9.9 ±2.1

d

%lacZ staining	Erythroid large	Erythroid medium	B-cell lineage	Granulocytic lineage	Monocytic lineage
wild-type	3	3	1	6	1
Btk^{lacZ} male					
#1	55	19	81	63	92
#2	53	16	90	61	91
Btk$^{lacZ/+}$ female					
#1	20	7	31	33	45
#2	23	7	39	27	46
#3	24	9	37	25	44
GATA-1 tg. Btk$^{lacZ/+}$ female					
#1	35	12	48	34	56
#2	26	9	37	30	46
#3	24	7	35	22	39

Figure 2 - GATA-1 transgene is subject to X-inactivation. **(a)** Western blot of nuclear extracts from 13.5 dpc fetal livers, with anti-GATA-1 antibody N6. DMSO-induced control (PEV3) and GATA-1 overexpressing (PEV3-GATA-1) MEL cell samples are included. Position of myc-tagged transgene-derived GATA-1 (trans.GATA-1) and endogenous GATA-1 (endo.GATA-1) are indicated. Cyclin A2 used as loading control. **(b)** RNA-FISH on 12.5 dpc disaggregated fetal liver cells. Cells stained for GATA-1 (green) and Xist (red). Representative cells shown. Percentage of transgene-derived GATA-1 positive cells, number of embryos and standard deviations indicated. **(c)** Percentage of erythroid cells and percentage of lacZ$^+$ erythroid cells in 13.5 dpc fetal livers from female GATA-1 transgenic mice mated to Btk^{lacZ} males. Number of embryos and standard deviations are indicated. **(d)** Percentage of lacZ$^+$ staining cells in haematopoietic lineages of six week old mice. Erythroid cells are arbritrarily divided into large and medium-sized based on forward scatter (FSC) value. Note the GATA-1 transgenic Btk$^{lacz/+}$ female £1 which express lacZ in a high number of erythroid precursors also expresses lacZ in a higher than expected number of cells in the B-cell and monocytic lineages. This reflects the normal variance of the binomial distribution of X-inactivation balance in early pluripotent precursors.

To determine which differentiation stage is affected in the transgenic males we carried out burst-forming unit-erythroid (BFU-E, reflecting early committed erythroid progenitors) and colony-forming unit-erythroid (CFU-E, reflecting a later stage in erythroid differentiation) assays. The same number of colonies in BFU-E and CFU-E assays were formed from both wild-type and transgenic 12.5 dpc and 13.5 dpc fetal livers (data not shown). CFU-E colonies develop from single cells[6] and do not require accessory cells to form[21], so the effect of GATA-1 overexpression on female transgenic precursors when no longer in close contact with non-overexpressing cells could be addressed. In male transgenic CFU-E colonies, the production of small late differentiated erythroid cells is inhibited (Fig. 4a) and after 24 hours approximately 40% of erythroid cells were dead, compared to 20% in wild-type cultures (Fig. 4b). Female transgenic CFU-E cultures had an intermediate phenotype (Figure 4a and 4b), identical to that found when wild-type and male transgenic cells were mixed before or after culture at a 1:1 ratio. This shows that female GATA-1 overexpressing erythroid precursors are intrinsically defective and behave identically to transgenic male erythroid precursors when removed from the fetal liver.

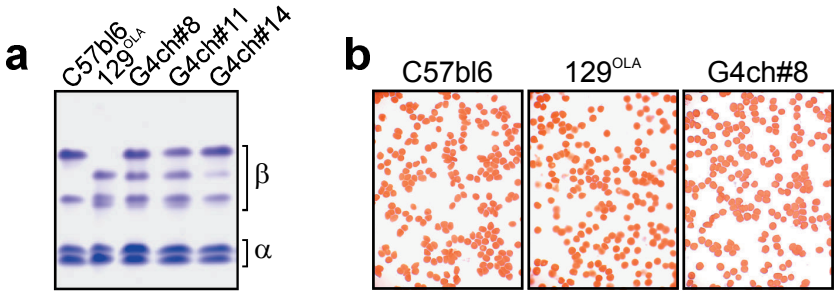
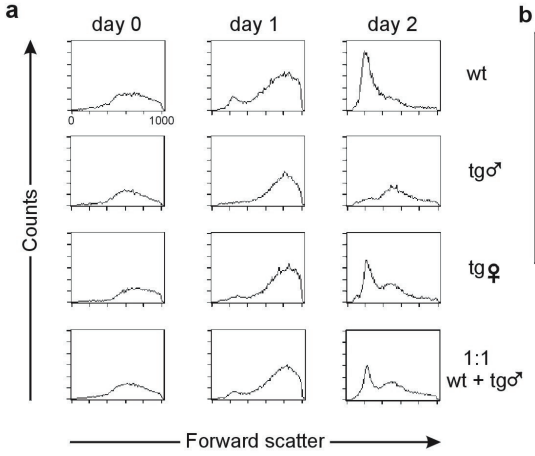


Figure 3 - Cells overexpressing GATA-1 contribute to erythroid lineage in adult chimaeric mice. **(a)** Triton-acid-urea gel of lysed blood from C57bl6 (host-blastocyst, Hb single), 129^{OLA} (ES cell strain, Hb diffuse) or clone G4 derived chimeras (G4ch#8, 11 and 14). Indicated are the positions of the alpha-type (α) and beta-type globin (β) chains. **(b)** Morphology of the blood derived from C57bl6, 129^{OLA} and G4 chimera #8 mice.

Gene function is "cell autonomous" when a cell displays a phenotype that corresponds to its genotype, regardless of the genotype of surrounding cells. Gene function is defined as "cell nonautonomous" when a cell exhibits a phenotype that does not correspond to its genotype[2]. In mosaics (that is. female transgenics and male chimeras), the phenotype of GATA-1 transgenic cells is wild-type. Therefore, the function of the GATA-1 transgene is cell nonautonomous. The conventional interpretation would be that GATA-1 overexpressing erythroid cells are normal and that the GATA-1 transgene causes a defect in a non-erythroid cell normally supporting erythropoiesis. If so, then all erythroid precursors in female transgenics would behave identically *in vivo* and *in vitro*; however, this is not the case (Fig. 4). Thus, the conventional interpretation of cell nonautonomy is incorrect.

Perhaps the mutant erythroid cells produce a negative factor inhibiting differentiation and this is diluted in mosaics. To be cell nonautonomous, however, expression of such a negative signalling factor would have to be the only defect in the GATA-1 overexpressing cells. Considering the biochemical effects of GATA-1 overexpression in erythroid cells[5,8,27], this is unlikely. As GATA-1 overexpressing erythroid cells are intrinsically defective, they must be responding to a signal reversing these defects in mosaics. We propose wild-type cells produce

a positive factor activating the differentiation of GATA-1 overexpressing cells. We tentatively name this activity "red cell differentiation signal" or REDS. The cells producing REDS must be absent or reduced in transgenic males. Definitive erythropoiesis occurs in erythroblastic islands[4]. These contain a central macrophage surrounded by erythroid cells at all stages of maturation, with immature cells close to the macrophage and mature cells near the edge of the island[3,4] (Fig. 5). The obvious candidate source of REDS is mature erythroid cells, as this is the only cell population clearly reduced in the transgenic males. Merely allowing wild-type fetal liver cells to contact GATA-1 overexpressing cells by mixing them in liquid culture does not restore differentiation to the latter (data not shown), indicating that disruption of the structure of the erythroblastic island also disrupts REDS activity. We suggest mature erythroid cells on the periphery of the island are the source of REDS. In mosaics, late erythroid cells overexpressing GATA-1 are juxtaposed with REDS-producing wild-type mature cells in the same erythroblastic island and/or the neighbouring island. This overcomes the defects induced by high GATA-1 levels and allows the final stages of erythroid maturation to proceed. In male transgenic mice there is no such juxtaposition. Death ligands expressed by mature erythroid cells can induce the degradation of GATA-1[7]. It is therefore possible that death ligands are a component of REDS. In supporting of this, death ligands partially reverse the effect of GATA-1 overexpression *in vitro* (data not shown).



b

	% dead erythroid cells		
	day 0	day 1	day 2
wildtype (n=9)	9 ±2	18 ±2	15 ±6
GATA-1 tg. male (n=5)	11 ±3	37 ±4	42 ±7
GATA-1 tg. female (n=4)	13 ±7	27 ±3	29 ±3

Figure 4 - GATA-1 overexpressing CFU-Es fail to differentiate and undergo apoptosis *in vitro*. **(a)** Representative histogram plots of FSC (indicating relative cell size) of viable erythroid cells from CFU-E assays performed on disaggregated fetal liver cells of 13.5 dpc embryos. Cells harvested before culture (day 0), after 24 hours (day 1) and 48 hours (day 2) of culture. Shown is the curve obtained by mixing transgenic male and wild-type samples in a 1:1 ratio after different periods of culture. **(b)** Percentage of dead erythroid cells in same samples as *a*. Owing to a background of dead cells with a FSC of less than 327 (using our instrument settings), present in all CFU-E samples, cells with a FSC of less than 327 were excluded from this analysis.

There is one precedent for a signalling mechanism similar to the one we propose. *C. elegans* larvae null for the IGF receptor homologue DAF-2 enter a state of diapause rather than develop into adulthood, however, DAF-2 null mosaic animals can become adult with all cells differentiating into adult tissues. Significantly, an adult phenotype is not associated with

an obligatory requirement for DAF-2 activity in a particular cell. Consequently, secondary signals operate to ensure that all cells adopt the same developmental fate[2]. Furthermore, our observations have important implications in interpreting other cell nonautonomous defects. For example, loss of the retinoblastoma protein (pRb) results in a cell nonautonomous inhibition of erythropoiesis. In accordance with the conventional interpretation of cell nonautonomy, it was concluded that pRb null erythroid cells are normal and that pRb function is required in stromal cells supporting erythropoiesis[14,29]. This conclusion may be incorrect, since transplantation studies suggest that pRb is required in erythroid cells[12]. This discrepancy remains unexplained.; however, one resolution may by that the effect of pRb loss in erythroid cells is reversed by a signal supplied by wild-type cells, similar to the one that restores the normal differentiation of erythroid cells overexpressing GATA-1.

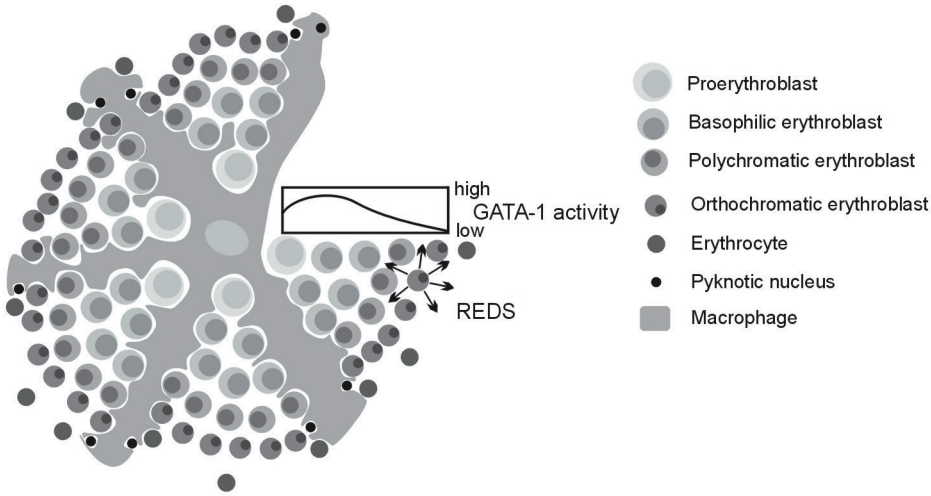


Figure 5 - A simplified model of the erythroblastic island. The position of the central macrophage and the arrangement of the erythroid precursors (as indicated by the key) within the macrophage's cytoplasmic extensions are shown. The processes of pyknosis, enucleation and phagocytosis of expelled pyknotic nuclei are represented. Arrows indicate the putative signal REDS, being produced by late differentiated erythroid cells. Graph indicates the proposed process of GATA-1 regulation late in the differentiation process.

Methods

Plasmids, probes and primers

Plasmids PEV3-GATA-1 and PEV3 have been described[9]. Details of the cloning steps to produce the puromycin resistant version of PEV3-GATA-1 are available on request. Integration of PEV3-GATA-1 in ES cells and mice was screened by Southern blot using a 1kb internal probe corresponding to the 5' end of the GATA-1 minigene. DNA-FISH and RNA-FISH was performed using a FITC-labelled 4.3 kb fragment spanning exon 2 to exon 6 of the GATA-1 gene, a Texas-Red labelled 21 kb human beta-globin LCR probe and a Texeas-Red labelled 6.5 kb fragment corresponding to the *Xist* RNA (a gift of N. Brockdorff). Embryos were sexed by PCR using primers specific for the Zfy gene[16].

Western blotting

Nuclear extracts were prepared as described[1]. Each sample (5µg) was subject to electrophoresis through a 10% SDS polyacrylamide gel, transferred onto nitrocellulose and probed with anti-GATA-1 antibody N6 (Santa Cruz) or anti-cyclin A2 antibody C-19 (Santa Cruz) and an appropriate secondary antibody before detection using chemiluminesence.

ES cells

The puromycin resistant version of PEV3-GATA-1 was linearized with *Pvu1* and electroporated into 129^{OLA}-derived ES cells as previously described[17] and individual clones selected in 1µg/ml puromycin.

Mice

Chimeric mice were generated by injecting ES cell clones generated as above into C57bl6 blastocysts. Chimeras were then bred with wild-type FVB males and screened by coat colour for germ-line transmission. Transgenic females were then mated to FVB males or Btk^{lacZ} males and sacrificed during gestation or allowed to go to term.

Blood and fetal liver cell analysis

Blood and/or disaggregated fetal livers were collected from embryos or adults and prepared on slides by cytocentrifugation. Slides were stained with neutral benzidine and a modified Giemsa-like stain.

Haemaglobin analysis

Globin chain representation in chimeric animals was analysed as described[14].

DNA- and RNA-FISH

DNA-FISH was done as described[15]. RNA-FISH was performed on disaggregated fetal liver cells as described[28].

CFU-E assay

CFU-E assays were done as described[30]. Fetal livers were disaggregated into single cells by passage through a 100µm mesh and plated at a density of $3x10^5$ cells per ml in methylcellulose containing 1U/ml Epo. Colonies were grown for times indicated and then collected and washed with PBS to remove residual methylcellulose before staining.

FACS analysis

Single cell suspensions of bone marrow, fetal livers and cultured cells were prepared as above, stained and analysed by FACS as described[21], with $5x10^4$ events taken per sample. We used the following antibodies and stains: R-PE-conjugated TER119 antibody (Pharmingen), 7-aminoactinomycin-D (7AAD, Molecular Probes BV), fluoroscein-di-b-D-galactopyranoside (FDG, Molecular Probes BV), biotin-conjugated ER-MP20 antibody, cychrome-conjugated CD45R/B220 (Pharmingen) and Tricolor-streptavidin secondary antibody (Calatag Laboratories, CA). Cell populations were divided as follows: non-viable ($7AAD^+$), erythroid ($TER119^+$), large erythroid ($TER119^+/FSC^{high}$), medium-sized erythroid ($TER119^+/FSC^{medium}$), B-cell lineage ($B220^+/ER\text{-}MP20^-/TER^-$), granulocytic ($ER\text{-}MP20^+/TER119^-$) and monocytic ($ER\text{-}MP20^{high}/TER119^-$).

Acknowledgements

We thank Rene Bernards for his critical evaluation, discussions and materials contributing to this work. We also thank Marieke von Lindern, Ton Verkerk, Vincent Lui, Ruud Delwel, Sandra Verbakel, Gaetano Zafarana, Nynke Gillemans, Dies Meijer, Theo Stijnen, Elaine Dzierzak, Eric Noteboom, Ron Kerkhoven, Jan-Herman Dannenberg, Hein te Riele and members of Rene Bernards' laboratory for advice, assistance and materials at various stages of the project. D.W. is supported by a long-term EMBO Fellowship. F.L. is supported by NWO (Netherlands). R.H. is supported by KNAW (Netherlands). NWO (Netherlands) and the EC supported this work.

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Chapter 3

Expression profiling of GATA1-overexpressing erythroid cells.

Rita Ferreira, George Garinis, Nynke Gillemans, Michael Moorhouse, David Whyatt, Frank Grosveld and Sjaak Philipsen

Ongoing work

Expression profiling of GATA1-overexpressing erythroid cells.

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We have previously shown that GATA1 overexpression in erythroid cells inhibits terminal erythroid differentiation, both *in vivo* and *in vitro*. However, specific GATA1 functions in terminal erythroid differentiation remain unclear. We employed cDNA microarray technology to analyse the expression profile of GATA1-overexpressing erythroid cells in an attempt to identify putative new GATA1 target genes.We started by analysing the gene expression profile of GATA1-overexpressing cells *in vivo*, using the GATA1 overexpressing transgenic mouse model previously generated in our laboratory. Transgenic female (TgF) embryos, expressing the transgene in approximately 50% of the erythroid cells, survive to adulthood but transgenic male (TgM) embryos, expressing the transgene in all the erythroid cells, die from anemia around embryonic day 13.5 (E13.5). Using E12.5 fetal livers from TgF, TgM and wild type (WT) embryos we identified 744 differentially expressed genes. Of these 18% were differentially expressed in both TgF and TgM. The remaining genes were differentially expressed in either TgF (26%) or TgM (56%). Differentially expressed genes were classified in agreement with their known role in particular physiological processes according to the Gene Ontology (GO) classification system. In a first analysis we identified particular physiological processes containing larger number of differentially expressed genes that correlate well with the known GATA1 functions in proliferation/differentiation, regulation of transcription and apoptosis.

Since variation is high between embryos we continued our analysis using and *in vitro* model system. GATA1-overexpressing (GATA1) and WT MEL cells were induced to differentiate and samples collected at day 0, 1,1.5, 2 and 3 of differentiation. With this approach 2704 genes were identified with statistically significant different levels of expression. These genes were also classified by gene ontology and interestingly the physiological processes containing the majority of the GO-classified genes were the same as in the *in vivo* analysis. However, in the *in vitro* analysis more genes were identified that are known to be involved in cell cycle control.

Our data indicate that that GATA1 is involved in several important cellular processes. Furthermore, we show that the experimental approach used in this analysis, i.e., the combined analysis of *in vivo* and *in vitro* systems, is reliable and thus provide a powerful new tool in the further analysis of the regulatory functions of GATA1 in terminal erythroid differentiation.

Introduction

GATA1 is the founding and best-studied member of the GATA family of transcription factors. Like all GATA transcription factors (GATA1 to 6) GATA1 is characterized by the presence of two zinc finger motifs that mediate DNA binding to (T/A)GATA(A/G) consensus sequences. Initially identified as an erythroid-specific transcription factor[23], GATA1 is now known to be expressed and play crucial roles in the generation and terminal differentiation of megakaryocytes[21], eosinophils[28] and mast cells[15].

GATA1 function in the erythroid lineage has been extensively studied but its role in

terminal erythroid differentiation remains unclear. Gene targeting studies revealed that GATA1 is essential for both primitive and definitive erythropoiesis[17] since GATA1 *null* erythroid precursors fail to mature beyond the proerythroblast stage[18] and die through apoptosis[25]. Consequently, GATA-1 *null* murine embryos die from severe anemia between embryonic day (E) 10.5 and E11.5[6].

We have shown previously that overexpression of GATA1 in erythroid cells inhibits terminal differentiation, both *in vivo* and *in vitro*[26,27]. Mouse erythroleukemia (MEL) cells overexpressing GATA1 under the control of the β-globin promoter and locus control region show defective differentiation in response to chemical induction by dimethyl sulphoxide (DMSO). These cells fail to undergo the proliferation arrest associated with differentiation, and to activate transcription of genes associated with late stages or erythroid differentiation, such as the β-globin and carbonic anhydrase form II genes[27]. A transgenic mouse line was generated in which GATA1 is overexpressed under the control of the β-globin promoter and locus control region, expressing the transgene at later stages of erythroid differentiation. The transgene was integrated on the X-chromosome, and therefore subject to X-inactivation. In this way, females express the transgene in ~50% of the erythroid cells while the males, where X-inactivation does not occur, express the transgene in all erythroid cells. Transgenic females (TgF) are able to survive to adulthood but transgenic males (TgM) die between E12.5 and E13.5 from lethal anemia[26]. We have shown that the defect in terminal erythroid differentiation is intrinsic to the overexpressing cells but it is cell-nonautonomous, since the overexpressing cells are able to mature to erythrocytes in the presence of wild-type cells. Embryos containing ~50% of the erythroid cells overexpressing GATA1 and the GATA1 gene inactivated in the remaining erythroid cells, showed a phenotype similar to the GATA1 overexpressing males. This confirms that terminal erythroid differentiation of GATA1 overexpressing cells is rescued by a homotypic signal from the wild–type erythroid cells present in the fetal liver[9].

The consequences of GATA1 overexpression both in vivo and in vitro support the notion that GATA1 plays a crucial role in the transcriptional regulation of key genes involved in terminal erythroid differentiation. Many erythroid-specific genes possess GATA binding sites in their promoters suggesting that GATA1 may directly regulate transcription of a large number of these genes[16]. For example, GATA1 is responsible for the regulation of heme biosynthesis genes[16,20], the erythropoietin and erythropoietin receptor genes[3,12,29] as well as other hematopoietic transcription factors, such as GATA2[7,24], EKLF[4] and TAL1[14]. GATA1 is also implicated in the prevention of apoptosis during differentiation since the pro-apoptic gene $Bcl\text{-}_{XL}$ has also been identified as a direct GATA1 target gene[8]. Furthermore, gene expression profiling studies performed during induced differentiation of the GATA1 null cell line G1E by reintroduction of GATA-1 identified a variety of potential direct and indirect GATA1 target genes. Among these genes are core cell cycle components, such as cyclin D2, Cdk 6 and cyclin kinase inhibitors p18 and p27, and genes involved in proliferation[20]. Abnormal expression of such genes can be a potential explanation for the defect in terminal erythroid differentiation observed in GATA1 *null* and overexpressing cells.

All the GATA1 target genes identified to date were based on comparisons of GATA1 *null versus* GATA1 expressing cells. Some of the these genes may be involved in the phenotype observed in GATA1 overexpressing cells but it is reasonable to assume that new target genes can be identified by analysis of the expression profile of GATA1 overexpressing cells. Therefore, we set out to identify new GATA1 target genes by comparing the expression profiles of wild type and overexpressing GATA1 erythroid cells using cDNA microarray technology. Comparison

between cells from E12.5 fetal livers from TgF, that survive to adulthood, and TgM, that die by E13.5, allow us to distinguish between direct GATA1 target genes and genes which expression is altered by secondary effects. For confirmation of the results obtained *in vivo*, we also compared the expression profiles of wild-type (WT) and GATA1 overexpressing (GATA1) MEL cells during DMSO-induced differentiation.

Results and Discussion

In vivo expression profiling of GATA1-overexpressing erythroid cells

Transgenic mice overexpressing GATA1 were generated by blastocyst injection of embryonic stem (ES) cells in which a GATA1 transgene under the control of the β-globin promoter and LCR was targeted to the X-chromosome (Fig. 1 A)[26]. In TgM, possessing a single X-chromosome, all erythroid cells overexpress GATA1 while in TgF, in which one of the X-chromosomes is randomly inactivated, approximately 50% of the erythroid cells express the transgene. Surprisingly, this leads to very distinct phenotypes in females and males. While the TgF are born normal, are fertile and possess normal life span, TgM die *in utero* by E13.5.

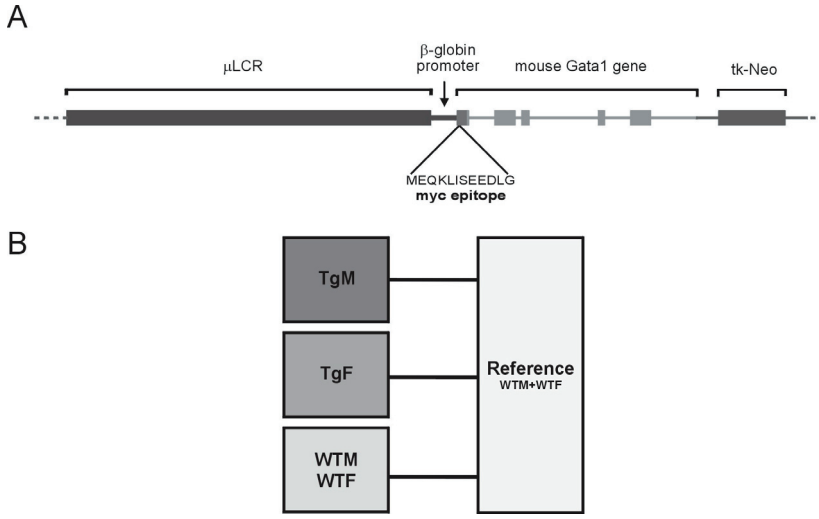


Figure 1 - GATA1-overexpressing transgenic mice **(A)** Schematic representation of the construct used to generate GATA1 overexpressing ES cells and transgenic mice. **(B)** Experimental design of expression profiling analysis GATA1 overexpressing mice. TgM-transgenic male; TgF-Transgenic female; WTM-wild-type male; WTF- wild-type female; Reference- pooled amplified RNA from WTM and WTF at a ratio 1:1.

To investigate the consequences of GATA1 overexpression in erythroid cells we performed an expression profiling analysis of both TgF and TgM. The fetal liver was the tissue of choice for these experiments since it is the main hematopoietic site during late embryonic development, composed of approximately 70% erythroid cells[9]. Fetal livers were collected at E12.5 to avoid secondary effects caused by the fact that transgenic males are dying by E13.5 and consequently cells are expected to express genes involved in hypoxia and apoptosis. To reduce the impact of variability between samples, transgenic and wild type littermates were used for the analysis. Total RNA from individual fetal livers collected from TgF, TgM and both wild-type

females (WTF) and males (WTM) was used for probe preparation. Poly-adenylated RNA from those samples was amplified by T7-mediated *in vitro* transcription, and 3µg of amplified RNA was used for probe preparation. Probes were labelled by direct incorporation of Cy3 or Cy5 dyes by reverse transcription and hybridised to cDNA microarray containing approximately 15000 murine cDNAs. A reference design was used for this experiment, as described in Figure 1B. Each individual sample was hybridised against a common reference sample composed of amplified RNA from WTF and WTM fetal livers RNA, pooled at a ratio 1:1. To avoid bias due to dye incorporation dye-reversed hybridisations were performed for each sample.

To identify the sex-independent differentially expressed genes in TgF and TgM, we performed independent one-way multivariante analysis of variance (ANOVA) between TgF and WT (WTF and WTM) and TgM and WT. Genes with a p value lower or equal to 0.05 ($p \leq 0.05$) and with a fold change equal or higher than 1.5 in at least three hybridisations were considered to be significantly different. To further eliminate differentially expressed sex-dependent genes, the same analysis was performed between WTM and WTF and the significantly different genes were then removed from the list of TgF and TgM differentially expressed genes. With this approach, 330 and 550 differentially expressed genes were identified in TgF and TgM respectively.

To confirm that the above results reflect significant rather than random changes in gene expression, we performed unsupervised hierarchical clustering of genes identified as significantly different (Fig. 2).

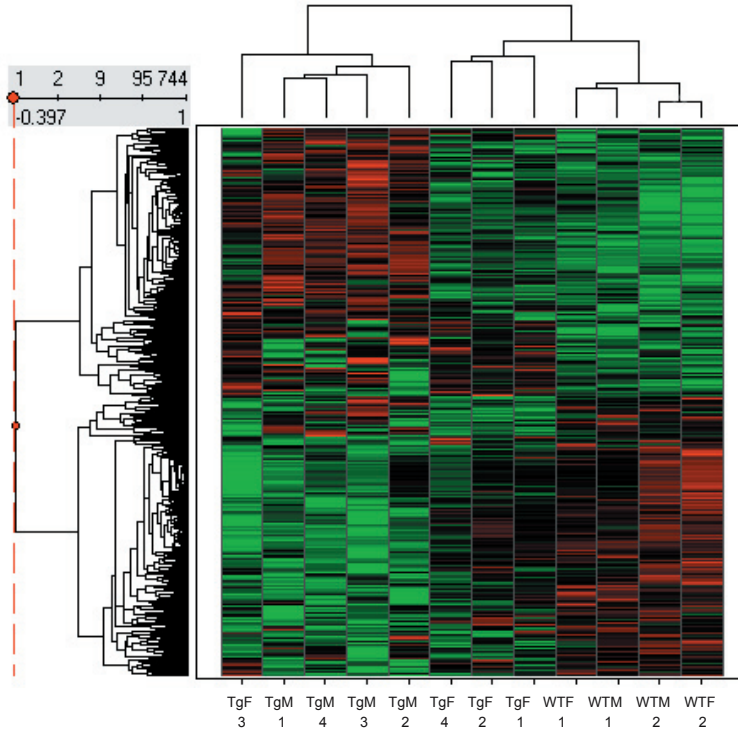


Figure 2 - Non-supervised hierarchical cluster of differentially expressed genes ($p \leq 0.05$, fold difference of ±1.5 in at least 3 experiments) between TgF and WT and TgM and WT.

WTF and WTM cluster together indicating that they are almost indistinguishable from each other upon removal of the sex-specific genes. In contrast, TgMs and TgFs cluster separately from each other. TgMs cluster separately from all the remaining samples, indicating that they are the most dissimilar, in agreement with the observed phenotype. Three out of the 4 TgFs cluster together and are more similar to the WTMs and WTFs that to the TgMs. The fourth TgF is more similar to the TgM. This correlates well with the observation that TgFs, due to the random nature of X-inactivation, show a more variable phenotype. Some TgF are indistinguishable from the WT littermates but others show a range of erythroid defects.

In order to reduce the complexity of the data set and confirm the clustering results, we employed a Principle Component Analysis (PCA) (Fig. 3). The PCA results are in good agreement with the clustering results. TgMs group together and are the most dissimilar from the WTs. Three of the TgFs group together and are more similar to the WTs than the TgMs. The fourth TgF is less similar to the WTs than the other three TgFs, and is more similar to the TgMs. Detailed analysis of GATA1 expression in the different samples shows that from all the TgFs, this one has the highest GATA1 expression (data not shown).

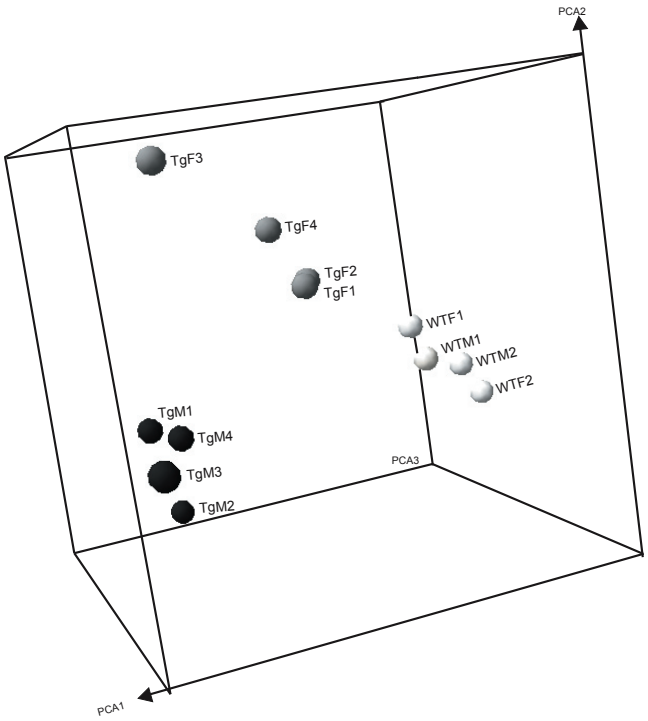


Figure 3 - Principal component analysis of differently expressed genes ($p \leq 0.05$, fold difference ±1.5 in at least 3 experiments) between TgF and WT and TgM and WT.

To identify the genes regulated by GATA1 we compared the differentially expressed genes in TgFs and TgMs to each other (Fig. 4). From the total number of differentially expressed genes (744) identified in TgFs and TgMs, 18% (136 genes) of the genes were common to both groups (TgMF), while 26% (194 genes) and 56% (414 genes) were exclusively found in the TgF and TgM groups, respectively. Differentially expressed genes common to TgFs and TgMs

are likely to reflect the direct effect of GATA1 overexpression in the erythroid cells. The large number of differentially expressed genes in TgMs will be partially due to secondary effects of GATA1 overexpression. It will also reflect differences in the cell population since terminal erythroid differentiation of GATA1-overexpressing erythroid cells in males is blocked. This does not happen in the TgFs due to the presence of wild type cells which rescue the terminal differentiation of the GATA1-overexpressing cells[26]. The relatively small number of genes differentially expressed in the TgFs may reflect variation between the different females, since they show variable phenotypes due to the random nature of X-inactivation, or to the influence of the wild-type cells on the differentiation of the GATA1 overexpressing cells.

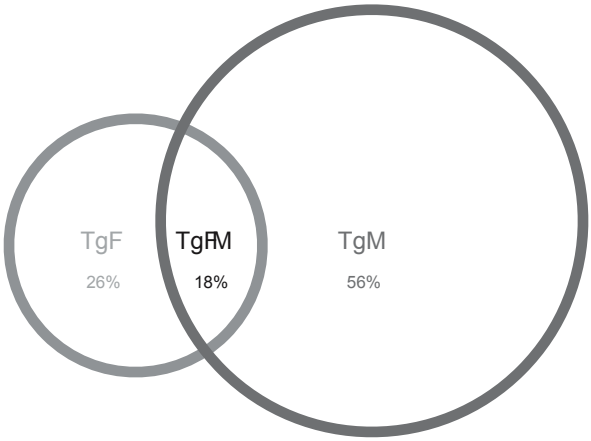


Figure 4 - Venn diagram representation of TgF and TgM biosets. Each circle represents the number of differently expressed genes ($p \leq 0.05$, fold difference of ±1.5 in at least 3 experiments) between TgF and WT (TgF) and TgM and WT (TgM). The percentage of genes shared between differently expressed genes (TgM TGF) is indicated.

To further validate the data obtained several differentially expressed genes were selected for further analysis by real-time quantitative (RQ)-PCR. These genes were chosen on the basis of their significance, fold change and/or their status as putative GATA1 target genes. This analysis was performed on non-amplified RNA samples to discard possible bias due to RNA amplification. TFE3 (Transcription Factor binding to immunoglobulin heavy chain Enhancer 3) and RUFY1 (Run and Fyve domain containing 1) transcripts are two of the most significant differentially expressed genes identified in this analysis. They have low p-values and are upregulated over 2-fold in TgFs and up to 7-fold TgMs. Furthermore these genes are found to be upregulated in 21 and 16 hybridisations, respectively, out of a total of 24 experiments. RQ-PCR analysis using primers specific for TFE3 and RUFY1 confirmed the expression profiles observed in the microarray experiments (Fig 5A). Both TFE3 and RUFY1 are overexpressed in TgFs (~ 2.5 fold) and TgMs (~ 6 fold). TFRC (Transferrin receptor) expression was moderately but significantly downregulated in TgMs (~3 fold) and 1.5 to 3-fold downregulation was seen in some TgFs, although not statistically significant. Samples analysed by RQ-PCR show a decrease in TCFR expression in both TgMs and TgFs indicating that the degree of TCFR downregulation varies between TgFs (Fig 5B). This suggests that TCFR is a direct target of GATA1 since GATA1 expression levels also varies between females due to the random nature of X-inactivation.

GATA binding sites are present in the LCR and promoter regions of the α-globin and β-globin gene clusters[10,19] but only a small reduction of the expression level of α-globin (HBA1) and β-major (HBB-B1) genes was observed in *in vitro* differentiated GATA1 *null* cells[24]. A reduction in the HBA1 expression was detected in TgMs and at lesser extent in TgFs, with a borderline significance (p value ranging from 0.04 to 0.02). RQ-PCR analysis confirmed that HBA1 is reduced about 2-fold in TgMs but no significant reduction was detected in TgFs (Fig. 5B). No significant reduction in HBB-B1 was detected in TgFs and TgMs, probably due to the fact that HBB-B1 is present in only a few spots on the microarray. Analysis of HBB-B1 expression by RQ-PCR revealed that HBB-B1 is expressed at lower level (~2 fold) in TgMs (Fig. 5B). Dowregulation of HB1A and HBB-B1 expression was confirmed by quantitative S1 protection assay (Fig 5C). Using this more sensitive assay, a small reduction in expression levels of HB1A and HBB-B1 was also detected in TgFs.

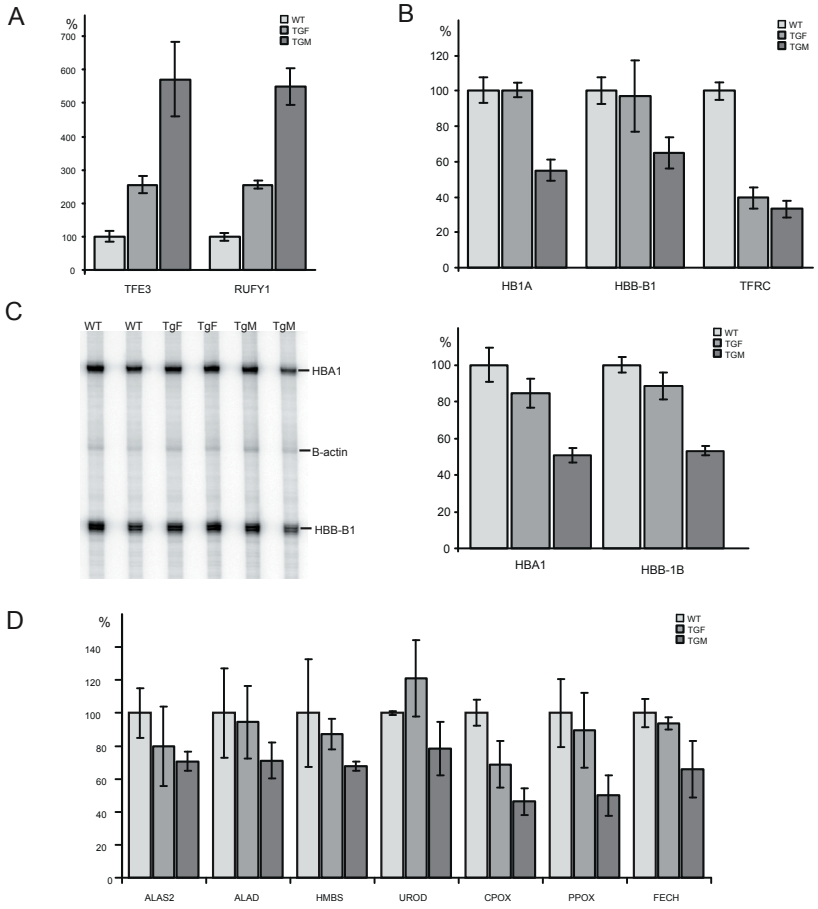


Figure 5 - Validation of microarray data by RQ-PCR and S1protection assay. **(A)** RQ-PCR analysis of TFE3 and RUFY1 expression in WT, TgF and TgM. **(B)** RQ-PCR analysis of HBA1, HBB-B1 and TFRC expression in WT, TgF and TgM. **(C)** S1 protection analysis of HBA1 and HBB-B1 expression in WT, TgF and TgM. **(D)** Analysis of the expression of genes involved in heme byosinthesis by RQ-PCR analysis.

Since GATA binding sites are present in regulatory sequences of genes involved in heme biosynthesis, we analysed the expression pattern of the genes involved in this pathway in more detail. Some of these genes, namely ALAS2, UROD, PPOX and FECH, are among the 15000 cDNA spotted on the microarrays used for this analysis. ALAS2 and UROD are downregulated in the TgMs while FECH is downregulated in both TgFs and TgMs. PPOx is also downregulated but at levels below the threshold of significance. RQ-PCR analysis confirmed that ALAS2 is slightly downregulated in the TgMs but not in the TgFs once more reflecting the large variability among TgFs. Other genes, such as UROD, CPOX, PPOX and FECH are also downregulated in TgMs by RQ-PCR. The effect on the biosynthesis of heme, however mild, may be either a consequence of the fact that GATA1 cells are unable to normally differentiate or a possible cause for their inability to differentiate.

The RQ-PCR and S1 analysis performed on a number of selected genes validates the results obtain with microarray analysis and discards the possibility of bias introduced by the amplification of the original RNA samples.

Gene ontology classification and network analysis of in vivo differentially expressed genes

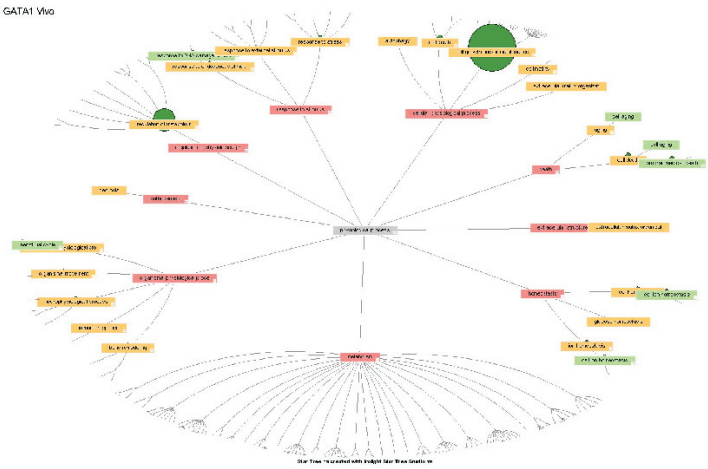
The interpretation of high throughput biological data is difficult due to the large amounts of data generated. In order to facilitate the understanding of the biological relevance of the data, functional categories, describing the full repertoire of biological processes according to the publicly available Gene Ontology (GO) classification system (www.geneontology.org), were assigned to the genes in each bioset (TgM, TgF and TgMF), beginning at the level of "Physiological Processes" (GO:0007582). To visualize these functional groups of significant genes we used a tool based on Star Tree™ visualization technology, in order to represent the data set within the tree-like structure of the GO classification system (see Figure 6A-B or "in vivo" and "star tree visualization" at http://michael.bioinformatix.nl/GATA1/).

In an attempt to identify 'over-represented' GO-classified biological processes we compared the number of pertinent genes in a given biological process to the total number of the relevant genes printed on the NIH 15K cDNA microarray for a particular biological process (Fisher exact test, $P \leq 0.05$, False detection rate (FDR) ≤ 0.1) using the publicly accessible software Ease [11]. Such analysis did not reveal any significantly over-represented processes, which suggests either that GATA1 is involved in a wide variety of functions or that the cDNA microarray platform is not the best platform to be used for this analysis.

However, comparison between the numbers of significantly expressed genes assigned to each GO-classified biological process may suggest a role for GATA1 in those processes. From the total number of differentially expressed genes identified (744) only 28% (208 genes) were assigned to physiological processes by GO-classification. The processes containing the largest numbers of genes were "cell growth and maintenance" (GO:0008151; 39%) followed by "protein metabolism" (GO:0019538; 28%), "Nucleic acid metabolism" (GO:0006139, 23%), "regulation of transcription" (GO:0045449; 19%) and "biosynthesis" (GO: 0009058; 16%). This distribution of genes assigned to physiological processes correlates well with known GATA1 functions in cell proliferation/differentiation, apoptosis, transcription activation/repression, and heme biosynthesis. Within the "cell growth and maintenance" process, the majority of the genes (60%, 24% of the total GO-classified genes) are categorized under "transport" (GO:0006810). More detailed analysis of the genes in this category reveals that the majority of these genes are differentially expressed exclusively in TgMs and therefore may not be direct GATA1 target

genes. Interestingly, many of these genes are either erythroid-specific, such as HBA1, which is dowregulated in the TgMs and in some TgFs, and/or with known functions in erythroid cells, such as TFRC (transferrin receptor), important for the heme biosynthesis.

A



B

Accession	Gene description	Similarity to gata-1	Similarity rank to gata-1	ANOVA
BG084969	Origin Recognition Complex, Subunit 2-Like (Yeast) (ORC2L)	5.153	468	0.01279
BG086482	Chromosome Condensation-Related Smc-Associated Protein 1; (CNAP1)	5.928	361	0.02715
AL043837	Spindlin (SPIN)	5.206	736	0.00104
BG068506	Cytoplasmic Polyadenylation Element Binding Protein 1; (CPEB1)	5.302	750	0.04510
BG068329	V-Myb Myeloblastosis Viral Oncogene Homolog (Avian)-Like 1; (MYBL1)	3.614	138	0.02509
BG086922	Checkpoint With Forkhead And Ring Finger Domains; (CHFR)	5.563	1579	0.01922
BG087882	Synaptonemal Complex Protein 1; (SYCP1)	4.042	174	0.01306
BG082192	B-Cell Translocation Gene 4; (BTG4)	3.461	128	0.04279

C

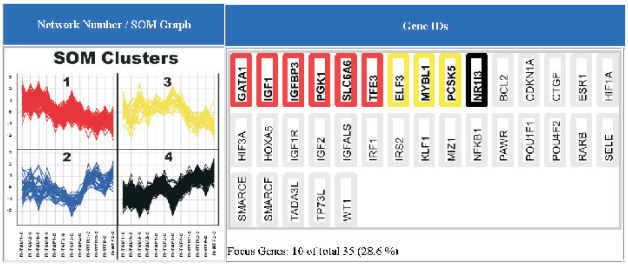


Figure 6 - Graphical representation of the interactive visualizations. **(A)** Analysis of GO-classified data using the StarTree View, allowing the three-dimensional representation of the full repertoire of biological processes within GO classification system. **(B)** Selection of any node from (A) allows interactive querying of expression profiles of the individual genes. **(C)** Visualization of significant genes classified according to gene networks in which they are most likely to participate based on known molecular interactions.

To further elucidate the structure and meaning of these expression patterns we annotated the genes whose products function in common pathways or "networks" as defined by the commercially available Ingenuity database (www.ingenuity.com). Each network was annotated by listing its statistical significance and the top functions associated with combinations of genes in that network (see Fig. 6C or "in vivo" and "pathway analysis" at http://michael.bioinformatix.nl/GATA1/). The most significant network (based on the network significance score) contained genes involved in the transformation, adhesion, proliferation and apoptosis of cells. The majority of genes within this network as similar or opposite expression pattern from GATA1, *i.e.* either are upregulated or downregulated in TgMs and, to a lesser extent, in TgFs. At least one of the genes in these network, BNIP3 has been implicated in apoptotic response induced by hypoxia suggesting that its upregulation may be a consequence of the phenotype rather that a direct effect of GATA1 overexpression[13]. Not surprisingly the network containing GATA1 is implicated in transcription, cell cycle progression, growth and differentiation, processes in which GATA1 is known to be involved. This network contains other genes, such as IGF1 (Insulin-Like Growth Factor 1) and IGFBP3 (IGF1 binding protein 3), which are important in fetal and adult growth and also in cancer. Other significant network contained genes involved in G1 progression, DNA damage and chromosome condensation. We have shown before that GATA1 overexpression in MEL cells prevents their arrest in G1 phase of the cell cycle [27]. Furthermore, deregulation of the process of chromosomal condensation may be one of the reasons why GATA1-overexpressing cells to fail to enucleate.

In vitro expression profiling of GATA1 overexpressing erythroid cells

Since embryos display some developmental variation, and the phenotype of TgF is also variable depending on X-inactivation, we decided to compare the expression profiles of WT and GATA1-oerexpressing MEL cells. MEL cells are transformed mouse erythroblasts that can be induced to differentiate with DMSO, thereby providing a synchronized system to study erythroid differentiation.

MEL cells were stably transfected with the same construct used for the generation of transgenic mice, expressing GATA1 under the control of β-globin promoter and Locus Control Region (GATA1) or vector alone (WT) as described[27] (Fig. 1A). Three independent GATA1 and two WT MEL cell populations were induced to differentiate with DMSO and samples were collected at day 0, 1, 1.5, 2 and 3 of differentiation. RNA isolated from GATA1 and WT populations at the different time points was amplified by T7-mediated *in vitro* transcription and used for probe preparation. Probes were labelled by direct incorporation of Cy3 or Cy5 dyes by reverse transcription and hybridised to cDNA microarray containing approximately 15000 murine genes.

A "time-vehicle control" approach was employed to eliminate potential variation between samples through time due to technical variations (Fig. 7A). Probes from each time of differentiation of the GATA1 populations were hybridised using the corresponding time of differentiation of the WT populations as the reference. Hybridisations were also performed between the two WT populations. To avoid bias due to dye incorporation dye-reversed hybridisations were performed for each sample.

Differentially expressed genes between WT and GATA1 MEL cell populations were identified by one-way multivariate analysis of variance (ANOVA). This analysis was performed for all the different days of differentiation and 2704 genes were identified as significantly

different ($p \leq 0.05$ and with a fold change ≥ 1.5 in at least 1 experiment). These significantly different genes were subjected to unsupervised hierarchical clustering analysis, revealing a good correlation between the time of differentiation and the different replicates (Fig. 8). WT cells, of all days of differentiation, cluster together with GATA1 cells at day 0 of differentiation indicating that they are very similar. The different GATA1 replicates cluster according to the day of differentiation: Day 1 and day 1.5 cluster separately but day 2 and day 3 cluster together. Since we compared RNA samples of corresponding differentiation times, GATA1-independent differences occurring during MEL cell differentiation are in principle taken out of the analysis. The clustering between WT controls and the different GATA1 populations at day 0, when the transgene is not expressed or expressed at very low levels, shows that the expression profiles of these cells are very similar. The observation that GATA1 replicates at day 1, day1.5 and day2-3 cluster separately indicates that GATA1 expression interferes dynamically with the expression profile of the cells during the first two days of differentiation, reaching a plateau by day 2-3. This correlates well with the expression pattern of the GATA1 transgene (Fig. 7B).

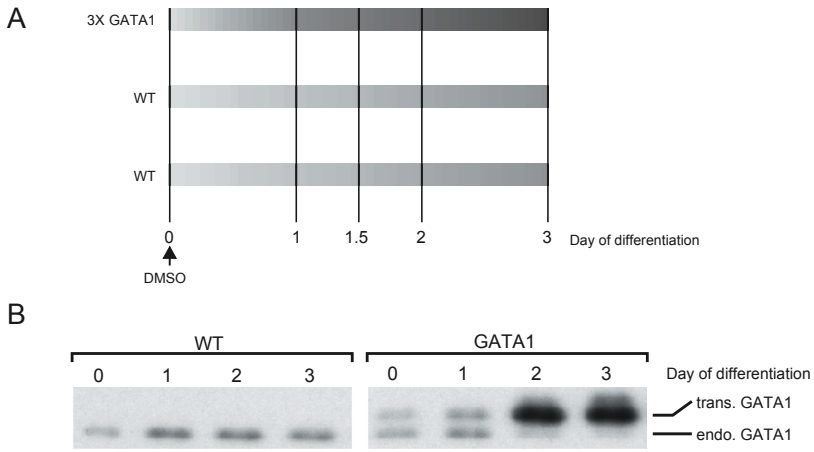


Figure 7 - GATA1-overexpressing MEL cells **(A)** Experimental design of expression profiling analysis GATA1 overexpressing MEL cells. **(B)** Western blot analysis of nuclear extracts from WT and GATA1 MEL cells throughout DMSO induced differentiation.

Gene ontology classification and network analysis of in vitro differentially expressed genes

Functional categories were assigned to the differentially expressed genes according to the publicly available Gene Ontology (GO) classification system (www.geneontology.org) and visualized using Star Tree™ (see "in vitro" and "star tree visualization" at http://michael.bioinformatix.nl/GATA1/).

We compared the numbers of significantly expressed genes assigned to each GO-classified biological process. From the total number of differently expressed genes identified (2704) only 33.4% (903 genes) were assigned to physiological process by GO-classification. The processes containing the largest numbers of genes were "cell growth and maintenance" (GO:0008151; 45%) followed by "protein metabolism" (GO:0019538; 29%), "Nucleic acid metabolism" (GO:0006139; 29%), "regulation of transcription" (GO:0045449; 15%) and

"biosynthesis" (GO:0009058; 15%). The increased numbers of genes assigned to processes correlate well not only with known GATA1 functions but also with the physiological process identified in the in *vivo* analysis. Within the "cell growth and maintenance" process, the majority of the genes are categorized under "transport" (GO:0006810; 47.3%, 21% of the total GO-classified genes), "cell cycle" (GO:0007049; 29.7%, 13.3% of the total GO-classified genes) and "cell organization and biogenesis" (GO:0016043; 28%, 12.5% of the total GO-classified genes). The number of differently expressed genes associated with cell cycle control is increased in the *in vitro* data when compared with the *in vivo* results. This may reflect the fact that DMSO-induced MEL cell differentiation is a synchronized process involving G1 arrest, while in the fetal liver the population of cells is mixed, potentially masking GATA1-dependent alterations to the cell cycle machinery.

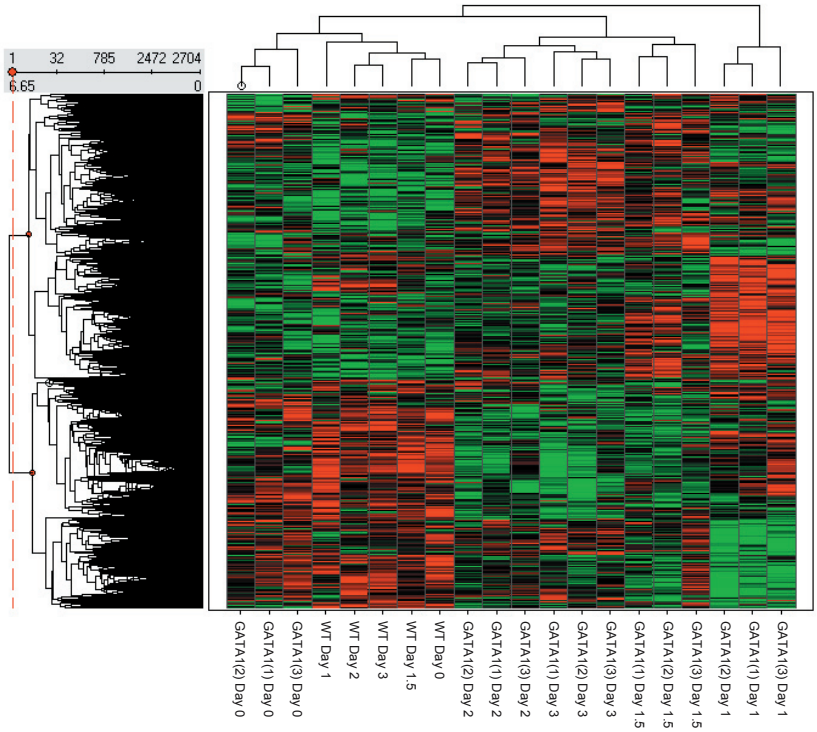


Figure 8 - Non-supervised hierarchical cluster of differentially expressed genes ($p \leq 0.05$, fold difference of ±1.5) between WT and GATA1 MEL cells throughout DMSO induced differentiation.

GO-classified differentially expressed genes between GATA1 and WT MEL cell populations were organized into "networks" as defined by the commercially available Ingenuity database (www.ingenuity.com). Each network was further annotated by listing its statistical significance and the top functions associated with combinations of genes in that network (see "in vivo" and "pathway analysis" at http://michael.bioinformatix.nl/GATA1/). The most significant network (based on the network significance score) contained genes involved in cell survival, proliferation, G1 progression. GATA1 itself is part of this network, which also includes

for example CCND2 (Cyclin D2) and CCND3 (Cyclin D3), which expression at is altered in MEL cells overexpressing GATA1 (data not shown). Other networks are implicated in cell cycle control, differentiation/proliferation, chromosome condensation, DNA modification (in particular DNA unwinding and methylation), transcription and transport. Under the category of transport we find important erythroid genes like α and β-like globins (HBA1 HBE1 and HBB-B2) among other erythroid-specific genes. One of the genes included in this category is ABCme (ABCB10) a known GATA1 target gene.

Conclusion

The data presented above, demonstrates that GATA1 is involved in several important cellular processes. The biological processes discussed above were selected on the bases of the percentage of differentially expressed genes under a particular gene ontology category but they correlate well with known GATA1 functions. This validates the quality of the data and data analysis and indicates that this data provide a good platform for future analysis of the regulatory functions of GATA1 in terminal erythroid differentiation.

Materials and Methods

Mice

Timed pregnancies were set with GATA1 overexpressing females [26] and FVB males. Fetal livers were isolated from E12.5 embryos. The embryos were genotyped by Southern blot using probes specific for GATA1 and for the Y-specific YMT gene.

Cells

C88 MEL cells were stably transfected with a construct expressing GATA1 under the control of the β-globin promoter and Locus Control Region (GATA1) or vector alone (WT) as described[27]. Three and two independent populations of GATA1 and WT MEL cell populations, respectively , were used in this study. These populations were expanded in DMEM medium containing 10% serum and antibiotics. Differentiation was induced by addition of 2% (v/v) DMSO for 3 days. Samples were collected at day 0, 1, 1.5, 2 and 3 of differentiation.

RNA isolation

Total RNA was isolated from WT and GATA1 overexpressing fetal livers and MEL cells using Tri-reagent (Sigma. RNA quantity and quality were assessed by spectrophotometry and by direct visualization of the RNA on 1% agarose gels containing formamide. Only samples with an OD 260nm/280nm ratio equal or superior to 1.8 and with a ratio of 2 between the 28S and 18S ribosomal RNA bands were used for probe preparation.

RNA amplification

A constant amount of total RNA (4μg) was used for amplification by T7 mediated *in vitro* transcription. In brief, double stranded cDNA was prepared by standard procedures using a T7dT primer (5'-GGC CAG TGA ATT GTA ATA GCA CTC ACT ATA GGG AGG CGG TTT TTT TTT TTT TTT TTT TTT TTT-3'[2]), *in vitro* transcribed using T7 MEGAscript Kit (Ambion) and purified using the RNeasy Kit (Qiagen)

Amplified RNA quantity and quality were assessed by spectrophotometry and by direct

visualization of the RNA on 1% agarose gels containing formamide. Samples with an OD 260nm/280nm ratio equal or superior to 1.8 and showing no signs of degradation were used for further processing.

cDNA labelling, hybridization and data extraction

Amplified RNA was labelled and hybridised to 15K cDNA microarrays, obtained from the Netherlands Cancer Institute (NKI), using protocols adapted from the National Institute of Aging (NIA, Bethesda, Maryland). In brief, cDNA was synthesized from 3μg amplified RNA by reverse transcription in the presence of Cy3-UTP or Cy5-UTP (Amersham). Cy3 and Cy5 labelled probes were purified using the QIAquick PCR purification Kit (Qiagen) and concentrated via Microcon YM-30 columns (Millipore). Purified probes from two samples were pooled and hybridised to cDNA microarrays in 25% formamide, 5X SCC, 0.01% SDS (containing poly d(A), yeast transfer RNA and COT-1 DNA), overnight at 42°C. Upon washing, hybridised microarrays were scanned using a laser confocal scanner (Scanarray Express HT, Perkin Elmer Inc, USA). Data was extracted with the Imagene software package version 5.0 (Biodiscovery Inc, CA, USA).

To account for possible dye-dependent bias, all amplified RNA samples were reversed labelled and reversed-dye hybridisations performed. Self-self hybridisations were also performed and any deviation from a ratio of 1 (centred to 0 for ^{2}log ratio data) was assigned to either dye effect or residual error. Dye-reversed hybridisations were considered technical replicates allowing gene-specific dye effects to be averaged out during clustering while excluding dye-dependent variances during the ANOVA analysis. In order to assure a balanced design, the same number of dye-reversed replicates was used for all samples.

Data processing and analysis

Data within each experiment (microarray) were normalized, per sub-array, using the Lowess normalization method. Data from dye-reversed hybridizations was combined by means of a weighted average. Normalization between experiments (between microarrays), from combined and non-combined dye-reversed data was obtained via the Z-score transformation.

One-way-analysis-of-variance (ANOVA) was employed to extract the statistically significant changes in mRNA expression levels. To account for the inherent variability as well as to compensate for the fact that dye bias is often not consistent, we performed this analysis on independent (non-averaged) microarray hybridizations. A *p*-value ($P \leq 0.05$) and fold change (+/- 1.5 fold change) criterion were set to alleviate the effect of significance errors while minimizing loss of significant information. Due to the expected high variation between *in vivo* (fetal liver) samples, statistically significant genes that had a fold change of +/- 1.5 in less than 3 (non–combined) experiments were removed from the analysis.

Non-supervised agglomerative hierarchical clustering, principal component analysis (PCA) and self-organizing maps (SOM) were performed on the statistically significant genes identified by ANOVA.

Z-score transformation, analysis of variance (ANOVA), hierarchical clustering, principal component analysis (PCA) and self-organizing maps (SOM) were performed by the Spotfire Decision Site software package 7.2 version 10.0 (Spotfire Inc., MA, USA).

Real time quantitative PCR

Four μg of total RNA was used for the reverse transcription reaction with oligo(dT)

(18mer) and random hexamers. Real-time quantitative (RQ)±PCR was performed in triplicate reactions on a Bio Rad iCycler machine, using the Eurogentec (Seraing,Belgium) SYBR green kit. Primers sets used: TFE3 (s 5'-acagaaggaacagcaacgc-3'; as 5'-aactcgtggttagggagagca-3'), RUFY1 (s 5'-ggttgaaaga tgacgaggcaa-3'; as 5'-acacacacgcactggctta-3'), TFRC (s 5'-cgttgttgaggcagaccttg-3'; as 5'-accctgatgactgagatggc-3'), HBA1 (s 5'-tttccagggcttcagctcc-3'; as 5'-tgattctga cagactcaggaag-3'), HBB-B1 (s 5'-atgccaaagtgaaggcccat-3'; as 5'-cccagcacaatcacgatc at-3'), ALAS (s 5'-cacctatgcttaaggagcca-3'; as 5'-cagaagcacacaggaaagca-3'), ALAD (s 5'-ctttgatctcaggactgctg-3'; as 5'-aacagctgcggtgcaaagta-3'), HMBS (s 5'-tacttctggcttccaagtgc-3'; as 5'-caaggtgaggcatatcttcc-3'), UROD (s 5'-atccctgtgccttgtatgca-3'; as 5'-aggttggcaattgagcgttg-3'), CPOX (s 5'-caatttgaagccagtccgtg-3'; as 5'-ctggactagaactccctttg-3'), PPOX (s 5'-attccagcttcagagctcag-3'; as 5'-tactgcagattcaccacagc-3'), FECH (s 5'-accagtgaccacattgagac-3'; as 5'-ggccttggagaacaatggat) and Cyclophiline (s 5'-tcaccatttccgactgtggac-3'; as 5'-acaggacattgcgagcagatg). Expression levels of cyclophiline were used for normalization.

Quantitative S1 protection assay

Quantitative S1 protection assay was performed on total RNA from fetal liver of E12.5 WT, TgF and TgM embryos has described[5,22]. Quantitation of the protected fragments was performed using the ImageQuant 5.2 software (Molecular Dynamics).

Gene Ontology classification and network analysis

All significant genes were subjected to GO classification (http://www.geneontology.org). Significant over-representation of GO-classified biological processes was assessed by comparing the number of pertinent genes in a given biological process to the total number of the relevant genes printed on the NIH 15K cDNA microarray for that biological process (Fisher exact test, $P \leq 0.05$, False detection rate (FDR) ≤ 0.1) using the publicly accessible software Ease [11].

Network data was generated through the use of Ingenuity Pathways Analysis. A detailed description of Ingenuity Pathways Analysis can be found at www.ingenuity.com.

Western blot

Total nuclear protein from MEL cells was extracted as previously described[1]. Protein concentration in the nuclear extracts was determined using the bicinchoninic acid protein assay (Pierce, Rockford, IL). For Western blotting analysis, 10 µg of total nuclear protein was loaded per lane and separated on 10% SDS-PAGE gels under reducing conditions and transferred to polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA). Blots were probed with rat anti-GATA1 mAb (N6) purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and HRP-conjugated goat anti-rat Ig (Dako, Glostrup, Denmark). Peroxidase activity was visualized by ECL using standard procedures.

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Chapter 4

Rescue of GATA1 *null* mice by GATA transgenes requires the correct spatio-temporal expression pattern

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Manuscript in preparation

Rescue of GATA1 *null* mice by GATA transgenes requires the correct spatio-temporal expression pattern.

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We have previously demonstrated that hematopoietic GATA proteins (GATA1, GATA2 and GATA3), when expressed under the control of GATA1 regulatory sequences (HRD), are able to rescue the lethal phenotype of the GATA1.05 knockdown mutation. Here we show that the same HRD-GATA transgenes are able to rescue the GATA1 *null* phenotype, thus eliminating the possibility that the remaining 5% of endogenous GATA1 expression in the GATA1.05 knockdown is required for the rescue by the HRD-driven GATA factors.

However, the same hematopoietic GATA proteins are unable to rescue the lethal GATA1 *null* phenotype when expressed under control of the β-globin promoter and locus control region (βLCR). The temporal expression of the βLCR driven GATA factor transgenes is distinct from that of the HRD driven transgene and the endogenous GATA1 gene. We show that the HRD driven transgenes and the endogenous GATA1 gene are upregulated during erythropoiesis but their expression levels out at the later stages of terminal erythroid differentiation. In contrast, βLCR transgene driven expression keeps rising during terminal differentiation. These results demonstrate that the correct spatio-temporal expression pattern of GATA factor, rather than the actual GATA factor expressed, is decisive for the appropriate execution of terminal erythroid differentiation program,.

Introduction

The GATA family of transcription factors is composed of 6 members (GATA1 to 6). These transcription factors are characterized by the presence of two zinc finger protein domains that mediate DNA binding to (T/A)GATA(A/G) consensus sequences[15]. The zinc finger motifs are highly homologous between the different GATA proteins, but very little amino acid sequence homology is found outside these domains. Within the GATA family, GATA1, GATA2 and GATA3 constitute a subfamily since all three are expressed in hematopoietic cells[39].

GATA1 is the founding member of the GATA family. Within the hematopoietic system, GATA1 is expressed in erythrocytes[16], megakaryocytes[19], eosinophils[43] and mast cells[19]. Gene targeting studies have shown that GATA1 is required for normal erythroid differentiation. Loss of GATA1 activity results in a developmental arrest at the proerythroblast stage, causing these cells to undergo apoptosis[40]. Consequently, GATA1 *null* mouse embryos die from severe anemia between day 10.5 and 11.5 of gestation (E10.5-E11.5)[10,27]. Paradoxically, overexpression of GATA1 in the erythroid lineage also causes a lethal anemia. GATA1 overexpressing embryos die around E12.5 and E13.5 owing to the failure of erythroid precursors to undergo

terminal differentiation[41]. This suggests that GATA1 activity is dynamically regulated during erythropoiesis.

The hematopoietic expression pattern of GATA2 overlaps substantially with that of GATA1. GATA2 is expressed in multilineage progenitors, mast cells and megakaryocytes[16,21]. The main hematopoietic expression site of GATA3 is in the T-cell lineage[11]. In addition, expression in multilineage progenitors has been inferred from the knockout phenotype[26]

These overlapping expression patterns suggest that these transcription factors may have redundant functions. However, gene knockout studies demonstrated that a given GATA protein does not compensate for the absence of another member of the GATA family. For instance, it has been shown that *in vitro* differentiation of GATA1 *null* embryonic stem (ES) cells results in a 50-fold increase of GATA2 mRNA in erythroid cells. However, these cells are still arrested in differentiation and eventually die by apoptosis[40]. In contrast to these results, several studies have shown that other GATA proteins can rescue the GATA1 *null* phenotype at least partially, when expressed under the control of GATA1 regulatory sequences. *In vitro* differentiation of GATA1 *null* ES cells was restored when GATA3 and GATA4 were expressed under the control of GATA1 promoter[3]. The knock-in of GATA3 cDNA into the GATA1 locus partially rescued the GATA1 KO mice phenotype[36]. The GATA3 knock-in mice were still embryonic lethal but they could survive up to E13.5 and presented an increased survival of erythroid precursor cells. In addition, we have shown that GATA2 and GATA3 transgenes under the control of GATA1 regulatory sequences can rescue the embryonic lethality of a GATA1 knockdown mutation[33]. Mice carrying this mutation express ~5% of the wild-type GATA1 levels and die at E12.5. The rescued mice survived into adulthood, although they were anemic and presented abnormal erythroid cells in peripheral blood. This suggests that the GATA1 protein performs functions in adult erythropoiesis that cannot be performed by the GATA2 and GATA3 proteins. Employing the GATA1 knockdown mutant leaves open the possibility that the remaining endogenous GATA1 contributes to the rescue phenotype. To address this question, we have now performed the rescue experiment in mice carrying a complete GATA1 *null* mutation.

The previous studies indicate that other GATA proteins are able to compensate for the absence of GATA1 when expressed under the control of GATA1 regulatory sequences. This raises the possibility that the spatio-temporal control of GATA activity is the most important parameter during erythroid differentiation, rather than the actual GATA factor expressed.

In the present study, we have tested this hypothesis by comparing the potential of GATA1, GATA2 and GATA3 transgenes to rescue the GATA1 *null* phenotype when expressed under the control of different regulatory sequences. We expressed these transgenes under the control of either the hematopoietic regulatory domain (HRD) of the GATA1 gene[23,33], or under the control of the β-globin promoter and Locus Control Region (β-LCR). The β-LCR is most highly active in late erythroid cells, as it recapitulates the expression pattern of β-like globin genes[6]. Thus, we have used the rescue of a GATA1 *null* mutation by HRD and β-LCR transgenes to demonstrate the importance of timing of GATA factor expression levels during erythroid development.

Results

Generation and characterization of transgenic mice

We have demonstrated previously that transgenic mice expressing GATA1, GATA2 and GATA3 under the control of GATA1 regulatory sequences are able to rescue erythropoiesis in GATA1.05 knockdown mice[33]. In order to ascertain the importance of the spatio-temporal

control of GATA1 expression during erythropoiesis we set out to check the potential of the same genes to rescue the GATA1 null phenotype when expressed under the control of the β-globin promoter and Locus Control Region (β-LCR). The β-LCR drives erythroid-specific expression of transgenes, recapitulating the expression pattern of β-like globin genes[6] and it has been used previously to rescue the lethal phenotype of the erythroid-specific transcription factor EKLF[34].

GATA1, GATA2 and GATA3 cDNAs were cloned in an β-LCR expression vector[22]. To facilitate the identification of the transgenic proteins all cDNA were tagged. A Myc-peptide tag was introduced at the start of the GATA1 open reading frame, while two and three HA-peptide tags were introduces at the GATA2 and GATA3 translation start site, respectively (Figure 1A). These constructs were used to generate transgenic mice. Several transgenic lines were obtained containing variable numbers of copies of the transgenes: Three β-LCR-GATA1 lines (A to C), containing one to two copies of the transgene; two β-LCR-GATA2 lines containing low (A) and high (B) copy numbers and three β-LCR-GATA3 lines of which one contained 2 copies (A) and the remaining (B and C) contained more than 7 copies (data not shown). The fact that no high copy number β-LCR-GATA1 were obtained from more than 50 founders analysed may be the result of embryonic lethality caused by overexpression of GATA1 in erythroid cells[41]. Heterozygous animals from all transgenic lines were born at Mendelian ratios and appeared normal.

The expression levels of the different GATA proteins in the different β-LCR transgenic lines were assessed by Western blot. The fetal liver is the main hematopoietic organ during late embryonic development where definitive erythroid cells are produced. Since by E12.5 it is mainly composed of erythroid progenitors (approximately 70% of all cells) we used the fetal liver as the source of erythroid cells to determine the expression levels of the transgenic GATA proteins. Western blot analysis of fetal liver cells from β-LCR-GATA1 transgenic lines revealed a variety of expression levels (Fig. 1B), but the expression of the transgenic protein was always lower than the endogenous GATA-1 protein. The low expression level of the β-LCR -GATA1 protein provides an explanation for the fact that these animals are viable, since overexpression of GATA1 blocks terminal erythroid differentiation and causes embryonic lethality due to anemia[41]. The β-LCR-GATA2 and β-LCR-GATA3 transgenic lines also express the GATA factors at different levels (data not shown); a direct comparison to the endogenous GATA1 levels is not possible in this case.

To check whether embryonic erythropoiesis is affected in the different β-LCR transgenic lines, E12.5 fetal livers were collected and the presence of erythroid precursors was assessed by flow cytrometric analysis using the erythroid marker TER119. TER119 starts to be expressed at the proerythroblast stage and is used as a marker for the erythroid lineage[14]. No significant difference in the percentage of TER119 positive cells was detected between wild-type and β-LCR transgenic fetal livers from all different transgenic lines (Fig. 1C). The differentiation potential of the erythroid precursors in these fetal livers was assessed in suspension cultures, an *in vitro* erythroid differentiation assay[13]. In this assay, fetal liver cells are cultured for two days in medium containing erythropoietin (Epo), insulin and hemin in small drops hanging from the lid of the culture dish. Terminal differentiation, assessed by enucleation, after two days of culture was determined by the cell size of the TER119 positive cells (FSC^{low}). No significant difference was observed in the percentage of enucleated cells at Day 2 between wild-type and β-LCR-GATA1 and β-LCR-GATA3 transgenic mice lines (Fig. 1D). In contrast, a small but significant reduction ($p<0.001$) in the percentage of enucleated cells is observed between wild-type and β-LCR-GATA2 transgenic mice with the highest expression of the transgene. This

result is in agreement with previously published data showing that ectopic expression of GATA2 in erythroid precursors arrests erythroid differentiation[4].

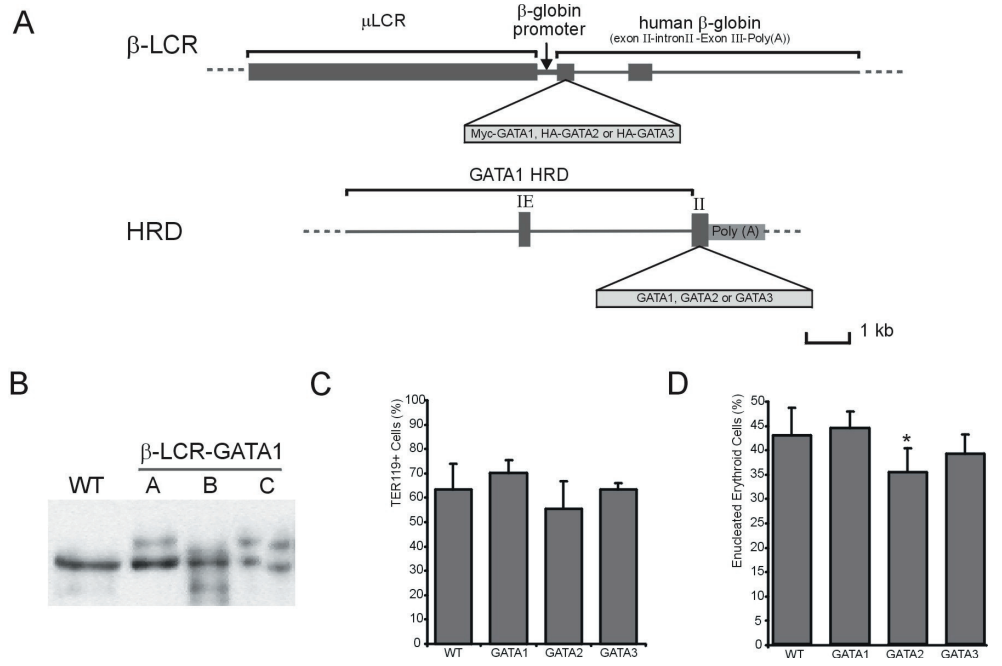

Figure 1 - Generation and characterization of β-LCR transgenic mice. **(A)** Schematic representation of the constructs used for the generation of HRD and β-LCR transgenic mice. **(B)** Western blot on E12.5 fetal liver cells from different β-LCR-GATA1 transgenic lines showing different expression levels of the GATA1 transgene. **(C)** Graph showing the percentage of $TER119^{+}$ erythroid cells in E12.5 fetal livers from wild-type and β-LCR transgenic lines, determined by flow cytometic analysis. Dead cells ($7AAD^{+}$) were excluded from the analysis. **(D)** Flow cytometric analysis of the percentage of enucleated (FCS^{low} $TER119^{+}$) erythroid cells in wild-type and β-LCR transgenic E12.5 fetal liver cells, after two days in suspension cultures. Cells were stained with the erythroid-specific surface marker TER119 and dead cells were excluded by staining with 7AAD. Enucleation was determined by cell size (FCS^{low}). * Significant difference ($p<0.01$) between βLCR-GATA2 and WT.

Comparison between HRD and βLCR driven transgene expression

To compare the expression profile of the transgenes under the control of the two distinct regulatory elements (Fig 1A), E12.5 fetal livers of HRD-GATA2 and β-LCR-GATA2 transgenic embryos were isolated and erythroblasts purified. These erythroblasts were expanded in liquid cultures containing dexamethasone (Dex), Epo and stem cell factor (SCF), for 3-4 days. Erythroblasts were then allowed to terminally differentiate by removing the Dex and SCF, increasing the Epo concentration and adding transferrin[7,38]. Samples were collected at several time points during erythroid differentiation until the stage prior to enucleation (Fig. 2A). As shown in Fig. 2B, no significant difference in the course of differentiation of cells different genotypes was observed during sampling. A reduction in enucleated cells was observed in β-LCR-GATA2 culture when compared with wild-type at later time points, in agreement with the results obtained from the suspension cultures. Nuclear extracts were prepared for Western blot

analysis of the expression of endogenous GATA1 and the GATA2 transgenes (Fig. 2C). The expression of nucleophosmin, a nucleolar phosphoprotein found at high levels in the nucleolus[9], was used to normalize for the amount of protein loaded.

The endogenous GATA1 protein is detected before induction of differentiation and increases during differentiation but its expression declines at the later stages of differentiation. The increase of the GATA-1 levels is mainly due to the increase of phosphorylated GATA-1 (Fig. 2C and data not shown). Like the endogenous GATA1 gene, the HRD-driven GATA2 transgene is expressed in undifferentiated erythroblasts and its expression increases moderately during differentiation. Contrary to endogenous GATA1 expression, a decline in expression at later stages of differentiation is not observed; this may be explained by differences in protein turnover between GATA1 and GATA2. The GATA2 transgene under the control of the β-LCR shows a very distinct temporal expression pattern from the endogenous GATA1. The transgenic protein is expressed at low levels in erythroblasts, greatly upregulated during differentiation, still increasing at the latest time point analysed here.

We conclude that HRD-driven expression mimics the endogenous GATA1 expression profile during terminal erythroid differentiation, while β-LCR-driven expression results in a very distinct pattern with increased expression during terminal differentiation.

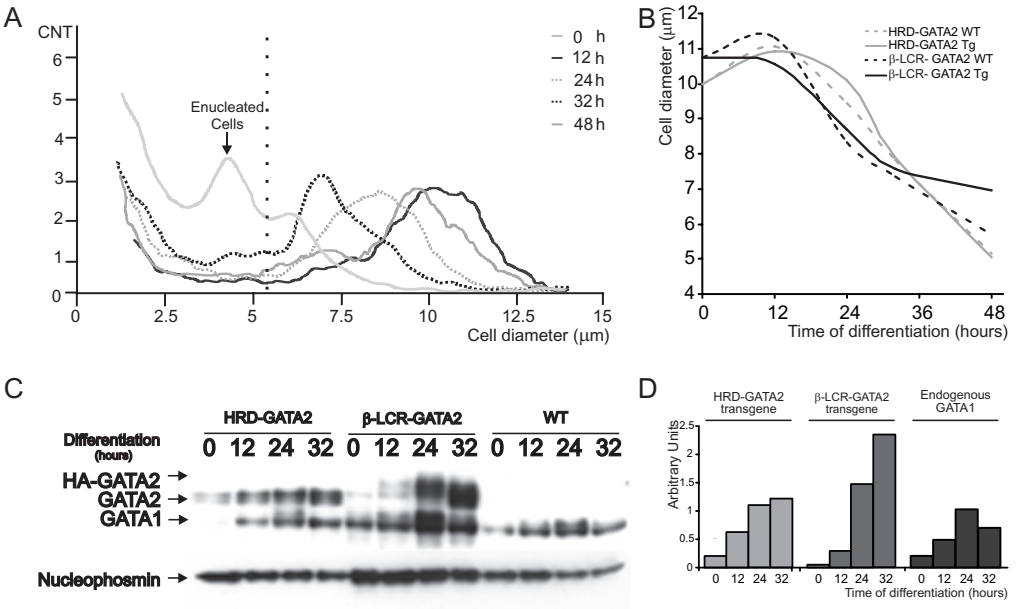

Figure 2 - HRD and β-LCR driven transgenes have distinct expression patterns during terminal erythroid differentiation. **(A)** Changes in cellular diameter during 48 hours in differentiation medium of a representative erythroblast culture. **(B)** Comparison between HRD-GATA2, βLCR-GATA2 and WT erythroblast cultures during differentiation. Reduction of cell diameter indicates progression in differentiation. **(C)** Western blot comparing the expression pattern of the GATA2 protein under the control of HRD and β-LCR. Top panel shows the expression of the GATA2 protein and the endogenous GATA1 protein and the bottom panel shows the staining of the same blot with an antibody against nucleophosmin, used as a loading control. **(D)** Graphic representation of the expression levels of the transgenic GATA2 proteins and endogenous GATA1 expression shown in Fig. 2B after normalization for nucleophosmin expression.

Rescue of GATA1 null mice by HRD and βLCR GATA1 transgenic mice

GATA1 KO mice were generated by breeding GATA1 conditional KO mice[18] with Zp3-Cre transgenic mice expressing Cre recombinase at early stages of embryogenesis. As expected[10], GATA1 KO male embryos, containing only one GATA1 allele at the X-chromosome (KO:Y), died between E10.5-E11.5 due to severe anemia while GATA1 KO females, heterozygous for the KO GATA1 allele (KO:X), showed transient anemia with most of them surviving to adulthood[10] (data not shown).

To confirm that the HRD-GATA1 transgene is able rescue the GATA1 *null* mutation, GATA1 KO females were mated with transgenic males from a high expressing HRD-GATA1 transgenic line. Seven GATA1 KO rescued males (HRD-G1/KO:Y) were identified out of a total of 41 newborn pups. All rescued mice developed normally, were fertile and showed no signs of anemia or thrombocytopenia (Table1). This demonstrates that GATA1 transgenes, when expressed under the control of the HRD, fully rescue the GATA1 *null* phenotype in the erythroid and megakaryocytic lineages. This also proves that the presence of the remaining 5% of endogenous GATA1 in GATA1.05 mice is not required for the rescue by the GATA1-HRD construct[23,33].

To verify to what extent βLCR-GATA1 transgenes are able to rescue the GATA1 *null* mutation, GATA1 KO females were mated with βLCR-GATA1 transgenic mice with different expression levels of the GATA1 transgene (Fig. 1B). In contrast to HRD-GATA1, no newborn βLCR-G1/KO:Y were obtained from these breedings (Table 2).

Table 1 - Peripheral blood indices from GATA1 KO rescued by the HRD-GATA1 transgene.

Genotype	Number of animals	Erythrocytes ($X10^4/\mu l$)	Hematocrit (%)	Hemoglobin (g/dl)	Platelets ($X10^4/\mu l$)
WT	5	967± 15	48.5±0.9	13.6±0.2	70.1±6.7
HRD-G1:GATA1.05:Y	3	1127±92	52.8±1.8	16.6±0.9	62.3±19.8
HRD-G1:KO:Y	4	1127±83	51.6±2.0	15.8±0.7	76.6±0.9

Table 2 - Genotype of progeny from GATA1 KO females and βLCR-GATA1 transgenic males.

Transgenic line	Age	Number of litters	Number of littermates	- Transgene			+ Transgene		
				WT	Y:KO	X:KO	WT	Y:KO	X:KO
βLCR-GATA1 (A)	E11.5	1	9	0	4*	1	1	0	3
	E12.5	1	9	1	0	2	4+1*	1*	0
	Adults	7	36	10	0	3	17	0	6
βLCR-GATA1 (B)	E12.5	1	11	1	2*	0	3	3	2
	E15.5	1	11	7	0	0	1	2	1
βLCR-GATA1 (C)	E12.5	2	19	8	0	5	5	2	1
	E14.5	1	9	4	0	0	1	1	2
	Adults	2	11	0	0	0	8	0	2

*Dead

Considering that GATA1 overexpression is embryonic lethal[41] we checked when the βLCR -G1/KO:Y embryos die during development. Progeny from intercrosses between GATA1 KO females and βLCR-GATA1 transgenic mice was analysed at different stages of embryonic development. Live βLCR-G1/KO:Y embryos were observed up to E15.5 from crosses between GATA1 KO females and males from two βLCR-GATA1 transgenic lines expressing the transgene at different levels (Fig. 1B and Table 2). No significant differences were observed with the two

βLCR-GATA1 transgenic lines analysed. At E12.5 βLCR-G1/KO:Y embryos showed normal morphology and were indistinguishable from the KO:X and βLCR -G1/KO:X, which survive to adulthood (Fig. 3A, upper panel). Also, no difference was observed between the embryonic blood of E12.5 βLCR-G1/KO:Y embryos and littermates, indicating that primitive erythropoiesis has been fully rescued by the expression of the βLCR-GATA1 transgenes (data not shown). In contrast, KO:X and βLCR-G1/KO:Y embryos have smaller and pale livers when compared with wild-type littermates, suggesting a defect in definitive erythropoiesis. βLCR-G1/KO:X embryos have a larger and less pale liver in comparison with KO:X embryos not expressing the transgene (Fig. 3A, lower panel).

By E15.5, βLCR-G1/KO:Y embryos are very pale, as well as a βLCR-G1/KO:X littermate, expressing the transgene (Fig. 3B, upper panel). Analysis of the embryonic blood reveals that βLCR-G1/KO:Y embryos have increased numbers of nucleated erythrocytes in circulation, comparable to the βLCR-G1/KO:X littermate (Fig. 3B, lower panel). The phenotype of this βLCR-G1/KO:X may be due to the random nature of X-chromosome inactivation. GATA1 KO females show variable phenotypes depending of the number of cell with a inactivated wild-type X-chromosome. It is apparent from the analysis of mutants expressing GATA1 at different levels that there is a threshold level of GATA1 necessary for normal definitive erythropoiesis[20,32].

Analysis of E12.5 fetal liver cells by flow cytometry, using the erythroid marker TER119, showed a reduction in the percentage of $TER119^+$ cells of ~50% in the KO:X and ~85% in the βLCR -G1/KO:Y by E12.5 (Fig. 3C). βLCR -G1/KO:X heterozygous for the βLCR-GATA1 transgene, do not show a significant increase in the percentage of $TER119^+$ cells. The differentiation potential of the fetal liver progenitors was analysed in vitro using the suspension culture system as described above. The percentage of enucleated erythrocytes in wild-type, KO:X and βLCR-G1/KO:X was not significantly different after two days in culture. However, the percentage of enucleated erythrocytes was severely impaired in βLCR -G1/KO:Y (Fig. 3C). This suggests that in the KO:X have a reduction of $TER119^+$ cells, correlated with the percentage of cells expressing GATA1, but the GATA1-expressing cells have competitive advantage over the GATA1 *null* cells compensating for the inability of GATA1 *null* cells to terminally differentiate.

The reduction in the percentage of $TER119^+$ cells in the KO:X and βLCR -G1/KO:Y raises the question of the identity of the $TER119^-$ cells in the liver. To address this question we analysed the expression of TER119 and CD71 (transferrin receptor) in cells from E15.5 fetal livers, before (Day 0) and after (Day 2) in vitro differentiation in suspension cultures. CD71 expression is high in early erythroid precursors that do not express TER119 yet, but its expression is gradually downregulated during terminal erythroid differentiation, where TER119 is expressed[30]. As shown in Figure 3D, in wild-type cells at Day 0 the majority of the cells in the liver express both CD71 and TER119 but at Day 2 the number of cells expressing only TER119 is increased, demonstrating that these cells are at a more differentiated stage. In βLCR-G1/KO:Y cells at Day 0, the number of $CD71^+TER119^+$ cells is decreased but the percentage of $CD71^+$ cells is increased indicating that these cells are early erythroid precursors, most likely CFU-e. We can therefore conclude that GATA1 null cells are arrested at a developmental stage prior to the proerythroblast stage, the stage at which TER119 starts to be expressed. After two days in suspension cultures, some of the $CD71^+TER119^+$ cells become $TER119^+$, indicating that they were able to progress in differentiation. However, the number of $CD71^+TER119^+$ and $CD71^+$ positive cells are increased, indicating an arrest in differentiation of the early erythroid precursors.

Taken together, these results show that the βLCR-GATA1 transgenes can rescue primitive erythropoiesis but are unable to rescue the definitive erythopoiesis. βLCR-G1/KO:Y survive up to E15.5 due to the presence of primitive erythrocytes in circulation but these cells are no longer present at later embryonic stages leading to embryonic lethality. The inability of the βLCR -GATA1 transgene to rescue definitive erythropoiesis can either be due to the reduced expression level of the transgene at the stage where GATA1 null erythroid progenitors are blocked in differentiation or to the high expression level of the βLCR -GATA1 transgene at latter stages of erythroid differentiation. These findings underscore the importance of the correct spatio-temporal expression of GATA1 during erythroid differentiation.

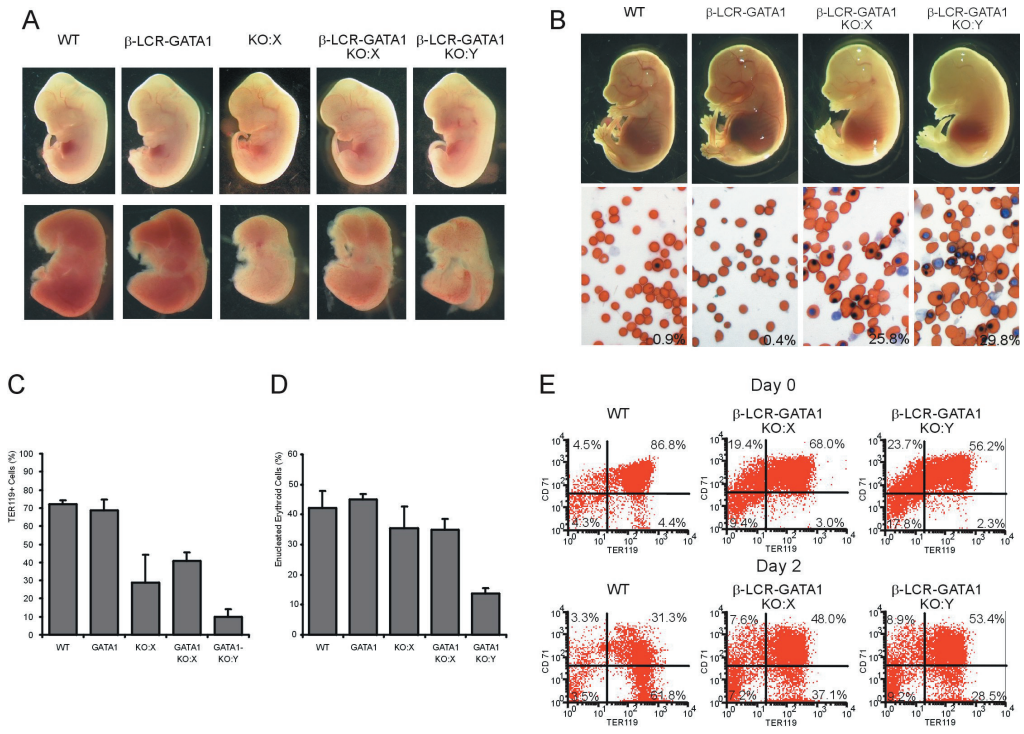

Figure 3 - Phenotype of β-LCR-GATA1 rescued GATA1 KO males. **(A)** The β-LCR-GATA1 transgene can rescue GATA1 KO embryos until E12.5. Top panel shows E12.5 embryos from different genotypes and the bottom panel shows the fetal livers isolated from these embryos. **(B)** The β-LCR-GATA1 transgene can rescue GATA1 KO embryos until E15.5. Top panel shows E15.5 embryos from different genotypes. Bottom panel shows erythroid cells from blood of embryos of different genotypes **(C)** Graph showing the percentage of $TER119^+$ erythroid cells in E12.5 fetal livers from βLCR-G1/KO:Y and littermates, determined by flow cytometric analysis. Dead cells ($7AAD^+$) were excluded from the analysis. **(D)** Graph showing the percentage of enucleated erythroid cells ($TER119^+$ FSC^{low}) in E12.5 fetal livers from βLCR-G1/KO:Y and littermates, determined by flow cytometric analysis. Dead cells ($7AAD^+$) were excluded from the analysis. **(E)** Graphs showing the percentage of cells expressing CD71 and TER119 in E12.5 fetal liver from βLCR-G1/KO:Y and littermates before (Day 0) and after (Day 2) *in vitro* differentiation in suspension cultures.

Rescue of GATA1 *null* mice by HRD and βLCR GATA2 and GATA3 transgenic mice

To test the potential of non-erythroid GATA transcription factors to rescue the GATA1 *null* phenotype, we analysed the offspring from crosses between GATA1 KO females and HRD- and βLCR- driven GATA2 and GATA3 transgenic males. Breeding between GATA1 KO females and a high copy number HRD-GATA3 transgenic lines gave rise to progeny containing HRD-G3/KO:Y, indicating that the HRD- driven transgenes are able to rescue the GATA-1 null phenotype, extending previous observations on the rescue of the GATA1.05 knockdown phenotype[23,33]. In contrast, no βLCR/KO:Y was obtained when GATA1KO females were crossed with males from 2 βLCR-GATA-2 and 3 βLCR-GATA3 transgenic lines (Table 3).

Next, we investigated the rescue potential of the βLCR-GATA2 and βLCR-GATA3 transgenic lines during embryonic development. Timed pregnancies were set up between GATA1 KO females and males from the different βLCR-GATA2 and βLCR-GATA3 transgenics, and embryos were collected at E12.5, E15.5 and E18.5.

Table 3 - Genotype of progeny from GATA1 KO females and βLCR-GATA2 and βLCR-GATA3 transgenic males.

Transgenic line	Age	Number of litters	Number of littermates	- Transgene			+ Transgene		
				WT	Y:KO	X:KO	WT	Y:KO	X:KO
βLCR-GATA-2 (A)	E12.5	5	65	16	9*	6	11	6	7
	E15.5	4	32	6	0	1	14	6	6
	E18.5	1	3	0	0	0	3	0	0
	Adults	3	19	7	0	4	6	0	2
βLCR-GATA-2 (B)	E12.5	3	33	8	3*	2	12	2*+2#	4
	Adults	3	15	5	0	1	7	0	2
βLCR-GATA-3 (A)	E12.5	2	22	5	2*	3+1*	3	4*	4
	Adults	2	10	1	0	2	7	0	0
βLCR-GATA-3 (B)	E12.5	2	19	3	1*	1	8	4*	2
	Adults	10	53	7	0	3	32	0	11
βLCR-GATA-3 (C)	E11.5	1	6	0	0	0	4	1#	1
	E12.5	1	10	0	0	0	4+1*	2*	3

*-Dead #-Retarded growth

A direct correlation was observed between the rescue potential of the GATA2 transgene and the expression level of the protein. βLCR–G2/KO:Y embryos were present at both E12.5 and E15.5 in litters from crosses between GATA1 KO animals and the βLCR-GATA2 transgenic line with highest transgene expression, Line A (Table3). At E12.5, βLCR-G2/KO:Y embryos were indistinguishable from KO:X embryos but could be distinguished from their wild-type littermates by the presence of a relatively pale fetal liver (Fig. A, left panel). By E15.5 βLCR-G2/KO:Y were easily distinguishable from their littermates due to their overall paleness (Fig. 4, right panel). No βLCR-2/KO:Y embryos were detected at E18.5. In progeny from crosses between GATA1 KO and the lower expressing βLCR-GATA-2 transgenic line, line B, βLCR-2/KO:Y embryos were only detected at E12.5 and these embryos showed severe growth retardation or were already dead (Table2). No alive βLCR-G3/KO:Y embryos were detected at E12.5 among the progeny of crosses between GATA1 KO females and the different βLCR-GATA3 transgenic lines (Table 3). A live βLCR–G3/KO:Y embryo was identified in an E11.5 litter, but this embryo showed severe growth retardation, reinforcing the idea that βLCR-GATA3 is unable to rescue, even partially, the GATA1 *null* phenotype.

More detailed analysis of the βLCR-G2/KO:Y indicated that, although these animals are alive, fetal erythropoiesis is impaired. Analysis of the number of $TER119^{+}$ cells present in

the fetal livers at E12.5 showed that βLCR-GATA2 expression in the βLCR-G2/KO:X does not have a significant effect on the numbers of TER119 positive cells. Like in KO:X a reduction of about 50% in the percentage of TER119^{+} cells is observed in βLCR-G2/KO:X. The defect in the TER119^{+} cells is even more pronounced, reaching over 80%, in the βLCR-G2/KO:Y (Fig. 4C). The differentiation capacity of the fetal liver erythroid progenitors was assessed in suspension cultures. After 2 days in culture the expected reduction in differentiated erythroid cells was observed in βLCR-GATA2 transgenics. Once again a normal percentage of differentiated KO:X cells was observed after two days in culture but this percentage was reduced in the βLCR-G2/KO:X female, reflecting again the negative effect of GATA2 overexpression in erythroid differentiation. The percentage of enucleated erythroid cells was severely reduced in the βLCR-2/KO:Y cells. Analysis of the fetal blood at E15.5 revealed the presence of large numbers of nucleated erythroid cells in circulation. The number of nucleated cells is increased in KO:X and βLCR-G2/KO:X but it is extremely elevated in βLCR-G2/KO:Y (Fig 4D). This shows that primitive erythropoiesis was rescued, allowing the male embryos to survive until midgestation. However, they develop a fatal anemia when they become dependent on definitive fetal liver erythropoiesis.

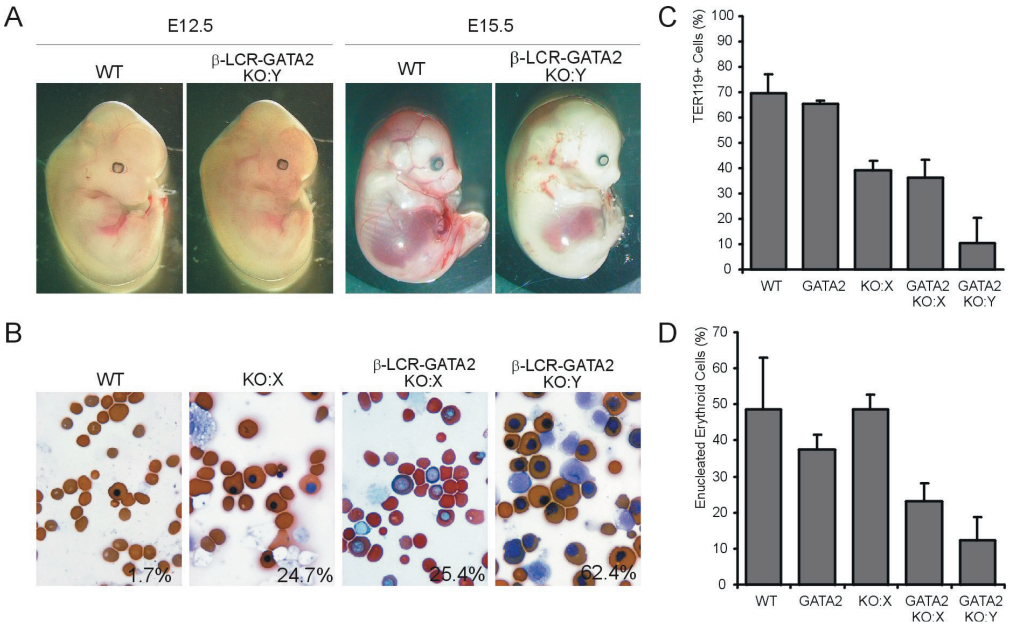

Figure 4 - Phenotype of β-LCR-GATA2 rescued GATA1 KO males. **(A)** The β-LCR-GATA2 transgene can rescue GATA1 KO embryos until E12.5. Left panel shows E12.5 embryos and right panel shows E15.5 embryos with the indicated genotypes. **(B)** Cytospins from embryonic blood of E15.5 embryos. **(C)** Graph showing the percentage of TER119^{+} erythroid cells in E12.5 fetal livers from βLCR-G2/KO:Y and littermates, determined by flow cytometric analysis. **(D)** Graph showing the percentage of enucleated erythroid cells (TER119^{+} FSC^{low}) in E12.5 fetal livers from βLCR-G2/KO:Y and littermates, determined by flow cytometric analysis. Dead cells (7AAD^{+}) were excluded from the analysis.

Discussion

The results presented in this paper clearly demonstrate that the proper spatio-temporal expression of GATA transcription factors is crucial for normal erythroid differentiation. When hematopoietic GATA transcription factors are expressed under the control of GATA1 regulatory sequences (HRD), mimicking the endogenous GATA1 expression, a full rescue of the GATA1 null phenotype is achieved. In contrast, when these factors are expressed under the control the β-globin promoter and locus control region (β-LCR), which is expressed later in erythroid development and remains high at the later stages of terminal erythroid differentiation, the lethality of the GATA1 *null* phenotype is not rescued.

HRD but not βLCR transgene expression mimics the endogenous GATA1 expression pattern

To compare the expression profile of the HRD and β-LCR driven GATA transgenes during erythroid differentiation we isolated primary erythroblasts from E12.5 fetal livers of transgenic embryos and analysed the expression profile of the transgenic proteins during *in vitro* differentiation. The culture method used allows us to maintain and expand the erythroblast population by inhibiting erythroid differentiation with the glucocorticoid hormone dexamethasone[37]. After 3 to 4 days a pure erythroblast culture is obtained that can then be induced to differentiate by removal of dexamethasone and addition of growth factors required for erythoid differentiation[7,38]. This culture system provides us with a controlled way of analysing the cellular changes that occur during terminal erythroid differentiation in settings closely resembling the *in vivo* situation.

To minimize the interference of enucleation in the quantification of the expression levels of the proteins, we collected samples at different time points prior to enucleation. In this way, the changes observed in protein levels in the latest stages of differentiation cannot be attributed to the loss of the nucleus.

Analysis of the expression of endogenous GATA1 protein during GATA1 terminal erythroid differentiation clearly demonstrates that GATA1 is expressed at the erythroblast stage, prior to induction of differentiation. Its levels increase during the first 24 hours after induction of differentiation, but declines afterwards. The reduction in GATA1 levels during the latest stages of erythroid differentiation has been inferred previously in different experimental settings[12,31]. Interestingly, the increase in GATA1 levels coincides with the increase in phosphorylated GATA1. GATA1 is phosphorylated at seven serine residues[5] and it has recently been reported that GATA1 phosphorylation is regulated through a MAP kinase pathway[35] suggesting that GATA1 phosphorylation may alter or regulate GATA1 function during erythroid differentiation, in response to receptor-mediated signaling.

Comparison of the expression profiles from HRD-GATA2 and β-LCR-GATA2 transgenes shows that the two transgenes have very distinct expression patterns. HRD-GATA2 expression is observed already prior to induction of differentiation while β-LCR-GATA2 only can be detected 12 hours after induction of differentiation, in agreement with the expression of β-globin. Both HRD and β-LCR-GATA2 expression levels increase during erythroid differentiation but while HRD-GATA2 shows only a moderate increase, β-LCR-GATA2 shows a very sharp increase in the levels of the protein and expression keeps rising even during the latest stages of differentiation. Contrary to what was expected, a decrease in the levels of HRD-GATA2 during the later stage of differentiation was not observed. This may reflect differences

in the turnover time of GATA1 and GATA2 proteins.

βLCR-driven transgenes are unable to rescue the lethal GATA1 *null* phenotype

Analysis of the progeny from GATA1 KO females with transgenic mice revealed, as expected, that HRD-driven transgenes were able to fully rescue GATA1 KO males from embryonic lethality. These animals were normal and did not show any signs of anemia or thrombocytopenia.

Despite the observation that β-LCR-GATA1 and β-LCR-GATA2 transgenes were capable of rescuing GATA1 KO males from lethality up to E15.5, no rescued GATA1 KO male (β-LCR/KO:Y) was born. Analysis of the blood from β-LCR/KO:Y embryos at E15.5 showed increased numbers of nucleated erythroid cells in circulation, suggesting that these animal are alive due to the presence of primitive erythrocytes in the blood stream. The death of these embryos during the later stages of development can be attributed to the disappearance of the primitive erythroid cells, which normally occurs by E16[25]. Rescue of primitive, but not definitive, erythropoiesis has been observed in studies with different GATA1 mutants suggesting that the requirements for lineage-specific transcription factors are not so strict during primitive erythropoiesis[24,29].

When compared to wild-type littermates, GATA1 KO females (KO:X and β-LCR/KO:X) show approximately 50% reduction in TER119$^+$ cells in the fetal liver. This suggests that GATA1 KO cells, which in the females comprise ~50% of the cells due to X-chromosome inactivation, are arrested in differentiation at a stage prior to the expression of TER119. This reduction is more pronounced in the males, reaching 85-90%. A more detailed analysis, using the TER119 and CD71 cell markers, revealed that the percentage of TER119$^+$ CD71$^-$ cells, representing the later stages of erythroid differentiation, is clearly reduced in βLCR/KO:Y. This reduction is compensated by an increase in the percentage of CD71$^+$ cells and, at a lesser extent, of CD71$^-$TER119$^-$ cells. This is in agreement with the report from Suzuki and colleagues[31] in which they demonstrate that GATA1 is expressed at very low levels in BFU-Es (CD71$^-$cells) but starts to be expressed at higher levels in CFU-Es (CD71$^+$cells) and thereafter (CD71$^+$TER119$^+$). This reinforces the idea that GATA1 KO erythroid cells are arrested at a stage prior to the expression of TER119, most likely at the CFU-E- to proerythroblast stage, in agreement with previous reports[28,40].

Analysis of the differentiation potential of the fetal liver cells of βLCR/KO:Y and βLCR/KO:X showed that after 2 days of differentiation the percentage of enucleated cells in KO:X and βLCR-G1/KO:X is similar to the wild type cells while in KO males only about 10-15% of the cells enucleate. This suggests that, in the females, cells expressing GATA1 have a competitive advantage over the GATA1 KO cells and are able to compensate for the differentiation deficiency of those cells. In βLCR/KO:Y cells expressing GATA1 are absent and cells expressing β-LCR-driven transgenes are not able to compensate for the loss of endogenous GATA1 due to the differences in the spatio-temporal expression pattern of the transgene.

The βLCR-GATA2 rescue potential is dependent on the expression level of the transgene, since only the βLCR-GATA2 transgenic line with the highest GATA2 expression rescued primitive erythropoiesis. Interestingly, after two days of differentiation in suspension culture, βLCR-GATA2:KO:X erythroid cells show a reduced percentage of enucleated cells compared with KO:X, not expressing the transgene. This is due the inhibitory effect of GATA2 in erythroid differentiation[4]. The same reduction is observed in βLCR-GATA2 expressing cells, when compared with wild-type cells.

βLCR-GATA3 fails to rescue both primitive and definitive erythropoiesis in GATA1 KO males since no βLCR-G3/KO:Y was found alive at E12.5. This lower rescue potential of GATA3 is consistent with previous observations. A knock in of GATA3 cDNA into the GATA1 locus only rescued embryos until E13.5[36], suggesting a defect in both primitive and definitive eythropoiesis. Furthermore, HRD-GATA3 was able to rescue the GATA1.05 knockdown mutant from embryonic lethality, but newborn mice were anemic and died earlier than wild-type and and GATA1.05 mutants rescued with HRD-GATA1 and HRD-GATA2 transgenes[33]. The difference in rescue potential may be due to differences at protein level between the GATA1 and GATA3 proteins[42]. The inability of βLCR-GATA3 to rescue primitive erythropoiesis may also be associated with the fact that endogenous GATA3 is never expressed in the erythroid lineage and abnormal high expression of this transcription factor may by harmful for these cells or interfere with the endogenous activity of other transcription factors.

Thus, we concluded that GATA1 is dynamically regulated during terminal erythroid differentiation (Fig. 5). It must be expressed at high levels in earlier progenitors but its expression must be downregulated in late progenitors for terminal erythroid differentiation to occur.

Deregulation of GATA1 expression, either by reducing expression levels in early progenitors or by overexpressing it in late progenitors, leads to embryonic lethality. This emphasises that the appropriate expression pattern of the hematopoietic GATA factors is an important determinant of their biological function.

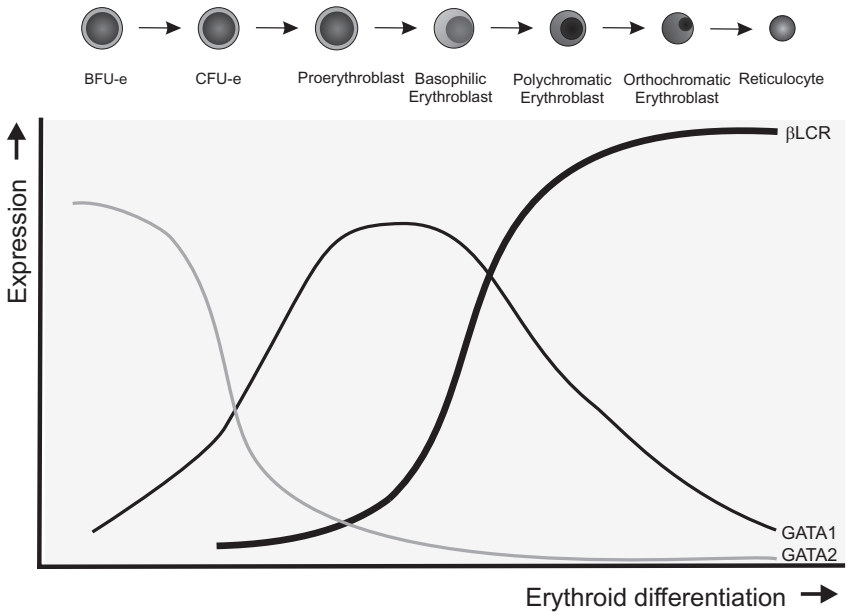

Figure 5 - Model for GATA1 and GATA2 expression during erythroid differentiation.GATA2 is expressed in early erythroid progenitors but its expression is downregulated when GATA1 starts to be expressed. GATA1 is expressed at high levels in early erythroid precursosr but it is downregulated in late erythroid precursors.

Materials and Methods

Generation and genotyping of mice

GATA1 *null* mice were generated by breeding mice harbouring a floxed GATA1 allele[18] with transgenic mice expressing Cre under the control of the zona pellucida 3 (Zp3) gene promoter[17]. For detection of the GATA1 recombined allele, genomic DNA was analysed by PCR using primers for the GATA1 gene (*5'G1*: 5'-CGCCGAGCTGTGTGTAGTAA-3' and *3'G1*: 5'-TTCCTGTTTCTCCTCCTCCG-3') located 5' and 3' of the first loxP site respectively and a primer located in the GFP gene (*GFP*: 5'-GGTGCTCAGGTAGTGGTTG-3'). GATA1 primers generate a 1.4kb product correspondent to the floxed GATA1 locus while 5'G1 and GFP produce a 2.8kb product correspondent to the recombined GATA1 locus.

Myc-tagged GATA1, 2xHA-tagged GATA2 and 3xHA-tagged GATA3 cDNAs were cloned in the PEV3 vector, containing human β-globin promoter and Locus Control Region[22,8]. β-LCR transgenic mice were generated by microinjection of PEV3-GATA1, PEV3-GATA2 and PEV3-GATA3 constructs in fertilized eggs. The genotype of these mice was assayed either by Southern blot using specific probes for the cDNAs or by PCR using primers specific for human β-globin sequences (βIVS2-s: CAGTGTGGAAGTCTCAGGATCC; βIVS2-as GAATGGTGCAAAGAGGCATGA), present in the vector.

HRD transgenic mice were generated and genotyped as described[33].

Histological staining

Fetal blood, fetal liver cell suspensions or cultured erythroblasts were spun onto glass slides and air-dried. Slides were stained with 1% O-dianisidine (Sigma) in methanol and Differential Quick Red and Blue staining as described [2].

Erythroid suspention cultures

Fetal livers, dissected from E12.5 mouse embryos, were disrupted and cells were grown in suspension[13]. In brief, fetal liver cells were seeded in Dulbecco's Modified Eagle's Medium (DMEM) 20% fetal calf serum (FCS), 200 μM hemin chloride (Sigma), 2 u/ml erythropoietin (Epo; Eprex, Janssen-Cilag), 5μg/ml Insulin and 100μM β-mercaptoethanol (Sigma), at a density of $2.5x10^6$ cells/ml. Cells were grown for 2 days in 20μl drops (containing $5x10^5$ cells) hanging from the lid of the culture dish.

Primary mouse erythroblast cultures

Primary erythroblasts, obtained from fetal livers of E12.5 transgenic mice, were maintained in serum-free medium (StemPro-34™; Life Technologies) supplemented with 0.5 u/ml human recombinant Epo (Epo; Eprex, Janssen-Cilag), 100 ng/ml murine recombinant stem cell factor (SCF; R&D Systems), and 10^{-6} M dexamethasone (Dex; Sigma) as described [7,38]. Erythroblast cultures were expanded, keeping cell densities at $1x10^6$ cells/ml, by daily addition of medium with fresh growth factors.

Terminal differentiation was triggered by washing the erythroblast in proliferation medium in PBS and reseeding them at 1 to $2x10^6$ cells/ml in serum-free medium, supplemented with 5 u/ml Epo and 1mg/ml iron-saturated human transferrin (Sigma). Cell numbers and size distributions were determined using an electronic cell counter (CASY-1, Schärfe-System, Reutlingen, Germany) and differentiation was assessed by the size reduction of the cells.

FACS analysis

After 2 days in suspension culture, cells were collected and incubated with PE-conjugated anti-mouse TER119 (BD Pharmingen), CD71 (BD Pharmnigen) and 7-aminoactinomycin-D (7AAD; Molecular Probes). Fifty thousand cells were analysed by FACS and dead cells ($7AAD^+$) were removed from the analysis. Enucleation of erythroid cells was evaluated by the expression of TER119 ($TER119^+$) and reduction of cell size (FSC^{low}).

Western blot

Total nuclear protein from fetal liver cells or cultures erythroblast was extracted as previously described [1]. Protein concentration in the nuclear extracts was determined using the bicinchoninic acid protein assay (Pierce, Rockford, IL). For Western blotting analysis, 20 to 50 μg of total nuclear protein was loaded per lane and separated on 10% SDS-PAGE gels under reducing conditions and transferred to polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA). Blots were probed with rat anti-GATA1 mAb (N6) and polyclonal rabbit anti-GATA2 (H-116) purchased from Santa Cruz Biotechnology (Santa Cruz, CA) or mouse monoclonal against B23 nucleophosmin (a kind gift by Pui K. Chan, Baylor College of Medicine, Houston, TX). Second-step reagents were HRP-conjugated goat anti-rat Ig and goat anti-rabbit Ig (Dako, Glostrup, Denmark). Peroxidase activity was visualized by ECL using standard procedures.

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Chapter 5

Enforced expression of GATA-3 during T cell development inhibits maturation of CD8 single-positive cells and induces thymic lymphoma in transgenic mice

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J. Immunol.(2001) 167:715-723

Enforced Expression of GATA-3 during T Cell Development Inhibits Maturation of CD8 Single Positive Cells and Induces Thymic Lymphoma in Transgenic Mice

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The zinc-finger transcription factor GATA-3 is of critical importance for early T cell development and commitment of Th2 cells. To study the role of GATA-3 in early T cell development, we analyzed and modified GATA-3 expression *in vivo*. In mice carrying a targeted insertion of a *lacZ* reporter on one allele, we found that *GATA-3* transcription in $CD4^+CD8^+$ double positive thymocytes correlated with the onset of positive selection events, i.e., TCRαβ upregulation and CD69 expression. *LacZ* expression remained high (~80 % of cells) during maturation of CD4 single positive (SP) cells in the thymus, but in developing CD8 SP cells the fraction of *lacZ*-expressing cells decreased to <20%. We modified this pattern by enforced GATA-3 expression driven by the *CD2* locus control region, which provides transcription of GATA-3 throughout T cell development. In two independent *CD2-GATA3*-transgenic lines, ~50% of the mice developed thymic lymphoblastoid tumors that were $CD4^+CD8^{+/low}$ and mostly $CD3^+$. In tumor-free *CD2-GATA3*-transgenic mice the total numbers of CD8 SP cells in the thymus were within normal ranges, but their maturation was hampered, as indicated by increased apoptosis of CD8 SP cells and a selective deficiency of mature $CD69^{low}HSA^{low}$ CD8 SP cells. In the spleen and lymph nodes, the numbers of $CD8^+$ T cells were significantly reduced. These findings indicate that GATA-3 supports development of the CD4 lineage and inhibits maturation of CD8 SP cells in the thymus.

Introduction

In the thymus early $CD4^-CD8^-$ double negative (DN) precursors develop into mature CD4 or CD8 single positive (SP) T cells following a tightly regulated program of cellular diffe rentiation[15,39,53,57]. The DN population is generally subdivided into four distinct developmental stages, defined by differential expression of the surface makers IL-2Rα chain CD25 and phagocyte glycoprotein 1 CD44[19]. Precursor T cells rearrange their *TCRβ* genes during the $CD25^+CD44^-$ DN stage, and only those cells that produce a functional TCRβ protein proceed via a proliferative phase to the $CD25^-CD44^-$ DN stage[15,27,37]. These cells rapidly up-regulate CD4 and CD8, and start to rearrange their *TCRα* genes. After successful *TCRα* rearrangement, TCRαβ-bearing immature cells are selected for MHC recognition during the process of positive selection [21,28,41]. Concomitantly, developing T cells will undergo lineage commitment to ensure the correlation of the TCR specificity for MHC class I with the CD8 lineage and for MHC class II with the CD4 lineage[29,39,64]. In addition, potential self-reactive T lymphocytes are eliminated by selection against self-recognition within the MHC context[51].

T cell development is regulated by a large number of transcription factors[9,30]. One of the transcription factors critically involved in T cell development is GATA-3, which was originally identified in the T cell lineage as a protein that binds to the *TCRα* gene enhancer[26]. GATA-3 is a member of a family of transcription factors that bind a GATA-consensus motif through a highly conserved C_4 zinc-finger binding domain[67]. Mice with a targeted deletion of GATA-3 display massive internal bleeding and central nervous defects and die between embryonic day 11 and 12 due to noradrenaline deficiency[35,47]. *GATA-3*$^{-/-}$ fetuses that were pharmacological rescued by feeding catechol intermediates to pregnant females displayed severe thymic hypoplasia at fetal day 16.5[35]. GATA-3 expression is abundant in the developing central nervous system, adrenal gland and kidney. Within the hematopoietic system GATA-3 expression is confined to T lymphocytes[4,18,24,44,47]. In mature T helper cells, GATA-3 has been shown to be essential for Th2 differentiation[14,70,71], and has been implicated in the regulation of locus accessibility of the *IL-4, IL-5* and *IL-13* genes by chromatin remodeling[2,3,46].

The *GATA-3* gene is expressed in common lymphoid progenitors and in the earliest CD25$^-$CD44$^+$ DN progenitors in day 12 fetal thymus[4,22]. Antisense GATA-3 oligonucleotides inhibited T cell development from fetal liver precursors in fetal thymic organ cultures, indicating the critical importance of GATA-3 for early T cell development[22]. Moreover, *RAG-2*$^{-/-}$ complementation experiments *in vivo* demonstrated that the development of *GATA-3*$^{-/-}$ embryonic stem (ES) cell-derived T cell precursors is arrested at or before the DN stage[62]. In such *GATA-3*$^{-/-}$*/RAG-2*$^{-/-}$ chimeric mice, the GATA-3-deficient ES cells contributed significantly to nonhaematopoietic tissues and to the erythroid, myeloid and B cell lineages. In chimeric mice generated by injection of GATA-3-deficient *lacZ*-expressing ES cells in wild-type blastocysts, we previously showed that *GATA-3*$^{-/-}$ ES cells did not contribute to the T cell lineage, not even to the earliest subset of CD25$^-$CD44$^+$ DN thymic progenitors[24].

Because *GATA-3*$^{-/-}$ cells display a block before the earliest T cell progenitor, few data are available on the role of GATA-3 during T cell development in the thymus. Using mice with an insertion of a *lacZ* reporter in the *GATA-3* gene on one allele (*GATA-3*$^{+/nlslacZ}$), we examined the proportion of *GATA-3*-expressing cells as a function of T cell development[24]. We found significant *GATA-3* expression at the earliest DN stage in the thymus. The two waves of *TCRβ* and *TCRα* gene recombination were associated with low proportions of *lacZ*$^+$ cells. The stage of rapidly proliferating CD44$^-$CD25$^-$ DN cells, which insulates these two periods of *TCR* rearrangement, was characterized by a large proportion of *lacZ*-expressing cells. The proportion of *lacZ*$^+$ cells increased again as double positive (DP) cells progressed into CD4 or CD8 SP cells. The presence of significant proportions of *lacZ*$^+$ cells within the CD8 SP T cell subpopulation in the thymus was in strong contrast with the almost complete absence of *lacZ* expression in mature CD8$^+$ T cells in the periphery[24].

The differential regulation of *GATA-3* gene expression in the CD4 versus the CD8 lineage prompted us to investigate its expression during positive selection and CD4/CD8 lineage commitment in the thymus in more detail. We analyzed the *GATA-3*$^{+/nlslacZ}$ mice, using additional markers for the maturation stages of DP and SP cells, including CD3, TCRαβ, heat stable antigen (HSA), CD62 L-selectin (CD62L) and particularly CD69, which is typically induced by TCR signaling and therefore marks cells that are in the process of positive selection[7,17,49,61,68]. In addition, we investigated the functional role of GATA-3 during T cell development *in vivo* by the generation of transgenic mice with enforced *GATA-3* expression driven by the human *CD2* locus control region (LCR), which provides expression of the *GATA-3* transgene throughout T cell development[20].

Results

GATA-3 expression is strongly induced during positive selection of developing T cells

We have previously quantified the GATA-3 expression profile during T cell development in vivo, by placing a *lacZ* reporter gene, containing a nuclear localization signal, under direct GATA-3 transcriptional control. In these *GATA-3*$^{+/nlslacZ}$ mice GATA-3 directed *lacZ* expression was analyzed by flow cytometry using fluorescein-di-β-D-galacto-pyranoside (FDG) as a β-galactosidase substrate, and differential expression of *GATA-3* in DP (~16% lacZ^{+} cells), CD4 SP (~84%) and CD8 SP (~33%) cells was found[24]. Since it has been shown that double positive T cells differentiate into mature single positive T cells via a series of phenotypically distinct subpopulations, reflecting the multistage process of positive selection and CD4/CD8 lineage commitment[29,39,64], we investigated *GATA-3* gene expression in these subpopulations in more detail.

Upon MHC/TCRαβ interaction in DP cells, the surface expression of the CD69 marker is upregulated[7,49,61,68], followed by a down-regulation of the CD4/CD8 co-receptor surface expression[39]. Therefore, we analyzed *lacZ* activity in conjunction with surface expression of CD4, CD8 and CD69 (Fig. 1). The majority of DP cells (~85%) did not express CD69 on the cell surface, and in this CD69^{-} DP population *lacZ* was expressed in ~19 % of cells. By contrast, within the CD69^{+} DP subpopulation, *lacZ* was expressed in ~54% of the cells. After subsequent downregulation of co-receptor expression and transition into the CD4^{low}CD8^{low} subpopulation, ~80% of the CD69^{+} cells expressed *lacZ* (Fig. 1). The CD4^{low}CD8^{low} cells have been shown to subsequently enhance CD4 expression[39], thereby developing into the CD4^{+}CD8^{low} subset, which still contains precursors for both CD4 and CD8 single positive T cells[31,39,40,60]. *LacZ* expression was present in ~86% of these CD4^{+}CD8^{low} CD69^{+} cells (Fig. 1). In addition, we found that in the DP, CD4^{low}CD8^{low} and CD4^{+}CD8^{low} subpopulations, *lacZ* expression correlated with the expression levels of TCRαβ or CD3 on the cell surface (Shown for TCRαβ in Fig.1A). These results indicated that the induction of *GATA-3* transcription coincides with CD3 and TCRαβ upregulation and CD69 expression in DP cells.

GATA-3 gene expression is downregulated after commitment to the CD8 lineage

Bipotential CD4^{+}CD8^{low} CD69^{+} T cells differentiate into either CD4 or CD8 SP cells by shutting down expression of the reciprocal coreceptor gene[31,39,40,60]. Final maturation of SP thymocytes is accompanied by downregulation of CD69 and HSA on the cell surface and induction of high-level expression of CD62L and CD44[5,8,17].

As shown in Fig. 1, *lacZ* expression was present in ~87% of the cells committed to the CD4 lineage (CD4^{+}CD8^{-}CD69^{+}TCRαβ^{high} cells) and in ~78% of the more mature CD4^{+} cells with a CD69^{-}HSA^{low} surface profile. By contrast, during the maturation of CD8 lineage cells, ~40% of the CD69^{+}HSA^{+} and only ~16% of the mature CD69^{-}HSA^{low} CD8^{+}TCRαβ^{high} cells expressed *lacZ*. Likewise, *lacZ* expression was found to be significantly downregulated in mature CD44^{+} and CD62L^{+} CD8^{+} cells (data not shown). The intensities of the fluorescence signals show that the *lacZ* expression levels per cell increased slightly during the maturation process of CD4 SP cells, whereas CD8 SP cells displayed lower and more heterogeneous *lacZ* expression levels (Fig. 1A).

When the T cells leave the thymus, the proportions of GATA-3^{+} cells decrease to ~20% of the CD4^{+} and to <1% of the CD8^{+} T cell populations in the spleen and lymph nodes[24]. For the

$CD4^+$ lineage cells in the spleen, we did not observe a clear correlation between GATA-3 and the expression of the HSA, CD69, CD44, CD62L or CD25 surface markers, which are instrumental to specify subpopulations of naive, activated or memory T cells[13,59].

As summarized in Fig. 1B, the proportions of *GATA-3* expressing cells were low in $CD3^-TCR\alpha\beta^-CD69^-$ DP cells (~19%), and increased at the onset of positive selection events, characterized by upregulation of CD3 and TCRαβ surface expression and induction of CD69. The proportions increased to ~86% at the stage of the last uncommitted subset of $CD4^+CD8^{low}CD69^+$cells, and remained high for the most mature thymic $CD69^-CD4^+$ subpopulation. By contrast, commitment to the CD8 lineage was associated with downregulation of GATA-3 expression, resulting in <20% $GATA\text{-}3^+$ cells within the mature population of $CD69^-CD8^+$ cells.

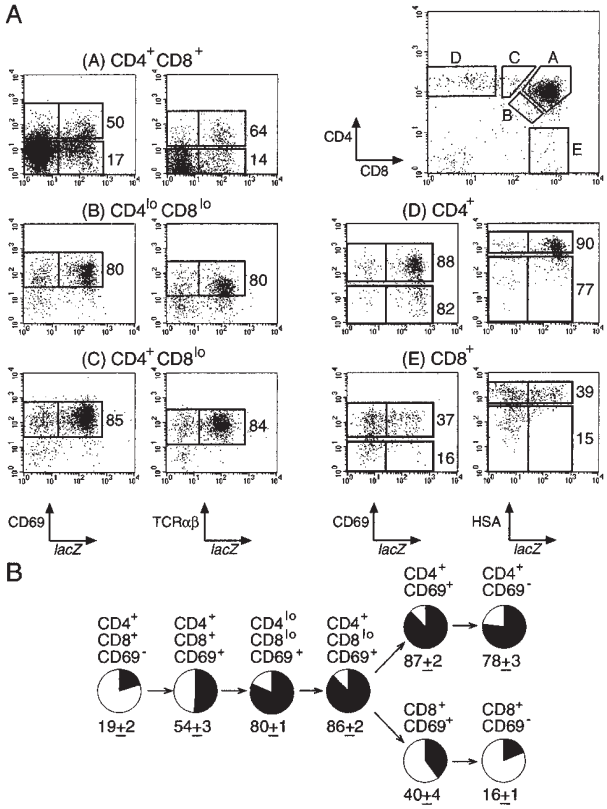

Figure 1 - Analysis of *lacZ* expression in thymocytes from *GATA-3*$^{+/nlslacZ}$ mice by four-color flow cytometry. **(A)**Thymus cell suspensions were loaded with the b-galactosidase substrate FDG and subsequently stained with anti-CD4, anti-CD8, in combination with anti-CD69, anti-TCRab or anti-HSA antibodies. Cells were analyzed for the expression of CD4 and CD8, the indicated subpopulations A-E were gated, and analyzed for *lacZ* expression and the fourth surface marker (CD69, TCRab or HSA). The numbers indicate the percentage of *lacZ* expressing cells in the subpopulations analyzed. All samples are lymphocyte gated by forward and sideward scatter. **(B)** Overview of the proportions of *lacZ* expressing cells in the indicated thymocyte subpopulations in *GATA-3*$^{+/nlslacZ}$ mice. The numbers are mean values ± SD (n=3). The background percentages of b-galactosidase positive cells, as determined in wild-type control mice, was <1% in all subpopulations.

Transgenic expression of GATA-3 driven by the human CD2 LCR

To modify GATA-3 expression *in vivo*, transgenic mice were generated in which the murine *GATA-3* gene, 5' tagged with 3 HA epitopes, was expressed under the control of the human *CD2* LCR[72]. Two independent *CD2-GATA3* transgenic lines, tgA and tgB, were established that appeared to contain comparable numbers of transgene copies (data not shown). No differences were found between the two lines in any of the performed analyses. The offspring did not manifest developmental defects or any increased susceptibilities to infectious disease or malignancies for over 9 months of age, with the exception of the observed thymic lymphomas discussed below.

Expression of the *CD2-GATA3* transgene was analyzed in various lymphoid tissues by comparing transgenic and non-transgenic littermates. Western blotting experiments were performed on nuclear protein extracts from 2-3 month-old mice, using a mouse mAb specific for GATA-3 and a polyclonal antibody specific for the HA-tag. The endogenous *GATA-3* gene encodes a ~47 kD protein that was detected in nuclear extracts from thymus and spleen of wild-type as well as *CD2-GATA3* transgenic mice (Fig. 2A). The anti-GATA-3 antibody also detected a slightly larger ~51 kD band in the thymus samples from mice of both transgenic lines. Comparison of the intensities of the ~47 and ~51 kD GATA-3 specific bands in these nuclear protein extracts from thymus samples, showed that the expression level of the 3HA-GATA-3 transgene encoded protein was similar to that of the endogenous GATA-3 protein. In contrast, in the spleen samples the ~51 kD GATA-3 specific band was very weak or absent (Fig. 2A). The ~51kD band in the thymus extracts of transgenic mice was also recognized by antibody against the HA-tag, but the expression levels of transgene encoded GATA-3 protein in the spleen and lymph node extracts were very low and often almost undetectable by Western blotting analyses (Fig. 2B).

To further investigate differential expression of GATA-3 in the individual stages of T cell development, intracellular flow cytometry experiments were performed, using the mouse monoclonal antiserum specific for GATA-3. Although this technique is limited by a background signal of the GATA-3 antibody, it allows a comparison of GATA-3 expression levels in non-transgenic and *CD2-GATA3* transgenic mice in separate T cell subpopulations. In the wild-type animals, the GATA-3 levels were low in DP cells, increased during positive selection in $CD4^{low}CD8^{low}$ cells, and were high in CD4 SP cells (Fig. 2C), consistent with our findings in the *GATA-3*$^{+/nlslacZ}$ mice (Fig. 1). Expression of the *CD2-GATA3* transgene was determined by comparison of the mean fluorescence intensities of intracellular GATA-3 staining in histogram overlays of transgenic and non-transgenic mice, revealing substantial GATA-3 overexpression in most thymocyte subpopulations (Fig. 2C). GATA-3 protein levels were uniformly higher in DP, $CD4^{low}CD8^{low}$, $CD4^{+}CD8^{low}$ and CD4 SP thymic subpopulations from *CD2-GATA3* transgenic mice, as compared to wild-type mice. By contrast, for the CD8 SP cells in the thymus and the $CD4^{+}$ or $CD8^{+}$ T cells in the spleen, GATA-3 levels in the *CD2-GATA3* transgenic were close to those observed in wild-type littermates. Therefore, these findings confirm the very low expression levels of the transgene encoded GATA-3 protein in peripheral T cells that were observed in the Western blotting experiments. As CD2 surface expression in the individual T cell subpopulations in thymus and spleen was comparable (data not shown), the observed modulated GATA-3 protein expression profile in the transgenic mice does not appear to reflect the activity of the *CD2* LCR. (Fig. 2B).

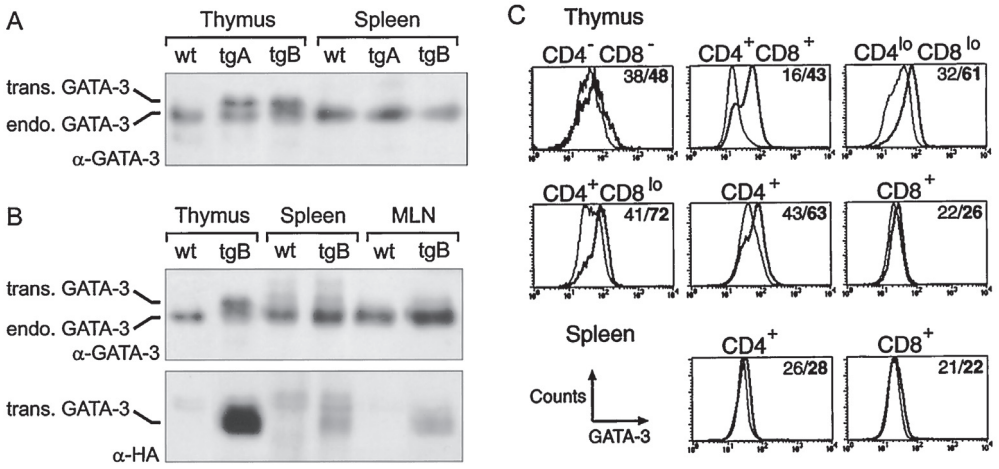

Figure 2 - Expression of GATA-3 in lymphoid organs of wild-type and CD2-GATA3 transgenic mice.**(A)** Western blotting analyses of GATA-3 protein expression in total nuclear protein extracts from thymus and spleen from wild-type (wt) and CD2-GATA3 transgenic mouse lines (tgA and tgB), as detected by anti-GATA-3 antibodies (~47 kD band: endogenous GATA-3; ~51kD band: transgenic GATA-3).**(B)** Western blotting analyses of GATA-3 protein expression in nuclear extracts from the indicated tissues from wild-type and CD2-GATA3 tgA mice. GATA-3 is detected by anti-GATA3 antibodies (upper half) or anti-HA antibodies (lower half). In the α-HA blot, thymus and spleen cell extracts from both non-transgenic and CD2-GATA3 transgenic mice displayed two weak background bands, just above the ~51 kD HA-GATA-3 band. **(C)** Cell suspensions were stained for surface CD3, CD4, and CD8 expression, and subsequently for intracellular GATA-3 protein. The indicated T cell subpopulations were gated and analyzed for GATA-3 expression. The results are displayed as histograms of CD2-GATA3 transgenic mice (bold lines), together with those of non-transgenic control mice (thin lines). $CD4^-CD8^-$ populations were gated on $CD3^-$ cells. $CD4^{lo}CD8^{lo}$ cells and $CD4^+CD8^{lo}$ were gated on $CD3^+$ cells. The numbers indicate the mean fluorescence intensities in nontransgenic (normal type) and CD2-GATA3 transgenic mice (bold type). Data shown are representative of 6 mice examined within each group.

Collectively, these data show that the presence of the *CD2-GATA3* transgene resulted in a modification the expression pattern of GATA-3 during T cell development, without extreme overexpression of GATA-3 protein in any of the thymic subpopulations. Especially in the DP population, which normally show little GATA-3 expression, the presence of the *CD2-GATA3* transgene strongly increased the GATA-3 protein levels.

CD2-GATA3 transgenic mice have decreased $CD8^+$ T cell numbers in the periphery

To analyze the effect of the *CD2-GATA3* transgene on T cell development, we examined the T cell populations in thymus, spleen and mesenteric lymph nodes from 2-3 month-old *CD2-GATA3* transgenic mice and non-transgenic littermates by flow cytometry (Fig. 3). In the *CD2-GATA3* mice the sizes of the main thymocyte subpopulations, the DN, DP and SP cells, were within the normal ranges, indicating that the enforced GATA-3 expression did not dramatically impede thymocyte development (Fig. 3). Moreover, thymus cellularity was not significantly different between transgenic mice ($99 \pm 34 \times 10^6$, n= 21) and non-transgenic littermates ($103 \pm 37 \times 10^6$, n= 14). No significant differences were detected between *CD2-GATA3* transgenic mice and normal littermates within the DN subpopulations as defined by differential CD44 and

CD25 expression (data not shown). In contrast, the *CD2-GATA3* transgenic mice had fewer $CD8^+$ T cells (~50% of control) in spleen and lymph nodes (Shown for spleen in Fig. 3). The residual transgenic $CD8^+$ T cells present exhibited a more heterogeneous CD8 expression and higher CD3 expression on the cell surface. The numbers of $CD4^+$ T cells in the periphery were comparable between the two groups of mice.

Taken together, these results indicated that enforced expression of GATA-3 did not result in detectable adverse effects on $CD4^+$ T cell development in 2-3 month-old *CD2-GATA3* transgenic mice. In contrast, mature CD8 SP cells manifested decreased survival, either within the thymus or shortly after leaving the thymus.

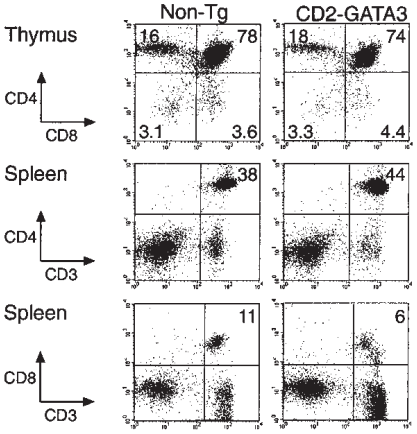

Figure 3 - Phenotype of *CD2-GATA3* transgenic mice. T cell development in the presence of the *CD2-GATA3* transgene results in reduced numbers of peripheral $CD8^+$ cells. Flow cytometric analyses of the thymus and spleen of 2-month-old wild-type and *CD2-GATA3* mice. Single-cell suspensions were stained with anti-CD3, anti-CD4 and anti-CD8 antibodies. Results are displayed as dot plots of lymphocyte gate cells; percentages of total cells within the indicated quadrants are given. Data shown are representative of over 20 mice examined within each group.

CD2-GATA3 transgenic mice develop thymic lymphomas

When the *CD2-GATA3* transgenic mice were followed up to 9 months of age, ~50% (26 out of 51) developed thymic lymphomas (Fig. 4A). Typically, these lymphomas were noticed as mice displayed respiratory distress at the age of 6-8 months, but in three cases such animals were observed at ~3 months of age. Tumor frequencies in the two independent transgenic lines were similar, while tumors were not seen in non-transgenic littermates. Several animals with a thymic lymphoma exhibited enlargement of spleen or lymph nodes. Lymphoma cells were found to be present in the spleen, liver, lymph nodes, and kidney, indicating that the thymic lymphomas metastasized to the periphery. This was confirmed by the presence of identical clonal *TCR*β rearrangement patterns in Southern blotting analyses, using probes specific for $J_{\beta1}$ or $J_{\beta2}$ gene segments (Fig. 4B). In a fraction of the tumors analyzed, we observed $J_{\beta2}$ restriction fragment patterns that would be consistent with biclonality (see Fig 4B, TL5). Often particular restriction fragments were lost in metastases, suggesting ongoing *TCRβ* rearrangement or deletion (see Fig. 4B, compare thymus and lymph node of TL2).

When tumor cell samples from thymus, spleen, or lymph node were analyzed for the expression of GATA-3 in western blotting experiments, high levels of transgenic HA-tagged

GATA-3 were observed, often accompanied by high endogenous GATA-3 expression (Fig. 4C). The ratio between transgenic and endogenous GATA-3 varied, not only between individual tumors, but also between different metastases of a single tumor (see Fig. 4C, compare mesenteric lymph node and spleen of TL 9).

Flow cytometric analyses demonstrated that the thymic lymphomas consisted of $CD4^+$ lymphoblasts with variable levels of CD8 coexpression. Fig. 5A shows four examples of thymic lymphomas ($CD4^+CD8^{+/low}$ cells), with different metastases in lymph nodes and spleen, showing the variability of surface CD4 and CD8 expression on the malignant cells. Immunohistochemical examination of thymic tumor tissue sections confirmed that the tumors mainly consisted of $CD4^+CD8^+$ lymphoblasts. Most of the tumors contained areas that had lost expression of CD8, and sometimes also CD4. A network of MHC class II negative fibroblasts supported these lymphoblasts, while characteristic structures of epithelial cells expressing cortical or medullar cell markers were absent (data not shown).

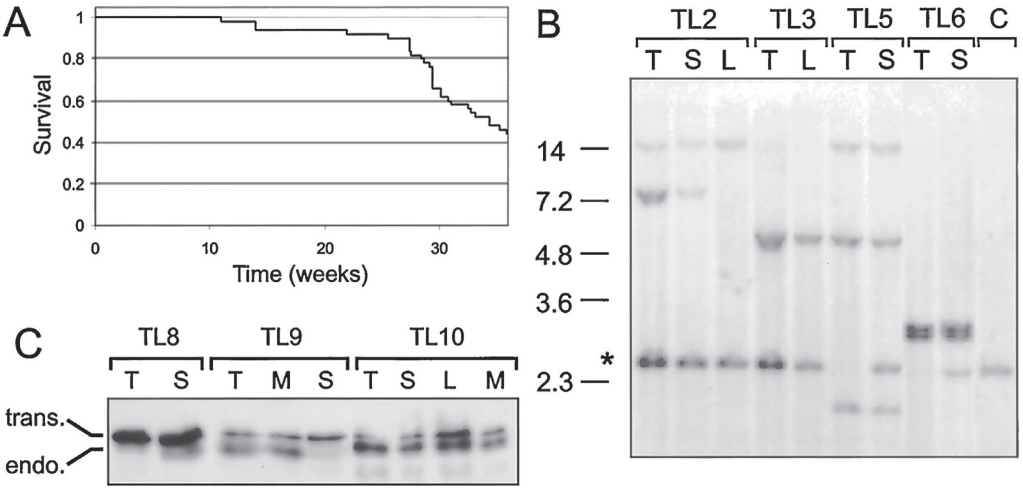

Figure 4 - Characteristics of thymic lymphomas in *CD2-GATA3* transgenic mice. **(A)** Survival of wild-type mice (gray line, n=42) and *CD2-GATA3* transgenic mice (black line, n=51), followed for 38 weeks in a Kapplan-Meyer curve as fraction of the total numbers of mice. **(B)** Southern blotting analysis of *TCR*β rearrangements in various lymphoblastoid tumor samples. *Eco*RI digests were hybridized to a TCR $J_{\beta 2}$ probe. T= thymus; S= spleen; L= lymph node. An asterisk indicates the position of the germ-line 2.4 kb *Eco*RI fragment; on the left the positions of λ, X BsteII restriction fragments are indicated in kb. **(C)** Western blotting analyses of GATA-3 protein expression in total nuclear extracts from the indicated tumor tissues, as detected by anti-GATA-3 antibodies. Trans.= ~51 kD transgenic GATA-3; endo.= ~47 kD endogenous GATA-3; T= thymus; S= spleen; M= mesenteric lymph node; L= axillary lymph node.

Among *CD2-GATA3* transgenic mice that did not exhibit outward signs of illness, nor manifested a macroscopically visible thymic tumor at ~3 months of age, we found evidence for early stages of tumor development in 6 out of 32 cases (~ 19%). In flow cytometric analyses of thymus cell suspensions the $CD4^+CD8^{+/low}$ subsets contained atypical fractions of $CD3^+$ lymphoblastoid cells with high FSC characteristics, suggestive of tumor growth. In the example shown in Fig. 5BC, the lymphoblastoid cells had a $CD3^+$ $CD4^+CD8^{lo}$ phenotype and expressed high levels of GATA-3 protein, as determined by intracellular flow cytometry. In

these lymphoblastoid cells, CD69 expression was variable (data not shown).

These findings indicate that dysregulation of GATA-3 expression results in the formation of lymphoblastoid tumors at a specific stage of thymic development, *i.e.* the $CD4^{+}CD8^{+/low}$ thymocyte subpopulation.

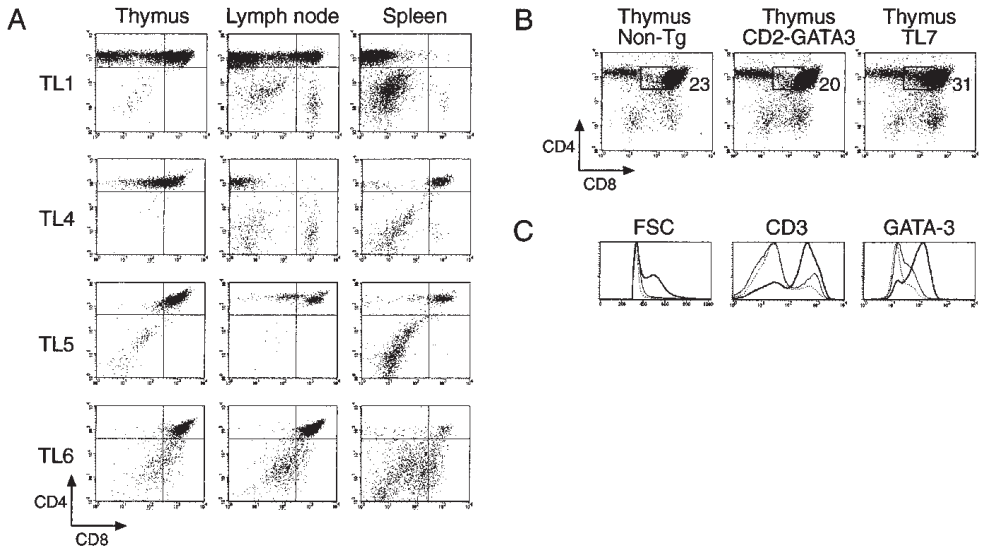

Figure 5 - Surface profile of lymphblastoid tumor cells in *CD2-GATA3* transgenic mice.**(A)**Flow cytometric analyses of four different $CD4^{+}CD8^{+/lo}$ thymic lymphoma primary tumor samples, as well as metastases present in lymph node and spleen. **(B)**Identification of an atypical GATA-$3^{hi}CD3^{+}CD4^{+}CD8^{lo}$ lymphoblastoid cell population in the thymus of a *CD2-GATA3* transgenic mouse, indicative for a thymic lymphoma (TL7). Non-transgenic and tumor-free *CD2-GATA3* transgenic mice are shown as controls. Results are displayed as dot plots for CD4 and CD8. The given percentages of the gated $CD4^{+}CD8^{lo}$ populations are of all thymocytes. **(C)** Analysis of forward scatter (FSC), CD3 and intracellular GATA-3 expression in the gated $CD4^{+}CD8^{lo}$ thymocyte subpopulation shown in panel B. The results are displayed as histograms of the *CD2-GATA3* transgenic TL7 mouse (*bold lines*), together with those of a non-transgenic (*dashed lines*) and a tumor-free *CD2-GATA3* transgenic mouse (*thin lines*). Cell suspensions were stained for CD3, CD4, and CD8, and subsequently for intracellular GATA-3. All samples are lymphocyte/lymphoblast gated by forward and sideward scatter.

GATA-3 enhances TCRαβ upregulation during positive selection

As we observed a correlation between GATA-3 expression and TCRαβ or CD3 surface levels in *GATA-3*$^{+/nlslacZ}$ mice, we investigated these parameters in the thymocyte subpopulations of the *CD2-GATA3* mice (Fig. 6A). The expression of CD69 in the DP, $CD4^{low}CD8^{low}$ and $CD4^{+}CD8^{low}$ subpopulations was similar in *CD2-GATA3* and wild-type mice. In contrast, the proportions of TCRαβ^{high} or $CD3^{high}$ cells were significantly increased in *CD2-GATA3* mice, particularly in $CD69^{+}CD4^{lo}CD8^{lo}$ subpopulation (Shown for TCRαβ expression in Fig. 6A). In the more mature fractions of $CD4^{low}CD8^{+}$ and SP cells, the expression levels of CD3 and TCRαβ were similar in transgenic animals and wild-type littermates.

We noticed that in the *CD2-GATA3* transgenic mice, the cells within the DP subpopulations had increased average forward scatter values, closer to those of normal SP cells (Fig. 6B). The increased size of *CD2-GATA3* transgenic DP cells did not reflect an enhanced

activation status of these cells, as we failed to detect activated cells with high Th2 cytokine production[14] in immunohistochemical analyses of the thymuses of *CD2-GATA3* mice. We also did not find evidence for a direct effect of transgenic GATA-3 on the cell cycle in DP cells, as flow cytometric analyses, using anti-CD4, anti-CD8 and To-Pro3, did not reveal differences in the cell cycle between *CD2-GATA3* transgenic animals and their wild-type littermates (data not shown). The development of CD3/TCRαβ^{low} DP into CD3/TCRαβ^{high} SP cells is normally accompanied by an increase in the average cell size. Therefore, the findings of the small increase in DP cell size and the slightly accelerated upregulation of surface TCRαβ and CD3 expression in *CD2-GATA3* transgenic mice suggest that enforced GATA-3 expression may influence the kinetics of positive selection.

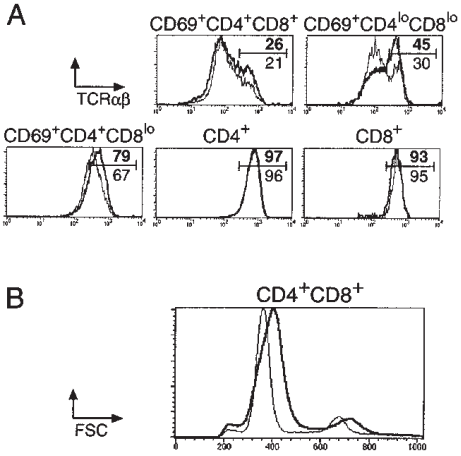

Figure 6 - The *CD2-GATA3* transgene enhances TCRαβ upregulation during positive selection. **(A)** Cell suspensions were stained for CD4, CD8 and CD69 expression, together with either CD3 or TCRαβ The indicated T cell subpopulations (see also Fig. 1A) were gated and analyzed for TCRαβ expression. The results are displayed as histograms of *CD2-GATA3* transgenic mice (*bold lines*), together with those of non-transgenic control mice (*thin lines*). The percentages shown are the fractions of the CD69^{+}CD4^{lo}D8^{lo} cells that are in the indicated TCRαβ^{hi} gate in wild-type mice (*below marker*) and *CD2-GATA3* transgenic mice (*above marker, bold type*). **(B)** The effect of the *CD2-GATA3* transgene on the cell sizes of the DP thymocyte subpopulation. Cell suspensions were stained for CD4 and CD8. DP cells were gated and analyzed for FSC; the results are displayed as histogram overlays of a *CD2-GATA3* transgenic (*bold line*) and non-transgenic control (*thin line*) mouse.

GATA-3 inhibits maturation of CD8 single positive T cells

As the reduction of peripheral CD8^{+} T cell numbers in the *CD2-GATA3* transgenic mice suggested increased cell death or hampered maturation of CD8 SP cells in the thymus, we analyzed the thymic CD8 SP compartment in more detail and specifically evaluated the final maturation steps of CD8 SP cells.

To analyze the extent of apoptosis in the SP subpopulations, we determined the fraction of cells that were annexin V-positive in *CD2-GATA3* transgenic mice and their non-transgenic littermates. In addition, we performed TUNEL assays in conjunction with surface CD4/CD8 staining. Using these techniques, we found that the thymuses of *CD2-GATA3* transgenic mice contained higher numbers of apoptotic cells, not only in the CD8 SP, but to some extent also in the CD4 SP subpopulations (Fig. 7AB).

t has been reported that final maturation of single positive T cells is accompanied by a downregulation of CD69 and HSA expression[5,17]. The enforced GATA-3 expression appeared to inhibit the final maturation of $CD8^+$ cells, as a selective deficiency of $CD69^{low}$ HSA^{low} cells was observed, when *CD2-GATA3* transgenic and wild-type littermates were compared (Fig. 7C). For $CD4^+$ cells the enforced GATA-3 expression only mildly affected the final thymic maturation steps. In addition, in the *CD2-GATA3* transgenic mice an increase in the surface expression of CD44, a marker for activated or memory T cells, was observed both in the CD4 and the CD8 SP population (Fig. 7C). This phenomenon was also seen in the mature $CD4^+$ and $CD8^+$ T cells in the spleen (see accompanying paper). Finally, the expression of L-selectin (CD62L), a marker which is expressed at high levels on naive T cells and which is essential for homing to peripheral lymphoid organs[58], was comparable in transgenic and non-transgenic animals. Therefore, the decrease in peripheral $CD8^+$ T cell numbers in *CD2-GATA3* transgenic mice cannot be explained by a reduced capacity of the mature CD8 SP T cells to leave the thymus.

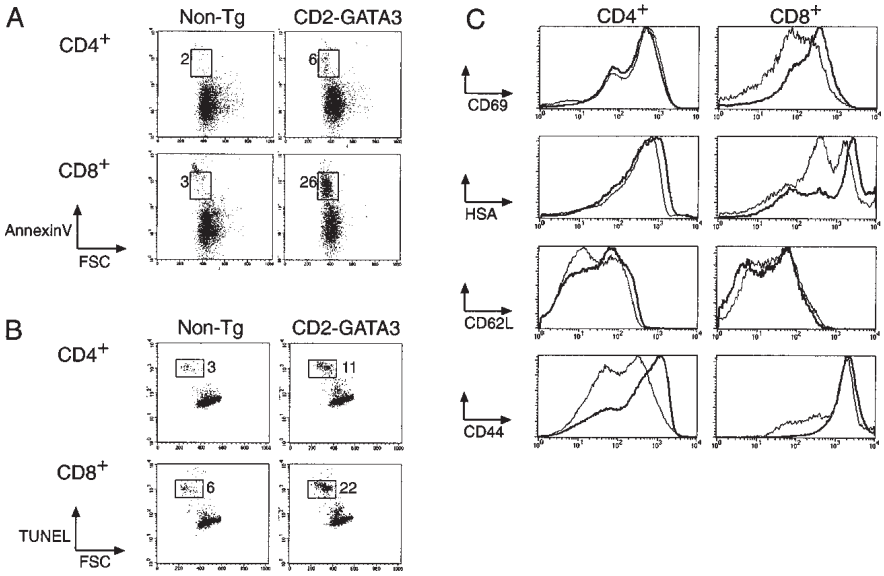

Figure 7 - Enforced expression of GATA-3 induces apoptosis and inhibits the maturation of $CD69^{lo}HSA^{lo}$ CD8 SP cells in the thymus. Thymus cell suspensions were stained for CD4, CD8 and Annexin V **(A)** or TUNEL **(B)**. Thymocytes were analyzed for the expression for CD4 and CD8, the indicated SP subpopulations were gated and analyzed for FSC and Annexin V or TUNEL. The numbers indicate the percentage of Annexin V-positive (A) or TUNEL-positive (B) cells in the subpopulations analyzed. **(C)** In four-color flow cytometry experiments, thymus cell suspensions were stained for CD4 and CD8 expression, together with anti-HSA and anti-CD69 or with anti-CD62L and anti-CD44. The CD4 and CD8 SP T cells were gated and analyzed for the expression of the indicated markers. The results are displayed as histograms of *CD2-GATA3* transgenic mice (*bold lines*), together with those of non-transgenic control mice (*thin lines*).

In summary, we observed a substantial increase of apoptotic CD8 SP cells and a decrease of mature $CD69^{low}HSA^{low}$ cells in the thymic CD8 SP subpopulation, as well as reduced numbers of $CD8^+$ T cells in the peripheral organs. These findings indicate that enforced GATA-3 expression resulted in a partial differentiation arrest of $CD8^+$ cells, associated with significant cell death in the thymus.

Discussion

In this report we have used two different mouse models to study the role of GATA-3 in early T cell development in vivo. We evaluated GATA-3 directed *lacZ* expression in *GATA-3*$^{+/nlslacZ}$ mice, and examined the effects of enforced GATA-3 expression throughout T cell development in *CD2-GATA3* transgenic mice, in which *GATA-3* transcription is driven by the *CD2* LCR.

Our findings implicate GATA-3 as a participant in the commitment process to the CD4 versus the CD8 lineage. First, we found that commitment to the CD8 T cell lineage coincided with downregulation of *GATA-3* expression. The most mature subpopulation of uncommitted thymocytes, the CD4^{+}CD8^{lo} subset, contained high numbers of *GATA-3* expressing cells. During the maturation of CD8^{+} cells in the thymus, *GATA-3* expression was gradually lost. By contrast, *GATA-3* expression remained high during differentiation of CD4^{+} cells in the thymus. Second, enforced GATA-3 expression inhibited the maturation of CD8^{+} cells. The CD8 SP fraction in the thymus contained increased numbers of apoptotic cells and exhibited a selective deficiency of mature CD69^{low}HSA^{low} cells. In the spleen and lymph nodes the numbers of CD8^{+} T cells were significantly reduced.

Enforced expression of GATA-3 did not appear to directly influence the CD4 versus CD8 lineage cell fate decision, as in the *CD2-GATA3* mice the percentages of CD4 and CD8 SP cells in the thymus were in the normal ranges. Although the molecular mechanisms underlying the developmental choice between CD4 and CD8 T cell fates are not known, they are thought to depend on differences in signal strengths of the MHC class I-CD8 and MHC class II-CD4 interactions. The influence of signaling molecules on lineage commitment is supported by the finding of differentiation towards the CD4 lineage in a gain-of-function extracellular signal-related kinase (Erk)-2 mutant, and in Csk or C-Cbl deficient mice[42,54,56]. Activated Notch transmembrane receptor or Bcl-2 overexpression were shown to promote differentiation to the CD8 lineage, probably by rescue from apoptosis and development along the CD8 lineage of cells that have a very low affinity MHC-interaction, which would normally die by neglect[11,38,52].

Our data point at a role for GATA-3 in the maturation of the cells once commitment has occurred. There is a progressive decline of *GATA-3* expression during CD8 lineage maturation, and the enforced GATA-3 expression impaired cell survival in the most mature CD8 lineage cells. Furthermore, peripheral CD8^{+} T cells from *CD2-GATA3 Tg* mice manifested functional defects in IL-2 and IFNγ production (See accompanying paper). In this context, there is a striking parallel with Th1/Th2 differentiation, where GATA-3 is expressed in naive peripheral T cells, followed by a substantial increase during Th2 development and a gradual downregulation during Th1 development[70,71]. The Th2 phenotype is initiated by IL-4 signaling, and by the action of GATA-3 becomes stable over time and independent of extrinsic factors, such as IL-4[1,2,46]. Retroviral tagging of naive progenitors with GATA-3 provided direct evidence for instructive differentiation, rather than selective outgrowth of committed Th1 or Th2 cells[14]. It was further shown that GATA-3 generates stability of Th2 commitment by chromatin remodeling of Th2-specific cytokine loci, associated with a positive autoactivation pathway, which is a recognized mechanism contributing to cell fate determination[46]. Concomitantly, GATA-3 inhibits Th1 development by repressing IL-12Rα expression and, as a result, IL-12 induced IFNγ production[45]. Assuming a parallel role for GATA-3 in CD4/CD8 and Th1/Th2 development, we propose that GATA-3 is involved in the stabilization of the distinct gene expression profiles in committed CD4 cells, while for the full maturation of CD8 T cells, GATA-3 expression

needs to be downregulated. Alternatively, GATA-3 may affect lineage commitment indirectly by inducing higher TCRαβ expression levels (Fig. 6A), thereby increasing the intensity of the signal delivered to DP cells, which has been shown to skew development toward the CD4 lineage[25,69]. A mechanism by which the influence of enforced expression of GATA-3 on CD4/CD8 commitment is directly related to the presence of GATA-3 recognition sites in the *CD8*α promoter[32] can also not be excluded. In that case GATA-3 would have to directly repress CD8 expression in mature cytotoxic T cells.

It is at present not clear why in *CD2-GATA3* mice the levels of transgene encoded GATA-3 protein are downregulated in CD8 SP cells and peripheral T cells. GATA-3 levels may be subject to posttranslational regulation, as indicated by the presence of caspase-mediated degradation of the closely related transcription factor GATA-1 in immature erythroid cells[10]. However, such mechanism should apply to both endogenous and transgene-encoded GATA-3. Therefore, the presence of the HA-tag would then affect posttranscriptional regulation.

Further experiments will be needed to identify the critical target genes for GATA-3 in early T cell development. Intriguingly, GATA recognition sequences are present in the *Notch4* promoter region[33]. If *Notch* genes would be regulated by GATA-3, this could explain the parallels that exist between the *in vivo* function of GATA-3 and Notch. Both genes are essential for the development of the first stage of T cell development, and not for any other haematopoietic lineage[24,50,62]. Apart from the accelerated TCRαβ upregulation in developing $CD69^+$ thymocytes that progress from the DP to the $CD4^+CD8^{lo}$ stage, we did not see any effects on the surface expression of presumed GATA-3 target loci, such *as TCR*α, β, and δ or *CD8*α.

Our previous finding of low *GATA-3* expression during the two waves of *TCR* gene rearrangement, separated by a stage of high *GATA-3* expression, suggested a role of GATA-3 in the regulation of proliferation events associated with the essential coupling of V(D)J recombination activity to cell cycle[24]. However, the absence of any detectable effects of the *CD2-GATA3* transgene on the cell cycle would argue against such an essential role for GATA-3. Nevertheless, all thymic lymphomas in the *CD2-GATA3* mice characterized so far appeared to have originated at the DP stage, in which all *TCR*α locus gene rearrangements occur. Therefore, it remains possible that - in the presence of high levels of GATA-3 - oncogenic events, such as translocations, are mediated by aberrant use of the V(D)J recombination machinery, as has been found in V(D)J-recombination driven thymic lymphoma in mice deficient for the *ataxia telangiectasia* gene[34].

Alternatively, the oncogenic potential of GATA-3 could be related to the ability of GATA-3 to form a complex with the TAL-1 and LMO transcription factors, which are implicated in a large fraction of human T-cell acute lymphoblastic leukemias[43]. Normally TAL-1 and LMO are not expressed in the T cell lineage, but expression is induced by translocation events. It was recently shown that forced expression of GATA-3 *in vitro* potentiated the induction by the TAL-1 and LMO transcription factors of retinaldehyde dehydrogenase 2, which inhibits apoptosis of T cells by generating retinoic acid[43].

Thirdly, enforced GATA-3 expression probably leads to increased basal transcription of the *RAD50* gene, which is involved in chromosomal double-stranded break repair. Because of the localization of the *RAD50* gene within the *IL-4/IL-5/IL-13* Th2 cytokine gene cluster, an increase of basal *RAD50* transcription is observed in Th2 cells[2]. It is possible that in the *CD2-GATA3* transgenic T cells the increase might be more extreme, thereby resulting in destabilization of the MRE11-RAD50-NBS1 protein complex, which is essential for chromosome stability[48].

Finally, a more general mechanism might be responsible for the oncogenic effect

of GATA-3, as GATA factors have a key role in the regulation of development toward cell division and differentiation via the cell cycle machinery[65]. Recently several other GATA family factors have been implicated in various human tumors, e.g. GATA-2 in acute promyelocytic leukemia, acute myeloid leukemia and myelodysplastic syndrome, and GATA-4 in esophageal adenocarcinomas and malignancies of the gonads[36,63,66]. Further characterization of the tumor cells should identify the possible involvement of any of these oncogenic pathways in the origin of the thymic lymphomas in the *CD2-GATA3* mice.

In conclusion, this study adds to our knowledge of the function of GATA-3 in early T cell development, as we have established a correlation between GATA-3 expression and maturation towards the CD4 versus the CD8 lineage. We propose that in early T cell development, expression of GATA-3 is essential for the maintenance of CD4 cell lineage fate commitment, but inhibits CD8 differentiation. Inferred from the recent findings that GATA-3 acts a key regulator of Th2 development by stabilizing patterns of gene expression, it is attractive to hypothesize that in early T cell development GATA-3 would stabilize, by chromatin remodeling, the unique gene expression profiles that are characteristic for the CD4 lineage.

Materials and Methods

Mice

The *GATA-3*$^{+/nlslacZ}$ mice in which one *GATA-3* allele was replaced by a *lacZ* reporter have been described previously[24]. For the generation of the *CD2-GATA3* construct, the translation initiation site was mutated (ATG to GTG) in a murine *GATA-3* cDNA clone and 3 HA epitope tags were added together with a new ATG and Kozak's consensus sequence. Subsequently, the ~ 2 kb *mGATA-3* was cloned into a human *CD2* mini-gene Bluescript SK vector, with ~5 kb of *CD2* 5'promoter sequence and ~ 5.5 kb 3' *CD2* flanking sequences[72]. The latter contained the 3' untranslated sequence and poly(A) addition site of the *CD2* gene, as well as the LCR, which was shown to confer T-cell specific, copy-dependent, integration site independent expression in transgenic mice[20]. A 13.2-kb linear fragment was injected into pronuclei of FVB X FVB fertilized oocytes at a concentration of ~2 ng/µl. Founder mice were identified by genomic Southern blotting and crossed onto an FVB background. To determine the genotype of the subsequent generations, tail DNA was analyzed by Southern blotting of either *Eco*RI /*Xba*I double digests hybridized to a 2 kb *Hin*dIII *CD2* LCR probe[16], or *Eco*RI digests hybridized to a 800 kb partial *GATA-3* cDNA probe[44].

Western blotting analyses

Total nuclear protein extracts were prepared according to Andrews *et al*.[6]. Protein concentration in the nuclear extracts was determined using BCA protein assay (Pierce, Rockford, IL). For Western blotting analysis, 50 µg of total nuclear protein was loaded per lane and separated on 10% SDS-PAGE gels under reducing conditions and transferred to PVDF membrane (Immobilon-P, Millipore, Bedford, MA). Blots were blocked with 2% BSA in PBS pH 7.0, 0.05% Tween 20 and incubated with first- and second step reagents in 2% non-fat dry milk in PBS pH 7.0, 0.05% Tween 20. The mouse anti-GATA-3 mAb HG 3-31 and the polyclonal rabbit-anti-HA antibody Y11 were from SantaCruz Biotechnology (Santa Cruz, CA). Second-step reagents were horseradish peroxidase-conjugated goat-anti-mouse Ig and swine-anti-rabbit Ig from DAKO (Glostrup, Denmark). Peroxidase activity was visualized by enhanced chemi-luminescence, using standard procedures.

Flow cytometric analyses

The preparation of single-cell suspensions, determination of β-galactosidase activity using fluorescein-di-β-D-galactopyranoside (FDG), mAb incubations and three or four color cytometry have been described previously[23]. The following mAb were purchased from Pharmingen: FITC-conjugated anti-CD3ε and anti-TCRαβ, PE-conjugated anti-CD4 (L3T4), anti-CD24/heat stable antigen (HSA), anti-CD25 (clone 3C7), anti-CD62L and anti-CD69, CyChrome-conjugated anti-CD4, anti-CD8 and anti-CD44, biotinylated anti-CD4 and anti-CD8, APC-labeled anti-CD3ε and anti-CD4. Secondary antibodies used were PE-, TriColor, or APC-conjugated streptavidin (Caltag Laboratories, Burlingame, CA). FDG and To-Pro3 were from Molecular Probes Europe BV (Leiden, The Netherlands). FITC-labeled Annexin V was from Nexins Research BV, Hoeven, The Netherlands.

For intracellular detection of GATA-3 protein, cells were fixed and permeabilized using paraformaldehyde and saponin, as described[12] and subsequently incubated with the Hg-3-31 anti-GATA-3 MoAb (Santa Cruz, Dan Diego, CA) and FITC-labeled anti-mouse IgG1 (Pharmingen) as a second step.

Simultaneous two-color staining of membrane CD4 and CD8, combined with a TUNEL (TdT-mediated dUTP-biotin nick end labeling) technique to quantify apoptosis was performed, using fluorescein in situ cell death detection (Roche Molecular Biochemicals, Mannheim, Germany), as described[55].

Acknowledgements

We would like to thank Y. Allen, W. van Ewijk, J. Guy, B. Lambrecht, J. Mahabier, S. Philipsen, and H. Savelkoul for their assistance at several stages of the project.

This work was partly supported by Grant 3069 from the Estonian Science Foundation (to A.K.) as well as by the Royal Academy of Arts and Sciences (to R.W.H.).

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Chapter 6

Enforced expression of GATA-3 in transgenic mice inhibits Th1 differentiation and induces de formation of a T1/ST2 expressing Th2-committed T cell compartment in vivo.

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J. Immunol.(2001) 167:724-73

Enforced Expression of GATA-3 in Transgenic Mice Inhibits Th1-differentiation and induces the formation of a T1/ST2-expressing Th2 committed T cell compartment in vivo.

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The transcription factor GATA-3 is essential for early T cell development and differentiation of naive CD4+ T cells into Th2 effector cells. To study the function of GATA-3 during T cell mediated immune responses *in vivo,* we investigated *CD2-GATA3* transgenic mice, in which GATA-3 expression is driven by the *CD2* locus control region. Both in the CD4+ and the CD8+ T cell population the proportion of cells exhibiting a CD44hiCD45RBloCD62Llo antigen-experienced phenotype was increased. In *CD2-GATA3* transgenic mice, large fractions of peripheral CD4+ T cells expressed the IL-1 receptor family member T1/ST2, indicative of advanced Th2 commitment. Upon *in vitro* T cell stimulation, the ability to produce interleukin-2 (IL-2) and interferon-γ (IFNγ) was decreased. Moreover, CD4+ T cells manifested rapid secretion of the Th2 cytokines IL-4, IL-5 and IL-10, reminiscent of Th2 memory cells. In contrast to wild-type CD4+ cells, which lost GATA-3 expression when cultured under Th1-polarizing conditions, *CD2-GATA3* transgenic CD4+ cells maintained expression of GATA-3 protein. Under Th1 conditions, cellular proliferation of *CD2-GATA3* transgenic CD4+ cells was severely hampered, IFNγ production was decreased and Th2 cytokine production was increased. Enforced GATA-3 expression inhibited Th1-mediated *in vivo* responses, such as antigen-specific IgG2a production or a delayed type hypersensitivity response to KLH. Collectively, these observations indicate that enforced GATA-3 expression selectively inhibits Th1 differentiation and induces Th2 differentiation. The increased functional capacity to secrete Th2 cytokines, together with the increased expression of surface markers for antigen-experienced Th2 committed cells, would argue for a role of GATA-3 in Th2 memory formation.

Introduction

The CD4+ T helper (Th) lymphocytes develop into two functionally distinct subsets that can be distinguished on the basis of their cytokine production profile[32,38]. Th1 cells are characterized by the production of interferon-γ (IFNγ) and tumor necrosis factor β (TNFβ), whereas Th2 cells typically produce interleukin-4 (IL-4), IL-5, IL-10 and IL-13. Each subset mediates distinct effector functions *in vivo*. Th1 cells are predominantly involved in immune responses against intracellular pathogens, and are associated with autoimmune disease. Th2 cells are of importance in the defense against extracellular pathogens, and are implicated in atopy and allergic diseases[1,34,41].

Both Th1 and Th2 cells are derived from a common naive precursor1,3,34. Signaling pathways initiated by cytokines play a dominant role in driving the differentiation of

activated naive CD4+ T cells into either effector phenotype[4,38]. For instance, IL-12 induces the differentiation of naive Th cells to the Th1 effector phenotype[19,29,30,43], by activation of the transcription factor Stat4[21,48,52]. On the other hand, Th2 differentiation is mediated by Stat6 activation through IL-4 receptor engagement[18,22,24,42,44,47,50]. In response to chronic antigenic stimulation *in vivo*, progressive polarization of the cytokine responses ultimately leads to the commitment of T helper cells to mutually exclusive Th phenotypes, which are thought to be maintained independently of extrinsic factors[20,33].

Stat6 induces the expression of the transcription factors GATA-3 and c-Maf[23], which have been shown to be selectively expressed in a Th2-specific fashion[17,56,59]. Using Stat6-deficient cells it has been shown that, although IL-4 and Stat6 signaling may initially direct Th2 development, GATA-3 and c-Maf are capable of inducing the development of stabile Th2 commitment, independent of Stat636. *In vitro* differentiation into Th1 cells induces chromatin remodeling of the *IFNγ* locus and, conversely, the differentiation into Th2 cells induces remodeling of the *IL-4/IL-5/IL-13* locus[2,51]. Recently, GATA-3 has been shown to play an instructive role in directing Th2 differentiation[10].

During early T cell development, *GATA-3* gene expression is required for the development of the earliest T cell progenitors[14,16,53]. GATA-3 levels are low during the two phases of *TCR* gene rearrangement, but are high in the fraction of rapidly proliferating cells that insulates these two periods of *TCR* rearrangement[16]. GATA-3 expression remains high in CD4+ thymocytes, but progressively declines in CD8+ thymocytes (see accompanying paper). GATA-3 is detected in naive CD4+ T cells and expression levels increase substantially during Th2 differentiation[56,59]. GATA-3 expression has been shown to be indispensable for Th2 development, and is downregulated in response to IL-12-mediated Stat4 activation[35,59]. GATA-3 strongly transactivates the *IL-5* promoter, but appears to have only limited effects on *IL-4* gene transcription[40,57,59]. Retroviral introduction of GATA-3 during *in vitro* Th1 differentiation of naive CD4+ T cells, resulted in an inhibition of IFNγ production, independently of IL-4[11,35], and a downregulation of IL-12Rβ235, which normally accompanies Th-2 differentiation[49].

The manipulation of Stat6 and GATA-3 expression in Th1 and Th2 polarization cultures of wild-type or specific cytokine-deficient cells *in vitro* have added significantly to our understanding of the molecular basis of Th1/Th2 differentiation. However, limited data are available on the role of GATA-3 during immune responses in animal models, partly because the embryonic lethality of GATA-3 deficiency in mice precluded *in vivo* studies[37]. Analysis of transgenic mice with T cell-specific expression of a dominant-negative mutant of GATA-3 indicated that inhibition of GATA-3 activity reduced the key features of asthma, including Th2 cytokine levels, eosinophilia and IgE production[58].

To study the function of GATA-3 during T cell differentiation, we generated transgenic mice in which the expression of GATA-3 is under the control of the human *CD2* locus control region (see accompanying paper). In these mice, the enforced GATA-3 expression induced the development of thymic lymphomas of CD4+CD8+/lo T cells and inhibited the maturation of CD8 single positive (SP) cells in the thymus. Within the CD8 SP population in the thymus apoptosis was increased and the fraction of mature CD69loHSAlo cells was significantly reduced. The numbers of peripheral CD8+ T cells were ~50% of normal. These observations supported a role for GATA-3 in the regulation of CD4/CD8 lineage commitment (see accompanying paper).

To investigate how the enforced expression of GATA-3 affected T helper cell differentiation, we analyzed T cell-mediated immune responses *in vivo* in *CD2-GATA3* transgenic mice. The observations of a selective deficiency of antigen-specific IgG2a production and

severely reduced delayed-type hypersensitivity responses in these mice showed that enforced GATA-3 expression inhibited the differentiation of Th1 cells *in vivo*. Furthermore, the expression of surface markers specific for antigen-experienced Th2 cells, together with the increased functional capacity to secrete the Th2 cytokines, indicated that transgenic GATA-3 expression induced Th2 commitment and pointed at a role for GATA-3 in Th2 memory formation.

Results

Increased expression of antigen-experienced T cell surface markers in CD2-GATA3 transgenic mice

In two independent *CD2-GATA-3* transgenic lines expression of HA-tagged GATA-3 was under the control of the *CD2* locus control region. Due to the presence of this transgene, GATA-3 expression was significantly enhanced in the thymus, especially in the DP fraction. In these mice, the expression of transgenic GATA-3 in peripheral lymphoid organs was low (see accompanying paper). In the experiments described below, we did not detect any differences between the two independent lines, TgA and TgB.

To determine the effect of enforced GATA-3 expression on the development of the peripheral T cell compartments, we investigated the sizes of the CD4+ and CD8+ populations in *CD2-GATA3* transgenic mice and their non-transgenic littermates at different ages (Fig. 1A). In spleen and mesenteric lymph nodes, the numbers of CD4+ T cells in *CD2-GATA3* mice were either increased (3 weeks of age) or within normal ranges (12 weeks of age). In contrast, the CD8+ T cell populations were consistently reduced in number, to ~50% of normal, irrespective of the age analyzed (Fig. 1A). Despite the observed increased apoptosis in the fraction of mature single positive cells in the thymus (see accompanying paper), the *CD2-GATA3* transgenic mice were not found to be lymphopenic at any of the ages analyzed.

As GATA-3 is involved in the stabilization of the Th2 phenotype and the maintenance of Th2 cytokine expression, which are important features of T helper memory, we wanted to assess whether the enforced expression of GATA-3 had an effect on the development of memory T cells. Antigen activation induces the expression of CD44 and decreases CD45RB and L-selectin (CD62L) expression on the cell surface of T cells. As this profile of cell surface marker expression is maintained, even after cells have reverted to a quiescent state, it can be used to define antigen-experienced T cells8. As shown in Fig. 1BC, in *CD2-GATA3* transgenic mice both the CD4+ and the CD8+ T cell populations contained increased proportions of CD44hi, CD45RBlo and CD62Llo cells. This was found both in spleen and in lymph nodes, and pointed to the presence of elevated numbers of either recently activated or antigen-experienced T cells8. The expression levels of CD69 and IL-2Rα (CD25), which are markers of recently activated T cells28, were low in *CD2-GATA3* mice and non-transgenic littermates (Shown for CD25 in Fig.1AB), arguing against the presence of major fractions of recently activated T cells in *CD2-GATA3* mice. It has been shown that the proportions of T cells with an antigen-experienced phenotype increases with age, probably as a result of progressive antigen experience9,46. When we compared CD44 and CD62L expression at two different ages, 3 months and 7 months, we indeed found that the conversion to the CD44hiCD62Llo antigen-experienced T cell phenotype increased with age, both in CD2-GATA3 transgenic mice and in control mice (shown for CD62L in Fig. 1C).

Taken together, these results indicated that in the *CD2-GATA3* mice both the CD4+ and the CD8+ T cell population contained increased proportions of cells with an antigen-experienced

phenotype.

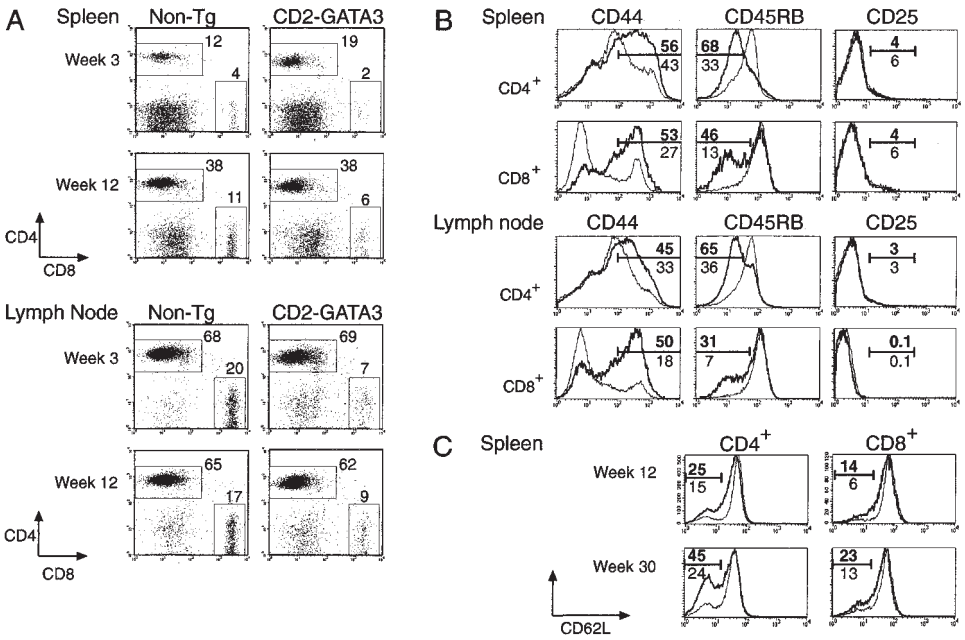

Figure 1 - Enforced GATA-3 expression is associated with increased numbers of T cells with a memory surface phenotype.(**A)** Single-cell suspensions from spleen and mesenteric lymph nodes from 3-week old and 12 week-old *CD2-GATA3* transgenic mice and non-transgenic littermates were analyzed for CD4 and CD8 expression. Surface CD4/CD8 profiles are shown as dot plots, in which the percentages of total lymphocytes within the indicated CD4+ and CD8+ gates are given. **(B)** Single-cell suspensions from spleen and mesenteric lymph nodes from 12 week-old *CD2-GATA3* transgenic mice and non-transgenic littermates were stained for CD4 and CD8, together with CD44, CD45RB or CD25. Total CD4+ and CD8+ T cell populations were analyzed for the indicated markers. Results are displayed as histograms of *CD2-GATA3* transgenic mice (*bold lines*) and those of non-transgenic littermates (*thin lines*). The percentages of activated/antigen-experienced cells ($CD44^{high}$, $CD45RB^{low}$ or CD25+) are indicated above the marker line in bold (*CD2-GATA3* transgenic mice) and below the marker line (non-transgenic littermates). Data shown are representative of at least 6 mice examined in each group. **(C)** The numbers of activated/antigen-experienced ($CD62L^{low}$) cells in *CD2-GATA3* transgenic and control mice at two different ages, 12 weeks and 30 weeks. The percentages are indicated as in panel B.

Enforced expression of GATA-3 results in increased numbers of T1/ST2 positive CD4+ T cells in the periphery

The IL-1R family member T1/ST2 is preferentially expressed on the surface of murine Th2 cells[5,25,54,55]. It was recently shown that CD4+ cells become T1/ST2 positive after repeated antigenic stimulation under Th2-polarizing conditions and that Th2 cytokine production precedes T1/ST2 expression[31]. Therefore, T1/ST2 expression appears to be a late event in Th2 commitment.

To investigate whether CD4+ T cells in *CD2-GATA3* mice exhibited preferential Th2 polarization and advanced Th2 commitment *in vivo* we evaluated surface expression of T1/ST2 (Fig. 2). In non-transgenic controls we found T1/ST2 expression on 5.1% $\pm$ 1.3 and 1.1% $\pm$

0.1 (n=5) of CD4+ T cells in spleen and mesenteric lymph nodes, respectively. In four-color labelings with CD4, CD8, CD44 and T1/ST2, it was shown that in non-transgenic mice T1/ST2 expression was largely confined to the CD44hi fraction of activated/memory CD4+ T cells (Fig. 2).

The CD2-GATA3 transgenic animals showed a significant increase in the proportions of T1/ST2+ cells: 31% ± 1 and 23% ± 2 (n=3) in spleen and mesenteric lymph nodes, respectively. This increase could not be attributed solely to the increased proportion of CD4+ T cells with a CD44hi activated/memory phenotype, as T1/ST2 was also found to be expressed on naive CD4+ T cells with a CD44lo surface profile (Fig. 2).

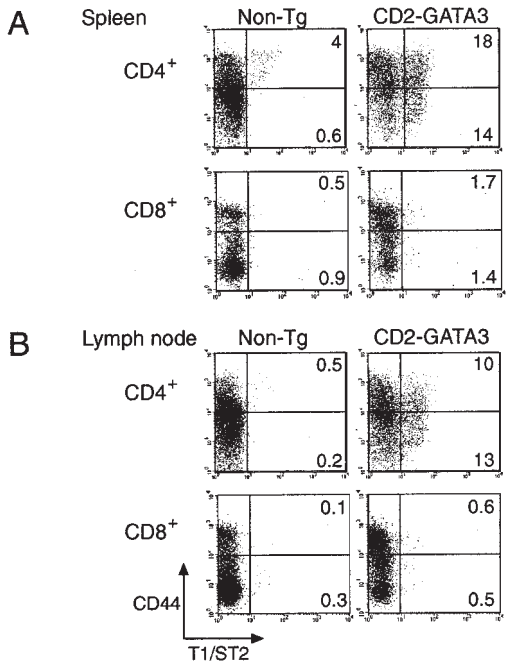

Figure 2 - Aberrant T1/ST2 surface expression in *CD2-GATA3* transgenic mice.Single-cell suspensions from spleen **(A)** and mesenteric lymph nodes **(B)** from 2-3 month-old *CD2-GATA3* transgenic mice and non-transgenic littermates were stained for CD4, CD8, CD44 and T1/ST2. CD4+ and CD8+ T cells were gated and analyzed for CD44 and T1/ST2 expression. Data are displayed as dot plots, and the percentages of gated cells within the indicated T1/ST2+ quadrants are shown.

When we analyzed T1/ST2 expression in the thymic subpopulations, we found induction of T1/ST2 on a small fraction of the CD4 single positive cells in *CD2-GATA3* transgenic mice: 6.0% ± 1.0 (n=3), compared with 0.4% ± 0.05 in non-transgenic mice (n=5). In contrast, T1/ST2 expression was not significantly induced on CD8 SP thymocytes (<0.5%). Consistent with the reported absence of T1/ST2 on the surface of CD8+ cells25,55, we found very low numbers of T1/ST2 positive cells in non-transgenic mice. However, some T1/ST2 expression was observed on CD8+ T cells in spleen (3.0% ± 0.8) and lymph node (1.7% ± 0.6) from *CD2-GATA3* transgenic mice (Fig. 2).

In summary, these data indicate that enforced expression of GATA-3 resulted in

significantly increased numbers of peripheral CD4+ T cells with an advanced Th2 committed T1/ST2+ phenotype, not only in the CD44hi activated/memory T cell compartment, but also in CD44lo naive T cells.

Increased ability to secrete Th2 cytokines in CD2-GATA3 transgenic T cells

As one hallmark of a memory cell population is the ability to secrete a wider diversity of cytokines8, we investigated the ability of peripheral T cells to synthesize various cytokines. After polyclonal *in vitro* stimulation of mesenteric lymph node cells, significant differences in the cytokine production profiles between wild-type and *CD2-GATA3* transgenic mice were observed. When intracellular cytokine expression was analyzed by flow cytometry, *CD2-GATA3* transgenic CD4+ and CD8+ T cells manifested decreased expression of IL-2 and IFNγ (Fig. 3A). In addition, the production of the Th2 cytokines IL-4, IL-5 and IL-10 was increased in *CD2-GATA3* transgenic T cells after 40 and 60 hours of culture (Fig. 3B).

The effect of enforced GATA-3 expression on T cell proliferation was studied in *in vitro* stimulations of purified CD4+ T cells. When 28thymidine incorporation was assessed at day 1 or day 2, the *CD2-GATA3* transgenic T cells manifested significantly reduced proliferation, as compared to non-transgenic T cells (Fig. 3C).

Collectively, the *CD2-GATA3* transgenic T cells were characterized by an increased production of Th2 cytokines and reduced production of IL-2 and IFNγ upon stimulation, suggesting the presence of an increased memeory compartment within the peripheral T cell population *in vivo*.

Expression of GATA-3 in CD2-GATA3 transgenic T cell cultures under Th1 polarizing conditions

Next, we performed in vitro Th1/Th2 polarization culture experiments on purified CD4+ T cells from spleen and lymph nodes to investigate whether the differentiation potential into T effector phenotypes was altered in the CD2-GATA3 mice. In these experiments, cells were stimulated with anti-CD3ε mAbs, either under "default" conditions (without additional cytokines or antibodies), Th1 polarizing conditions (in the presence of IL-12 and anti-IL-4 mAbs) or under Th2 polarizing conditions (in the presence of IL-4 and anti-IFNγ mAbs) for 4 days. Subsequently, the cells were washed and restimulated with anti-CD3ε mAbs for 3 days, without additional antibodies or cytokines.

First, we evaluated the GATA-3 expression in lymph node derived T cell cultures by Western blotting and intracellular flow cytometric analyses, using a mouse monoclonal antiserum specific for GATA-3 (Fig. 4AB). In non-transgenic and CD2-GATA3 transgenic CD4+ T cell cultures, GATA-3 protein was abundantly expressed both under default and Th2 polarizing conditions. GATA-3 protein could not be detected in non-transgenic Th1 cultures (Fig. 4A). In contrast, GATA-3 was expressed in CD2-GATA3 transgenic Th1 cultures, although the levels of expression were lower than in the corresponding Th2 or default cultures (Fig. 4A). The GATA-3 protein present had the apparent molecular weight of endogenous GATA-3, and no transgene encoded GATA-3 bands could be detected, using antibodies specific for the HA tag present in the transgenic GATA-3 protein.

Intracellular GATA-3 protein flow cytometric assays allowed a comparison of mean fluorescence intensity values in histogram overlays. Although these analyses are limited by a background signal of the GATA-3 antibody, they showed that GATA-3 protein levels were higher in the cultures of *CD2-GATA3* transgenic mice, when compared to those of the non-

transgenic littermates, irrespective of the culture conditions (Shown for Th1 and Th2 conditions in Fig. 4B).

Therefore, we conclude that due to the presence of the *CD2-GATA3* transgene, GATA-3 protein expression is maintained in CD4+ T cells that are cultured under Th1-polarizing conditions.

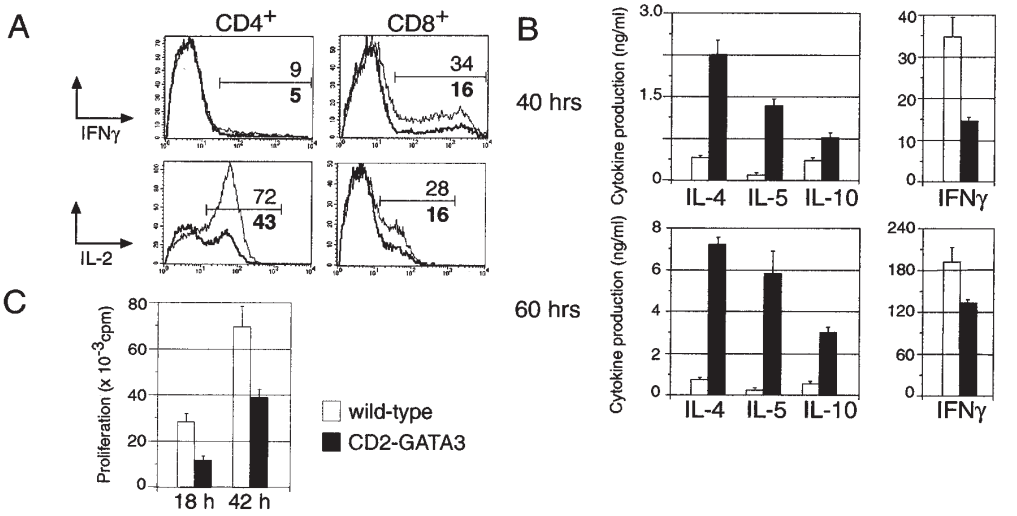

Figure 3 - Peripheral T cells from *CD2-GATA3* transgenic mice produce Th2 cytokines upon activation. **(A)** Decreased synthesis of IL-2 and IFNγ in *CD2-GATA3* transgenic T cells. Mesenteric lymph node T cells were stimulated with anti-CD3ε for 40 hours, and intracellular IL-2 and IFNγ was determined by flow cytometry, in conjunction with membrane expression of CD4 and CD8. Results are displayed as histograms of *CD2-GATA3* transgenic mice (*bold lines*) and those of non-transgenic littermates (*thin lines*). The percentages of cytokine-positive cells are indicated below the marker line in bold (*CD2-GATA3* transgenic mice) and above the marker line (non-transgenic littermates).**(B)** Th2 cytokine profiles of *CD2-GATA3* transgenic and control T cells. Mesenteric lymph node T cells were stimulated with anti-CD3ε for either 40 or 60 hours, as indicated. Open bars: wild-type mice; closed bars: *CD2-GATA3* transgenic mice. The levels of the indicated cytokines were measured in the culture supernatants by ELISA. **(C)** Proliferation, determined by [3H]thymidine incorporation, in response to anti-CD3ε stimulation of purified CD4+ T cells. Data are given as mean values ± standard deviation

The Th1/Th2 differentiation potential of CD2-GATA3 transgenic T cells in vitro

Cytokine production was evaluated in the Th1/Th2 polarized CD4+ T cell cultures from lymph nodes or spleen. *CD2-GATA3* transgenic CD4+ T cells produced normal amounts of IL-4, but higher levels of IL-5 and IL-10 in the default or Th2-polarized cultures, when compared to non-transgenic CD4+ T cells (Fig. 4C). In addition, when *CD2-GATA3* transgenic CD4+ T cells were cultured under Th1 polarizing conditions, they produced significantly increased amounts of IL-4, IL-5 and IL-10. Irrespective of the culture conditions, the production of IFNγ was significantly reduced in the *CD2-GATA3* transgenic CD4+ T cells, when compared to those from non-transgenic littermates (Fig. 4C).

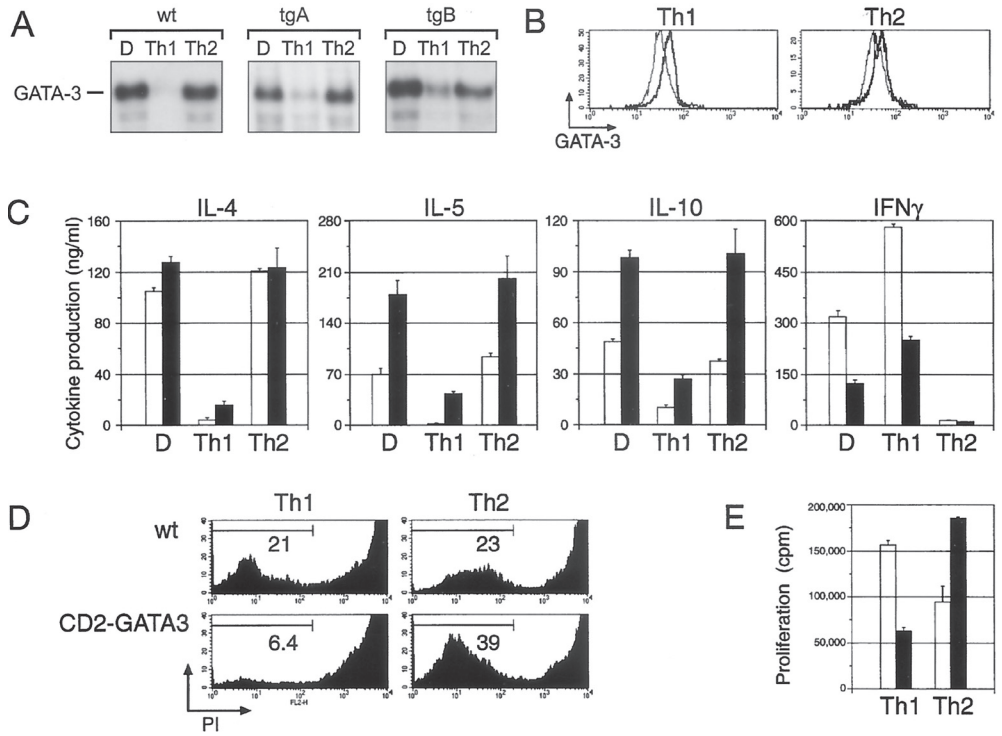

Figure 4 -The effect of enforced GATA-3 expression in CD4+ T cell cultures under Th1 and Th2 polarizing conditions. **(A)** Western blotting analysis, showing the expression of GATA-3 in purified CD4+ T cells from the indicated non-transgenic (wt) and *CD2-GATA3* (tgA and tgB) mice, cultured for 7 days under default (D), Th1 and Th2 polarizing conditions. **(B)** Expression of GATA-3 in *CD2-GATA3* transgenic (*bold lines*) and non-transgenic (*thin lines)* purified CD4+ T cells that were activated under Th1 or Th2 conditions as indicated. Cell suspensions were stained for surface CD4 and CD8 and subsequently for intracellular GATA-3 protein. Flow cytometry results are displayed as histograms of CD4+CD8- cells. **(C)** Cytokine production in supernatants of 7-day cultures of purified CD4+ T cells under default, Th1 and Th2 conditions, as indicated (Open bars: wild-type mice; closed bars: *CD2-GATA3* transgenic mice). **(D)** Cell viability after 7 days of culture under Th1- or Th2-priming conditions of purified CD4+ T cells of the indicated mice. Cell suspensions were stained for surface CD4 and CD8 and propidium iodide (PI). Results are displayed as histograms of CD4+CD8- cells. The percentages of viable (PI-) cells are indicated. **(E)** Proliferation, determined by [3H]thymidine incorporation, in response to stimulation with anti-CD3ε of purified CD4+ cells, under Th1 and Th2 conditions. Data are given as mean values ± SD (Open bars: wild-type mice; closed bars: *CD2-GATA3* transgenic mice). Data are representative of five repeat experiments.

The reduced IFNγ production by *CD2-GATA3* transgenic CD4+ T cells cultured under Th-1 polarizing conditions could either result from an inhibitory effect of GATA-3 on the differentiation of naïve cells into Th1 cells, or by an inhibition of the amount of IFNγ produced by differentiated Th1 effector cells. To distinguish between these possibilities, we assessed cell viability and proliferation in the T cell cultures. When analyzed by flow cytometry using propidium iodide, CD4+ T cells from CD2-GATA3 transgenic mice showed increased cell death under Th1 culture conditions at day 7, as compared with non-transgenic littermates (Fig. 4D). When [3H]thymidine incorporation was assessed at day 7, we observed a specific inhibitory

effect of enforced GATA-3 expression on cell proliferation in Th1 cultures (Fig. 4E). By contrast, the presence of the *CD2-GATA3* transgene enhanced viability and proliferation of CD4+ T cells in the Th2 cultures (Fig. 4DE).

Collectively, these observations demonstrate that although the presence of the *CD2-GATA3* transgene inhibited the proliferation of Th cells under Th1 polarizing conditions, considerable production of Th2 cytokines was still present. Furthermore, the enforced GATA-3 expression significantly supported proliferation and differentiation of Th2 effector cells in a Th2 environment.

GATA-3 expressing lymphoblastoid tumors express Th2 cytokines

The coordinate expression of IL4, IL5 and IL-13 is thought to be under the direct control of GATA-3, as GATA-3 specifically interacts with an intergenic DNAse I hypersensitivity site in Th2 cytokine locus that contains the *IL-4/IL-5/IL-13* gene cluster51. However, the mechanism by which GATA-3 would regulate IL-10 expression is unknown. The rapid production of IL-10 after anti-CD3 stimulation *in vitro* (Fig. 3C) would suggest that GATA-3 is directly involved in the regulation of *IL-10* gene expression. To further address this question, we examined lymphoblastoid tumor samples from *CD2-GATA3* transgenic mice. At the age of 9 months, ~50% of these mice developed thymic lymphomas that were CD4+CD8+/lo and expressed high levels of GATA-3 (see accompanying paper). Immunohistochemical analyses of thymic tumor tissues showed that most of the tumors contained areas where the lymphoblastoid cells had lost expression of CD8, and sometimes also CD4 (data not shown). Particularly in such areas very high expression of the Th2 cytokines IL-4, IL-5 or IL-10 was found (Fig. 5ABC). Moreover, when tumor cells were cultured in the presence of anti-CD3ε an extremely high production of Th2 cytokines, including IL-10, was observed (An example is shown in Fig. 5D). Proliferative responses were determined by [3H]thymidine incorporation and showed that CD2-GATA3 transgenic mice had enhanced *in vitro* recall responses (Fig. 6C).

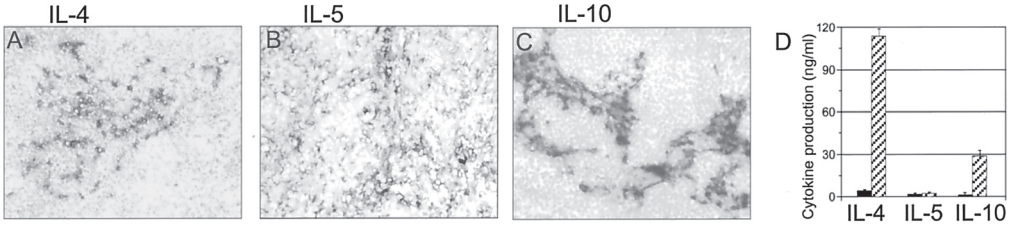

Figure 5 - Cytokine production by thymic lymphoblastoid tumor cells. Immunohistochemical analysis of three thymic lymphoblastoid tumor tissue samples from *CD2-GATA3* transgenic mice, showing areas with cells that produce high levels of **(A)** IL-4, **(B)** IL-5 and **(C)** IL-10. **(D)** Cytokine production in the supernatant of a 40-hour culture of lymphoblastoid tumor cells in the presence of anti-CD3ε. (Closed bars: *CD2-GATA3* transgenic lymph node cells from a tumor-free mouse; hatched bars: lymphoblastoid tumor cells from a lymph node).

In conclusion, the observed suppression of TNP-specific IgG2a production indicated that transgenic GATA-3 expression is sufficient to inhibit IFNγ-mediated antigen-specific class switching. In the *CD2-GATA3* transgenic mice the Th2-dependent induction of IgE was enhanced, while increased class switching to IgG1 was only

These findings show that high expression of GATA-3 in lymphoblastoid tumor cells is associated with high level production of Th2 cytokine production, suggesting that like the

IL4/IL-5/IL-13 locus, also the *IL-10* gene may be a direct target of GATA-3.

Enforced GATA-3 expression inhibits switching to IgG2a in an antigen-specific immune response

Serum levels of individual Ig isotypes are generally dependent on the Th1/Th2 balance. IL-4 primes mouse B-lymphocytes for switching to IgG1 and IgE, while IgG2a responses are induced by IFNγ45. When total serum Ig levels were determined in 2-3 month-old *CD2-GATA3* transgenic mice and non-transgenic littermates by ELISA, a selective increase in IgG1 was found in the *CD2-GATA3* transgenic animals (Fig. 6A). The levels of all other isotypes, including IgE, were similar in the two groups of mice (Fig. 6A and Fig. 6C IgE pre-immune values).

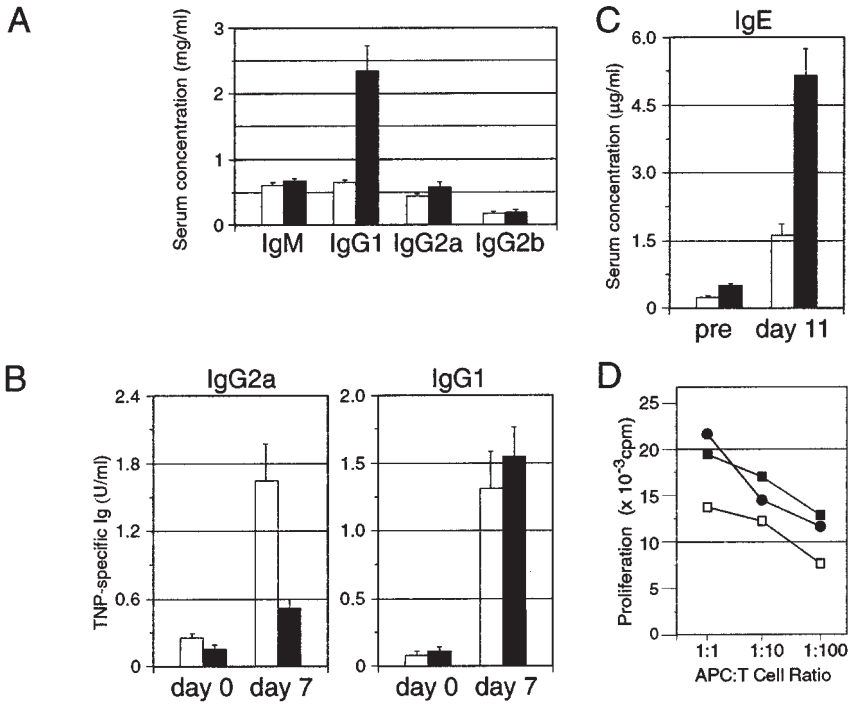

Figure 6 - Expression of the *CD2-GATA3* transgene affects Ig class switching in vivo. **(A)** Serum concentrations of Ig isotypes in unimmunized 2-3-month-old mice (open symbols: non-transgenic mice, n=15, closed symbols: *CD2-GATA3* transgenic mice, n=12). Data are shown as mean values ± standard errors. **(B)** For T cell dependent responses, serum concentrations of TNP-specific IgG1 and IgG2a were determined by ELISA, before (*day 0*) and after (*day 7*) i.p. booster injection of TNP-KLH in PBS (open bars: non-transgenic mice, n= 6, closed bars: *CD2-GATA-2* transgenic mice, n=6). **(C)** Total serum IgE levels in unimmunized animals (*pre*) and 11 days after i.p. injection of TNP-KLH on alum. **(D)** In vitro recall response of splenic T cells, 4 weeks after i.p. TNP-KLH immunization. Data shown are mean values for 4 wild-type mice (open squares), and 2 mice of each independent transgenic *CD2-GATA3* line TgA and TgB (closed symbols).

To analyze Ig class switching in a T cell-dependent response *in vivo*, mice were immunized i.p. with 10 µg TNP-KLH, which was precipitated on alum. After two months a

booster dose of 100 μg of TNP-KLH in PBS was given. *CD2-GATA3* transgenic mice showed a significantly decreased secondary TNP-response for the INFγ-dependent isotype IgG2a on day 7 after the booster injection, as measured in a TNP-specific ELISA (Fig. 6B). The TNP-specific levels of the other Ig isotypes elicited in this response were comparable between transgenic animals and wildtype littermates (shown for IgG1 in Fig. 6B). Also the primary IgG2a response to TNP-KLH *in vivo* was specifically decreased in the CD2-GATA3 transgenic animals (to ~15% of normal), as determined in the serum at day 7 and 14 after an i.p. immunozation with 100 μg TNP-KLH precipitated on alum (data not shown). The Th2-dependent induction of heavy chain class switch to IgE was determined at day 11 after i.p. injection of 10 μg TNP-KLH precipitated on alum. *CD2-GATA3* transgenic mice manifested elevated total serum IgE levels in this response (Fig. 6C).

Next, we directly tested the ability of *CD2-GATA3* transgenic T cells to respond to antigen after a previous i.p. immunization with TNP-KLH. CD4+ T cells were purified from spleens of immunized *CD2-GATA3* transgenic mice and control littermates 4 weeks after i.p. injection with TNP-KLH precipitated on alum and stimulated *in vitro* using wild-type irradiated APCs, which had been pre-incubated with KLH for 4 hours. observed in the total serum levels. In addition, CD2-GATA3 transgenic CD4+ T cells were shown to have an elevated *in vitro* recall response.

Enforced GATA-3 expression diminishes delayed type hypersensitivity response to KLH

To directly test whether enforced GATA-3 expression suppresses Th1-dependent immune responses in vivo, we assayed delayed type hypersensitivity (DTH) responses to the protein antigen KLH. Two-month-old mice were primed by i.p. injection of 100 μg KLH, and challenged on day 6 by injection of 25 μl PBS alone and 25 μl PBS containing 150 μg KLH in the left and right hind footpad, respectively. Twenty-four hours after the injections, footpad thickness was measured and the difference between the two footpads was calculated (Fig. 7). The KLH-induced footpad swelling was significantly reduced in CD2-GATA3 transgenic animals, as compared to the wild type littermates. This reduction of footpad swelling did not reflect delayed kinetics of the DTH response, as also at 48 hours after the injections footpad swellings were still essentially absent in the CD2-GATA3 transgenic mice.

These observations demonstrate that GATA-3 expression has a severe inhibitory effect on the Th1-mediated DTH response in vivo.

Discussion

Prior studies using Th1 and Th2 polarization cultures of wild-type and specific cytokine-deficient cells *in vitro* have identified GATA-3 as a master switch in Th2 development[10,11,35,36,51,56,59]. GATA-3 does not only induce the expression of Th2-specific cytokines, but also acts as a repressor of Th1 differentiation. Introduction of GATA-3 by retroviral infection into naive T cells strongly inhibited IFNγ production, independently of IL-4 expression[11,35].

To analyze the function of GATA-3 in an *in vivo* system, we investigated transgenic mice that expressed GATA-3 under the control of the *CD2* LCR. In these mice, the expression of transgenic GATA-3 in the peripheral T cells of the spleen or lymph nodes was very low (see accompanying paper). However, in contrast to wild-type $CD4^+$ cells, which lost GATA-3 expression when cultured under Th1-polarizing conditions, *CD2-GATA3* transgenic CD4+ cells

maintained expression of endogenous GATA-3 protein. The finding by Ouyang et al.36 that GATA-3 may, either directly or indirectly, activate its own expression, could be a mechanism by which low amounts of transgenic GATA-3 enhance the expression of endogenous GATA-3. This could explain our observation of increased GATA-3 protein levels in T cell cultures, while transgenic GATA-3 could not be detected in Western blotting analyses using antibodies to GATA-3 or the HA-tag (Fig. 4AB).

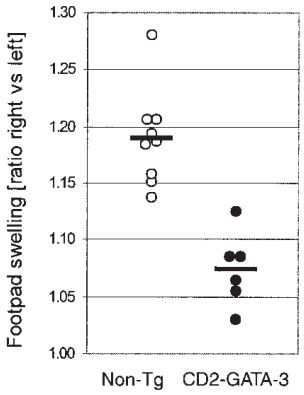

Figure 7 - Diminished DTH responses in *CD2-GATA3* transgenic mice. Ratios of footpad swelling of KLH-injected over PBS-injected footpads, in non-transgenic mice (open symbols, n=9) and *CD2-GATA3* transgenic mice (closed symbols, n=6) 24 hours after the injections.

Our analyses of the *CD2-GATA3* transgenic mice support the findings that GATA-3 expression inhibits Th1 development. The enforced GATA-3 expression inhibited Th1-mediated responses *in vivo*, including antigen-specific IgG2a production and DTH responses to protein antigen. In our Th1/Th2 polarization cultures, enforced GATA-3 expression under Th1-inducing culture conditions resulted in a reduction of cell survival, proliferation and IFNγ production. The additional findings of increased T1/ST2-expression in CD4+ T cells and elevated total IgG1 serum levels suggest that the presence of the *CD2-GATA3* gene drives T cells preferentially towards differentiation along the Th2 pathway. Therefore, we conclude that GATA-3 plays a dual role *in vivo* in the differentiation of naive T helper cells into Th2 cells, as it both represses Th1 differentiation and induced Th2 differentiation.

Various lines of evidence indicate that the enforced expression of GATA-3 may enhance Th2 memory cell formation. First, In *CD2-GATA3* transgenic mice, the peripheral T cell compartment contained a high proportion of cells with an antigen-experienced cell surface profile, defined as $CD44^{high}CD45RB^{low}CD62L^{low}$ and negative for CD25 and CD69. The ratio of naive versus memory-phenotype cells decreased with age, as normally seen in wild-type mice. The possibility that peripheral T cells obtained the antigen-experienced surface phenotype because of a homeostatic proliferation mechanism13 in the *CD2-GATA3* transgenic mice is unlikely, as these mice were not lymphopenic at any of the ages analyzed. Second, In *CD2-GATA3* transgenic mice the expression of the Th2-specific T1/ST2 marker within the CD44hi memory Th cell population in spleen and lymph nodes was increased by a factor ~6 and ~20, respectively. T1/ST2 marker expression is associated with advanced Th2 commitment, as it was shown to be expressed only after repeated antigenic stimulation under Th2-polarizing

conditions *in vitro*, with delayed kinetics compared with the kinetics of Th2 cytokines[31]. Third, *CD2-GATA3* transgenic T cells were rapidly induced to synthesize IL-4, IL-5 and IL-10 *in vitro,* whereas production of IL-2 was low, which is typical for memory Th2 cells[8]. Fourth, T cells from *CD2-GATA3* mice exhibited an increased recall response to TNP-KLH antigen *in vitro*. Finally, the selective increase of the total levels of the IL-4 dependent isotype IgG1 in the serum would also be consistent with increased Th2 memory formation.

It is presently not clear how GATA-3 would affect Th2 memory formation. GATA-3 may regulate the cell fate decision of activated CD4+ T cells, by reducing activation-induced cell death, in favor of Th2 memory cell formation. Alternatively, GATA-3 may facilitate the differentiation process of dividing effector T cells that are already committed to the memory cell fate. A third possibility is that GATA-3 would act as a survival factor for Th2 memory cells. This is not very likely, because survival alone does not appear to be sufficient for memory cell formation, as was shown by the absence of increased memory formation in Bcl-2 transgenic mice[39]. Further experiments are required to define GATA-3 targets that are involved in Th2 memory cell formation.

One of the molecules involved in Th2 memory development may be T1/ST2, as it is normally specifically expressed in the Th2 lineage within the compartment of CD44hi activated/memory T cells (Fig. 2). As cross-linking of T1/ST2 enhanced proliferaton of Th2 cells that were stimulated with sub-optimal concentrations of anti-CD3 mAb31, it is possible that the increased proliferation and cell survival of *CD2-GATA3* transgenic $CD4^+$ T cells in the *in vitro* Th2 polarization cultures originates from increased T1/ST2 expression. The increased expression of T1/ST2 in *CD2-GATA3* transgenic mice would argue for a direct regulation of *T1/ST2* transcription in T cells by GATA-3. The identification of three GATA elements in the minimal GATA-responsive *T1/ST2* promoter in mast cells[12] would support this hypothesis of a direct regulation of *T1/ST2* expression by GATA-3, independent of Th2-specific cytokines. Therefore, we hypothesize that GATA-3 is not only essential for instructive differentiation of naive Th cells into committed Th2 cells[10,35,36,56,59], but can also affect proliferation and survival of GATA-3 expressing CD4+ T cells through the induction of T1/ST2.

In conclusion, this study shows that enforced expression of GATA-3 inhibits Th1 function and induces Th2 commitment *in vivo*. Moreover, the increased expression of T1/ST2, the enhanced production of Th2 cytokines in response to T cell activation, and the elevated serum levels of IgG1 in *CD2-GATA3* transgenic mice argue for a role of GATA-3 in the formation of Th2 memory.

Material and Methods

Mice

The *CD2-GATA3* mice are described in the accompanying paper and were crossed on a uniform FVB background. To determine the genotype of the mice, tail DNA was analyzed by Southern blotting, as described in the accompanying paper.

Flow cytometric analyses

The preparation of single-cell suspensions, mAb incubations and three- or four-color cytometry have been described previously[15]. The following mAb were purchased from Pharmingen (San Diego, CA): FITC-conjugated anti-CD3ε, PE-conjugated anti-CD4 (L3T4), anti-CD24/heat stable antigen (HSA), anti-CD25 (clone 3C7), anti-CD62L and anti-CD69, CyChrome-

conjugated anti-CD4 (L3T4), anti-CD8 and anti-CD44, biotinylated anti-CD4 (L3T4), and anti-CD8, APC-labeled anti-CD3ε and anti-CD4. Anti-CD45RB (MB23G2) was a purified mAb conjugated to biotin, according to standard procedures. Secondary antibodies used were PE-, TriColor-, or APC-conjugated streptavidin (Caltag Laboratories, Burlingame, CA). The Th2-selective surface marker T1/ST2 (3E10, rat IgG1, kindly provided by A.J. Coyle, Cambridge, MA) was detected by secondary goat-anti-rat IgG-PE (Jackson Immunoresearch)25.

For intracellular detection of GATA-3 protein, cells were fixed and permeabilized using paraformaldehyde and saponin, as described[27] and subsequently incubated with the Hg-3-31 anti-GATA-3 MoAb (Santa Cruz, Dan Diego, CA) and FITC-labeled anti-mouse IgG1 (Pharmingen) as a second step. For three-color analysis, 0.5-1 x 105 events were scored using a FACScan analyzer (Becton Dickinson, Sunnyvale, CA). For four-color analysis, 105 - 2x106 events were scored using a FACS Calibur dual laser instrument (Becton Dickinson).

For intracellular detection of cytokines, cells were stimulated for 40 hours in the presence of mAb to CD28 (37.51; 5 μg/ml) in 96-well plates (106 cells/well) pre-coated with mAb to CD3ε (145 2C11; 10 μg/ml in PBS). Subsequently, cells were stimulated by adding PMA (50 ng/ml; Sigma) and calcium-ionophore (500 ng/ml; Sigma) for 5 hours. For the last three hours of the culture, BrefeldinA (10 μg/ml; Sigma) was added to the cells. Finally, cells were harvested and stained with CyChrome-labeled mAb to CD4 or CD8 (Pharmingen). Cells were fixed using 2% paraformaldehyde and stored up to 3 days at 4º C. Intracellular cytokine staining was performed using PE-labeled mAb to IL-2, IL-4 and IL-10 (Pharmingen) and APC-labeled mAb to IL-2, IL-5 and IFNγ (Pharmingen) according to the manufacturer's instructions.

Serum Ig detection and in vivo immunizations

Total serum Ig levels were determined by subclass-specific sandwich ELISA, as described previously[7]. Immunizations were done i.p. with 100 μg TNP-KLH precipitated on alum. Serum levels of TNP-specific Ig subclasses were determined by ELISA, using TNP-specific standards (IgG1, IgG2a and IgG2b) or TNP-specific reference serum samples (IgM and IgG3), as described[26].

Purification of CD4+ T cells and in vitro cultures

Single cell suspensions from spleen were incubated with biotinylated mAb to CD8 (YTS-169), CD11b/Mac-1 (M1/70), CD40 (FGK-45.5), B220 (RA3-6B2) and IgM (M41), followed by streptavidin-conjugated microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Using a Vario-MACS magnetic-activated cell sorter, $CD4^+$ T cells were purified according to the manufacturer's instruction to purity >95%. The $CD4^+$ T cells were cultured for up to 5 days in the presence of IL-2 (50 U/ml) on 96 well plates pre-coated with 10 μg/ml anti-CD3 (145 2C11) mAb.

Purified CD4+ T cells were polarized into Th-1 and Th-2 effector cells in a total volume of 200 μl for 4 days in the presence of 5 μg/ml anti-CD28 (37.51) and 50 IU/ml IL-2 on 96-well plates, which were pre-coated with 10 μg/ml anti-CD3ε (145 2C11)16. Th1-polarizing cultures included 5 ng/ml rIL-12 (R&D Systems, Minneapolis, MN) and 10 μg/ml neutralizing mAbs to IL-4 (11B11). Th2 polarized cells were cultured in the presence of 10-50 ng/ml rIL-4 and 10 μg/ml neutralizing mAbs to IFNγ (XMG1.2). After 4 days of culture, the cells were thoroughly washed and transferred to new anti-CD3 coated 96-well plates, and cultured in the presence of IL-2, without addition of further cytokines or neutralizing antibodies.

To measure DNA synthesis during T cell cultures, cells were pulsed with [3H]thymidine

for ~16 hours, harvested and counted using standard methods. Cytokine levels in culture supernatants were determined by ELISA using the Opteia kit for IL-4, IL-5, IL-10 and IFN-γ (Pharmingen) according to the manufacturer's instructions. Expression of GATA-3 protein was evaluated using a Western blotting procedure, as described in the accompanying paper.

DTH responses

DTH responses were performed essentially as described by Cua et al.[6]. In short, mice were immunized i.p. with 100 μg KLH in 250 μl PBS and on day 6 they were challenged with 150 μg of KLH in 25 μl PBS in the left hind footpad. The right hind footpad was in injected with a vehicle control (25 μl PBS). Responses were quantified 24 and 48 hours after the challenge, by measuring the difference in footpad thickness between the KLH- and the PBS-injected footpads.

Immunohistochemistry

Tissue samples were embedded in OCT compound and frozen 5μm cryostat sections were acetone fixed and single labelings were performed using standard procedures7. The monoclonal antibodies biotinylated anti-IL-4 (11B11) and alkaline phosphatase-conjugated anti-IL-5 (TRFK5) were purified hybridoma supernatants and conjuagated according to standard procedures. Biotinylated anti-IL-10 (SXC1) was purchased from Pharmingen.

Acknowledgements

This work was partly supported by Grant 3069 from the Estonian Science Foundation (to A.K.) as well as by the Royal Academy of Arts and Sciences (to R.W.H.).

We would like to thank W. van Ewijk, D. Hijdra, J. Mahabier, S. Philipsen and V. de Vries for their assistance at several stages of the project and G.Z. Tau for critical reading of the manuscript. We thank A.J. Coyle for provision of the anti-T1/ST2 antibody.

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Chapter 7

Discussion

Discussion

Understanding how hematopoietic stem cells (HSC) can give rise to the variety of cells composing the hematopoietic system has been the goal of many researchers over the years. Despite great progress over the last decades many tantalizing questions remain to be answered.

Hematopoiesis occurs by a succession of lineage-commitment steps associated with a restriction of differentiation potential and the establishment of lineage-specific expression profiles. The establishment of such expression profiles relies on lineage-specific transcription factors to regulate the expression of particular genes. Therefore, a strict regulation of the expression and function of such transcription factors is essential to ensure the differentiation into the different hematopoietic lineages. A variety of lineage-specific transcription factors have been identified which are crucial for different lineage-commitment steps during hematopoiesis[5,6,25,30].

In this thesis we focus on the importance of transcription regulation for the specific function(s) of the hematopoietic GATA transcription factors. To test the importance of accurate spatio-temporal expression of the GATA transcription factors we used transgenic mouse models where the different GATA factors are expressed under the control of heterologous promoters, thus leading to an expression pattern that differs from that of the endogenous gene. From the analysis of the phenotypes of these transgenic mice and the ability of some of these transgenes to rescue the absence of the endogenous gene, we can conclude that tight dynamic regulation of GATA transcription factors is essential for their normal function in hematopoiesis.

GATA1 and GATA2 are dynamically regulated during erythropoiesis

GATA1 and GATA2 are expressed in both primitive and definitive erythroid cells and are essential for normal erythropoiesis. GATA1 *null* primitive and definitive erythroid progenitors fail to progress in differentiation beyond the proerythroblast[26,27]. Consequently, GATA1 *null* mice die from anemia between embryonic day (E) 10.5 and E11.5 due to the impaired primitive erythropoiesis[13]. Mutations in GATA1 regulatory sequences leading to a reduced expression of the GATA1 gene revealed a direct correlation between the amount of protein produced and the severity of the phenotype. GATA1.05 mice, expressing approximately 5% of endogenous GATA1 protein die around E12.5 due to impaired primitive erythropoiesis[32]. However, about 10% of the GATA1 low mice, expressing about 20% of the endogenous GATA1 protein are born alive and a small number survive to adulthood[18]. These mice are anemic at birth but eventually recover and show a normal life span. GATA2 null mice also die from anemia around E10.5 due to impaired production of primitive erythroid cells. In fact, GATA2 is a key regulator of proliferation and differentiation of stem cell/multipotential precursors. Thus, GATA2 *null* cells fail to contribute to any hematopoietic lineage[36].

Both GATA1 and GATA2 expression must be tightly regulated for normal erythropoiesis to occur. Deregulation of either of these transcription factors has serious consequences for the development of erythroid cells (Fig.1).

We show in Chapter 2[40] that erythroid-specific GATA1 overexpression leads to a failure of terminal erythroid differentiation and subsequent embryonic death between E12.5 and E13.5 due to severe anemia. This, together with the known phenotypes of knockout and knockdown mutations in the GATA1 gene, suggested that GATA1 levels must be high at the early stages of erythroid differentiation but must decline in the later stages in order for terminal differentiation to occur. This model is reinforced by the data presented in Chapter 4 where we show that

GATA1 transgenes, under the control of a heterologous promoter (β-LCR), are unable to rescue definitive erythropoieisis in GATA1 null mice, whereas the same transgene under the control of GATA1 regulatory sequences is able to do so. Detailed analysis of endogenous and transgene encoded GATA1 during terminal erythroid differentiation revealed that indeed these GATA1 transgenes are expressed in a very distinct way. In contrast with endogenous GATA1, β-LCR GATA1 transgenes are expressed in later erythroid progenitors and their expression remains high, preventing normal terminal differentiation.

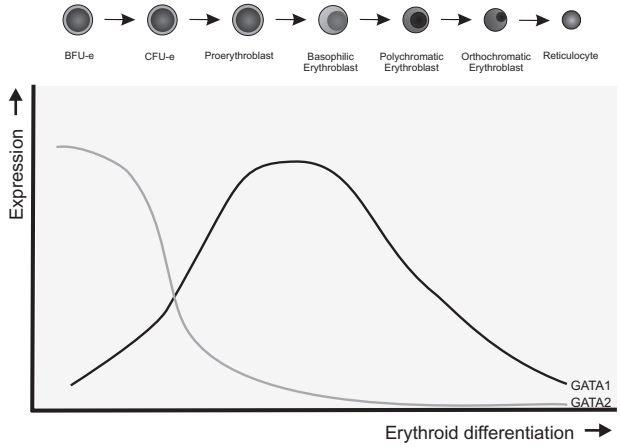

Figure 1 - Model of GATA1 and GATA2 expression during erythroid differentiation.

In agreement with previously published data[4], we also show in Chapter 4 that GATA2 overexpression in erythroid cells leads to a defect in terminal erythroid differentiation. Overexpression of GATA2 in late erythroid progenitors leads to a blockage in terminal erythroid differentiation, and the severity of this differentiation block correlates with the expression level of the transgene. These results support a model in which GATA2 downregulation in erythroid precursors correlates with GATA1 upregulation in the same progenitors Is there a reference for this model[21]. This suggests a cross-regulation between GATA1 and GATA2 where GATA2 activates transcription of the GATA1 gene and the GATA1 protein represses the expression of GATA2 allowing erythroid differentiation to proceed. This hypothesis is supported by several data including the upregulation of GATA2 expression in GATA1 *null* cells[38] and the observed repression of GATA2 trancription during GATA1-dependent differentiation of G1E GATA1 *null* erythroid precursors[29]. A recent report[15] provides evidence that GATA1 can specifically bind regulatory sequences of the GATA2 gene, displacing GATA2 bound to the same sequences. Moreover, Rodriguez and colleagues[28] have recently shown that the repressive complex GATA1/Fog1/MeCP1 binds to GATA2 regulatory sequences in vivo

Interestingly, GATA2 transgenes expressed under the control of GATA1 regulatory sequences, but not under its own or other heterologous promoter, can rescue the GATA1 null phenotype[33] (Chapter 4) indicating that expression of one and the same transcription factor can have different outcomes depending on when and at what level it is expressed. This idea is reinforced by the fact that GATA3 is also able to rescue the GATA1 null phenotype when expressed under the control of GATA1 regulatory sequences but not of the β-globin LCR (Chapter 4).

In an attempt to understand why GATA1 overexpression in late erythroid precursors impairs terminal erythroid differentiation we performed gene expression profiling of the GATA1 overexpressing erythoid cells (Chapter 3). Differentially expressed genes were identified, using both *in vivo* (fetal livers from transgenic mice) and *in vitro* (MEL cells) systems, and classified in agreement with their known role in particular physiological processes. The majority of these genes are involved in biological processes such as regulation of transcription, proliferation, differentiation, apoptosis and cell cycle control. All these processes are known to be affected by GATA1. The data obtained with this analysis proved to be reliable and a good model system for more detailed analysis in the regulatory functions of GATA1 during terminal erythroid differentiation. Similar strategies should also be helpful to understand how GATA1 and GATA2 factors are regulated during erythropoiesis. Cultured primary fetal liver erythroblasts can provide a good model system for such analysis. Primary fetal liver cells can be grown in the presence of glucocorticoid hormones, allowing for the expansion of erythroblasts. Induction of terminal differentiation of these erythroblasts can be achieved through removal of the glucocorticoid hormones and addition of appropriate growth factors[12,37]. This culture system provides an opportunity to collect samples over time, enabling us to analyse the expression profile of GATA1 and GATA2 and its downstream target genes, throughout terminal erythroid differentiation. Furthermore, this culture system allows us to use knockout or transgenic mice as the source of erythroblasts. Expression profiling of GATA1 null erythroid cells expressing other GATA transgenes, either under the control of endogenous or heterologous promoters, can also prove to be valuable to understand the functional overlap between these factors and the impact of correct spatio-temporal expression of such genes in terminal erythroid differentiation.

GATA3 is dynamically regulated during T-cell development

GATA3 has a more restricted expression pattern than GATA1 and GATA2 in the hematopoietic system since it is exclusively expressed in T-cells[42]. Like GATA1 and GATA2, GATA3 is dynamically regulated during cellular differentiation (Figure 2). A variety of reports have demonstrated that GATA3 is required at distinct stages of T-cell differentiation and deregulation of its expression levels has severe consequences for the progression of differentiation.

The first suggestion that GATA3 is necessary at different stages of T-cell development came from a detailed analysis of GATA3 expression during T-cell development in the thymus[17]. This report shows that GATA3 is expressed at low levels during the CD4 CD8 double negative (DN) stage but is upregulated during TCRβ rearrangement. GATA3 expression is maintained in $CD4^+$ T-cells but its expression is gradually downregulated in $CD8^+$ T-cells. The requirement of GATA3 in the DN stage of thymic T-cell development, in particular for TCRβ expression and β-selection, was later demonstrated[22]. Later in thymic T-cell differentiation, GATA3 is again required for the generation of $CD4^+$ T-cells[22]. We show in Chapter 5[20] that deregulation (*i.e.* overexpression) of GATA3 at this stage inhibits the maturation of $CD8^+$ T-cells and induces thymic lymphomas.

GATA3 is also implicated in the lineage selection of $CD4^+$ T-cells upon activation in the peripheral lymphoid organs. GATA3 is selectively expressed in the Th2 subset of activated $CD4^+$ T-cells[43,44] and is essential for the maintenance of the Th2 phenotype[23]. We also show in Chapter 6[19] that GATA3 downregulation in the Th1 lineage is critical for the differentiation of naïve $CD4^+$ T-cells.

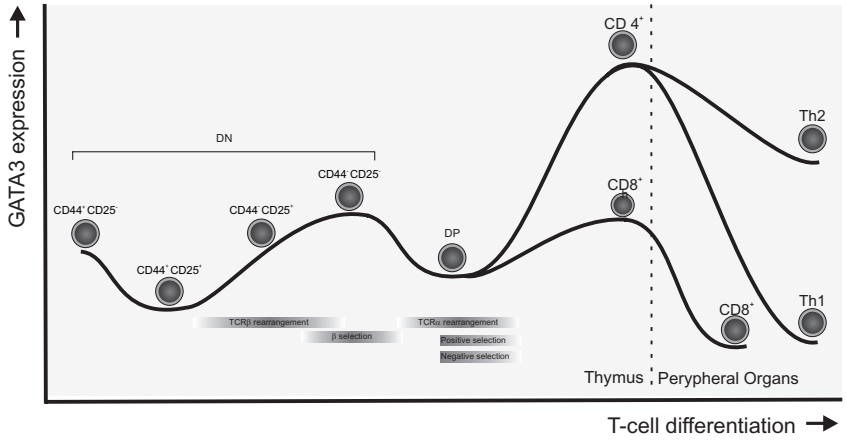

Figure 2 - Model of GATA3 expression during T-cell differentiation.

In the last few years a new GATA3 conditional knockout has been generated that allows deletion of the GATA3 gene at precise stages of T-cell differentiation[22]. The use of such conditional knockout models, together with transgenic mouse models in which GATA3 is expressed under the control of heterologous promoters, provide new opportunities to deepen our insight in the importance of spatio-temporal expression of the GATA3 gene. Expression profiling of lymphocytes from such models will be a valuable tool in our quest to understand the functional role of GATA3 in T-cell lineage commitment.

Regulation of GATA transcription factors activity

The activity of lineage specific transcription factors, like any other protein, can be regulated in multiple ways. Regulation at the transcriptional level is one way to ensure that these transcription factors have a correct spatio-temporal expression pattern. The importance of correct spatio-temporal expression is underscored by a number of experiments presented in this thesis. Both absence and overexpression of GATA1 in erythroid precursors, impairs terminal erythroid differentiation and leads to fatal anemia. Furthermore, forced expression of GATA3 throughout T-cell differentiation induces T-cell lymphomas and inhibits differentiation of specific T-cell lineages, such as $CD8^+$ and Th1. In addition to transcriptional control, gene expression can be regulated at several other levels.

Increasing evidence is accumulating for the importance of post-translational modifications as a mean of controlling transcription factor function. Despite the fact that the *in vivo* functions of many of these post-translational modifications are still unclear, increasing numbers of such modifications are being identified in a growing number of tissue specific transcription factors.

GATA1, GATA2 and GATA3 can be acetylated *in vivo* and acetylation appears to be crucial for their normal function[2,16,31]. However, how acetylation affects GATA factor function is still not clear. GATA1 acetylation seems to stimulate its transcriptional activity[2,3], increase its DNA-binding activity, at least in the avian system[3], and has also been implicated in its targeting for degradation through ubiquitination (J. Boyes, personal communication). Acetylation of

GATA2 results in increased DNA-binding affinity and transcriptional activity through synergy with p300, the acetyltransferase involved in the acetylation. Acetylation of GATA3 does not affect its DNA-binding activity but its acetylation level directly correlates with transcriptional activity[41]. Other transcription factors, such has EKLF, p53, and E2F1, have been reported to be acetylated and acetylation is in many cases associated with increased DNA binding activity, increased transcriptional activation potential, protein stability and/or alterations in interactions with co-factors (reviewed by Bannister and Miska[1]).

The hematopoietic GATA factors can also be phosphorylated[7,10,34] but, again, the implication of phosphorylation in the function of these transcription factors is unclear. It is possible that phosphorylation modulates the activity of such transcription factors by interfering with DNA binding, transactivation potential and interaction with co-factors, but reports are scarce and sometimes contradictory[10,24,34,35]. Phosphorylation of GATA transcription factors seems to be regulated by cytokine-mediated signalling through the MAPK pathway [34,35]. Phosphorylation was also implicated in nuclear-cytoplasmic transport and protein degradation of other transcriptions factors (reviewed by Whitmarsh and Davis[39]).

SUMO is a ubiquitin-like polypeptide that can be conjugated to lysine residues of proteins[14]. Several transcription factors, such as the glucocorticoid receptor, P53, and SP3, are known to be SUMOylated. SUMOylation seems to influence transcription factor function in several ways, including cellular localization, reduction of transcriptional activation potential and prevention of protein degradation (through competition with ubiquitin for the lysine residues). Recent reports show that both GATA1 and GATA2 can be SUMOylated[8,9] but the function of this modification remains to be identified.

Another important player in transcription factor regulation is the interaction with co-factors and/or regulatory complexes. A variety of proteins have been identified as interaction partners of the GATA transcription factors and include other transcription factors, non-DNA binding co-factors, chromatin-remodelling factors, and proteins involved in cell cycle regulation.

GATA1 is known to interact with a diverse range of proteins. GATA1 interacting proteins include the co-factor FOG1, several transcription factors (including EKLF, SP1, LMO2, Pu.1), the tumor suppressor protein RB, and the acetyltransferases CBP and P300. The interaction with each of these proteins was identified individually and the experimental procedures are laborious and overall reveal only a small amount of information. Recently a new one-step purification technique for isolation of protein complexes was developed in our laboratory. This technique consists in tagging the protein of interest with a small biotinylated tag followed by purification of the tagged protein and its interacting partners using streptavidin-coated beads. Purified proteins are then identified by mass spectrometry. Using this method several new and already known GATA1 interacting proteins were identified[28]. Interestingly, some of these proteins seem to be part of well-characterised complexes involved in transcription activation or repression. Using the same system it will be interesting to isolate complexes with, for example, GATA mutants for particular post-translational modifications. Comparing complexes identified with wild type and mutated GATA proteins may reveal specific functions of a particular posttranscriptional modification in the context of a GATA factor in specific cell lineages.

Assuming that GATA proteins show different post-translational modifications and/or can interact with distinct protein complexes in different progenitor cells it would be of considerable

interest to identify such modifications/complexes throughout differentiation. For example, in Chapter 4 we show that GATA1 becomes phosphorylated[12] hours after the induction of terminal erythroid differentiation suggesting an alteration in its function. Functional analysis of transgenic mice models where GATA transcription factors are unable to be post-translationally modified by, for example, phosphorylation, acetylation or SUMOylation, may prove to be a very efficient method of unraveling the specific functions of such modifications. Furthermore, the simple purification system mention above can be employed not only in cell lines, but also in transgenic mice[11].

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Summary

The hematopoietic system is composed of a variety of cells, whose activity is essential for the normal functioning of an organism. Erythrocytes, or red blood cells, transport oxygen and carbon dioxide throughout the body, platelets are essential for coagulation and white blood cells (lymphocytes, granulocytes and macrophages) are responsible for the protection of the organism against pathogens. All these different cells originate from a single cell type, the hematopoietic stem cell (HSC), through a process denominated hematopoiesis.

To understand how the HSC can originate so many different cell type has been the aim of many scientists over the years. Advances in molecular biology tools allowed the gathering of vast amounts of information about the hematopoietic system and the process of hematopoiesis. However, many questions remain without answers.

The HSC gives rise to the different hematopoetic cell lineages via a series of steps. HSCs are rare cells that have the capacity to duplicate themselves (self-renewal) as well as to give rise to all the different hematopoietic cell types (pluripotency). The descendants of the HSC are still able to give rise to all hematopoietic lineages but they lose the ability to self-renew. These cells will further differentiate into other cells that can give rise to an increasingly restricted number of hematopoietic lineages until they reach a stage were they can only differentiate into a single lineage. Such process is called lineage-commitment and its accuracy is essential for the normal function of the hematopoietic system.

How this lineage commitment occurs is as yet not clear. It is known that it is dependent on environmental cues as well as, at least partially, on stochastic events. The identity of each cell is dependent on their particular gene expression profile. Each cell expresses certain genes that are responsible for its specific characteristics. Transcription factors, proteins able to bind DNA and regulate the transcription of particular genes, are essential for the production of such expression profiles. There are two broad types of transcription factor: general transcription factors, which are present in every cell, and lineage-specific transcription factors, present only in particular cell lineages. Lineage-specific transcription factors are the main proteins responsible for the expression of genes in specific cell lineages, and are therefore responsible for their unique gene expression profiles. A variety of transcription factors has been identified which are crucial for the different lineage-commitment steps during hematopoiesis.

To generate the appropriate gene expression profiles, the function of these transcription factors must be tightly regulated. Expression of particular genes in the inappropriate cell lineage or at the wrong time may have severe consequences. Transcription factors functions, like any other protein, can be regulated at transcriptional level, controlling the expression of the protein, or after the protein is produced by modulating its activity.

This thesis is focused on the importance of the transcription regulation of the GATA hematopoietic transcription factors. Three of the six members of the GATA family of transcription factors are expressed in the hematopoietic system. GATA1 is expressed in the erythroid, megakaryocytic, eosinophil and mast cell lineages. GATA2 in expressed in early multilineage precursors and in the erythroid and mast cell lineages while GATA3 is expressed exclusively in T-lymphocytes.

In the studies presented in this thesis transgenic mice, were used where the expression of these genes is altered, to analyse the importance of the correct spatio-temporal regulation of these genes for the differentiation of the hematopoietic lineages were they are expressed. In

Chapter 2 we show that overexpression of GATA1 in erythroid cells impairs terminal erythroid differentiation leading to anemia, resulting in embryonic death. Detailed analysis of the gene expression profile of GATA1-overexpressing erythroid cells, presented in Chapter 3, reveals that GATA1 overexpression interferes with important physiological processes. To demonstrate the importance of timing of GATA factor expression during erythroid development, we used the rescue of a GATA1 *null* mutation by GATA1, GATA2 and GATA3 transgenes under the control of different promoters in Chapter 4. GATA1, GATA2 and GATA3 expressed under the control of GATA1 regulatory sequences, therefore mimicking the endogenous GATA1 expression, are able to rescue the GATA1 *null* phenotype. However, when placed under the control of β-globin regulatory sequences, resulting in expression at high levels in late stages of erythroid differentiation, the GATA transgenes fail to rescue the GATA1 *null* phenotype. Chapters 5 and 6 show the consequences of GATA3 overexpression throughout T-cell differentiation. GATA3 overexpression in transgenic mice inhibits the maturation of specific subsets of T-cells, and induces thymic lymphomas.

Taken together, the studies presented in this thesis demonstrate that hematopoietic GATA transcripton factors are dynamically regulated during hematopoiesis. They also emphasize the importance of a correct spatio-temporal expression of these GATA transcription factors for their function in the different hematopoietic lineages.

Samenvatting

Bloed bestaat uit een aantal verschillende celtypes, die nodig zijn voor het optimale functioneren van een organisme. Erytrocyten of rode bloedcellen, transporteren zuurstof en kooldioxide door het lichaam; bloedplaatjes zijn essentieel voor de bloedstolling, en de witte bloedcellen (lymfocyten, granulocyten en macrofagen) zijn verantwoordelijk voor de bescherming van het organisme tegen ziektekiemen. Al deze verschillende celtypen komen voort uit dezelfde voorlopercel, de hematopoietische stamcel (HSC), in een proces dat hematopoiese genoemd wordt.

Vele wetenschappers hebben de afgelopen jaren experimenten gedaan om te leren hoe de HSC in staat is om al die verschillende cellen te produceren. De ontwikkelingen in de moleculaire biologie hebben het mogelijk gemaakt om enorme hoeveelheden informatie te verkrijgen over het hematopoietische systeem en het proces van hematopoiese. Toch blijven er nog vele vragen over voor verder onderzoek.

De HSC produceert de verschillende hematopoietische celtypen in een aantal stappen. De HSC is een weinig voorkomende cel, die zichzelf kan vermenigvuldigen (zelf-vernieuwing) maar ook alle celtypen in het hematopoietische systeem kan maken. De HSC is een "alleskunner", een eigenschap die "pluripotentie" wordt genoemd. Het vermogen om zelfvernieuwing te ondergaan is de eerste eigenschap die verloren gaat als de HSC hematopoietische cellen gaat produceren. Deze eerste afstammelingen kunnen nog wel alle celtypen in het bloed maken. Na verdere celdelingen zullen deze cellen zich in toenemende mate specialiseren, om uiteindelijk een stadium te bereiken waarin ze nog maar één celtype kunnen maken. Deze toewijding aan één bepaald celtype wordt "lineage commitment" genoemd.

Hoe lineage commitment in z'n werk gaat is nog niet duidelijk. Het is bekend dat het afhankelijk is van omgevingsfactoren, en ook op z'n minst gedeeltelijk van toeval. De identiteit van iedere cel wordt bepaald door de genen die actief zijn, het genexpressieprofiel. Transcriptiefactoren, eiwitten die aan DNA binden en de expressie van genen beïnvloeden, spelen een sleutelrol in de productie van genexpressieprofielen. Er zijn grofweg twee verschillende types transcriptiefactoren: algemene transcriptiefactoren die in elke cel aanwezig zijn en celtype-specifieke transcriptiefactoren die alleen in bepaalde celtypes voorkomen. Celtype-specifieke transcriptiefactoren zijn met name verantwoordelijk voor het produceren van de genexpressieprofielen die karakteristiek zijn voor een bepaald soort cel. Er is een groot aantal transcriptiefactoren bekend die cruciaal zijn voor het nemen van lineage commitment stappen in het hematopoietische systeem.

De activiteit van dergelijke transcriptiefactoren is onderworpen aan diverse controle mechanismen. Dit is nodig omdat de expressie van bepaalde genen in verkeerde celtypes drastische gevolgen kan hebben. Het falen van deze controle mechanismen kan bijvoorbeeld leiden tot het ontstaan van kanker. De activiteit van transcriptiefactoren kan gecontroleerd worden door de hoeveelheid ervan te regelen op expressie-niveau en door het moduleren van de factoren.In dit proefschrift heb ik mij geconcentreerd op transcriptiefactoren die tot de GATA factor familie behoren. Drie van de zes GATA factor familieleden komen tot expressie in het hematopoietische systeem. GATA1 komt tot expressie in de erytroïde cellen (rode bloedcellen), megakaryocyten (bloedplaatjes), eosinofielen en mest cellen (types witte bloedcellen). GATA2 komt voor in vroege voorlopercellen, mogelijk zelfs in de HSC, erytroïde en mest cellen. GATA3 komt uitsluitend voor in T-lymphocyten. De factoren hebben heel belangrijke functies

in de cellen waar ze tot expressie komen.

Voor de studies beschreven in dit proefschrift heb ik gebruik gemaakt van transgene muizen waarin de expressie van deze transcriptiefactoren veranderd is, zodat het belang van het juiste expressiepatroon in een bepaald celtype en gedurende de ontwikkeling van de cellen bestudeerd kan worden. In Hoofdstuk 2 beschrijf ik dat overexpressie van GATA1 in erytroïde cellen de laatste stappen in de ontwikkeling van deze cellen blokkeert, wat resulteert in een dodelijke bloedarmoede. De gedetailleerde analyse van de genexpressieprofielen in deze cellen, beschreven in Hoofdstuk 3, onthult dat overexpressie van GATA1 leidt tot de verstoring van belangrijke fysiologische processen in de cel. Om het belang van de juiste hoeveelheid GATA factor op de juiste tijd en plaats gedurende de ontwikkeling van rode bloedcellen aan te tonen, heb ik onderzocht in hoeverre GATA1, GATA2 en GATA3 transgenen de afwezigheid van GATA1 kunnen compenseren. (Hoofdstuk 4). Hierbij heb ik gebruik gemaakt van transgenen die gecontroleerd worden door GATA1 regulerende elementen, waardoor het *bona fide* expressiepatroon van GATA1 wordt gereproduceerd. Met dit construct kan de afwezigheid van GATA1 door zowel GATA1, GATA2 als GATA3 vrijwel geheel gecompenseerd worden. Daarnaast heb ik transgenen gebruikt met β-globine regulerende elementen. Deze transgenen komen later tijdens de erytroïde ontwikkeling hoog tot expressie. Hiermee kan de afwezigheid van GATA1 niet gecompenseerd worden. In Hoofdstuk 5 en 6 beschrijf ik de consequenties van GATA3 overexpressie in de ontwikkeling van T-lymfocyten. Dit resulteert in remming van de rijping van specifieke types T-lymfocyten, en induceert lymfomen in de zwezerik, het orgaan waar T-lymfocyten geproduceerd worden.

Het werk beschreven in dit proefschrift demonstreert dat de activiteit van de hematopoietische GATA transcriptiefactoren zeer dynamisch gereguleerd wordt tijdens de hematopoiese. De resultaten benadrukken dat expressie op de juiste tijd en plaats van het grootste belang zijn voor het correct uitvoeren van de functies van deze GATA transcriptiefactoren in de verschillende hematopoietische celtypes.

Sumário

O sistema hematopoiético é composto por diferentes tipos de células cujas funções são cruciais para o funcionamento normal do organismo. Os eritrócitos, ou glóbulos vermelhos, transportam oxigénio e dióxido de carbono atravs do organismo, enquanto que as plaquetas são responsáveis pela coagulação do sangue e os glóbulos brancos (leucócitos) são responsáveis pela protecção do organismo contra patogenios. Surpreendentemente, todas estas células são originadas a partir de um único tipo de célula, a célula estaminal hematopoiética (CEH), por um processo denomimado hematopoiese.

Desde há muito que cientistas tentam compreender como é que uma única célula pode dar origem a tão grande variedade de células. Recentemente, avanços tecnológicos ao nível da biologia molecular tem permitido a recolha de uma grande quantidade de dados acerca do funcionamento do sistema hematopoiético e da hematopoiese. No entanto, muitas perguntas continuam sem resposta.

As CEH dão origem às diferentes células hematopoiéticas através de um processo hierárquico que envolve uma sucessão de passos bem definidos. CEHs são células extremamente raras, caracterizadas pela capacidade de duplicação (autoregeneração) e de diferenciação em todas as linhagens hematopoiéticas (pluripotência). As células directamente descendentes da CEH mantêm a pluripotência mas perdem a abilidade de autorenovação. Durante o processo de diferenciação estas células perdem progressivamente as suas características de pluripotência, ficando cada vez mais restritas a uma única linhagem celular. A precisão com que este processo decorre tem consequências drásticas no funcionamento normal do sistema hematopoiético.

O processo gradual de diferenciação da CEH em todas as linhagens hematopoiéticas tem como base alterações ao nível do perfil de expressão génica, definido como o conjunto de genes expressos em cada célula. A expressão destes genes é regulada por proteínas, denominadas factores de transcrição, capazes de interagir com o DNA e activar ou reprimir a expressão de certos genes. Cada célula produz um conjunto específico de factores de transcrição. Alguns destes factores estão presentes em todos os tipos celulares enquanto que outros são expressos apenas em certas linhagens celular. Em conjunto estes factores são responsáveis pelo perfile de expressão génica de cada célula. Até ao momento, foram identificados vários factores de transcrição cujas funções são cruciais para o processo da hematopoiese.

Os factores de transcrição regulam a expressão génica mas, por sua vez, tem também de ser regulados. Tal como qualquer proteína, o seu funcionamento pode ser regulado de diversas formas. Esta tese foca-se na regulação destes factores ao nível da transcrição. Três dos seis factores de transcrição que fazem parte da família GATA, são expressos em células do sistema hematopoiético. GATA1 é expresso em eritrócitos, megacariócitos, eosinófilos e mastócitos. GATA2 é expresso em percursores hematopoiéticos enquanto que GATA3 é expresso apenas em linfócitos T.

Nos estudos apresentados nesta tese foram utilizados ratos transgénicos, onde a expressão destes factores foi geneticamente alterada, de forma a analisar a importância da regulação espaço-temporal destes factores de transcrição na diferenciaçãoo de várias linhagens hematopoiéticas. Nos capítulos 2 e 3 demonstra-se que a sobrexpressão de GATA1 provoca uma interrupção na differenciação de eritrócitos e estes animais morrem durante a gestação devido a anemia. Pelos resultados apresentados no capítulo 4 conclui-se que a correcta espressão espaço-temporal é mais importante do que o factor de transcrição expresso (GATA1, GATA2 ou GATA3). Finalmente nos capítulos 5 e 6 demonstra-se que a sobrexpressão de GATA3 em

linfócitos T também tem consequências na maturação destas células e causa o desenvolvimento de linfomas.

Em conjunto, os resultados apresentados nesta tese demonstram que a expressão espaço-temporal dos factores de transcrição hematopoiéticos pertencentes a família GATA é essencial para a sua função em várias linhagens hematopoiéticas.

List of abbreviations

AGM	Aorta-Gonad-mesonephros
APC	Antigen presenting cell
BFU-e	Burst forming unit erythroid
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CFU-e	Colony forming unit erythroid
CLP	Common lymphoid precursor
CMP	Common myeloid precursor
CMLP	common myeloid lymphoid progenitor
Dex	Dexamethasone
DN	Double negative ($CD4^-CD8^-$)
DNA	Deoxyribonucleic acid
DP	Double positive ($CD4^+CD8^+$)
E	Embryonic day
Epo	Erythropoietin
ES cells	Embryonic stem cells
FACS	Fluorescence-activated cell sorter
GMP	Granulocyte/macrophage precursor
HRD	Hematopoietic regulatory domain
HS	Hipersensitive site
HSC	Hematopoietic stem cell
IL	Interleukin
kb	Kilo base
kD	Kilo Dalton
KO	Knockout
LCR	Locus control region
MEL	Mouse erythroleukemia
MBP	Myeloid/B-cell precursor
MEP	Myeloid/erythroid precursor
MLP	Multilineage precursor
MTP	Myeloid/T-cell precursor
mRNA	Messenger ribonucleic acid
PCR	Polymerase chain reaction
SCF	Stem cell factor (also called Kit ligand or steel factor)
SP	Single positive ($CD4^+ CD8^-$ or $CD4^-CD8^+$)
TCR	T-cell receptor
TER119	Erythroid cell marker
TFRC	Transferrin receptor or CD71
Th1	T-helper cell type 1
Th2	T-helper cell type 2

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Acknowledgments

First of all I would like to thank Frank for giving me the opportunity to work in his laboratory and fulfil my dream of being a researcher in the field of molecular biology. I am also grateful for all your advice and suggestions.

Sjaak, thank you for allowing me to work in your group and for everything you taught me. It has been a great experience to work with you. Thank you for being always so optimistic (seeing bands where no one else could see them) in contrast to me who is always pessimistic about everything. Thank you for the great effort that you put in helping me with writing this thesis and by always believing that I could finish on time.

I also would like to thank the present and past members of lab 1075/667 and lab 1079 for just being there and making my stay in the lab so enjoyable.

I have to specially acknowledge Nynke without whom the lab would not be the same. Thank you not only for teaching and helping me over the years but also for being always so happy and energetic. I enjoyed very much our, sometimes long, conversations.

Dave, despite you not being around anymore, I still want to thank you for everything that you taught me and for all our discussions, about science but also about everything else. Especially, thank you for rescuing me on my first day in the lab, when I didn't know what to do and you came and showed me how to genotype mice.

Robbert and Albert, I also would like to thank for their help and suggestions.

I also have to acknowledge several people that helped me in the completion of this thesis.

Marieke, thank you for your help with not only the erythroblast cultures but also for always being willing to help me with everything that I asked you for and more.

Rudi, thank you for all the help with different aspects of this thesis, for helping me to understand the immune system and the development of T-cells and especially for reading and correcting the introduction.

George Garinis and Michael Moorehouse, I appreciate all your efforts in the analysis of the microarray data. Thank you for taking time from your own work to help me.

I must also acknowledge Prof. Yamamoto for inviting me to visit his lab and everyone there for making me feel so welcome. Thank you Kinuko and Ritsuko for sending me all the mice, antibodies, data, etc and Osamu for teaching me to differentiate ES cells.

The members of the inner doctoral committee, Dies, Nils and Ruud, thank you for reading my thesis so fast and for all your comments and remarks that improved it so much.

Ton de Wit and John Kong-a-san, thank you for all the help with the mice. I really appreciate all your help trying to teach me to do microinjections. I was really bad at it but you never gave up trying to teach me. John, thank you for all the microinjections that you did for me.

I must not forget to thank all the personal from the EDC, in particular Patrick, Danielle and Vincent, for all the help. Thank you all.

I also must thank Marike, Jasperina and the "computer guys" for all your prompt help. Marike, I have to thank you also for your assistance on my arrival in Rotterdam. I don't know what I would have done without your help to sort out all the paper work I needed to stay here.

Now in a more informal way, I would like to thank all the people with whom I had fun all these years. I will not mention names because of the risk of forgetting someone. You know who you are. Thank you. Special thanks to the members of Elaine's lab for letting me be almost a member of your lab and for always inviting me to go for lunch with you.

Sandra, obrigada pela tua companhia e por seres uma verdadeira amiga, tão longe de casa, da familia e dos amigos.

Mãe, Pai, Luis, Avó, Avô e Madrinha, obrigada pelo vosso apoio.

Dominik, thanks for everything. It would have been impossible to live away from home all these years without you.

Finally, thank you Roy and Mehrnaz for allowing me to share such an important event with you. It has been great to be able to share the good and bad moments with you over the last few months. It has been a lot of work but we still had fun together. Good luck in the future.