The devil is in the level

The role of the transcription factor GATA-1 in erythropoiesis

Het venijn zit in de hoeveelheid

De rol van de transcriptiefactor GATA-1 in erythropoiese

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Promotor(en):	Prof. dr. F.G. Grosveld
Overige leden:	Prof. dr. E.A. Dzierzak
	Prof. dr. I.P. Touw
	Prof. dr. J.A. Grootegoed
Copromotor(en):	Dr. J.N.J. Philipsen

Cover: Karola van Rooyen

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Voor Fanny, Levi & Okkie2.

Contents

Abbreviations

A	Adenine
7AAD	7-aminoactinomysin-D
AGM	aorta-gonad-mesonephros
ATP	Adenosine triphosphate
BFU-E	burst forming unit erythroid
BM	bone marrow
bp	base pair(s)
BRE	TFII-B recognition element
C	Cytosine
Cdk	cyclin dependent kinase
cDNA	complementary deoxyribonucleic acid
CBP	CREB binding protein
Cdk	cyclin dependent kinase
CFU-E	colony forming unit erythroid
CFU-GEMM	colony forming unit granulocyte erythrocyte macrophage megakaryocyte
CFU-S	colony forming unit spleen
CREB	Cyclic AMP responsive element binding protein
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dpc	days past coitus
DPE	downstream promoter element
EKLF	erythroid krüppel like factor
Epo	erythropoietin
ER	estrogen receptor
ES cells	embryonic stem cells
FasL	Fas ligand
FIDL	Fanny is de leukste
FCS	fetal calf serum
G	Guanine
GFP	green fluorescent protein
HAT	histone acetyl transferase
HbS	sickle cell hemoglobin
HDAC	histone deacetylase
HPFH	Hereditary Persistence of Foetal hemoglobin
HS	hypersensitive site
HSC	hematopoietic stem cell
hsp	heat shock protein
IL	interleukin
IRES	internal ribosomal entry site
IVS	intervening sequence, intron
kb	kilo base
kDa	kilo Dalton
KL	Kit Ligand
LBD	ligand binding domain
LCR	locus control region
LIF	leukemia inhibitor factor
lox	locus of recombination (x)
MEL	mouse erythroleukemia
ml	milliliter
αMRE	major regulatory element (of the mouse alpha globin locus)
mRNA	messenger ribonucleic acid
MTG	monothioglycerol
NLS	nuclear localisation signal
nm	nanometer (10^{-9} meter)

NO	nitric oxide
nt	nucleotide
4-OH-T	4-hydroxy tamoxifen
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PIC	Pre initiation complex
Rb	retinoblastoma
RNA	ribonucleic acid
rpm	rotations per minute
SCF	stem cell factor (= Kit Ligand or steel factor)
SCL	stem cell leukemia gene
SNO	S-nitrosothiol
Sp1	specific protein 1
T	Thymidine
TAF	TBP associated factor
tal-1	T-cell acute leukemia (=SCL)
TBP	TATA box binding protein
UTR	untranslated region
ZP3	Zona Pelusida 3
µl	microliter (10^{-6} liter)

Scope of the thesis.

This thesis describes the experiments done to understand the role of the transcription factor GATA-1 in erythropoiesis and Sertoli cells.

In **Chapter 1** hematopoiesis, erythropoiesis and the GATA transcription factor family are introduced. Hematopoiesis is the process of the formation of blood cells from hematopoietic stem cells via differentiated progenitors. Erythopoiesis is the process of the formation of the red blood cells, that if disturbed leads to anaemia. The transcription factor GATA-1 is essential for erythropoiesis beyond the proerythroblast stage. Transcription factors are proteins that bind DNA and are required for regulation of gene transcription. GATA-1 is thought to be important for the expression of all known erythroid-specific genes. However, these target genes are not completely silenced and are sometimes expressed at considerable levels in GATA-1 deficient mice. The precise role of the protein is however still elusive.

In the second part of the introduction the GATA factor family is introduced. Most GATA factors are co-expressed with family members for at least some time during a wide variety of differentiation processes. Some clues to the function of the GATA-1 protein might therefore be obtained from studies of other family members.

In erythroid progenitors GATA-1 and GATA-2 proteins are expressed. It has been suggested that a relative high level of the GATA-1 protein, compared to GATA-2 protein, would instruct erythroid progenitors to terminally differentiate. To test this hypothesis we did experiments in which we overexpressed GATA-1 in these erythroid progenitors in mice. The results of these experiments are described in **Chapter 2** and reveal that although GATA-1 is essential, it should be downregulated during terminal differentiation. Cells overexpressing the GATA-1 protein are blocked late in differentiation. This defect is overcome when overexpressing cells are in a mixed environment with wild type cells in vivo. The wild type cells appear to provide a signal to the GATA-1 overexpressing cells instructing them to differentiate. The nature of this signal, which we called red cell differentiation signal or REDS, is still unknown and the search for this signal is ongoing.

In **Chapter 3** the generation of conditional GATA-1 knockout mice, using the Cre-loxP system, is described. These mice were made to reveal more of GATA-1's secrets. These mice would make it possible to study the GATA-1 knockout after the embryonic stage.

In **Chapter 4** the conditional knockout was used to make a tissue-specific knockout of GATA-1 in the testis, the only site of expression of GATA-1 outside the hematopoietic system. Deficiency of the GATA-1 protein in the testis did not result in any obvious phenotype. Consequently GATA-1 is not essential in the testis.

In **Chapter 5**, a new method to culture and differentiate erythroid progenitors in a quick and small scale assay is introduced. This assay has several practical advantages over other culture methods, thus adding a useful tool to the arsenal of the molecular hematologist.

Chapter 6 is a general discussion of the work described in this thesis, and suggests several future directions that could be taken to follow-up on the present studies of GATA-1 function.

Chapter 1

Introduction: hematopoiesis, erythropoiesis and the transcription factor GATA-1

Hematopoiesis

Hematopoiesis is the process of formation of blood cells. There are at least eight types of blood cells that are very different in their appearance and function. In a simplified categorization they can be divided into white and red blood cells and platelets. The white blood cells are the lymphocytes, granulocytes (leukocytes, eosinophils, neutrophils) monocytes/macrophages and mast cells. They are the soldiers of the immune system and protect us against bacteria, viruses and other micro organisms. The platelets are small cell fragments that are required for hemostasis. The red blood cells or erythrocytes are small biconcave cells that transport oxygen and carbon dioxide through the body. They take up oxygen and release carbon dioxide in the lungs and do the reverse in the other tissues. All the different blood cells originate from a small population of rare cells, the hematopoietic stem cells (Lemischka et al. 1986).

The hematopoietic stem cells

Stem cells have the potential to self-renew and to give rise to every type of mature blood cells (Ploemacher 1997). The latter ability is called pluripotency. The process of stem cells undergoing changes to become a different type of cells is called differentiation. The accepted model is that when hematopoietic stem cells divide, a proportion of the daughter cells remains stem cells thus forming a continuous source of hematopoietic cells, and other daughter cells enter the differentiation process. During this process they divide many times so that one stem cell can be the source of a large number of mature blood cells. During differentiation the cells go through many different stages in which they change morphology and functional characteristics, gradually losing the potential to become any type of blood cell.

The idea of a pluripotent stem cell was first postulated in the beginning of the twentieth century when improvements in histological techniques enabled scientists to discriminate between various cells (Bondurant and Koury 1998). At that time it was already known that the site of blood formation in adults is the bone marrow (Neumann 1868). In this tissue there appears to be a continuous sequence of cells changing in morphology and number. It was proposed that as cells increase in number, they also change in morphology. This model has been further refined and has led to the concept of the hematopoietic tree, a schematic representation of the different routes cells can follow to become mature blood cells. Formal proof of the existence of stem cells has come from retroviral marked bone marrow transplantations in lethally irradiated animals (Lemischka et al. 1986) (Ford et al. 1956).

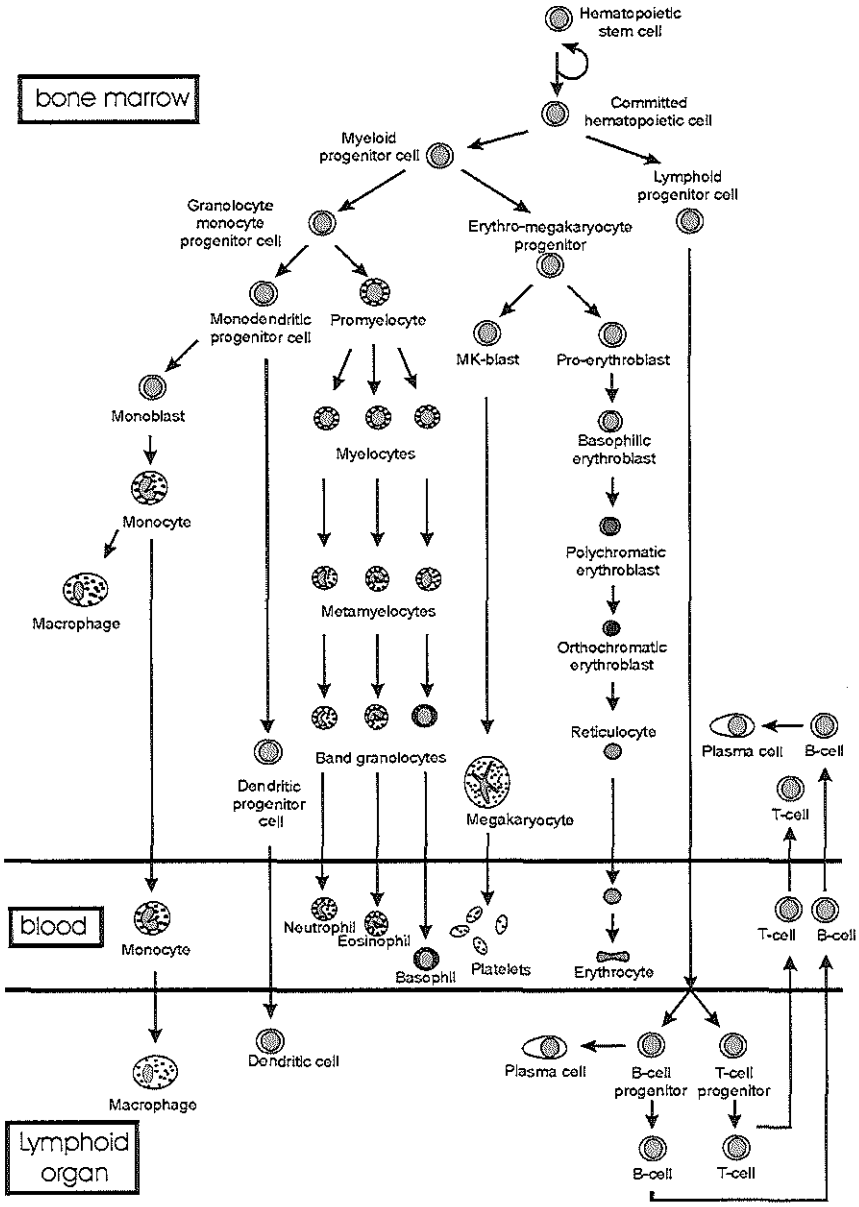


Figure 1: The hematopoietic tree. The different pathways starting from the stem cell leading to the mature blood cells are shown. Between the different stages, the cells divide so that a small number of stem cells can give rise to a large number of mature cells.
(Adapted from (Bondurant and Koury 1998))

The committed hematopoietic progenitor cells

The first 'offspring' of the stem cells that have started to differentiate, the committed hematopoietic progenitor cells, are cells that can still form different blood cell types but are not capable of long term reconstitution of hematopoiesis in lethally irradiated recipients. As cells progress in the differentiation tree the cells become more restricted to specific lineage choices. These progenitors can be monitored by culturing them in semi-solid media with the appropriate mix of growth factors (Metcalf 1989). During culture the progenitors form colonies and the mature cells from these colonies can be analysed. More immature cells give rise to several cell types in their colonies. The progenitors that give only one cell type in their colonies are called the single lineage progenitors, these are the most mature progenitors (Bondurant and Koury 1998).

Regulation of the differentiation process.

The hematopoietic system is very tightly regulated. Because it must respond to the physiological demands of the body, most blood cells need to be to replenished constantly due to cell death and turn-over of cells. Factors, such as loss of cells through bleeding, low oxygen levels or an infection, also create a demand for new hematopoietic cells. Hematopoiesis is regulated through signalling molecules, the hematopoietic growth factors (HGF)(Metcalf 1989; Lotem and Sachs 2002). These signalling molecules can come from blood cells themselves, but also from different tissues.

Most of these signals like for instance erythropoietin (Epo) or granulocyte colony stimulating factor (G-CSF) are bound by receptors on the cell membrane by which a signal is transmitted a to the inside of the cell (Mulcahy 2001). This signal is usually passed on via a cascade of phosphorylations / dephosphorylations of other proteins like the Janus kinase family of tyrosine kinases (JAK) and the STAT family proteins whose activity depends on their phosphorylation status. This results in regulation of gene expression, survival, or apoptotic pathways that may stimulate or inhibit a cell to commit to a specific lineage (Kirito et al. 1997; Bittorf et al. 2000; de Koning et al. 2000).

A very basic question is: how does a stem cells commits itself to become a progenitor to a specific cell type? For this enigma two models have been proposed, the deterministic and the stochastic model. The deterministic model says the commitment is a predetermined fate. An example of proof given for this model are the repopulation experiment with daughter HCS cells of clonally derived hematopoietic stem cells, showing similar characteristics (Muller-Sieburg et al. 2002). In the stochastic model, gene expression and therefore commitment occurs in a probabilistic manner. The probability of the gene expression can

however be influenced by factors like transcription factor concentration and cis-regulatory elements (Ko 1991). Examples of stochastic gene expression are X-inactivation and globin gene expression (Panning and Jaenisch 1998; de Krom et al. 2002). In the case of globin expression in fetal liver cells, it is also shown that although the expression is stochastic, the expression pattern can be inherited in daughter cells (de Krom et al. 2002). This may be also the case with genes effecting the characteristics of the HSC in the example of the deterministic model (Muller-Sieburg et al. 2002).

Stem cell plasticity

The dogma that bone marrow stem cells can only give rise to hematopoietic cells and not to other tissues has recently been challenged (Nolta and Jordan 2001). In transplantation experiments bone marrow stem cells have been shown to possess tissue plasticity as they contributed to neuronal, muscle and liver tissue (Gussoni et al. 1999; Petersen et al. 1999; Brazelton et al. 2000; Mezey et al. 2000; Theise et al. 2000). Vice versa neuronal and muscle stem cells have given rise to hematopoietic cells (Bjornson et al. 1999; Jackson et al. 2001).

There are three theories to explain these phenomena (Nolta and Jordan 2001). First, the 'two stem cell' theory that explains these results by the presence of neuronal, liver or muscle stem cells in the bone marrow, that are isolated together with the hematopoietic stem cells. Second, the trans-differentiation theory, that says that a stem cell restricted to one fate can differentiate to a stem cells of a different tissue. Third, the totipotent stem cell theory, in which a uncommitted stem cell can generate different tissues. More research is needed to determine which theory is correct. For example with genotypical marking of stem cells by retroviral insertion can discriminate between the 'two stem cell' theory and the other theories.

As a cautionary note, recent experiments suggest that, rather than stem cell plasticity, cell fusion could be the basis of the above observations.(Terada et al. 2002; Wurmser and Gage 2002) (Ying et al. 2002) Rigorous karyotyping of the grafted cells will be necessary to resolve this issue.

De-differentiation

Besides stem cells plasticity there are reports of committed progenitor cells that are able to 'de-differentiate'. The report where pax-5 -/- pre-B-cells can be re-directed to erythroid, myeloid, and lymphoid cell lineages suggest that cells can go up in the hematopoietic hierarchy (Fig.1) (Schaniel et al. 2002). Another step further is a report on the reprogramming of committed mouse myotubes by ectopic expression of the transcriptional repressor msx1, reverting them to cells with a de-differentiated phenotype. These cells could be induced to

differentiate into cells expressing chondrogenic, adipogenic, myogenic, and osteogenic markers(Odelberg et al. 2000). If de-differentiating is also possible for other committed cell types, this could have important clinical consequences. Moreover, it changes the currently accepted dogma that cell-commitment is an irreversible process (Lemischka 2001) (Cantor and Orkin 2001) (Domen and Weissman 1999).

Hematopoiesis during development

During development the site of hematopoiesis changes several times. The first site are the blood islands of the yolk sac during early embryonic development, 7-11 days postcoitum (dpc) in the mouse (Dzierzak et al. 1998). This first wave of blood formation is referred to as primitive hematopoiesis and mainly consist of production of red blood cells. Around 11 dpc the main site of hematopoiesis changes to the fetal liver and to a lesser extent the fetal spleen. From now on it is referred to as definitive erythropoiesis. Definitive fetal liver erythrocytes expel their nucleus and express the fetal globin genes in humans.

The origin of the definitive or adult hematopoiesis is still under debate as there are two main models. One model claims that definitive hematopoiesis originates from cells that have migrated from the blood islands in the yolk sac to the Aorta-Gonads-Mesonephros region (AGM) and the fetal liver (Palis and Yoder 2001). A second model says that at least two independent hematopoietic sites generate blood cells de novo during development: the yolk sac, which produces the transient embryonic hematopoietic system, and AGM region as this is the first site from which definitive hematopoietic stem cells can be isolated (Fig. 2) (Dzierzak 1999). Although the first model seems likely since hematopoietic activity appears first in the yolk sac, experiments in avian and amphibian species have clearly shown that this wave of primitive extraembryonic erythropoiesis is transient and that definitive hematopoiesis is almost entirely from intraembryonic origin (Dieterlen-Lievre 1975; Beaupain et al. 1979; Dieterlen-Lievre and Martin 1981; Turpen et al. 1981). Furthermore the first site to find multipotential hematopoietic progenitors is the intraembryonic splanchnopleura (7.5 dpc) and the first HSCs are detected in the AGM (10 dpc) (Godin et al. 1993) (Muller et al. 1994). Therefore, although ultimate proof is still lacking, the second model is the most probable.

Around birth the site of hematopoiesis changes to the bone marrow and the spleen in mouse.

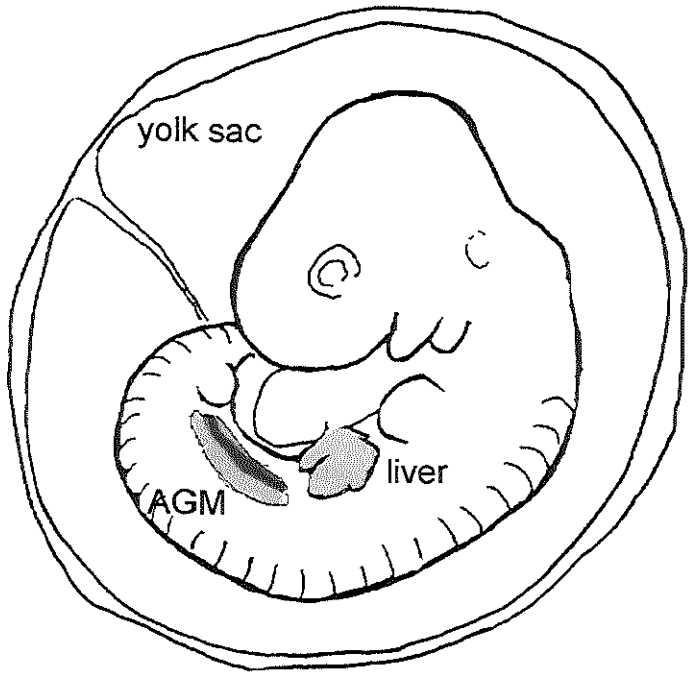


Figure 2: The site of erythropoiesis during embryonic development. Yolk sac, AGM and fetal liver. In adults the major site of hematopoiesis is the bone marrow.
(Taken from (Dzierzak and Medvinsky 1995))

Erythropoiesis: the formation of erythrocytes

The erythrocyte

The definitive erythrocyte is a small cell of about 7-8 μm in diameter with the characteristic shape of a flat ball indented from two opposing sites also described as biconcave disk (Figure 3) (Evans and Fung 1972). Erythrocytes transport oxygen (O2) from the lungs through the body and exchange it for carbon dioxide (CO_2) which is transported back to the lungs, where it is exchanged for oxygen again. Since this is the major function of erythrocytes they are completely specialized for this purpose. At the final stage of maturation the erythrocyte sheds its nucleus, mitochondria and ribosomes.

The shape enables the erythrocyte to efficiently exchange O_2 and CO_2, because it has the maximum surface area for the volume of cytoplasm (Lenard 1974). Furthermore, this shape is more able to deform than a sphere, a property essential for an erythrocyte as it has to

move through the smallest capillaries to reach the remote regions of tissues (Schmid-Schonbein 1975). The resilience of the erythrocytes to the stress of circulation is due to the structural organization of the membrane, the membrane proteins and the cytoskeleton. The reader is referred to review articles for further information on the erythroid membrane (Disscher&Carl 2001 and Takakuwa Y, 2000).

The O_2/CO_2 transporter protein hemoglobin predominates the cytoplasmic proteins of a erythrocytes.

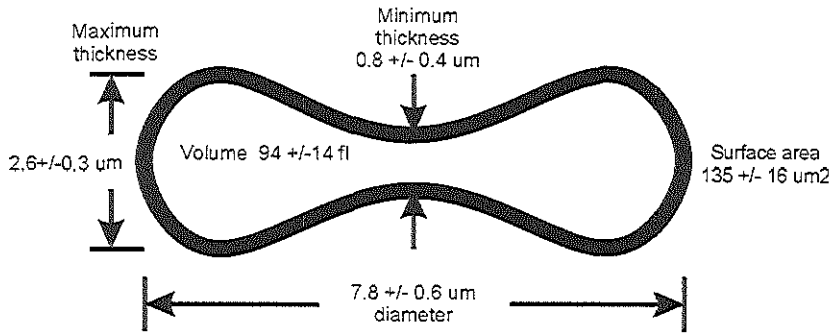


Figure 3: Cross section of a definitive erythrocyte in isotonic solution (Taken from from (Evans and Fung 1972))

Hemoglobin

The hemoglobin protein binds a prosthetic haem group that is essential for the binding of oxygen. The heam group is a protoporphyrin IX ring with a iron ion (Fe^{2+}) bound in the middle (Perutz et al. 1960). Hemoglobin protein functions as a tetramer consisting of two β-like and two α-like globin proteins. In mice there are four β-like chains (εy, βH1, β_{major} and β_{minor}) and three α chains (ζ, α_1 and α_2) expressed. In human there are five β chains (ε, Gγ, Aγ, δ and β) and three α chains (ζ, α_2 and α_1) expressed (Fraser et al. 1998).

During development different isoforms are expressed and different combinations can be formed (figure 4B and 4C). The embryonic and fetal hemoglobins have a higher affinity for oxygen and are therefore capable of taking the oxygen from the mother's adult hemoglobins. After birth only the adult globins are expressed.

The α- and β-globin genes are expressed from two distinct genetic loci on chromosome 16 and 11 respectively . Although the genes are very homologous, the loci differ

in the way they are regulated . The order of the genes in the genetic loci resembles the order in which they are expressed during development (Fig 4A) (Fraser et al. 1998). The major regulatory element of α-globin expression is the αMRE (in humans hypersensitive site-40, HS-40), an element that can give high expression in a transgene but not in a copy number dependent fashion as has been observed with the major regulatory element of the β-globin locus, the β-globin locus control region (Higgs et al. 1998; Grosveld 1999).

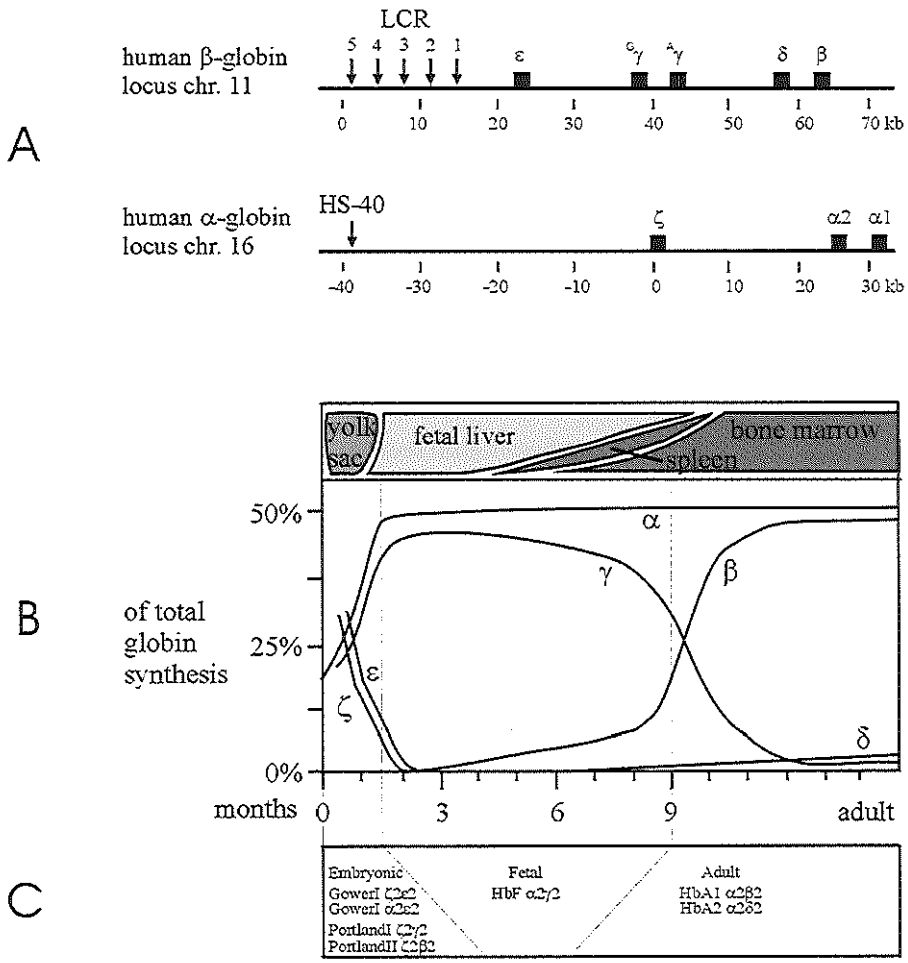


Figure 4: (A) schematic representations of the α-and β-loci, (B) the site of erythropoiesis and the expression of the individual globin genes during development and (C) the embryonic, fetal adult globins can form different hetero-tetramers (all in human).
(Taken from (Weatherall and Clegg 1981))

Besides oxygen, hemoglobin also transports nitric oxide (NO). Nitric oxide is produced by vascular endothelium and relaxes smooth muscle cells surrounding the vessels, thereby controlling blood flow and pressure. Hemoglobin acts as a scavenger of nitric oxide thereby inactivating the NO (Gross and Lane 1999). Furthermore, S-nitrosylation of NO to a very conserved cysteine (β-hemoglobin Cys93 in human) and release of NO as a S-nitrosothiol (SNO) in the nucleus tractus solitarius in the brainstem, is important in the regulational control of respiration in response to hypoxia (Lipton et al. 2001; Lipton 2001)

Erythropoiesis

The process of the production of erythrocytes is called erythropoiesis and occurs, in adults, almost exclusively in the bone marrow. As all blood cells, the erythrocyte is a terminally differentiated descendent of the hematopoietic stem cell. The erythroid progenitors together with the erythrocytes are called the erythron. This name contributes to the concept and understanding of blood as a tissue. (Boycott 1929). Erythropoiesis is highly controlled process because the supply of oxygen has to be sufficient at all times. Homeostasis of the erythron is necessary in response to changes in the physiological situation such as a low oxygen level atmosphere or blood loss . These changes lead to reduced tissue oxygen tension in the kidney. In reaction to this the kidney secretes the glycoprotein hormone Erythropoietin (Epo) that stimulates growth, differentiation and survival of erythroid progenitors thus increasing the number of mature erythrocytes (Koury and Bondurant 1988; Krantz 1991) (Lin et al. 1985).

The number of erythrocytes is also influenced by breakdown. The average lifespan of an erythrocyte is 60 days in mice and 120 days in human. Ageing of red blood cells is characterized by loss of enzymatic activity, a less flexible cell membrane and higher cell density. Damaged and worn-out cells are removed by the spleen and the liver. The iron is reutilised and the globin is degraded to amino acids (Dessypris 1998).

<u>The erythroid progenitors</u>

Stem cells and early erythroid progenitors can not be morphologically distinguished. The existence of stem cells and early erythroid progenitors can demonstrated by several *in vitro* and *in vivo* assays including repopulating assays in lethally irradiated mice, cobble stone area assay and colony assays in semi solid media (Heimfeld and Weissman 1992). Four progenitor cells are defined, in order from immature to more mature: the CFU-S, the CFU-GEMM, the BFU-E and the CFU-E. The CFU-S, colony forming unit spleen, is a cell that can form

colonies in the spleens of lethally irradiated mice (Medvinsky et al. 1993). The CFU-S is a multipotent cell but does not have a long-term repopulating capability (Bondurant and Koury 1998). The CFU-GEMM is a multi-lineage progenitor that can be demonstrated with an *in vitro* colony assay in semi-solid medium. The CFU-GEMM can give rise to granulocytes, erythrocytes, megakaryocytes and macrophages (Johnson and Metcalf 1977). The BFU-E, burst forming unit erythroid, represents a more committed cell that forms large colonies under the influence of Epo, interleukin-3 and stem cell factor in an *in vitro* colony assay with semi-solid medium. These colonies contain mature erythrocytes (Metcalf and Nicola 1984). A more differentiated cell is the CFU-E, colony forming unit erythroid, that forms small colonies (8-64 cells) in a short-time *in vitro* culture in semi-solid medium (3-4 days) (Wong et al. 1986). The CFU-E is very sensitive to and dependent on Epo for its survival and is more frequently found in cell cycle than the BFU-E (70-90% versus 0-25% in S-phase) (Gregory and Eaves 1978). The CFU-E is the earliest erythroid progenitor cell that increases proliferation in response to Epo (Iscove 1977). It is believed that this cell has the highest number of Epo-receptors on its cell membrane (Krantz et al. 1987).

Later erythroid progenitors can be morphologically distinguished because the cells shrink during differentiation, partly due to the reduction of the size of the nucleus (Dessypris 1998). The earliest morphologically identifiable progenitor is the proerythroblast or pronormoblast. In some reports the term normoblast is used instead of erythroblast to distinguish it from megaloblast, a pathological erythroblast. The proerythroblast (14 to 19 μm), with visible nucleoli in its nucleus, is already taking up iron for hemoglobin synthesis. The presence of the iron containing protein ferritin in pinocytotic vesicles and siderosomes is the marker to distinguish proerythroblasts from other immature cells such as myeloblasts and lymphoblasts (Dessypris 1998).

The next stage is the basophilic erythroblast. The nucleoli in the nucleus are no longer visible, hemoglobin synthesis has started and the cell has shrunk a little (12 to 17 μm). The chromatin is starting to become more condensed. The name is derived from its basic cytoplasm that contains a large amount of globin and ribosomal RNA. This gives the cell a blue appearance in standard histological staining procedures (Beug et al. 1982).

The cells further differentiate to polychromatic erythroblasts. This cell type already contains a high concentration of hemoglobin protein and has shrunk further (12 to15 μm). This cell still has large amounts of RNA in its cytoplasm but the hemoglobin (acidophilic) gives it a red/beige appearance after staining (Dessypris 1998).

A step further in the differentiation is the orthochromatic or acidophilic erythroblast. This cell is further reduced in size (8 to 12 μm) and has an eccentric, pyknotic nucleus

containing clumped and compressed DNA. After extrusion of the pyknotic nucleus the cell is called a reticulocyte. It is thought that in the bone marrow the nucleus is squeezed out as the reticulocyte transfers through the endothelial cell layer into the sinusoidal capillary space. This is however not a requirement as cells can also enucleate in culture. The expelled nucleus is phagocytosed by a macrophage. The reticulocyte is still a sphere and contains some mitochondria, ribosomes, RNA and the Golgi complex. These features disappear upon maturation, which takes around 24 hours. The size is comparable to the erythrocyte (7-8 μm). Reticulocytes mature in the bone marrow and in the peripheral blood where their number is about 1% of the number of erythrocytes (Dessypris 1998).

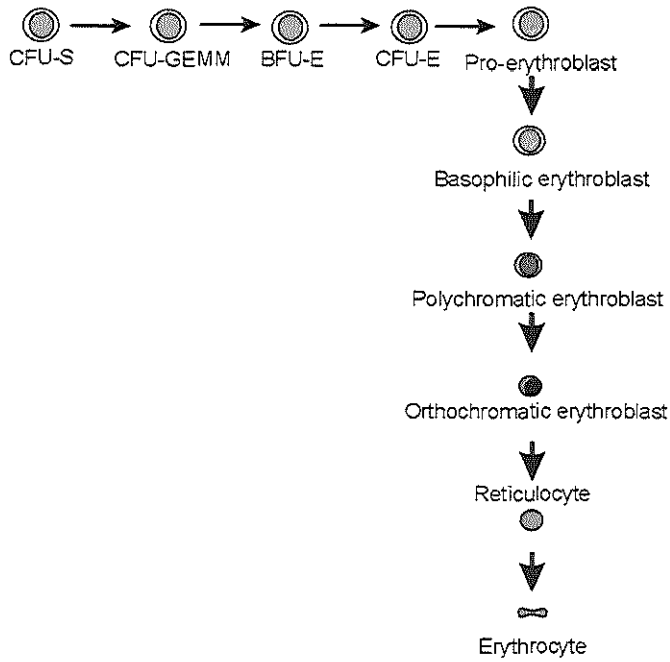


Figure 5: a schematic representation of the subsequent stages of erythroid differentiation (Adapted from (Dessypris 1998))

The erythroblastic island

The environment of maturing erythroid progenitors is the erythroblastic island. This is a sponge-like structure composed of one or two central macrophages in close contact with erythroid progenitors in different stages of differentiation lying in the invaginations of the macrophages, with immature cells close to the macrophages and mature cells to the edge of the structure

(Bessis et al. 1983). The macrophage is believed to interact with the progenitors in several ways, supplying undefined nutrients directly and via rhopheocytosis (internalisation of vacuoles of the macrophage). Besides this, the macrophage phagocytises the expelled nuclei (Allen and Dexter 1982). Although erythroid progenitor cells can mature *in vitro* without the formation of an erythroblastic island, this structure appears to be an important feature *in vivo*, as such a close association between the erythroid progenitors and the macrophages influences local concentrations and gradients of growth factors, intercellular transport and the impact of mechanical forces (Bessis et al. 1978; Mel 1991).

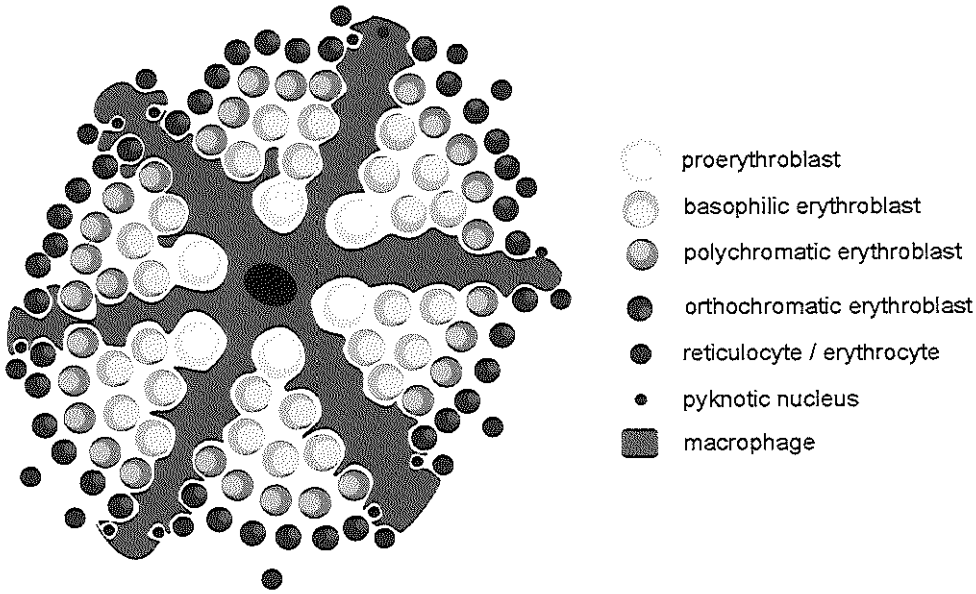


Figure 6: a simplified model of the erythroblastic island. The position of the central macrophage and the arrangement of the erythroid progenitors within the macrophage's extensions are shown. The process of pyknosis, enucleation and phagocytosis of the expelled nuclei are represented.

Disorders of red blood cells

Abnormalities associated with red blood cells can basically be caused by two problems, too many red blood cells (polycythaemia) or too few cells (anemia). The latter is a major cause of mortality worldwide (Erslev 2001).

Polycythaemia

Polycythaemia or erythrocytosis is the overproduction of erythrocytes. Polycythaemia can be divided in two diseases, polycythaemia vera and erythremia .In polycythaemia vera, not only the number of red blood cells is higher, but also that of the granulocytes and platelets. The erythroid progenitors grow without the need for Epo or are responsive to a very low amount of this hormone (Erslev 2001). In erythremia only the number of red cells is elevated. This can be caused by an inherited mutations, for example mutants of the Epo receptor leading to a abnormal response of the erythroid precursors (Kralovics et al. 1997).

Anemia

Anaemia is defined as a reduced concentration of hemoglobin or erythrocytes in the blood. Anaemia can be the result of lack of production or excessive breakdown of red blood cells, or lack of a functional protein, usually hemoglobin. Anaemia is often secondary to renal failure, liver disease, poor diet or uptake of nutrients, chronic inflammatory and malignant diseases like leukaemias and other cancers. It can also be caused by an inherited mutation (Lee 1998).

Aplastic anaemia is a lack of blood cell production caused by aplasia of the bone marrow. Examples are the hereditary Fanconi anaemias and the Josephs-Diamond-Blackfan syndrome. Fanconi anaemia is a recessive inheritable disease, that shows a reduction of all blood cells. For Faconi anemia, eight gene complementation groups have been described (d'Apolito et al. 1998). An aplastic anaemia only affecting the erythrocytes (red cell aplasia) is the Josephs-Diamond-Blackfan syndrome in which there is a great reduction or almost complete absence of nucleated erythroid precursors but plentiful granulocytes and megakaryocytes (Wang et al. 1978). The defect underlying this disease, a reduced response to Epo, is thought to be a defect intrinsic to the progenitor (Tsai et al. 1989a).

Chronic red cell aplasia is often associated with autoimmune disorders. The red cells are selectively destroyed via T-cell mediated destruction or via antibodies produced by the B-cells (Krantz and Kao 1967). Aplastic anaemia can also be caused by direct damage to the bone marrow by radiation or cytotoxic drugs (Lee 1998).

Another cause of anaemia is the production of non-functional erythrocytes. This can be due to shortage of a component or substance that is needed for proper red blood cell production.

Iron deficiency is the most common cause of anaemia, as iron is an important part of the hemoglobin proteins. Vitamin B12 or folate deficiency disturbs the maturation of erythroblasts. The maturation of the nucleus is delayed compared to the cytoplasm. The cause

of this is defective DNA synthesis. The origin of this deficiency can be lack of folate in the diet, poor absorption, transport and/or storage and loss of the nutrients.

Sideroblastic anaemia is characterised by abnormal erythroblasts with a ring of iron granules around the nucleus. It is caused by a defect in haem synthesis. It can be hereditary (mutations in genes encoding enzymes of the haem synthesis pathway) but also acquired (drugs, alcohol, lead poisoning or other marrow disorders) (Lee 1998)

Haemolytic anaemias are the anaemias that result from an increase in the rate of red cell destruction. The normal lifespan of a red cell in humans is 120 day but in this anaemia the cells sometimes survive only a few days. The cause can be hereditary. In these cases the synthesis of membranes, membrane proteins, enzymes of red cell metabolism or hemoglobin is defective in some way. Acquired causes can be an autoimmune response, direct damage to the erythrocytes or infection. An example is Paroxymal Nocturnal Hemoglobinuria (PNH) in which the erythrocytes have acquired an abnormal sensitivity for complement mediated lysis because of mutation(s) in membrane proteins (Parker and G.R. 1998).

In erythroleukemia the cells have a normal Epo response but have lost the capacity to make functional red blood cells. The bone marrow has enormous numbers of dysplastic erythroblasts (Roggli and Saleem 1982; Means Jr 1998).

Hemoglobinopathies

These disease conditions, caused by defects in the globin genes, are very common especially in the (sub)tropical areas in the world because heterozygosity for these diseases offers some protection against malaria (Luzzi 1991). Hemoglobinopathies can be separated in diseases in which there is a lack of production of hemoglobin (thalassemias) and diseases in which a mutated form of hemoglobin is produced such as sickle cell anaemia.

The thalassemias are the result of a deletion of a gene or a regulatory sequence in either the α- or the β-globin cluster. α thalassemia is often caused by deletion of one or more copies of the α genes whereas the majority of the β thalassemias are point mutations. Lack of α-globin is lethal during gestation whereas lack of β-globin can be tolerated due to the _-globin expression (Weatherall 2001).

Sickle cell anaemia is caused by a single point mutation in the β-hemoglobin gene that causes an amino acid change in the protein (Glu6→Val6). This minimal change has dramatic consequences because sickle hemoglobin (HbS) polymerises and becomes insoluble when exposed to low oxygen tension. The formed polymers deform the erythrocyte that thereby looses its deformability and can now block capillaries, causing infarction of various organs.

There are several other milder mutations called hemoglobin C, D an E that become more apparent in combination with HbS or β-thalassaemia (Beutler 2001).

A condition called Hereditary Persistence of Fetal Hemoglobin (HPFH) in which elevated levels of fetal _-globin are expressed during adulthood is often found together with β-thalassemia or sickle cell anaemia. HPFH does not have any harmful effects and fetal hemoglobin can substitute for adult β-globin. For β-thalassaemia and sickle cell anaemia a possible therapy against the effects of the mutations could be the renewed use of the fetal globin genes that are normally shut down after birth (Forget 1998). Changing the expression of the globin genes is one of the goals of current research in the hemoglobin field. Therefore it is important to know how gene expression is controlled.

Control of gene expression

Classifications of distinct cell types within an organism are most often based upon appearance and function while the genetic information in all cells of an organism is, with very few exceptions, identical. The specific properties of a cell are generally established by differential gene expression. Some genes are expressed in almost every cell; such genes are usually involved in general processes and are therefore called housekeeping genes.
Tissue specific genes are expressed in specific subset of tissues or cell types and their protein products often have a very specialized function for that tissues or cell types.

The control of expression of protein-encoding genes includes the following steps: transcription of DNA to primary RNA transcripts, processing of primary RNA transcripts to messenger RNAs (mRNAs), transport of mRNAs from the nucleus to the cytoplasm, stabilisation / degradation of mRNAs, control of mRNA translation to protein and control of protein activity through post-translational modifications and the modulation of protein stability. Regulation of transcription is the first important step in the control of gene expression.

General transcription machinery

Transcription is defined as the process of synthesis of a single-stranded RNA copy of a gene. The enzymes responsible for RNA synthesis are the RNA polymerases I, II and III, large protein complexes formed by multiple polypeptides. RNApol I and III synthesise RNAs with structural or catalytic roles such as ribosomal RNAs. The mRNAs for proteins are synthesized by RNApolII. It is estimated that about 1/3 of the genome is transcribed into primary transcripts, but only 1.5% consists of coding exons. Because only a small portion of the

chromosomal DNA is transcribed to mRNA the RNA polymerases must work very selectively.

In vivo, RNApolII only transcribes DNA after it has been tethered to a start region of a gene called the promotor. This is done by the general transcription factors that bind to specific sequences in the promotor including the TATA box, the TFIIB recognition element (BRE), the initiator (Inr) and the downstream promoter element (DPE). These general transcription factors are TF II-A, -B, -D, -E, -F and -H. The first step consists of the binding of TFII-D to a DNA element called the TATA box or to the down stream promoter element, DPE (Burke and Kadonaga 1997). TFII-D is a protein complex composed of TBP (TATA Binding Protein) and the TAFIIs (TBP-associated factors). Binding to the TATA box is mediated by TBP, while the TAFIIs are required for binding to the DPE. After TFII-D has bound, TFII-B can bind to the TFII-B recognition element BRE, thus further enhancing protein-protein and protein-DNA interactions at the core promoter (Lagrange et al. 1998). Next, RNApolII binds together with TFII-F. Finally, TFII-E and TFII-H are bound. The RNApolII and the general transcription factors together form the pre-initiation complex (PIC). TFIIH must phosphorylate the carboxy-terminal domain of the large subunit of RNApolII in order to releases RNApolII from the promoter, thus initiating transcription. For more detailed reviews the reader is referred to (Buratowski 1994; Green 2000).

Transcription factors

Transcription is further regulated by the presence of transcription factors. This is achieved by binding to specific regulatory sequences in the DNA and via protein-protein interactions. These regulatory sequences are located in promoters, enhancers and locus control regions of genes. Transcription factors are classified by the structural motifs that they use to bind DNA and/or proteins. These motifs include the homeodomain, the zinc-finger, the winged-helix or forkhead, the leucine-zipper, the helix-loop-helix domain, the runt homology domain, the ETS domain, the HMG-box. Transcription factors are able to interact with cofactors and proteins from the general transcription factor machinery and thereby stimulate or inhibit transcription initiation.

The first step in transcription is unwinding of the chromatin, the compact structure of the DNA with histone proteins. Folding of the DNA is essential for these long molecules to fit into the relatively small nucleus. The basic units of chromatin are the nucleosomes, approximately 200 bp od DNA wrapped around an octamer of two set of histone proteins H2A, H2B, H3 an H4. The nucleosomes can be organized in higher order structures that make the DNA less accessible for the general transcription machinery.

Transcription factors can also assist in opening up chromatin. This can be achieved by covalent modifications of the histone proteins, for instance phosphorylation, methylation and acetylation, and by an ill-defined ATP-dependent mechanism termed chromatin remodelling that does not involve covalent modifications of proteins and DNA.

Methylation of cytosine residues in promoters of genes correlates in general with transcriptional inactivity. This modification has a permanent character and is therefore thought to have an important role in irreversible silencing of genes. As a rule, transcription factors lack catalytic domains required for these biochemical reactions. Transcription factors usually act by recruiting complexes of co-factors that do contain these catalytic activities. These mechanisms have recently been reviewed in detail by Rice, Marmorstein and Wade (Marmorstein and Roth 2001; Rice and Allis 2001; Wade 2001).

Hematopoietic transcription factors

A class of transcription factors has been identified that can bind to regulatory sequences like promoters, enhancers and locus control regions (LCR) important for hematopoietic cell development and gene expression. Upon binding they enhance or inhibit transcription of their target genes. Binding to other proteins is an essential property because they can form either bridging complexes or docking sites.

The first tissue-specific transcription factor described was the zinc-finger protein GATA-1 (Wall et al. 1988). A single transcription factor however is not responsible for a differentiation program of a lineage. It is the combination of transcription factors present and their interactions that is more crucial. For this reason blood cell differentiation has been called "a party for transcription factors", as interaction partners might change as a party evolves (Sieweke and Graf 1998). For example the cofactor and acetyltransferases CBP can interact with a wide variety of transcription factors such as GATA-1 and c-myb, but only bipartite complexes with GATA-1 and CBP or c-myb and CBP can be shown. This suggest that mutual inhibition the transcriptional activity of GATA-1 and c-myb, is caused by the exclusive binding of GATA-1 or c-myb to CBP (Takahashi et al. 2000b).

Null mutant and transgenic mice have been instrumental in functional studies on the role of transcription factors in hematopoiesis (see Table 1) (Shivdasani and Orkin 1996). Some of the most important factors are mentioned below. The basic helix-loop-helix transcription factor Tal-1 (SCL, TCL5), overexpressed in T-cell acute lymphoblastic leukaemia (ALL), is essential for yolk sac and adult hematopoiesis (Porcher et al. 1996). The null mutant mice die in utero (9.5 dpc) (Robb et al. 1995; Shivdasani et al. 1995a). The zinc-

finger protein GATA-2 is another transcription factor that is important for all hematopoietic cells. The null mutant dies around 10-11 dpc . GATA-2 is thought to be required for the expansion of early hematopoietic cells (Tsai et al. 1994). The leucine-zipper protein c-myb and the runt homology domain transcription factor AML-1, that is the most frequent target of chromosomal rearrangements in human leukemia, are essential for definitive but not primitive hematopoiesis (Mucenski et al. 1991) (Okuda et al. 1996). Transcription factors that are important for specific hematopoietic lineage include Pu.1 (monocytes, granulocytes and B and T lymphocytes) (Scott et al. 1994), GATA-3 (T-lymphocytes) (Hendriks et al. 1999), NF-E2 (platelets) (Shivdasani et al. 1995b), c-fos (osteoclasts) (Johnson et al. 1992), Ikaros (B- and T lymphocytes, NK cells) (Boggs et al. 1998) and GATA-1 (erythrocytes)(Pevny et al. 1995).

Hematopoietic transcription factors

DNA binding motif	hematopoietic phenotype of the null mutant mice	reference
zinc-finger:		
GATA-1	block in erythropoiesis and megakaryopoiesis	(Pevny et al. 1991)
GATA-2	yolk sac and adult hematopoiesis defect	(Tsai et al. 1994)
GATA-3	expansion of T-cell progenitors	(Pandolfi et al. 1995)
EKLF	lack of β-globin expression	(Nuez et al. 1995; Perkins et al.
1995)		
	and erythroid differentiation defect	
FOG-1	block in erythropoiesis and megakaryopoiesis	(Tsang et al. 1998)
Ikaros	B&T lymphocytes an NK cells	(Boggs et al. 1998)
homeodomain:		
Lhx2	definitive erythropoiesis	(Porter et al. 1997)
HoxA3	thymocyte defect	(Su and Manley 2000)
HoxA9	defects in myeloid, erythroid, and lymphoid hematopoiesis	(Izon et al. 1998)
HoxB6	increased number of erythroid progenitors	(Kappen 2000)
HoxC8	less BFU-E, CFU-GM	(personal communication E.van den Akker)
leucine-zipper		
ATF4	transient fetal anemia	(Masuoka and Townes 2002)
E2F-1	T-cell defect	(Field et al. 1996)
E2F-2	T-cell defect	(Murga et al. 2001)
E2F-4	various mature hematopoietic cell types defects	(Rempel et al. 2000)
C/EBP-α	block in neutrophilic differentiation	(Zhang et al. 1997)
C/EBPβ	lymphoproliferative disorder	(Screpanti et al. 1995)
C/EBP_	impaired granulopoiesis, myelodysplasia	(Lekstrom-Himes and Xanthopoulos
1999)		
C/EBP_	NK-cell maturation defect	(Kaisho et al. 1999)
c-myb	definitive but not primitive hematopoiesis defect	(Allen et al. 1999)
c-fos	osteoclast and macrophage defect	(Johnson et al. 1992)
junB	myeloproliferative defect	(Passegue et al. 2001)
MAD1	granulocyte differentiation defect	(Foley et al. 1998)
NF-E2 (p45)	platelet formation	(Shivdasani et al. 1995b)
Stat5a	granulocyte-macrophage defect	(Feldman et al. 1997)
Stat5b	proliferative defect natural killer cells	(Imada et al. 1998)
the helix-loop-helix domain		
tal-1	yolk sac and adult hematopoiesis defect	(Shivdasani et al. 1995a)
E2A	B-cell defect	(Zhuang et al. 1994)
runt homology domain		
Runx1	definitive but not primitive hematopoiesis	(Okuda et al. 1996)
CBFβ	definitive but not primitive hematopoiesis	(Sasaki et al. 1996)

ETS domain		
Pu.1	multiple hematopoietic lineages affected	(Scott et al. 1994)
Ets-1	Natural killer cells	(Barton et al. 1998)
Lim domain		
Lmo2/Rbtn-2	yolk sac and adult hematopoiesis defect	(Yamada et al. 1998b), (Warren et al. 1994)
HMG-box		
TCF-1	T-cell defect	(Castrop et al. 1995)
Steroid receptor		
Glucocorticoid receptor 1	stress erythropoiesis	(Bauer et al. 1999)
Rel domain		
c-Rel	Multiple hemopoietic defects and lymphoid hyperplasia	(Kontgen et al. 1995) (Gerondakis et al. 1996)
relA	increasede TNFα sensitivity macrophages	(Beg and Baltimore 1996)
relB	dendritic cell defect	(Burkly et al. 1995)
nfkb1	non-activated B-cell survival defect	(Grumont et al. 1998)
nfkb2	proliferative humoral response defect	(Franzoso et al. 1998)

Table 1: Transcription factors with hematopoietic phenotypes of the null mutant.

GATA transcription factor family

GATA-1 is a Zn-finger transcription factor. The Zn-finger motif is a protein domain that was first recognised as a DNA binding domain (Miller et al. 1985). Later it was found that Zn-finger motif can also mediate protein-protein interactions. In the Zn-finger motif the polypeptide chain is wrapped around a Zn^{2+} ion that is bound by either cysteine and/or histidine thus stabilizing the 3D structure of the protein. When depicted in two-dimensions this domain looks like a finger. Proteins can contain multiple Zn-finger domains or different types of Zn-finger proteins resulting in several classes of Zn-finger proteins (Klug and Schwabe 1995).

GATA-1 is the founding member of the GATA transcription factor family characterized by their two typical Zn-fingers (Tsai et al. 1989b). The family consists of six members, GATA-1 to GATA-6. These proteins can all bind to a consensus sequence (A/T)GATA(A/G) with highly conserved Cys-X_2-Cys-X_{17}-Cys-X_2-Cys Zn-finger domains (Merika and Orkin 1993; Whyatt et al. 1993). Outside the Zn-finger domains, conservation between the different GATA factors is poor. The family is divided into two subgroups; the hematopoietic and the non-hematopoeitic group. GATA-1, 2 and 3 are usually defined as the hematopoietic members as all three are expressed in hematopoietic cells. However all three are also expressed in a number of other tissues; in particular GATA-2 and -3 play essential roles in tissues outside the hematopoietic system such as placenta and developing central nervous system (Ito et al. 1993; Pandolfi et al. 1995; Ma et al. 1997; Nardelli et al. 1999). GATA-4, -5 and –6 are expressed in overlapping patterns in tissues such as the intestine, lung and heart but not in hematopoietic cells (Molkentin 2000).

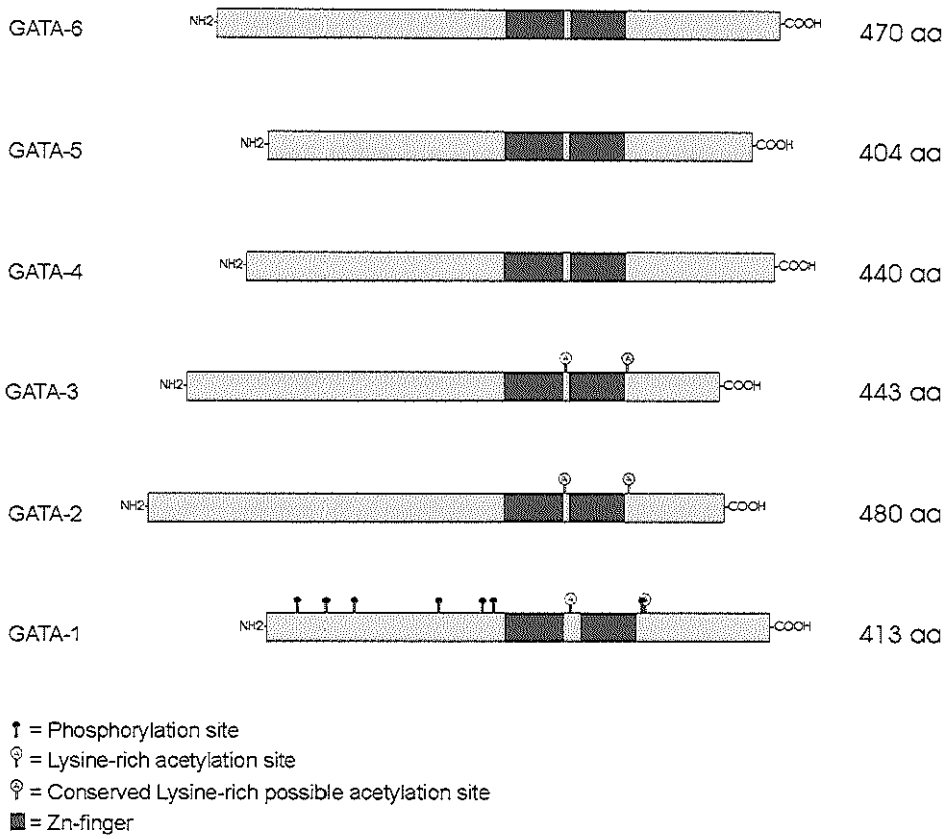


Figure 7: the GATA transcription factor family (mouse).

GATA-1

GATA-1, also known as NF-E1, NF-1, Ery-1 and GF-1, was first discovered as a protein with binding activity for the 3' region of the β-globin locus (Wall et al. 1988). After the gene was cloned (Tsai et al. 1989b) it was found that the gene is located on the X-chromosome in both mouse and human (Zon et al. 1990). The protein is expressed in primitive and definitive erythroid cells (Fujiwara et al. 1996) and it is thought to influence transcription of all erythroid genes since putative GATA binding sites are found in the promoters of all erythroid-specific genes studied. Besides erythroid cells the GATA-1 protein is also expressed in megakaryocytes, mast cells, eosinophils and in the Sertoli cells of the testis (Ito et al. 1993) (Yomogida et al. 1994).

The GATA-1 deficient mouse showed that GATA-1 is essential for red blood cell development (Pevny et al. 1991; Fujiwara et al. 1996). *In vitro* ES cells differentiation studies with GATA-1 deficient ES cells pointed out that the erythroid cells did not progress beyond the proerythroblast stage (Weiss et al. 1994; Pevny et al. 1995). This arrest was also confirmed for primitive erythroid cells in GATA-1 deficient mice *in vivo* (Fujiwara et al. 1996). Surprisingly, typical erythroid genes like the β-globin genes were activated to some extent in the GATA-1 deficient mice (Fujiwara et al. 1996).With knockdown mutation in the GATA-1 regulatory DNA sequences selective loss of megakaryocytic-specific expression could be achieved which caused a defect in the maturation of megakaryocytes (Shivdasani et al. 1997).

As all GATA factors, GATA-1 has two Zn-fingers of which the C-terminal finger is essential for DNA binding and the N-terminal contributes to the stability of the binding but is not able to bind DNA on its own. The N-terminal finger is however essential for interaction with FOG-1 (see below). A domain claimed to be necessary for transcriptional activity was shown to be unimportant for rescuing the knockout phenotype in transgenic mice (Shimizu et al. 2001).

Diseases related to GATA-1 malfunction

The known human diseases related to GATA-1 malfunctioning are single amino acid substitutions in the N-terminal Zn-finger causing an inhibition of the interaction of the GATA-1 protein with the cofactor FOG-1. These mutations cause thrombocytic defects and milder erythroid defects (Nichols et al. 2000; Freson et al. 2001; Mehaffey et al. 2001).

Target genes of GATA-1

All erythroid specific genes studied have GATA sites in their transcription regulatory sequences. This group of genes includes erythroid transcription factors, anti-apoptotic genes, erythroid membrane protein genes and genes important for hemoglobin synthesis such as enzymes of the haem synthesis-pathway and the globins.

While the role of GATA-1 in erythropoiesis may be due to transcriptional activation of such target genes, a number of putative target genes like the Epo receptor are normally expressed in GATA-1 deficient proerythroblasts (Weiss et al. 1994). The globin genes are also expressed in erythroid cells deficient in GATA-1, although at a reduced level (Fujiwara et al. 1996). One possible explanation is that GATA-2 takes over some of the activity of GATA-1, as GATA-2 expression is up regulated in GATA-1 deficient cells (Weiss et al. 1994). This idea is supported by experiments in which a lethal GATA-1 mutant, expressing

~5 % of the normal level of GATA-1, is rescued by a GATA-2 overexpressing transgene driven by a GATA-1 promotor (Takahashi et al. 2000a). Thus, there appears to be redundancy in GATA transcription factor function.

GATA-1 as stimulator of differentiation

In the absence of GATA-1, erythroid progenitors *in vivo* and *in vitro* do not progress beyond the proerythroblast stage (Pevny et al. 1995). Experiments with a GATA-1 deficient erythroid cell line G1E have shown that an inducible GATA-1 protein can rescue the differentiation program of these cells (Weiss et al. 1997).

GATA-1 as survival factor

GATA-1 deficient erythroid progenitor cells undergo apoptosis (Pevny et al. 1995). This suggests a role for GATA-1 as survival factor. GATA-1 is able to activate the EpoR promotor and Epo signalling is important for erythroid progenitor survival (Zon et al. 1991; Silva et al. 1996). However, EpoR is expressed normally in GATA-1 deficient mice (Fujiwara et al. 1996). One of the target genes of GATA-1 is Bcl-x_L (Gregory et al. 1999). This is a long splice variant of the Bcl-x gene that codes for an anti-apoptotic protein essential for erythroid differentiation. Since Bcl-x_L expression is low in GATA-1 deficient cells, it is a good candidate for the survival factor activated by GATA-1.

GATA-1 as regulator of the cell cycle

Differentiation of erythroid cell is associated with cell cycle arrest. When assuming that GATA-1 is a transcription factor stimulating differentiation factors it might be expected that GATA-1 expression would stimulate cell cycle arrest. However when GATA-1 is overexpressed in murine erythroleukemia (MEL) cells, an erythroid cell line, the opposite occurs. GATA-1 stimulates the cell cycle of MEL cells apparently by activating the crucial G1/S transition. In addition the overexpressing MEL cells fail to activate differentiation markers in response to the chemical inducer DMSO (Whyatt et al. 1997). In non-erythroid cell GATA-1 has also been found to affects the cell cycle as it elongates S-phase in NIH3T3 fibroblasts, possibly by introducing premature G1/S transition (Dubart et al. 1996). Mice that overexpress GATA-1 during the late phase of erythroid differentiation die around 12.5 dpc due to anaemia caused by inhibition of terminal erythroid differentiation (Chapter 2).

Collectively, these findings indicate that the control of GATA-1 activity is crucial for erythroid differentiation pathway.

Regulation of GATA-1 activity

GATA-1 activity in vivo may be modulated by several different mechanisms including: transcriptional control, translational control, interactions with co-factors, post translational modifications and protein degradation. These will be reviewed here.

GATA-1 transcriptional control

The GATA-1 transcription unit contains two alternative first exons that are non-coding (IT and IE) and five coding exons (II to VI). The GATA-1 gene is thought to have two distinct promoters which are alternatively spliced to the coding exons (Ito et al. 1993). Exon IT is primarily used in Sertoli cells of the testis and exon IE in erythroid cells. Since the second exon harbours the start of translation, the proteins expressed in erythroid cells and the testis are identical. The testis-specific promotor and first exon (exon IT) are located 8 kb upstream of the erythroid specific first exon (exon IE). Both promoters harbour GATA sites that are required for proper promotor function and hence a positive feedback loop was suggested but a promoter study in GATA-1 null cells and overexpression of GATA-1 in MEL cells do not support a regulatory loop (Tsai et al. 1991; McDevitt et al. 1997; Onodera et al. 1997b; Whyatt et al. 2000). Interestingly the GATA-1 promoters lack a TATA motif (Hannon et al. 1991).

The first intron, between the erythroid first erythroid exon IE and exon II, has regulatory sequences important for proper expression of the gene in the hematopoietic system (Onodera et al. 1997a). A palindromic sequence in the first intron of the chicken gene resembles a binding site for the steroid hormone super family. This motif is protected by fingerprinting in brain but not in erythroid cells and might represent a negative regulatory motif (Hannon et al. 1991). Furthermore, seven BGP1 sites and a c-myb site are found the chicken GATA-1 promoter (Hannon et al. 1991). In the mouse, a duplicated CACC motif was found (Simon et al. 1992). Despite these detailed analyses of the GATA-1 promoter, many questions regarding the complex transcriptional regulation of the GATA-1 gene remain unanswered.

Figure 8: schematic representation of the gene intron-exon organisation

Translational control

In K562 and MEL cells two isoforms of GATA-1 protein are detected, GATA-1 and GATA-1s. The difference these two proteins is the lack of a 83 aa N-terminal region in GATA-1s. This smaller form of GATA-1 can arise from alternative usage of the translation initiation sites in the GATA-1 mRNA (Calligaris et al. 1995). Alternative translation starts have been described for other transcription factors as a mechanism to regulate the activity by changes in the ratio of two isoforms (Calkhoven and Ab 1996). However the levels of expression of this alternative form in vivo is much lower than that of the full length protein suggesting that this is not a relevant mechanism for the regulation of activity.

GATA-1 expression is been thought to be affected by RNA stability that has been shown to change in *in vitro* differentiation of human erythroid cell lines (Morceau et al. 1996). It is known however that the first intron of GATA-1 is important for proper expression of the protein and RNA stability may play a role in this (Onodera et al. 1997a).

Interactions with cofactors

It has been shown that GATA-1 can interact with several other transcription factors that are either ubiquitously or erythroid specifically expressed. The most important factors are discussed here.

The first indications of collaboration between GATA-1 and other factors came from the observation that in many erythroid-specific regulatory regions like promoters and the β-globin Locus Control Region (LCR), GATA binding sites are found in close association with AP-1/NF-E2 and Sp-1 like binding sites. The Sp-1 like binding sites, GC and GT/CACC motifs, were proven to be important for proper function of core segments of the LCR. In artificial constructs no single motif suffices for the function of these LCR segments (Philipsen et al. 1993; Gillemans et al. 1998). Furthermore, since the LCR can control β-globin gene transcription over large distances, it is thought that protein-protein interactions between factors bound to these motifs and the promoters of the globin genes can loop out the intervening DNA and bring the LCR in contact with the promoters of the globin genes. This would imply that the LCR is a docking site for transcription factors forming a holocomplex that interacts with one of the β-globin genes (Hanscombe et al. 1991; Wijgerde et al. 1995; Dillon et al. 1997).

Sp1 is a ubiquitously expressed Zn-finger protein thought to be important for the expression of many genes. Sp1 and EKLF can bind to the same sites. EKLF is a transcription

factor with Zn-fingers homologous to the Sp1 Zn fingers, but it is an erythroid-specific transcription factor. The GATA-1, Sp1 and EKLF proteins can function synergistically on erythroid specific promoters non-erythroid *drosophila* S2 cells. EKLF and Sp1 can physically interact with GATA-1 via their Zn-finger regions *in vitro*. Via this interaction EKLF and Sp1 can recruit GATA-1 to a synthetic promotor and vice versa in an transient transfection assay (Merika and Orkin 1995; Gregory et al. 1996).

The knockout of Sp1 showed that Sp1 is essential for early embryonic development. Embryos die around day 9.5 of gestation due to a broad range of abnormalities. The globin genes in the null mutants are activated although transcription might be reduced (Marin et al. 1997). The knockout of EKLF dies around day 12.5 due to anaemia (Nuez et al. 1995). EKLF is required for the activation of β-globin expression in vivo (Gillemans et al. 1998).

Friend of GATA-1 (FOG-1) is a nine Zn-finger protein, that binds to the N-terminal Zn-finger of GATA-1, -2 and -3 mainly via its 6th Zn-finger (Tsang et al. 1997) (Fox et al. 1998). FOG-1 fingers 1,5 and 9 also contribute to binding (Fox et al. 1999). FOG-1 is co-expressed with GATA-1 and although no FOG-1 DNA-binding activity is known, GATA-1 and FOG-1 can synergistically activate hematopoietic-specific promotor constructs and cooperate during megakaryocytic and erythroid development (Tsang et al. 1997). The importance of the interaction is underscored by the report of a dyserythropoietic anemia and trombocytopenia caused by a mutation in the GATA-1 gene that abolishes the binding of FOG-1 to GATA-1 (Nichols et al. 2000). Experiments with GATA-1 mutants that can not bind FOG-1 but do bind DNA have shown that FOG-1 interaction with GATA-1 is essential for GATA-1 function (Crispino et al. 1999).

The FOG-1 null mutant mouse has a similar phenotype to the GATA-1 null mutant mouse with regard to erythroid development but differs considerably in megakaryocytic development as these cells fail to develop (Tsang et al. 1998). Surprisingly, a mutant GATA-1 transgene lacking the N-terminal finger is able to rescue the primitive erythropoiesis of the GATA-1 knockdown mouse, suggesting that the interaction between GATA-1 and FOG-1 is not essential for primitive erythropoiesis and that FOG-1 functions independently at this stage (Shimizu et al. 2001).

The existence of another FOG protein, FOG-2, has brought up the idea that interactions with FOG proteins are a common theme in the regulation of GATA factors. FOG-2 is expressed at low levels in the majority of tissues and at higher levels in heart and skeletal muscle. It can also interact with GATA-1 (Holmes et al. 1999). FOG-2 can interact with GATA-4 thereby repressing its transcriptional activity (Tevosian et al. 1999) (Lu et al. 1999;

Svensson et al. 1999). Furthermore it has been shown that FOG in Xenopus can act as a repressor of GATA-1 and erythropoiesis (Deconinck et al. 2000). The general idea is that FOGs can be activators but also repressors of GATA factors depending on the promotor and the cofactor environment. The repression of GATAs via FOGs is at least partially mediated via CtBP2 (C-teminal Binding Protein) that is able to bind to a PLDLS domain present in several Zn-finger proteins (BKLF,EVI-1, AREB6, ZEB and FOG) and repress their transactivation potential (Fox et al. 1999; Holmes et al. 1999; Svensson et al. 2000; Fossett et al. 2001) (Turner and Crossley 1998). This interaction between FOG-1 and CtBP is however not essential for normal erythropoiesis yet a FOG-2 mutant lacking the CtBP2 binding domain is no longer functional (Katz et al. 2002) (Fossett et al. 2001).

In Drosophila the FOG homologue U-Shaped, Ush, functions primarily as a repressor and not as a activator or synergistic partner (Fossett et al. 2001). Many questions about how FOG and GATA cooperate remain unanswered.

GATA-1 can also bind the Rb protein and overexpression of GATA-1 results in inactivation of Rb by hyperphosphorylation through an unknown mechanism (Whyatt et al. 1997). This observation was surprising as it was believed that high levels of GATA-1 expression induce differentiation by arresting the cell cycle in G1. Rb phosphorylation however inactivates Rb and releases the E2F transcription factors and this is essential for G1/S progression (Harbour and Dean 2000).

The Rb null matant has a neuronal and erythroid phenotype and the mice die around day 14.5 of gestation (Jacks et al. 1992; Lee et al. 1992). These defects are cell nonautonomous as Rb -/- ES cells can contribute to all tissues in chimaeric mice and only show certain histological defects including cataracts, hyperplasia of the adrenal medulla, and enlarged cells in the cerebellum and the liver but no defects in the erythrocytes (Williams et al. 1994). This is remarkably similar to phenotype of the GATA-1 overexpressing mice (Whyatt et al. 2000). GATA-1 overexpressing mice also show a cell nonautonomous inhibition of erythropoiesis. This is further described below and in chapter 2 of this thesis.

The Lim domain protein Rbtn2 is also thought to cooperate with GATA-1 based on similarities of the knockout phenotypes and the expression of the two proteins (Warren et al. 1994; Silver and Palis 1997). Rbtn2 may function as a scaffold protein since no DNA binding activity has been found. Formation of complexes involving Rbtn2, GATA-1, Tal-1 and E47 has been shown (Osada et al. 1995; Osada et al. 1997). Also a complex involving GATA-1, Tal-1 and E2A, and Ldb1/NLI has been discovered (Wadman et al. 1997). It is suggested that

variation in the amounts of these complexes can be important in the regulation of erythropoiesis.

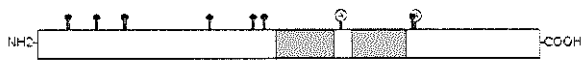
The Ets-domain transcription factor Pu.1 (or Spi-1) is important for myeloid and B-cells commitment. It is thought to promote myeloid differentiation by suppression of non-myeloid genes like GATA-1 and by activation of its own promoter and turning on the three myeloid CSF receptors (M, GM, and G) (Nerlov and Graf 1998). Furthermore, overexpression of Pu.1 blocks erythroid differentiation. It has been suggested that overexpression of Pu.1 results in reduced DNA binding by GATA-1 (Yamada et al. 1998a; Zhang et al. 2000). Similarly GATA-1 is thought to inhibit the activation of targets of Pu.1 (Zhang et al. 1999; Nerlov et al. 2000). In addition it has also been shown that Pu.1 and GATA-1 can physically interact and thus, the balance between GATA-1 and Pu.1 might be an important regulatory step in the differentiation of myeloid and erythroid cells (Rekhtman et al. 1999).

Post translational modifications

The activity of may proteins is controlled by covalent post-translational modifications. A wide variety of post translational modifications is known such as acetylation, phosphorylation, methylation and glycosylation. These modifications can control activity, specificity, stability and the localisation of a modified protein (Han and Martinage 1992).

Two cofactors of GATA-1, P300 and CBP, are almost identical acetyltransferases that are connected with transcription and cell transformation. These proteins interact with a many different proteins including general transcription factors and tissue-specific transcription factors. This suggests a pivotal role in transcription, cell growth, transformation and development (Goodman and Smolik 2000). These proteins can bind to GATA-1 and acetylate the GATA-1 protein, thereby stimulating transcriptional activity (Blobel et al. 1998; Boyes et al. 1998). It has also been suggested that acetylation is required for differentiation of a GATA-1 null erythroid cell line G1E when transfected with a GATA-1-expressing retrovirus (Hung et al. 1999). The acetylation of chicken GATA-1 by p300 increases DNA binding activity in vitro and in transfected MEL cells. This increase in binding capacity might be caused by a conformational change which could also influence the interaction of GATA-1 with other proteins (Boyes et al. 1998). This effect has not been seen with mammalian GATA-1 (Hung et al. 1999).

Phosphorylation is a common mechanism for regulation of protein activity for many cellular processes including cell cycle control, transcriptional activity, specificity and signal transduction (Whitmarsh and Davis 2000). GATA-1 can be phosphorylated on seven serines (Crossley and Orkin 1994). Of these seven serine residues, six (aa 26, 49, 72, 142, 178, 187) are phosphorylated in uninduced MEL cells and the seventh (aa 310) is increasingly phosphorylated upon DMSO induction. Mutations in GATA-1 that replace the serines for alanines did not change DNA binding affinity, DNA bending, or the transcriptional activity of GATA-1 on a reporter construct transfected in NIH3T3 cells. Another report shows that in K652 cells phosphorylation of GATA-1 does influence DNA binding and confirms the absence of such an effect in MEL cells (Partington and Patient 1999). Though Ser 310 is located in a classical nuclear localisation signal, it does not influence nuclear transport. This is unlike what is seen in chicken cells, where upon differentiation, cGATA-1 was increasingly phosphorylated and transported into the nucleus (Briegel et al. 1996). The discrepancy between these two results may be explained by the difference between avian and mammalian erythropoiesis. In avian erythropoiesis, the erythrocytes remain nucleated. The conservation between the avian and mammalian GATA-1 proteins is much lower than among the mammalian GATA-1 proteins. Another reason could be that the cells used are not comparable in their developmental stage and as a consequence, GATA-1 is mainly nuclear in MEL cells, whereas in the chicken cells it is mostly cytoplasmic.



= Phosphorylation site
= acetylation site
= Zn-finger

Phosphorylation sites: aa 26, 49, 72, 142, 178, 187 and 310
Lysine rich acetylation sites: aa 245 - 252, 308 - 316
Zn fingers: 204 - 228, 258 - 282

Figure 9: Schematic representation of GATA-1 with the Zn-fingers, phosphorylation and acetylation sites.

In conclusion, the few reports on GATA-1 phosphorylation have been contradicting and thus its role in the regulation of GATA-1 function is still unclear. It might be that phosphorylation does play a role in mammalian cells, for instance in regulating the interaction between GATA-1 and other proteins like the cofactor CBP. It is known that phosphorylation of p53 is of great importance for the interaction of CBP and p53 (Lambert et al. 1998). Phosphorylation of the GATA-1 protein might be required very early in erythroid differentiation, a question which could not be addressed with the methods used so far. Knockin mutant mice with substitution of serine 310 might elucidate whether or not phosphorylation of this amino acid is important.

GATA-1 protein degradation

The level of transcription factors can be regulated by the rate of synthesis and degradation. Protein degradation can, like protein synthesis, be a very controlled mechanism of regulation as in the case of the addition of ubiquitin and subsequent degradation (Desterro et al. 2000). Protein degradation is important feature of apoptosis and in mediated by Caspase proteins that can be activated by stimulated death ligand receptors (Budihardjo et al. 1999).

Erythroblasts express several death receptors: Fas (CD95), tumour necrosis factor (TNF) receptor, TRAIL receptor-1 (DR4) and TRAIL receptor-2 (DR5), especially at the basophilic erythroblast stage (Rusten and Jacobsen 1995; De Maria et al. 1999a; De Maria et al. 1999b). Erythroid progenitors acquire a transient sensitivity to death ligands until the orthochromatic erythroblast stage. It has been shown that these more mature erythroblasts express the ligand for Fas, Fas-ligand (FasL). The death ligands TNF-α, Trail and Fas have been shown to negatively regulate erythropoiesis through Caspase-3-mediated cleavage of GATA-1 (De Maria et al. 1999b). These data have led to the proposal of a model with a negative feedback mechanism in the erythroblastic islands. Upon accumulation of mature erythroblasts, FasL levels increase leading to GATA-1 cleavage in the erythroid progenitors. This reduction of GATA-1 activity results in differentiation arrest of these immature erythroblasts (Orkin and Weiss 1999) (De Maria et al. 1999a).

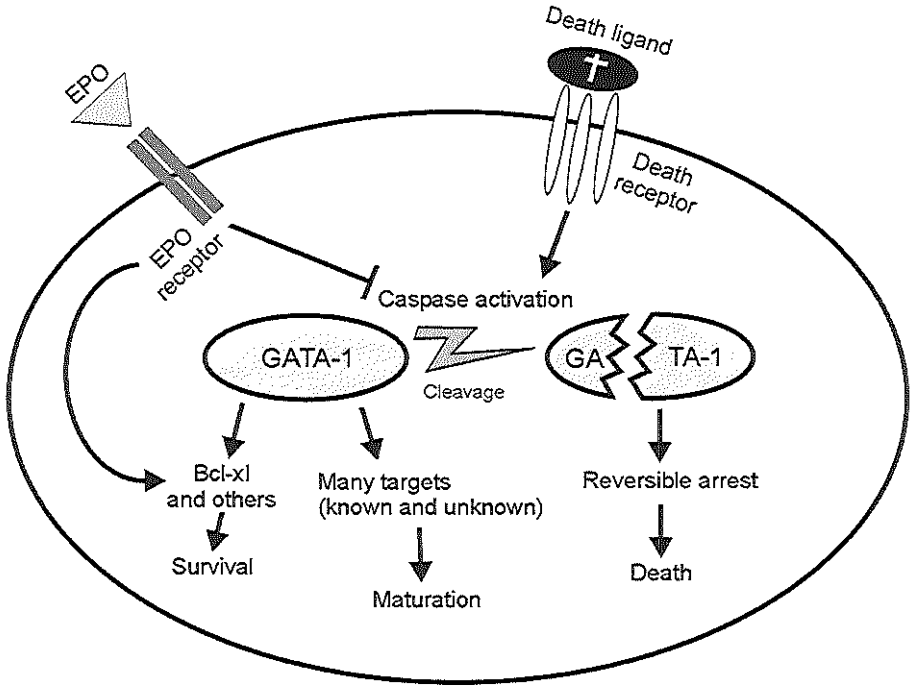


Figure 10: The negative feedback model of immature by mature erythroid progenitor cells via death ligand signalling, caspase activation and GATA-1 degradation.
(Adapted from (Orkin and Weiss 1999))

There are however a few questions that remain unanswered. Why is the caspase cleavage site not conserved in an evolutionary close organism like the mouse? Is this a common way to regulate the activity of GATA factors? What is the in vivo relevance of the breakdown of GATA-1 ? It is for instance known that in death ligands besides inducing apoptosis, can also have effects on proliferation and differentiation (Baud and Karin 2001). Furthermore it has been shown that caspase activation is required for terminal erythroid differentiation *in vitro*, inhibition of caspases leads to a block at the basophilic erythroblast stage (Zermati et al. 2001).

We suggest that GATA-1 activity is dynamically regulated during differentiation, because in mice overexpressing GATA-1 erythroblasts are blocked in terminal maturation. This block is alleviated in mice that overexpress GATA-1 heterocellularly. We propose a model in which a signal, REDS for red cell differentiation signal, expressed by the mature

erythroblast, reduces the GATA-1 activity in the immature erythroblast thereby allowing these cells to complete differentiation (Fig.11) (Chapter 2).

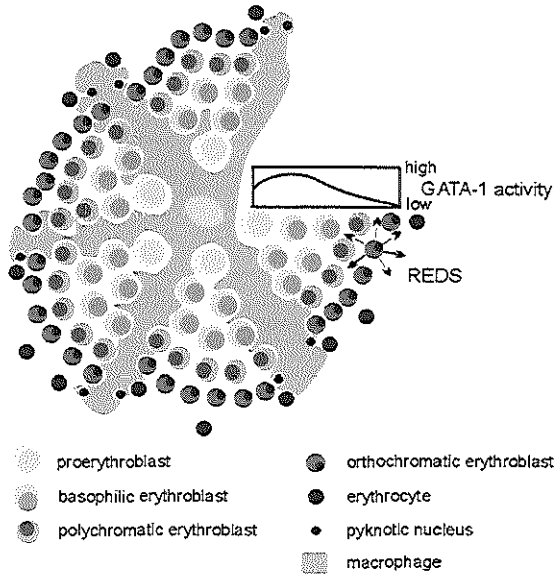


Figure 11: A simplified model of the erythroblastic island. The position of the central macrophage and the arrangement of the erythroid progenitors within the macrophage's extensions are shown. The process of pyknosis, enucleation and phagocytosis of the expelled nuclei are represented.

The other GATA transcription factor proteins

GATA-2

GATA-2 is expressed in the early hematopoietic progenitors, early erythroid cells, mast cells and megakaryocytes . Other sites of expression are the placenta and the developing central nervous system where it overlaps with GATA-3 expression (Ma et al. 1997). A suggested mechanism how GATA-2 influences transcription is via regulation of chromatin accessibility by associating with histone deacetylases HDAC3 and HDAC5 (Ozawa et al. 2001).

The mouse null mutant showed that GATA-2 is essential for all hematopoiesis (Tsai et al. 1994). The GATA-2 -/- mice die between 10 and 11 dpc due to severe anaemia, but already look pale at day 9.5. Although the primitive erythrocytes look normal they are reduced in number two to seven-fold. In chimaeras GATA-2 -/- ES cells did contribute to all

tissues except the definitive haematopoietic lineages. In vitro differentiation of GATA-2 -/- ES cells revealed a profound deficit in definitive hematopoietic stem cells or progenitor cells largely related to poor expansion of the cellular pool in response to hematopoietic growth factors. It has been proposed that GATA-2 regulates genes controlling growth factor responsiveness or the proliferative capacity of early hematopoietic cells. (Tsai et al. 1994). It is known that in pluripotent stem cells the GATA-2 level is higher than in more differentiated cells and this supports the idea that GATA-2 is down regulated during differentiation (Orlic et al. 1995). GATA-1 may play a role in this process in the erythroid lineage because GATA-2 is upregulated ~50-fold in GATA-1 -/- erythroid cells (Weiss et al. 1994). Overexpression studies have shown that high GATA-2 levels inhibit differentiation and also block proliferation (Heyworth et al. 1999; Persons et al. 1999) (Briegel et al. 1993)(R. Rottier unpublished data).

In the placenta GATA-2, together with GATA-3, regulates the synthesis of placental hormones (Ma et al. 1997). In the central nervous system GATA-2 and GATA-3 also have overlapping expression patterns in some areas. GATA-2 is expressed from 9 dpc onwards and analysis of the GATA-2 -/- mice showed that the expression of GATA-2 in the developing CNS of the mouse embryo is essential for GATA-3 expression. Loss of GATA-2 leads to defects in neurogenesis with a notable lack of ventral neuronal progenitors(Nardelli et al. 1999; Pata et al. 1999).

Finally, partial rescue experiments showed that GATA-2 is also important for proper urogenital development (Zhou et al. 1998).

GATA-3

GATA-3 was first identified as a transcription factor binding to the enhancer of the TCRα enhancer (Ho et al. 1991). The expression pattern of GATA-3 very complex as it is first expressed in the ectoplacental cone and the trophoblast cells, after day 10.5 dpc in T-lymphocytes, the central and peripheral nervous system, otic and optic vesicles, cochlear endothelial cells, kidney, liver, adrenal gland, mesonephros, Wolfian duct, ureteric bud, whisker follicles and the primary palate (Oosterwegel et al. 1992; George et al. 1994; Lakshmanan et al. 1999). In transgenic experiments using YACs as big as 625 kb (450 kb 5' and 150 kb 3'of the gene), no complete expression pattern could be achieved, showing that some of regulatory elements of the GATA-3 gene are not in close proximity of the actual coding sequence (Lakshmanan et al. 1999).

GATA-3 haplo-insufficiency has been associated with the hypoparathyroidism, sensorineural deafness, renal anomaly syndrome in humans (HDR) (Van Esch et al. 2000;

Van Esch and Devriendt 2001). In contrast mice heterozygous for the GATA-3 mutation are fertile and appeared to be normal. Homozygous mutant embryos die between day 11 and 12 dpc and display massive internal bleeding, marked growth retardation, severe deformities of the brain and spinal cord, and gross aberrations in fetal liver hematopoiesis(Pandolfi et al. 1995). An indication of the actual cause of death was found by a failed rescue experiment with a transgene that did not express in the thymus and the symphathoadrenal system (Lakshmanan et al. 1998). This experiment showed that loss of GATA-3 leads to reduced accumulation of tyrosine hydroxylase (Th) and dopamine β-hydroxylase (Dbh) in the sympathic nervous system (SNS) which leads to reduced noradrenaline levels. The GATA-3 mutation-induced lethality could be partially averted by feeding pregnant GATA-3 +/- intercrossed female mice catechol intermediates (Lim et al. 2000). This temporal rescue revealed roles for GATA-3 in some other tissues and organs that could previously not be detected. These older embryos had renal hypoplasia and developmental defects in structures derived from cephalic neural crest cells such as the tongue, mandible, tooth primordia and semicircular canals (Lim et al. 2000).

In chimearic mice GATA-3 null cells can contribute to the brain, kidney and lungs but not to the T-cells (Hendriks et al. 1999). Thus, although it has been suggested that GATA-3 could be involved in the process of intra-embryonic stem cell generation, of all the hematopoietic cells GATA-3 is only essential for T-cell development (Manaia et al. 2000) (Ting et al. 1996). Furthermore, GATA-3 is thought to be important for the expansion of T-cell progenitors and for the control of subsequent proliferation steps, as GATA-3 expression appears to be coupled to the cell cycle (Hendriks et al. 1999). The level of expression during T-cell development is influencing the commitment process, as enforced expression of GATA-3 inhibits CD8 single positive cells and induces the formation of the Th2–committed T-cell compartment and Th2-specific cytokines (Nawijn et al. 2001b) (Zheng and Flavell 1997; Nawijn et al. 2001a). The regulation of GATA-3 expression is therefore a central issue in T-cell development. A lymphoid-specific protein ROG, repressor of GATA, is induced in Th1 cells. It interacts with GATA-3 and represses its function (Miaw et al. 2000). Acetylation of GATA-3 also regulates its function and has impact on the survival and homing capacity of the T-cells in secondary lymphoid organs (Yamagata et al. 2000).

GATA-4

During embryogenesis GATA-4 is expressed in the visceral yolk sac endoderm, in the cardiogenic mesoderm and subsequently in the heart, in proximal and distal gut, testis, ovary

and liver (Morrisey et al. 1996). Expression persists in the adult heart, ovary, testis, liver and small intestine (Arceci et al. 1993).

Heterozygous GATA-4 null mice have no phenotype but homozygous GATA-4 null mice arrest in development between 7 and 9.5 dpc due to severe developmental abnormalities. They lack a primitive heart tube and foregut and develop partially outside the yolk sac (Molkentin et al. 1997). It has been suggested that GATA-4 is essential for terminal cardiac differentiation, but GATA-4 null ES cells contribute normally to the three heart layers in chimeric embryos and the mutant cardiomyocytes in such embryos have a fully differentiated phenotype (Narita et al. 1997). It has been suggested that GATA-5 and GATA-6 are partially compensating for the absence of GATA-4. In the absence of GATA-4, the level of GATA-6 is significantly upregulated *in vivo* and *in vitro* in differentiated ES cells (Kuo et al. 1997). GATA-4 is thought to be required for the migration or folding morphogenesis of the precardiogenic splanchnic mesodermal cells at the level of the anterior intestinal portal (Molkentin et al. 1997).

GATA-5

GATA-5 is expressed during development in precardiac mesoderm and throughout the heart until 16 dpc, the developing lung, urogenital ridge, the bladder and the gut and postnatally only in the intestine, stomach, bladder and the lungs (Morrisey et al. 1997).

GATA-5 is the only GATA transcription factor without a embryonic lethal phenotype of the null mutant. The GATA-5 deficient male mice are undistinguishable from wild type mice, the females exhibit urogenital abnormalities that included vaginal and uterine defects and hypospadias (Molkentin et al. 2000). As GATA-5 overlaps in expression with either GATA-4 or GATA-6 or both, its function is probably overlapping for most targets. Despite this, it might be influencing the expression of some genes, for example during heart formation (Kakita et al. 1999; Morimoto et al. 1999) or in stomach (Sakamoto et al. 2000). Also GATA-5 -/- / GATA-4 +/- or GATA-5 -/- / GATA-6 +/- compound mutant mice showed no extra phenotype, thus one copy of the other GATAs is sufficient for proper development (Molkentin et al. 2000). On the other hand in zebrafish GATA-5 is required for expression of myocardial genes and for the formation of the heart tube, similar to the role of GATA-4 in mice (Reiter et al. 1999). This suggests that during evolution most of the function of GATA-5 has been taken over by GATA-4.

GATA-6

GATA-6 is first expressed before implantation in the blastocyst, followed by expression in the developing heart, gut, lungs, liver, aorta, urogenital ridge, arterial smooth muscle cells, the stomach and the testis. In most of these tissues it persists after birth (Morrisey et al. 1996; Morrisey et al. 1998) (Kiiveri et al. 1999).

The knockout showed that GATA-6 is essential for early embryonic development. It is embryonic lethal shortly after implantation (5.5 dpc) (Koutsourakis et al. 1999). Furthermore experiments with chimeric mice and embryonic lung-explant cultures showed that GATA-6 is required for normal branching morphogenesis and late epithelial cell differentiation (Keijzer et al. 2001). It has been suggested that GATA-6 should be down regulated during heart formation as overexpression leads to an excess of cells, thickening of the myocardial muscle and a block in differentiation (Gove et al. 1997). This might be similar to GATA-1 overexpression in erythroid cells (Chapter 2).

Summary GATA transcription factor family

The transcription factors of the GATA-family are essential for the proper development of several tissues in vivo (Pevny et al. 1991; Tsai et al. 1994; Pandolfi et al. 1995; Molkentin et al. 1997) (Koutsourakis et al. 1999) . It has been shown that GATA factors are important regulators of development and are suggested to be 'master' regulators of the choice between differentiation and proliferation (Tsai et al. 1994; Whyatt et al. 1997; Whyatt et al. 2000) (Gove et al. 1997; Hendriks et al. 1999).

Tissues known to express GATA transcription factors often express more than one GATA factor. These GATA factors are then often separately expressed at consecutive periods but with an intermediate period with overlapping expression. Examples are erythroid progenitors (GATA-1 and -2) , Sertoli cells (GATA-1, 4 and -6), nervous system (GATA-2 and –3) and heart and gut (GATA-4,5 and –6). It has been suggested that GATA transcription factors, that overlap in expression pattern, can regulate each others transcription and that the switch in the expression of one GATA factor to the other, is essential for the progression of differentiation. This is probably an oversimplification and so far it has not been convincingly demonstrated that a GATA factor can act as a regulatory switch of differentiation, as several overexpression studies never resulted in an enhanced or accelerated differentiation process but rather in sustained proliferation and a block in differentiation.

Other studies suggest that it does not matter which GATA factor is expressed but that the level and the timing of expression of a GATA factor is more important for proper differentiation. The biochemical properties of the factors may be largely interchangeable.

There are a number of reports that support this possibility. In a primitive murine myeloid cell line megakaryocyte differentiation can be induced by GATA-1 and –2, and only the C-terminal GATA-1 Zn-finger is essential for differentiation to occur (Visvader et al. 1992; Visvader et al. 1995). The GATA-1 null mutant can be partially rescued by a knock-in mutation of GATA-3 in the GATA-1 locus (Tsai et al. 1998). Takahashi et al showed that a lethal GATA-1 promotor knockdown mutation can be largely rescued by transgenes expressing GATA-2 or -3 under control of GATA-1 regulatory elements.The interchangeability of the different proteins suggests that expression at the right time, place and level are the very important parameters for GATA function (Takahashi et al. 2000a).
In addition to this there are many similarities between the mechanisms used by the GATAs as they can all interact with CBP/P300 (GATA- 1 (Blobel et al. 1998), GATA- 2, 3 and 4 (Blobel et al. 1998), GATA-5 (Kakita et al. 1999),GATA-6 (Wada et al. 2000)) and FOG-1 or-2 (Tsang et al. 1997). Recently interaction between the peroxisome proliferator activated receptor (PPAR) binding protein (PBP) and GATA-1,-2,-3,-4 and -6 was described (Crawford et al. 2001). This interaction was suspected after comparison of the PBP -/- mice with the GATA factor -/- mice. PBP is an important coactivator of PPAR_ and several other nuclear receptors and transcriptional complexes where it is thought to function as a bridging factor (Crawford et al. 2001). Although a great deal has been learned about the biological roles of the GATA factors, many interesting and fundamental questions remain. How are they regulated, how are they linked to cell cycle regulation and differentiation, what are the essential target genes and what is the composition and the importance of the complexes in which they are present? The search for answers continues and with the help of new technology of the emerging genomics, proteomics and bioinformatics fields we may solve these questions, and undoubtedly ask new ones.

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Chapter 2

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

David Whyatt, Fokke Lindeboom, Alar Karis, Rita Ferreira, Eric Milot, Rudi Hendriks, Marella de Bruijn, An Langeveld, Joost Gribnau, Frank Grosveld and Sjaak Philipsen

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Abstract

GATA-1 is a tissue-specific transcription factor essential for erythropoiesis [Pevny, 1991 #1][Weiss, 1994 #2]. We demonstrate that GATA-1 overexpression in erythroid cells inhibits their differentiation, leading to a lethal anaemia. Using X-inactivation of a GATA-1 transgene and chimeric animals, we show that this defect is intrinsic to erythroid cells and yet nevertheless cell nonautonomous. Usually, cell nonautonomy is thought to reflect aberrant gene function in cells other than those that exhibit the phenotype [Rossant, 1998 #3]. Based on 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 [Pevny, 1991 #1][Weiss, 1994 #2], since GATA-1 null proerythroblasts undergo apoptosis [Weiss, 1995 #4] and reduced GATA-1 levels inhibit proerythroblast differentiation [Takahashi, 1998 #5]. 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 beta-globin locus control region linked to the beta-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 [Whyatt, 1997 #6]. Embryonic stem (ES) cell clones overexpressing GATA-1 also generate erythroid colonies inhibited in terminal differentiation [Whyatt, 1997 #6]. 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 (unpublished observations, R.Ferreira and D.Whyatt). These results may explain our failure to produce a transgenic line of mice via conventional microinjection of PEV3-GATA-1, since loss of erythroid differentiation would be lethal *in vivo*.

To circumvent this, we exploited the process of X-inactivation [Kuroda, 1997 #7]. An 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, approximately 30% of GATA-1 null heterozygous females die from 15.5 days post

coitus (dpc) with severe anaemia. In survivors, anaemia is transient and recovery is thought to be due to *in vivo* selection of normal progenitors [Fujiwara, 1996 #8][Tsai, 1998 #9].

DNA fluorecence-in-situ-hybridisation (DNA-FISH) screening of male ES cells stably transfected with PEV3-GATA-1 demonstrated localisation 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.

Transgenic females were mated and progeny examined. Transgenic females are indistinguishable from wild-type littermates. Male transgenics are anaemic from 12.5 dpc and dead by 14.5 dpc (Figure 1a and 1b). Backcrossing 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 (Figure 1c).

The fetal liver of transgenic male embryos, the site of definitive erythropoiesis at 13.5 dpc, is normal in size, cell number and contains similar numbers of apoptotic cells (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 (Figure 1c), confirming that fetal liver erythropoiesis is defective. Surprisingly, female transgenics display no alteration in the morphology of cells in the fetal liver and have normal numbers of enucleated erythrocytes at this stage (Figure 1c). Expression of putative GATA-1 target genes such as α-globin is unchanged in female transgenics, since RNA-FISH detection of α-globin nascent transcripts on 13.5 dpc fetal liver cells shows a normal number of cells transcribing α-globin (72% compared to 73% in controls). Furthermore, co-staining shows that half of these cells are positive for transgene-derived GATA-1 transcripts (data not shown), suggesting the transgene is sensitive to X-inactivation.

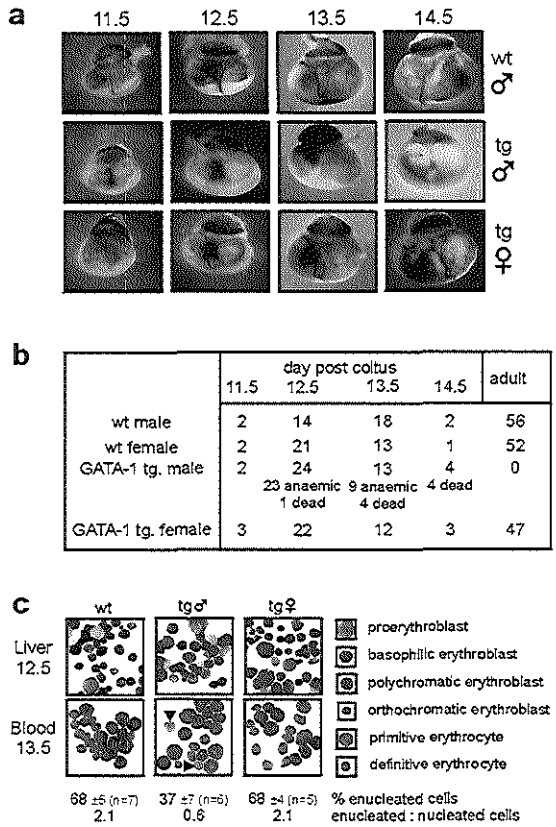


Figure 1: GATA-1 overexpression *in vivo*. *a*, Representative wild-type (wt) males and transgenics (tg) of either sex, 11.5 dpc to 14.5 dpc, yolk sacs intact. *b*, Genotype and phenotype of embryos and adults, excluding F1. Death was scored by lack of heartbeat, pallor as anaemia. *c*, Disaggregated 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). Beneath is percentage enucleated erythrocytes (>300 events/embryo), number of independent embryos and standard deviations. Ratio of enucleated to nucleated erythroid cells is shown.

Consistent with this interpretation, Western blot analysis demonstrates a higher level of transgene-derived GATA-1 protein in male versus female transgenic embryos (Figure 2a). RNA-FISH using a GATA-1 probe demonstrates that at 12.5 dpc, 66% of transgenic male fetal liver cells are positive for transgene-derived GATA-1 nascent transcripts (appearing as a

bright nuclear dot), while 32% of transgenic female cells are GATA-1 positive (Figure 2b). This method did not detect endogenous GATA-1 transcripts. Co-staining for Xist RNA shows a single punctate signal in male fetal liver cells, corresponding to transcription from the Xist allele on the active X chromosome. Female cells also contain a larger area of accumulated Xist RNA on the inactive X chromosome (or Barr body) [Panning, 1997 #10][Sheardown, 1997 #11]. Apparent co-localisation of Barr body and GATA-1 signals occurs 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.

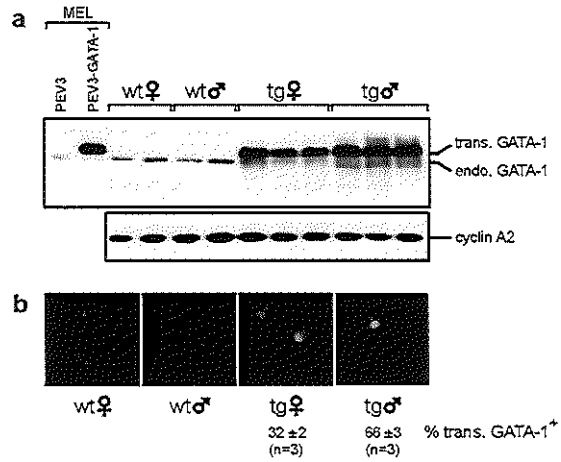


Figure 2: GATA-1 transgene is subject to X-inactivation. *a*, Western blot of nuclear extracts from 13.5 dpc fetal livers, with anti-GATA-1 antibody N6. DMSO-induced control (PEV3) and GATA-1 overexpressing (PEV3-GATA-1) MEL cell samples are included. Position of myc-tagged transgene-derived GATA-1 (trans.GATA-1) and endogenous GATA-1 (endo.GATA-1) 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.

Survival of transgenic mice is not dependent on being female *per se*, since a transgenic XXY male (where X-inactivation occurs [Kuroda, 1997 #7]) is viable. One could argue that the observed phenotype is due to 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 accumulates to lower levels than PEV3-GATA-1 derived protein and consequently induces 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).

To examine the relative contribution of GATA-1 overexpressing cells to the erythroid lineage, transgenic females were bred with males containing a lacZ gene insertion in the X-inactivation sensitive Bruton's tyrosine kinase (Btk^{lacZ}) locus [Hendriks, 1996 #12]. We find the same number of lacZ positive erythroid cells in the fetal livers of 13.5 dpc $Btk^{lacZ/+}$ and compound Btk^{lacZ+}/GATA-1 transgenic females (Figure 3a). Adult Btk^{lacZ} males or females express lacZ in 50% or 25% of large erythroid precursors in the bone marrow respectively. Compound Btk^{lacZ+}/GATA-1 transgenic females also express lacZ in 25% of large erythroid precursors (Figure 3b).

a

	%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

b

%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 3: GATA-1 transgene is subject to X-inactivation. a, 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 indicated. b, 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) expressing 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.

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. Since 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, chimeric animals were generated with clone G4 cells (which are male and express the GATA-1 transgene pancellularly). These cells contribute up to 50% of the erythrocytes, as assayed by globin chain isoform analysis (Figure 4a) and confirmed by glucose phosphate isomerase analysis (data not shown). These cells are morphologically normal (Figure 4b). Furthermore, analysis of the blood of female transgenic mice demonstrates no effect on other blood cell parameters, including red cell number, cell volume and haemoglobin content (data not shown).

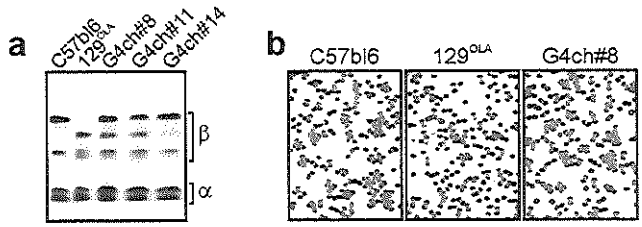


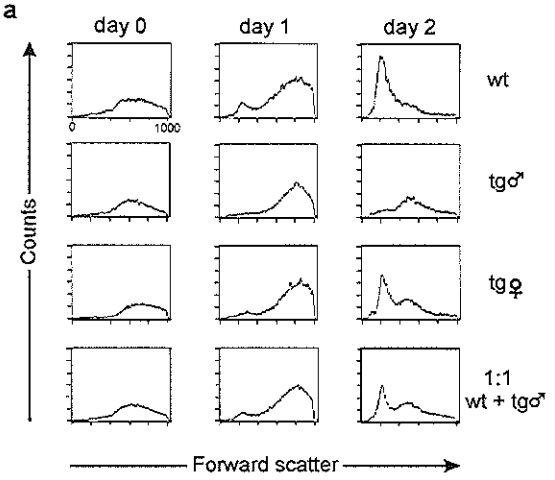
Figure 4: GATA-1 overexpressing CFU-Es fail to differentiate and undergo apoptosis *in vitro*. *a*. Representative histogram plots of FSC (indicating relative cell size) of viable erythroid cells from CFU-E assays performed on disaggregated fetal liver cells of 13.5 dpc embryos. Cells harvested before 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*. Due to a background of dead cells with a FSC <327 (using our instrument settings), present in all CFU-E samples, cells with a FSC<327 were excluded from this analysis.

To determine the differentiation stage which is affected in the transgenic males, 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 were performed. The same number of colonies in BFU-E and CFU-E assays are 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 [Cormack, 1976 #13] and do not require accessory cells to form [Sawada, 1989 #14], 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 (Figure 5a) and after 24 hours approximately 40% of erythroid cells are dead, compared to 20% in wild-type cultures (Figure 5b). Female transgenic CFU-E cultures have an intermediate phenotype (Figure 5a and 5b), identical to that found when wild-type and male transgenic cells are mixed before or after culture at a 1:1 ratio. This demonstrates that female GATA-1 overexpressing erythroid precursors are intrinsically defective and behave identically to transgenic male erythroid precursors when removed from the fetal liver.

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 [Apfeld, 1998 #15]. In mosaics (ie. 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 (Figure 5). Thus, the conventional interpretation of cell nonautonomy is incorrect.

a



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 5: 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*. Due to a background of dead cells with a FSC <327 (using our instrument settings), present in all CFU-E samples, cells with a FSC<327 were excluded from this analysis.

Possibly the mutant erythroid cells produce a negative factor inhibiting differentiation and this is diluted in mosaics. However, to be cell nonautonomous, 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 [Whyatt, 1997 #6][Dubart, 1996 #16][Briegel, 1996 #17], this is unlikely. Since GATA-1 overexpressing erythroid cells are intrinsically defective, they must be responding to a signal reversing these defects in mosaics. Therefore, 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 [Bessis,

1983 #18]. 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 [Bessis, 1983 #18][Bernard, 1991 #19] (Figure 6). The obvious candidate source of REDS is mature erythroid cells, since 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 [De Maria, 1999 #20]. It is therefore possible that death ligands are a component of REDS. Supporting this, death ligands partially reverse the effect of GATA-1 overexpression *in vitro* (data not shown).

There is one precedent for a signalling mechanism similar to that 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 was not associated with an obligatory requirement for DAF-2 activity in a particular cell. Consequently, this study suggested that secondary signals operate to ensure that all cells adopt the same developmental fate [Apfeld, 1998 #15]. 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 [Williams, 1994 #21][Maandag, 1994 #22]. This conclusion may be incorrect, since transplantation studies suggest that pRb is required in erythroid cells [Hu, 1997 #23]. This discrepancy remains unexplained. However, one could resolve these conflicting data by suggesting that the effect of pRb loss in erythroid cells is reversed by a signal supplied by wild-type cells, similar to that which restores the normal differentiation of GATA-1 overexpressing erythroid cells.

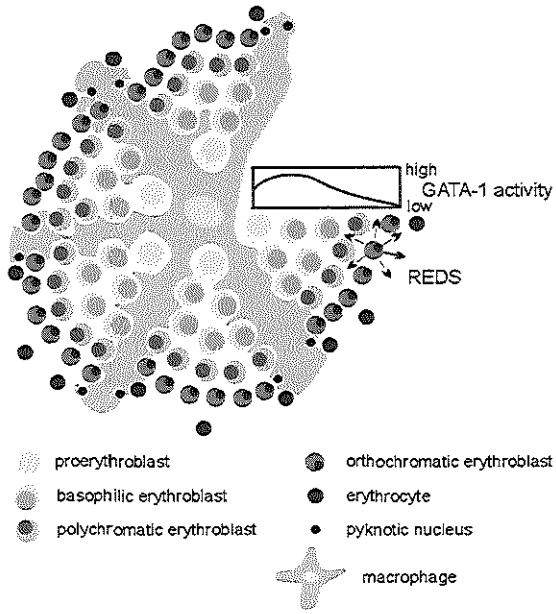


Figure 6 A simplified model of the erythroblastic island. The position of the central macrophage and the arrangement of the erythroid precursors (as indicated by the key) within the macrophage's cytoplasmic extensions are shown. The process of pyknosis, enucleation and phagocytosis of expelled pyknotic 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 previously described [Elefanty, 1996 #24]. 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 (gift of N. Brockdorff, London). Embryos were sexed by PCR using primers specific for the Zfy gene [Nagamine, 1989 #25].

Western blotting

Nuclear extracts were prepared as previously described [Andrews, 1991 #26]. 5μg of each sample was subject to electrophoresis through a 10% SDS/polyacrylamide gel, transferred onto nitrocellulose and probed with anti-GATA-1 antibody N6 (Santa Cruz, CA, cat# sc-625) or anti-cyclin A2 antibody C-19 (Santa Cruz, cat# sc-596) 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 [Pandolfi, 1995 #27] 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.

Hemoglobin analysis

Globin chain representation in chimeric animals was analysed as described previously [Maandag, 1994 #22].

DNA- and RNA-FISH

DNA-FISH was performed as previously described [Mulder, 1995 #28]. RNA-FISH was performed on disaggregated fetal liver cells as previously described [Wijgerde, 1995 #29].

CFU-E assay

CFU-E assays were performed as previously described [Wong, 1986 #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 FACS analysed as described previously [Sawada, 1989 #14]. $5x10^4$ events taken per sample. Antibodies and stains used: 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 ($ER\text{-}MP20^{high}/TER119^-$).

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Chapter 3

A conditional knockout allele of the GATA-1 gene

Fokke Lindeboom, Alar Karis, Frank Grosveld and Sjaak Philipsen

Abstract

To elucidate the function of the transcription factor GATA-1 beyond 10.5 dpc, a conditional knockout allele was generated, using the Cre-loxP system. The GATA-1 coding exons were bracketed by two loxP sites yielding the "floxed" GATA-1 locus. We fused Cre with the ligand-binding domain (LBD) of the estrogen receptor containing a mutation that renders it sensitive to activation by 4-hydroxy Tamoxifen. A μLCR/β-globin promoter construct was used to express the Cre protein or the inducible Cre-LBD protein in erythroid cells. We found that Tamoxifen induction resulted in a very low frequency of recombination, suggesting that Cre-LBD is an inefficient recombinase, or that it was not high enough expressed. We obtained germline transmission of the GATA-1 null allele through breeding of the floxed GATA-1 mice to a transgenic mouse line expressing Cre under the zona pellucida 3 promoter.

Introduction

The GATA transcription factor family consists of six members (GATA-1 to 6). The family can be divided in two groups, the hematopoietic (GATA-1, -2 and -3) and the heart and gut group (GATA-4, -5 and -6) (Weiss and Orkin 1995; Molkentin 2000). The members share highly homologous Zn-finger DNA binding domains, and they all bind to the consensus binding site (T/A)GATA(A/G). There is little sequence conservation outside the DNA binding domain. Most members are important for differentiation and proliferation of specific cell types (Pevny et al. 1991; Tsai et al. 1994; Pandolfi et al. 1995; Molkentin et al. 1997; Koutsourakis et al. 1999; Molkentin et al. 2000).

GATA-1 is a tissue-specific transcription factor expressed in erythrocytes, megakaryocytes, eosinophils, mast cells and in the Sertoli cells of the testis (Ito et al. 1993). GATA-1 is essential for the progression of erythroid precursor cells beyond the proerythroblast stage (Pevny et al. 1991; Fujiwara et al. 1996). The GATA-1 null mutant mouse is embryonic lethal around day 10-11 of gestation. To further investigate the role of GATA-1 later in development and in the different tissues in which GATA-1 is expressed we generated a conditional knockout allele of the GATA-1 gene using the Cre-loxP recombination system. This approach is based on the observation that the bacteriophage P1 Cre recombinase protein excises the DNA between two directly repeated 34 bp recognition sequences called loxP sites.(Sauer 1998). We inserted two loxP sites followed by a splice acceptor site and GFP reporter gene in the GATA-1 locus by homologous recombination in mouse ES cells. Upon the action of Cre, the coding exons of the GATA-1 gene are deleted and transcripts from the

first, non-coding, exon of the GATA-1 gene are spliced onto a splice acceptor site provided by the GFP reporter.

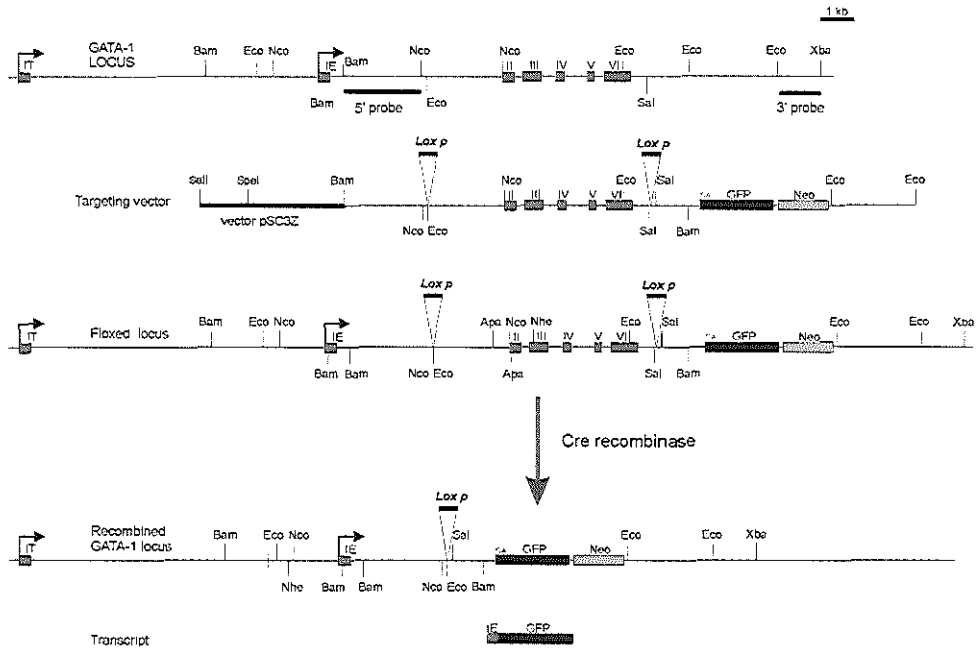
To gain control over the activity of Cre, we constructed a Cre protein fused to the ligand-binding domain of the estrogen receptor (Cre-LBD). Fusion to the ligand binding domain confers hormonal control because of binding to heat shock protein hsp90 thereby inhibiting nuclear transport and/or activity of the Cre protein (Scherrer et al. 1993). We used a mutant LBD that is sensitive only to the exogenous ligand 4-hydroxy-Tamoxifen (4OH-T) (Littlewood et al. 1995), thus rendering the activity of Cre dependent on the presence of 4OH-T (Chambraud et al. 1990). Transgenic mice were generated that express the Cre-LBD under the transcriptional control of the β-globin μLCR and promoter. Compound transgenic mice were made containing the CreLBD transgene and the floxed GATA-1 allele. *In vivo* administration of Tamoxifen, even for a prolonged period of over three months, did not result in any detectable recombination of the GATA-1 locus. However, when we differentiated fetal livers cells in hanging drop cultures (Chapter 5) in the presence of 4OH-T, we observed 4OH-T dependent Cre-mediated recombination of the floxed GATA-1 locus. The frequency was very low thus precluding the analysis of the GATA-1 knockout phenotype in fetal liver cells. We conclude that the Cre-LBD protein is an inefficient recombinase or that the CreLBD was not high enough expressed; this is supported by our observation that the expression of Cre with the same β-globin μLCR/promoter construct results in quantitative recombination of the floxed GATA-1 locus in the fetal liver. To generate GATA-1 null mutant mice we bred the floxed GATA-1 mice though the female germline of a transgenic line expressing Cre under the promoter of the Zona Pelucida 3 promoter (zp3 Cre) (Lewandoski et al. 1997). The zp3 transgene, the Cre protein is expressed in the oocyte, this resulted in the establishment of a mouse line carrying a true *null* mutation for the GATA-1 gene.

Results

Construction of the floxed GATA-1 locus

Two loxP sites were inserted in the GATA-1 locus. The targeting construct (Fig. 1A) was designed with the first loxP site inserted in the first intron between exon Ia and Ib, and the second was placed after exon VI. At the 3' site of this loxP site, a splice acceptor sequence and a GFP gene were placed, with the aim to render GFP expression, after recombination, dependent on transcription of the GATA-1 gene from the endogenous non-coding first exons.

A



B

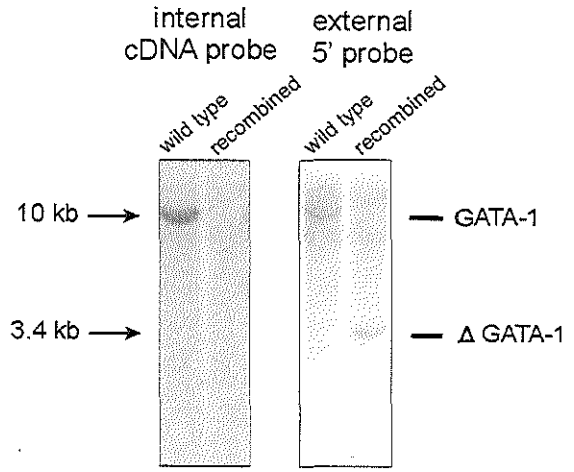


Figure 1: (A) GATA-1 locus, targeting vector, floxed locus, recombined locus and the GFP transcript of the recombined locus, (B) ES cell clone screened for the loss of the GATA-1 gene. Genomic DNA was cut with BamHI, blotted and hybridised with an external 5' probe and an internal probe (Fig. 1)

Analysis of the ES cells

ES cells, of male origin, were electroporated with the linearised (digestion with SpeI) knockout construct and grown in medium containing G418. After 10 days, 300 clones were picked and screened for the presence of the desired homologous recombination event with a external 3' probe flanking the right homologous arm (Figure 1A). Approximately 10% had recombined correctly at the 3' end of the construct. Positive clones were karyotyped and two clones, with the correct number of chromosomes, were electroporated with a plasmid expressing Cre, under the transcriptional control of the PGK promoter and with a hygromycin resistance gene. These ES cells were selected for hygromycin resistance and clones were picked screened for the recombination at the GATA-1 locus (Figure 1B).

To assess whether the floxed GATA-1 locus is still fully functional, an ES cell line containing the floxed GATA-1 locus, a recombined clone derived from this line, and wild type ES cells were subjected to *in vitro* differentiation to the erythroid lineage (Weiss et al. 1994). The differentiated wild type and floxed GATA-1 ES cells formed colonies of hemoglobin producing cells which looked identical, while the differentiated knockout cells hardly showed formation of any colonies of hemoglobinised cells (Fig. 2). This leads to the conclusion that in the GATA-1 lox ES cells the GATA-1 locus is still functional and that we successfully deleted it in the GATA-1 KO ES cells.

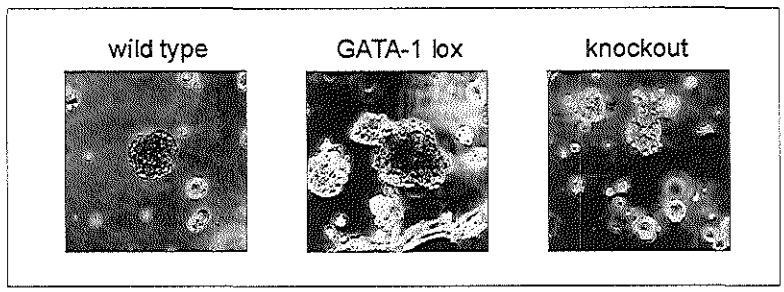


Figure 2: In vitro differentiated wild type, GATA-1 floxed and GATA-1 KO (GATA-1 floxed/ PGK Cre compound) ES cells.

Chimaeric mice

The floxed GATA-1 ES cells were injected into C57/bl6 blastocysts and chimaeric mice were born. These mice transmitted the floxed GATA-1 allele to their offspring. Male F2 mice carrying the floxed GATA-1 allele survived, showing that GATA-1 is expressed at physiological levels from the floxed allele.

Null mutant mice

Transgenic mice with the Cre protein expressed under the control of the zona pellucida 3 gene promoter were bred with the floxed GATA-1 mice to generate GATA-1 null mutant mice. Female offspring with both the Cre transgene and the floxed GATA-1 locus transmitted the recombined GATA-1 locus to their offspring, but below the frequency expected according to Mendelian distribution. Out of 94 pups born, 8 were heterozygous females (8,5%). The expected ratio is 1out of 7 (14,3%). That less GATA-1 heterozygous females would be born could be predicted, as it has been reported that one third of the heterozygous females die *in utero* due to anaemia caused by random inactivation of the X chromosome bearing the normal allele (Fujiwara et al. 1996; Tsai et al. 1998).

To recombine the floxed GATA-1 allele in an erythroid-specific manner, mice were generated by breeding the floxed mice with mice expressing the Cre protein under the control of the β-globin μLCR and promoter (pEV-Cre mice). These erythroid knockouts (GATA-1 lox + / pEV-Cre males) were indistinguishable form the null mutant embryos and died by 11.5 dpc due to anemia (Fig. 3). Furthermore, GATA-1 lox +/- / pEV-Cre females were often anaemic and sometimes also dead due to variable X-inactivation (Fig 3.)

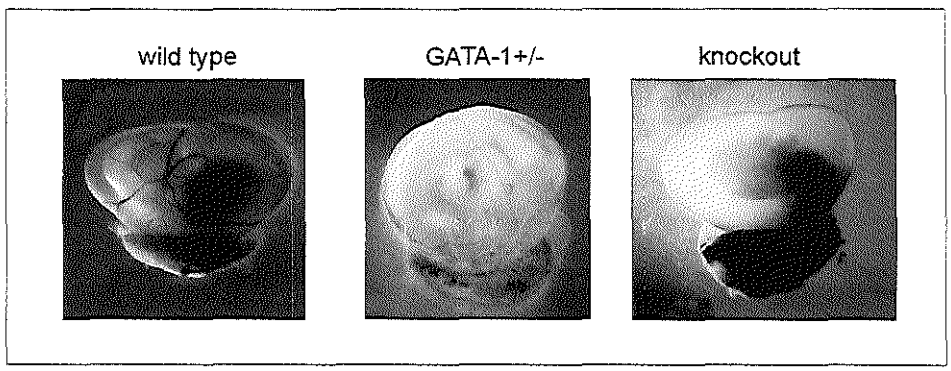


Figure 3: Wild type, GATA-1 +/- female and knockout male 12.5 dpc embryos.

Tamoxifen induced recombination

Transgenic mice were generated that express the Cre-LBD fusion-protein under the transcriptional control of the β-globin μLCR and promoter. Compound transgenic mice were made containing the CreLBD transgene and the floxed GATA-1 allele. To activate the Cre-LBD protein *in vivo*, GATA-1 lox + / CreLBD males mice were given drinking water with

100 µg/ml Tamoxifen and 1% ethanol (v/v). Hematological values the compound transgenic and wild type mice were measured after two and nine weeks. After nine weeks, the animals were sacrificed and spleen and bone marrow were isolated for DNA and RNA analysis. Spleens were weighed and no significant difference was measured (Table 1). Standard hematological values were also identical (Table 1).

	GATA-1lox+/ pEV CreLBD (n=5)	wild type (n=4)
Spleen (mg)	96 (±7)	83 (±20)
WBC ($x 10^9$)	7.7 (±2.6)	7.2 (±2.2)
RBC ($x 10^{12}$)	9.0 (±0.6)	9.8 (±0.6)
HBG (mmol/l)	9.0 (±0.8)	9.6 (±0.5)
HCT (l/l)	0.50 (±0.05)	0.54 (±0.03)
MCT (fl)	56 (±2)	55 (±3)
PLT ($x 10^9$)	1450 (±348)	1315 (±161)

Table 1: Spleen weight and hematological values after nine weeks of Tamoxifen administration. (WBC: white blood cell count, RBC: red blood cell count, HBG: Hemoglobin, HCT: hematocrit, MCV: mean cell volume, PLT: platelets)

No recombination of the floxed GATA-1 allele was detected by Southern blotting or with PCR. There were no differences in the FACS analysis of bone marrow cells after two days of differentiation in the hanging drop cultures (data not shown). This means that this recombination system is not sensitive enough for GATA-1 in erythroid cells *in vivo*. If any recombination was achieved it was too minimal to detect. It is furthermore very probable that erythroid precursors that might have recombined the GATA-1 locus underwent apoptosis and could not be detected. If this were happening at all, it did not influence the standard hematological values.

4OH-T induced recombination *ex vivo*

To determine whether recombination of the floxed GATA-1 allele could be achieved *ex vivo*, fetal liver cells were isolated from 12.5 dpc compound GATA-1 floxed +/PEV Cre-LBD embryos. The cells were used for hanging drop cultures (Chapter 5) with or without the addition of 4OH-T. DNA and RNA were isolated after 0,1, 2 and 3 days of culture and the cells were analysed by FACS for differentiation. Three different genotypes were used in this experiment: GATA-1 lox +/ Cre-LBD-/male, GATA-1 +/-/Cre-LBD +/-/female and GATA-1 lox +/Cre-LBD +/-/male. The results show that only the embryos heterozygous for the pEV Cre-LBD transgene are able to recombine the floxed GATA-1 allele, and that recombination is dependent on the presence 4OH-T (Fig. 4, lanes 9, 11, 12, 14, 15). After two days of culture a very minimal amount of recombination could be shown by PCR (Fig.4 lane 9). This increased after 3 days of culture (Fig. 4, lanes 11, 12). The recombined GATA-1 knockout PCR product is however preferentially amplified over the floxed GATA-1 PCR product as seen by using genomic DNA of GATA-1 +/- females (data not shown).

Thus Cre-LBD is functional and inducible by 4OH-T, recombination is at too low a level to be complete, estimated to be lower than <5% after two days of hanging drop culture.

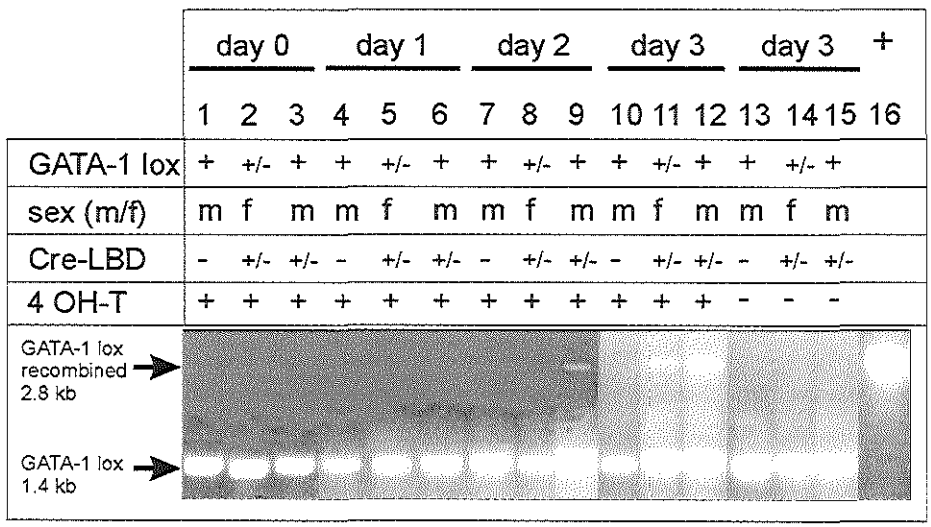


Figure 4: Ethidium bromide stained agarose gel of the products of a PCR on genomic DNA of erythroid precursor cells, cultured in hanging drops for 0, 1, 2, or 3 days, with or without the presence of 4OH-T. Three compound transgenic genotypes are present: GATA-1 lox +/ PEV Cre-LBD-/male (lanes 1, 4, 7, 10 and 13), GATA-1 +/-/Cre-LBD +/-/female (lanes 2, 5, 8, 11 and 14) and GATA-1 lox +/Cre-LBD +/-/male (lanes 3, 6, 9, 12 and 15). Lane 16 is a positive control PCR for the recombined locus. This is a representative of three experiments experiment, a total of 32 embryos were analysed.

After two days of hanging drop culture, RNA from $4x10^4$ cells was isolated and RT PCR performed and showed that GATA-1 mRNA levels are not affected by the addition of 4OH-T to the cultures (Figure 5). This can be explained by the minimal recombination shown by the genomic PCR (Figure 4).

To assess differentiation, the cells were harvested from the hanging drop cultures and subjected to FACS analysis. Viable erythroid cells were analysed for size by forward scatter as a measurement for differentiation. This did not reveal changes in the differentiation profiles between the genotypically different progenitors after addition of 4OH-T to the media (data not shown). Finally, BFU-E assays with and without 4OH-T also showed no difference between the genotypically different progenitors (data not shown).

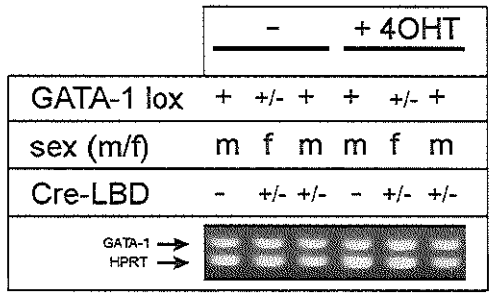


Figure 5 RT PCR for GATA-1 (product 297 bp) and HPRT (product 247 bp) on total RNA from erythroid cells after being cultured in hanging drops for two days. Male GATA-1 lox mice, with or without one inducible Cre allele, PEV CreLBD. Female mice were heterozygous for the GATA-1 lox allele and heterozygous for the inducible Cre allele, PEV CreLBD.

Discussion

To learn more about the function of the transcription factor GATA-1, an inducible knockout was designed using the Cre-loxP system. To get complete recombination from the oocyte onward the GATA-1 lox mice were transmitted trough a transgenic mouse line expressing Cre under the ZP3 promoter. An erythroid specific knockout was generated by breeding the floxed GATA-1 mice with transgenic mice expressing Cre under the control of the μLCR and the β-globin promoter. The activity of the Cre protein was made inducible by the fusion to the ligand-binding domain of the estrogen receptor rendering it sensitive to the exogenous hormone 4-hydroxy Tamoxifen. Transgenic mice were generated in which the expression of the inducible Cre protein was under the control of the μLCR and the β-globin promoter.

Unfortunately the activity of the inducible Cre protein was insufficient to generate a phenotype in mice or isolated cells.

There can be several reasons why the inducible Cre-LBD functioned so poorly. The sensitivity to 4OH-T could have been too low. After we started doing our experiments an improved version of the LBD was published that has a 3 to 4 times higher sensitivity for 4OH-T in cell lines (Feil et al. 1997) and up to 10 times higher in mice (Indra et al. 1999). Possibly, delivery of the 4OH-T to the bone marrow is ineffective. This could also have been a problem in a comparable system driven by the CMV promoter (Brocard et al. 1997). The issue of drug delivery does not apply when the cells are cultured in a dish and we were indeed able to show recombination in this experimental set-up. However, the majority of the cells still contained the floxed GATA-1 locus. A formal possibility is that the progenitors are rapidly removed through apoptosis after recombination. However, if this were the case we should have seen recombination occurring in female cells in which the inactive X chromosome carried the floxed locus. A more likely explanation is that addition of the LBD interferes with the activity of the Cre protein also in the presence of a ligand. During the recombination process, a complex of four Cre molecules bound to two loxP sites must be formed. Proper spatial alignment of this complex is important to catalyze the recombination reaction. Since the LBD is very bulky, it may impair the efficiency of the reaction through steric hindrance. Nevertheless, there are examples in the literature describing systems where Cre-LBD is active in vivo and in cultured cells (Feil et al. 1997; Li et al. 2000; Vooijs et al. 2001). Other important parameters are suggested to be the distance between the two lox sites, the locus used, the expression of the Cre-LBD, the tissue and the expression of the target gene (Vooijs et al. 2001). In our case the distance between the lox sites is unfavourably large (10 kb). The Cre-LBD protein is expressed but the level of expression might have been too low and mosaic.

The concept of an inducible deletion of a gene is an excellent idea but the methods have to improve. Other ways to get inducebility have been tried, making use of the glucocorticoid receptor ligand binding domain (Brocard et al. 1998), ecdyson receptor (Sawicki et al. 1998), the Tet-system (St-Onge et al. 1996) or even a combination of the ER-LBD with the Tet-system (Chiba et al. 2000). Finally, an alternative way of delivering the Cre protein has been described. A fusion of Cre to a short membrane translocation sequence (MTS) can be offered to cells immediately by intraperitoneal injection (Chen and Behringer 2001; Jo et al. 2001) or simply by adding it to cells (Le et al. 1999).

Despite our disappointing results with the inducible Cre-LBD system, the floxed GATA-1 mouse is still an important tool. In Chapter 4, we describe the Sertoli cell-specific

knockout of GATA-1 through the specific deletion of the gene by a Cre transgene expressed under the control of the Desert Hedgehog promoter. In addition, the role of GATA-1 in megakaryocytes, mast cells and eosinophil cells can be investigated by breeding floxed GATA-1 mice with mice that express the Cre protein under the control of the appropriate regulatory sequences.

Materials and Methods

Construction floxed GATA-1 locus

The 3'homologous region is an EcoRI fragment 3' from the last exon isolated from the GATA-1 locus containing cosmid pTCF 3'mGATA-1. A larger BamHI/SalI fragment that contained all the coding exons was cloned into a low copy number plasmid to yield pSC3Z m GATA-1 genomic. In this construct, the 5'loxP site was inserted in an EcoRI site in the intron between the first erythroid non-coding exon IE and the second exon.
In plasmid pGT1.8 IRES βgeo the IRES and the lacZ gene were replaced (SacI-Xho/blunt) with a GFP gene from the plasmid phGFP-S65T (SacI-BamHI/blunt). The cassette with the splice acceptor, GFP and the Neomycin selectable marker was excised with SalI.
The 3'loxP site (XhoI fragment), the GFP cassette (SalI fragment) and the 3'homologous region (EcoRI fragment) were put together in pBluescript and taken out with SpeI and PvuI. This fragment was inserted in the SalI site (3'of the 6th and last exon) of the pSC3Z mGATA-1 genomic clone vector containing the 5'loxP site. The construct was linearised with SpeI.

ES cells

E14 ES cells were electroporated with the targeting construct and grown in medium containing G418 (Neomycin resistance selection). ± 300 clones were picked and screened by Southern blotting for homologous recombination in the GATA-1 locus. The genomic DNA was digested with XbaI and the blot was hybridised with probe 3' GATA-1 EcoRI-XbaI, which is adjacent to the 3' homologous region. The targeting efficiency was ~10%. Genomic integrity of ES cell clones was checked by karyotyping. Recombinant and wild type ES cells were transiently transfected with a Cre expression plasmid (pGK Hyg Cre, a kind gift from G.Weeda) and put on hygromycine selection. Screening for recombination was done by Southern blotting using a 5'GATA-1 probe (5' part of the construct BamHI-NcoI just before the 5'lox site, figure 1A).

Construction of the inducible Cre-LBD fusion protein.

Cre was recovered with PCR and cloned in pGEM-T (Promega). A BamHI site was created at the 3'end of the Cre coding region by PCR. A BamHI-EcoRI fragment of the estrogen receptor that comprised the ligand binding domain (LBD) with the mutation (Gly 525 →Arg) (a kind gift from M. Parker) was ligated in frame to the 3 end' of the Cre coding sequence. The fusion construct was taken out with Sal and SacII and pEV3 vector that uses the μLCR and the β-globin gene promoter to drive erythroid expression of inserted cDNAs (Needham et al. 1992).

Mice

Chimaeric mice were generated by injecting an ES cell clone with the floxed GATA-1 locus in C57/bl6 blastocysts. Chimeras were then bred with wild type C57/bl6 females and the offspring was screened by coat colour for germline transmission. Transgenic mice were generated by oocyte injection.

In vitro differentiation of ES cells

ES cell were grown in IMDM medium (+Glutamine) containing FCS (10%), non-essential amino acids(Gibco BRL), penicillin (100 U/ml final), streptomycin (100 μg/ml), β-mercaptoethanol (100 μM), monothioglycerol (150 μM) and leukemia inhibitory factor (LIF) (1000 U/ml) for 2 days and collected. They were then taken up in a differentiation medium which is the medium described above without LIF but with Epo (1 U/ml) and IL-3 (10ng/ml). The cell concentration was adjusted to $5x10^4$cells/ml. 20 μl droplets were pipetted on the inside of the lid of a bacterial dish and put back on the dish filled with PBS. After 2 days the cells have formed aggregates. They were collected and grown in the same medium for 4 additional days after which most of the cells have attached to the surface. The cells were trypsinised, taken up in differentiation medium with 3% methylcellulose, insulin (40 ng/ml) and stem cell factor (SCF) followed by incubation for 4-6 days.

Tamoxifen

Tamoxifen (Sigma) was dissolved in absolute ethanol at a concentration of 10 mg/ml. For *in vivo* treatment, this stock solution was added to the drinking water to obtain a final Tamoxifen concentration of 100 μg/ml.

Hematological analysis

Mice were bled by orbital bleeding and blood samples were analysed on a F800 microcell counter.

Hanging drops

Hanging drop cultures were performed as described in Chapter 5. Cells were cultured with or without the addition of 4OH-T to a final concentration of 5 μM). 15 drops were used for FACS analysis, 15 for DNA isolation and 30 for RNA isolation.

Detection of the knockout allele by PCR

For the detection of the knockout allele three PCR primers were used:

A (5'-CGCCGAGCTGTGTGTAGTAA-3'),

B (5'-TTCCTCTTTCTCCTCCTCCG-3') and

C (5'-GGTGCTCAGGTAGTGGTTG-3).

Primers A &B are in the GATA-1 locus, 5' and 3' of the first loxP site. Primer C is in the GFP sequence. These primers can generate three products:

A-B: 1.4 kb, floxed GATA-1 locus

A-C: +/- 10 kb, floxed GATA-1 locus

A-C: 2.8 kb, recombined GATA-1 locus (knockout)

We observed that the 2.8 kb product is preferentially amplified over the 1.4 kb product. A robust quantitative analysis of recombination efficiency is therefore not possible with these primers. Expand polymerase (Boehringer Mannheim) was used for 30 cycles (30 sec 94C, 1 min 60C and 3 min. 68 C).

RT PCR

Total RNA was isolated with the tri reagent (Sigma) from cells grown in hanging drop cultures . After oligo-dT primed cDNA synthesis, PCR was performed with the following primers:

GATA-1 sense: 5'-TCCTCTGCATCAACAAGCCCA-3',

GATA-1 anti: 5'-GTTGAGCAGTGGATACACCTG-3', product size 297 bp.

HPRT sense:5'-CACAGGACTAGAACACCTGC-3',

HPRT anti: 5'-GCTGGTGAAAAGGACCTCTC-3', product size 247 bp.

SuperTaq polymerase was used for 28 cycles (30 sec. 94C, 30 sec. 60C and 3 min. 72).

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Chapter 4

A tissue-specific deletion reveals that GATA-1 is not essential for Sertoli cell function.

Fokke Lindeboom, Nynke Gillemans, Alar Karis, Martine Jaegle, Dies Meijer, Frank Grosveld and Sjaak Philipsen

Abstract

GATA-1 is a transcription factor that is essential for the development of erythroid cells: GATA-1 null mutants die *in utero* due to the consequences of severe anemia. Outside the hematopoietic system, GATA-1 is expressed in the Sertoli cells of the testis. To elucidate the function of GATA-1 in the mouse testis, we made a Sertoli cell-specific knockout of the GATA-1 gene using the Cre-loxP system. In these experiments a normally functioning "floxed" GATA-1 gene was deleted in Sertoli cells *in vivo* by means of a Cre transgene driven by the Desert Hedgehog gene promoter. Surprisingly, morphology of the testis and spermatogenesis were not affected by deletion of GATA-1 in Sertoli cells, demonstrating that GATA-1 is not essential for testis development or spermatogenesis in the mouse.

Introduction

The GATA transcription factor family consists of six members (GATA-1 to -6). The family is divided in two groups, the hematopoietic (GATA-1,-2 and -3)(Weiss and Orkin 1995) and the heart and gut group (GATA-4,-5 and -6) (Molkentin 2000). The members share their highest homology in the Zn-finger DNA binding domain and they all bind to the consensus binding site (T/A)GATA(A/G). Outside the DNA binding domain they are quite different. Most members are important for the proliferation and differentiation of specific cell types (Introduction, this thesis).

GATA factors are thought to be important transcriptional regulators of several Sertoli cell-specific (Hales 2001; Tremblay and Viger 2001). These target genes have GATA sites in their promoters that are essential for optimal expression of reporter genes in transient transfection assays (Tremblay and Viger 2001). In Sertoli cells GATA-1, -4 and -6 are expressed (Ito et al. 1993; Yomogida et al. 1994).

Besides Sertoli cells GATA-1 is expressed in erythrocytes, eosinophils and mast cells (Evans and Felsenfeld 1989; Tsai et al. 1989; Martin et al. 1990; Romeo et al. 1990; Zon et al. 1993). GATA-1 is essential for erythroid precursor cells to progress beyond the proerythroblast stage (Pevny et al. 1991). The GATA-1 transcription unit contains two alternative first exons that are non-coding (IT and IE) and five coding exons (II to VI). The GATA-1 gene is thought to have two distinct promoters which are alternatively spliced to the coding exons (Ito et al. 1993). Exon IT is primarily used in Sertoli cells of the testis and exon IE in erythroid cells. Since the second exon harbours the start of translation, the proteins expressed in erythroid cells and the testis are identical. The testis-specific promoter and first exon (exon IT) are located 8 kb upstream of the erythroid specific first exon (exon IE). Both

promoters harbour GATA sites that are required for proper promoter function and hence a positive feedback loop was suggested but a promoter study in GATA-1 null cells and overexpression of GATA-1 in MEL cells do not support a regulatory loop (Tsai et al. 1991; McDevitt et al. 1997; Onodera et al. 1997b; Whyatt et al. 2000). In the mouse and the rat gene, erythroid and a testis specific promoter elements have been described (Onodera et al. 1997b). It has been suggested that GATA-1 is a developmental-stage and spermatogenic-cycle specific regulator of gene expression in Sertoli cells (Yomogida et al. 1994).

Although no GATA-1 target genes in the Sertoli cells have been found it is believed that the anti-müllerian Hormone (AMH), also known as müllerian inhibiting substance (MIS), is a possible target (Watanabe et al. 2000). A model has been proposed in which GATA-4 is involved as a positive regulator of AMH expression in the early fetal/perinatal pre-Sertoli cells while GATA-1 acts as a repressor of AMH expression at later stages (Beau et al. 2000; Watanabe et al. 2000). The expression patterns of the GATA factors and AMH conform to this model and, GATA-1 has also been shown to be able to bind to the AMH promoter in vitro (Ketola et al. 1999; Beau et al. 2000). Furthermore, two human males harbouring a mutation in the GATA-1 gene displayed cryptorchidism (Nichols et al. 2000); a phenotype also observed in mice overexpressing very high levels of AMH under the metallothionein-1 promoter (Behringer et al. 1990). However, cryptorchidism has not been reported for X-linked-thrombocytopenic males with different GATA-1 mutations (Freson et al. 2001; Mehaffey et al. 2001). Other Sertoli cell-specific candidate target genes of GATA-1 are the follicle-stimulating hormone receptor (FSHR), inhibin α subunit and inhibin/activin β-B-subunit. For FSHR, this is based on a conserved GATA site in the core promoter and the observations that this site is occupied in Sertoli cells as shown by *in vivo* footprinting and can bind GATA-1 in *in vitro* electro-mobility-shift assays (EMSA) (Kim and Griswold 2001). For inhibin α subunit and inhibin/activin β-B-subunit, the notion is based on transient transfection assays with a bacterial reporter gene driven by the corresponding promoters in a rat testicular Leydig tumor cell line MA-10 and mouse Sertoli cell line MSC-1 (Feng and Chen 1994) (Feng et al. 2000). In these studies, the GATA sites were essential for expression . In addition to this, the expression of the inhibin α subunit and the inhibin/activin β-B subunit occurs in a pattern similar to GATA-1 (Feng et al. 1989; Keinan et al. 1989; Krummen et al. 1989). The caveat of these experiments is that they were all performed in cell lines, and the results may therefore not reflect the *in vivo* role of GATA-1 appropriately.

The GATA-1 null mutant is embryonic lethal due to a block in erythropoiesis at the proerythroblast stage (Pevny et al. 1991; Fujiwara et al. 1996). To overcome this early embryonic lethality we made a conditional knockout allele (cKO) of the GATA-1 gene using

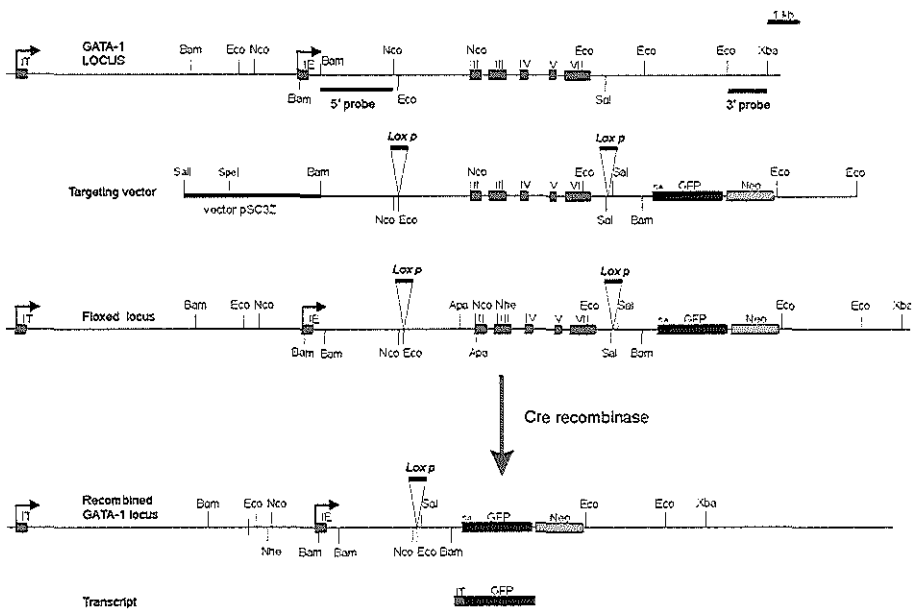
Cre-loxP (Sauer 1996). To delete the GATA-1 gene in Sertoli cells, we made use of a transgenic mouse line in which Cre expression is driven by the promoter of the Desert Hedgehog gene(Dhh). Dhh is expressed in Schann cells in the nervous system, vascular endothelium, endocardium and in Sertoli cells but not in hematopoietic cells (Bitgood et al. 1996). The expression of Dhh in Sertoli cells becomes apparent at 11.5 dpc, approximately one day after the formation of Sertoli cell precursors (Bitgood et al. 1996). This approach has enabled us to study the role of GATA-1 in the testis during development and in adult mice.

Results

Targeting of the lox sites in the GATA-1 locus

A targeting construct (Fig. 1a) was designed with the first loxP site inserted in an EcoRI site in the first intron, in between erythroid exon IE and exon II, and the second loxP after exon VI in the 3'flanking region of the gene. A splice acceptor sequence, GFP cDNA and neomycin selectable marker gene were placed 3' to the second loxP site, with the aim to activate GFP expression after Cre-mediated excision of the GATA-1 coding sequences. ES cells were electroporated with the targeting construct and grown in medium containing G418. Southern blot analysis of 300 clones showed that approximately 10% had recombined correctly at the 3' end of the construct. Several clones were transiently transfected with a Cre expression vector and screened for recombination. In one of these, we could demonstrate appropriate recombination (Fig. 1b). This clone was karyotyped, checked for correct number of chromosomes and used for blastocyst injections. Chimeric mice were born and mated with C57/Bl6. Offspring was checked for the conditional knockout (cKO) GATA-1 locus and backcrossed to FVB mice. In further generations, hemizygous and homozygous GATA-1 cKO mice were born according to Mendelian distribution. These mice were healthy and fertile with no apparent phenotype, indicating that expression of GATA-1 was not disturbed by the presence of two loxP sites and the GFP-neo cassette in the locus.

A



B

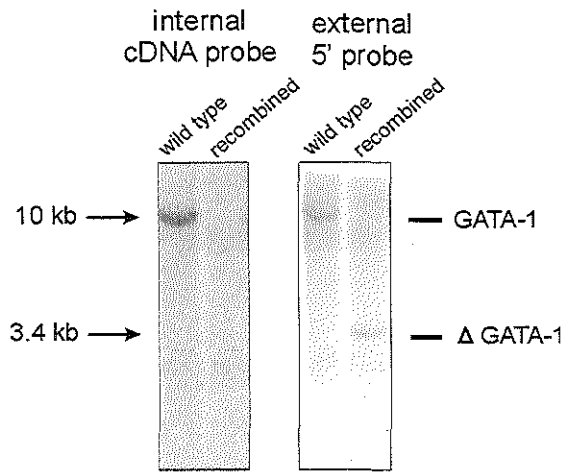


Figure 1: (A) GATA-1 locus, with the testis specific first exon (IT) used by the testis promoter approximately 8 kb upstream of the erythroid specific first exon (IE) used by the erythroid promoter. The coding exons (II –VI) are used in both mRNAs. After recombination with the targeting vector, the GATA-1 locus has two loxP sites and the splice-acceptor-GFP cassette inserted and is now called floxed. After recombination of the floxed GATA-1 locus the GATA-1 coding exons are deleted and the GFP transcript should be expressed. (B) Southern blot of genomic ES cell DNA after transient Cre transfection, digested with BamHI hybridised with an internal cDNA probe and the 5' GATA-1 probe, showing recombination of the floxed GATA-1 allele.

A Sertoli cell-specific null mutant for the GATA-1 gene.

To obtain mice that are Sertoli cell-specific null mutants for the GATA-1 gene, conditional knockout GATA-1 mice (cKO) were crossed with Dhh-Cre transgenic mice. In male offspring, Cre-mediated recombination at the GATA-1 locus occurred only in mice harbouring the Dhh-Cre transgene. However, we were unable to detect GFP expression with RT PCR or in cryosections (data not shown).

The absence of GATA-1 expression in the testis was checked by RT-PCR. No GATA-1 transcript could be detected in the GATA-1 cKO males with the Dhh-Cre transgene (Fig. 2).

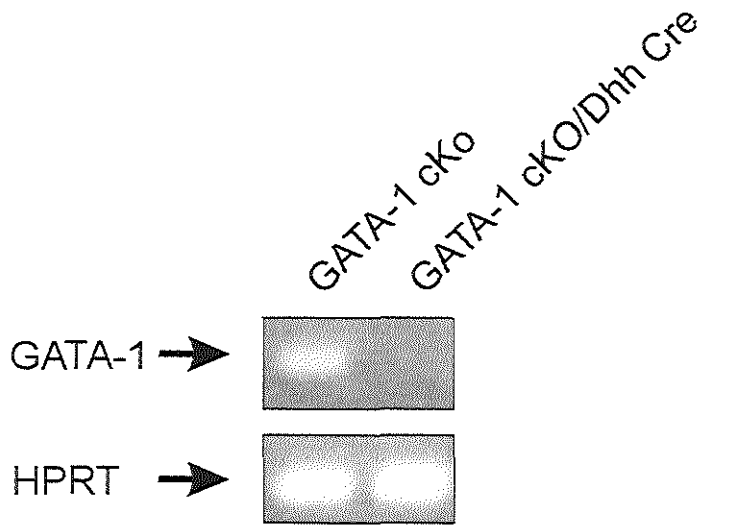


Figure 2: RT PCR for GATA-1 (297 bp) and HPRT (247 bp) on total testis cDNA from GATA-1 cKO and GATA-1 cKO/Dhh Cre male mice.

Fertility

No difference was found in breeding the 4 Sertoli cell-specific null mutants mice and their cKO littermates with wt FVB females. The Sertoli cell-specific null mutants gave rise to 8 litters had an average size (± 8 pups) and the distribution between males and females was equal (32-30 : 52-48%). Since the mice develop to maturity it can be concluded that the Dhh-Cre transgene does not express the Cre protein in erythroid precursor cells. The Sertoli cell-specific null mutants were willing to mate and the distribution of the several possible

genotypes was according to Mendelian law. Their fertility did not decrease as the mice aged (>1year).

Gonadal tissue and sperm counts

Testes, epididymides and seminal vesicles of GATA-1 cKO and Sertoli cell-specific GATA-1 knockout littermates of different ages were isolated and weighed and the gross morphology was compared. Sperm was isolated from the epididymides and cell numbers counted. Within the same age groups, no significant differences were found between any of the mice analysed (Table 1).

age (days)	ko/wt	n	testis (mg)	(sd)	epididymis (mg)	(sd)	seminal vesicles (mg)	(sd)	sperm count (x 10^6 per epididymus)	(sd)
24	wt	1	35.5	1.6	10.0	-	5.7	-	-	
	ko	3	42.1	3.9	10.5	2.1	6.4	1.3	-	
52	wt	1	67.5	21.9	23.2	-	52.3	-	18.0	
	ko	1	90.5	2.1	29.3	-	60.5	-	19.0	
74	wt	3	101.0	15.9	40.4	9.8	65.5	5.6	31.7	6.6
	ko	4	102.0	11.5	38.0	1.8	61.6	9.6	28.5	26.6

Table 1: A comparison of testes, epididymides, seminal vesicles and sperm counts of GATA-1 cKO and Sertoli cell-specific GATA-1 knockout littermates of different ages.

Expression of potential Sertoli cell-specific GATA-1 target genes

Real-time RT-PCR on RNA of testis from two-week-old KO and cKO mice was performed to compare the expression of suggested GATA-1 target genes AMH, FSHR, inhibin α-subunit and activin/inhibin βA and βB subunit. Also the expression of Star (steroidogenic acute regulatory protein) and Cyp19 (PII Aromatase) since these genes have essential GATA sites in their regulatory elements (Silverman et al. 1999) (Jin et al. 2000). As controls HPRT and cyclophiline primers were used (table 2). No significant differences could be detected leading to the conclusion that GATA-1 can be omitted for the proper expression of these genes.

Expression of other GATA family members

In erythroid GATA-1 null cells the transcription of GATA-2 is highly upregulated and this can imply some interchangeability between GATA factors (Weiss et al. 1994). Furthermore a

negative regulation of GATA-2 transcription by GATA-1 was suggested (Weiss et al. 1994). A transgene that expresses the GATA-2 cDNA under GATA-1 regulatory sequences can rescue the embryonic lethal phenotype of the GATA-1 knockdown mice (Takahashi et al. 2000). To see if there is such a relation between GATA factors in the testis, real-time RT-PCR on RNA of testis from two weeks old cKO and GATA-1 testis KO mice was performed to detect transcript levels of different GATA family members that are expressed. No difference in the levels of GATA-2, GATA-4 or GATA-6 between different genotypes were detected.

	cKO cycle threshold	(sd)	cKO Dhh Cre cycle threshold	(sd)
AMH	26.6	1.2	25.5	0.7
FSHR	25.3	0.3	24.7	0.6
inhibin α	23.5	0.4	24.1	0.6
inhibin βA	27.5	0.4	27.5	0.6
inhibin βB	22.8	0.1	23.1	0.1
STAR	23.7	0.2	24.3	0.2
Cyp19	28.1	0.1	26.8	0.4
GATA-2	27.6	0.4	26.4	0.6
GATA-4	24.2	0.4	23.8	0.3
GATA-6	24.7	0.2	24.6	0.3
HPRT	24.4	0.3	24.7	0.1
Cyclophiline	20.2	0.1	19.7	0.0

Table 2: Expression of possible target genes and GATA transcription factors measured with real-time PCR.

Discussion

Here, we describe the generation of a mouse line in which the GATA-1 gene has been flanked by loxP sites. After Cre-mediated recombination, the coding sequences of the GATA-1 gene are deleted and a GFP reporter gene is brought under the control of the GATA-1 erythroid- and testis-specific promoters. However, we have been unable to detect expression of the GFP reporter from the recombined locus, both in Sertoli- and erythroid cells (data not shown). Since expression of GFP was also negative by RT-PCR analysis, a likely explanation is that the part of the gene which is deleted by the recombination event contains important regulatory regions for the expression of the GATA-1 gene. The GATA-1 locus appears to contain multiple regulatory elements (Schwartzbauer et al. 1992; Trainor et al. 1995; Onodera et al. 1997a; Onodera et al. 1997b; Vyas et al. 1999). It is now known that at least one of these elements resides in the area deleted in the recombined locus (Onodera et al. 1997a); our data support the notion that this element is important for erythroid expression of the GATA-1 gene in the context of the endogenous locus. Apart from the testis-specific promoter, testis-specific enhancer elements of GATA-1 expression are not known; we suggest that such elements reside in the area deleted by Cre in the GATA-1 cKO locus described here. Most importantly, insertion of the loxP sites and the GFP-neo cassette gene did not appear to affect the expression of GATA-1 and the GATA-1 cKO mice developed normally.

Our data demonstrate that a Sertoli cell-specific knockout of GATA-1 does not result in an apparent phenotype based on the analysis of the expression of putative testis-specific GATA-1 target genes, testis development, spermatogenesis and male fertility. This is surprising since specific modulation of GATA-1 expression during testis development has been reported, and a number of potential target genes has been identified (Feng and Chen 1994; Yomogida et al. 1994; Feng et al. 1998; Beau et al. 2000; Feng et al. 2000; Watanabe et al. 2000; Kim and Griswold 2001). These target genes have been identified through *in vitro* promoter studies that utilize proximal promoter that stimulate the expression of reporter genes. These assays can only mimic the in vivo situation to a very limited extent for several reasons such as the possibility that important regulatory elements are omitted, the promoter not being in its normal chromatin environment and the cells used being transformed cells.

A testis promoter knockdown mutation in a mouse did not show a phenotype (Shivdasani et al. 1997). However, in these experiments it can not be excluded that residual GATA-1 expression from the erythroid promoter, masks the phenotype in Sertoli cells.

In the present study, we have used the Dhh-Cre transgene used to delete the GATA-1 gene in Sertoli cells. This promoter becomes active around 12.5 dpc in pre-Sertoli cells, long before GATA-1 expression is normally activated, 7 days *post partum* (dpp), in this cell

lineage (Bitgood et al. 1996; Beau et al. 2000). Thus, this is the first description of the consequences of a true GATA-1 *null* mutation in Sertoli cells. The absence of a notable phenotype could be explained by functional overlap with other GATA factors, most likely GATA-4 and GATA-6 because these are also expressed in Sertoli cells (Viger et al. 1998; Ketola et al. 1999). GATA-4 promotes the expression of several Sertoli cell-specific promoter constructs in tissue culture (Tremblay and Viger 2001). The model implicating GATA-1 in the downregulation of AMH expression predicts that the absence of GATA-1 in Sertoli cells would result in sustained high-level AMH expression directed by GATA-4. Aberrantly very high AMH levels may result in a phenotype, since overexpression of AMH in male mice can affect sexual development adversely (Behringer et al. 1990). However, we do not find any changes in AMH expression at 17 dpp in the GATA-1 mutants, and therefore conclude that if GATA-1 is involved in the physiological downregulation of AMH expression, it is not an essential regulator of this process at this timepoint.

Finally, it remains possible that the expression of GATA-1 in Sertoli cells is an unimportant whim of Nature with no functional consequences. This is unlikely since the expression is very specifically regulated and it is conserved between man and mouse (Yomogida et al. 1994). Furthermore, two human males bearing a GATA-1 mutation (V205M) displayed cryptorchidism (Nichols et al. 2000). Although our data suggest that loss of GATA-1 function may not be responsible for the observed cryptorchidism, an alternative explanation is that the V205M mutation has a dominant-negative effect, resulting in a more drastic phenotype than complete loss of GATA-1 activity in Sertoli cells. This suggests functional redundancy of GATA factors in Sertoli cell development. The work reported here sets the stage to address this issue through the analysis of compound Sertoli cell-specific knockout mutants of GATA factors, using the Dhh-Cre mice described in this paper.

Methods

Construction of the floxed GATA-1 locus

The 3'homologous region is an EcoRI fragment 3' from the last exon isolated from the GATA-1 locus-containing cosmid (pTCF 3'mGATA-1) A larger BamHI/SalI fragment containing all the coding exons was cloned into a low copy-number plasmid (pSC3Z m GATA-1 genomic). In this construct the 5'lox site was inserted in an EcoRI site in the intron between the first erythroid non-coding exon IE and the second exon.

In plasmid pGT1.8 Ires Bgeo the IRES and the lacZ gene were replaced (SacI-Xho/blunt) for a GFP gene from the plasmid phGFP-S65T (SacI-BamHI/blunt). The cassette with the splice acceptor, GFP and the Neo selectable marker was excised with SalI.
The 3'lox site (in XhoI), the GFP cassette (in SalI) and the 3'homologous region (in EcoRI) were put together in pBluescript and taken out with SpeI and PvuI. This fragment was inserted in the SalI site (3'of the 6th and last exon) of the pSC3Z mGATA-1 gene with the 5'lox site. The construct was linearised with SpeI and used to electroporate E14 ES cells.

ES cells

E14 ES cells were electroporated with the targeting construct and grown in medium containing G418 to select for integration of the neomycin gene. ± 300 clones were picked and screened by Southern blotting for homologous recombination in the GATA-1 locus. The genomic DNA was digested with XbaI and the blot was hybridised with probe 3' GATA-1 EcoRI-XbaI, that is adjacent to the 3' homologous region. Positive ES cell clones were transfected with a vector expressing Cre under the PGK promoter and a hygromycin resistance marker. Clones were screened for recombination by Southern blot analysis, using a 5'GATA-1 probe (5' part of the construct BamHI-NcoI fragment just before the 5'lox site).

Mice

Chimaeric mice were generated by injecting ES cell clones generated as above into C57/bl6 blastocysts. Chimearas were then bred with wild type C57/bl6 mice and screened for germline transmission. Transgenic mice were mated to FVB males.

RT-PCR

RNA was isolate with TRI REAGENT^{TM} (Sigma prod.nr. T9424).
5µ g of RNA were used for the reverse transcription reaction with oligo dT (18-mer) and random hexamers. The following oligonucleotides were used for RT-PCR and real time RT-PCR. Real-time PCR was preformed in triplo.

gene	sense and anti sense primer	product size (bp)
AMH	5'- AGCTGGACACCATGCCTTTC–3'	
	5'-AGGGTCTCTAGGAAGGGGTC–3'	88
Cyclophilin	5'-TCACCATTTCCGACTGTGGAC-3'	
	5'-ACAGGACATTGCGAGCAGATG-3'	120
Cyp 19	5'- TCGAGTACTTCCCTAAGCCC –3'	
	5'-GCCAAAAGGCTGAAAGTACC –3'	83

FSHR	5'- GCAAACTGGAGGCGGCAAAC –3'	
	5'- TCTGATCCCCAGGCTGAGTC –3'	97
GATA-1	5'-TCCTCTGCATCAACAAGCCCA-3'	
	5'GTTGAGCAGTGGATACACCTG-3'	297
GATA-2	5'-AGAACCGGCCGCTCATCAAG–3'	
	5'-GGTGGTGGTTGTCGTCTGAC–3'	92
GATA-4	5'-TCTCACTATGGGCACAGCAG–3'	
	5'-ACAGCACTGGATGGATGGAG–3'	82
GATA-6	5'-ATCCCAGAACCCATTCATCC–3'	
	5'-AGACCAAATGGCTCCCAGTG–3'	82
HPRT	5'-CACAGGACTAGAACACCTGC-3'	
	5'-GCTGGTGAAAAGGACCTCTC-3'	247
Inhibin-α mouse	5'-CTCCCAGGCTATCCTTTTCC-3'	
	5'-AGTGAAGAGGCCTTCCTCAG-3'	97
Inhibin-β-A	5'-AGCAGACCTCGGAGATCATC–3'	
	5'-CTGCCTTCCTTGGAAATCTC–3'	82
Inhibin-β-B	5'-AAACAATCCTTCGAGTGGCC–3'	
	5'-GGGTGTGTACGGACAGAAAAG–3'	98
StAR	5-GGAAGTCCCTCCAAGACTAAAC-3	
	5'-TGGTTGATGATTGTCTTCGG–3'	80

Sperm counts

Seminal vesicles were flushed with PBS and a fraction was counted under the microscope.

Acknowledgements

We thank An Langeveld for karyotyping, Axel Temmen for discussion, Geert Weeda for plasmid pGK Hyg Cre, Dave Whyatt for discussion and the pTCF 3'mGATA-1 and pSC3Z mGATA-1 genomic plasmids, Martine Jeagle and Dies Meijer for the Dhh-Cre transgenic mice.

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Chapter 5

A hanging drop culturing method to differentiate erythroid precursor cells

Fokke Lindeboom, Laura Gutierez, Rita Ferreira, Roy Drissen, Frank Grosveld, Sjaak Philipsen and David Whyatt

Abstract

We describe a suspension culture method to study the differentiation of primary erythroid precursor cells from fetal liver. With this system, differentiation of erythroid precursors occurs in very small volumes of culture media. The method is very useful when the number of cells is a limiting factor, and when many different media or conditions have to be tested, e.g. in screens for novel pharmaceutical compounds. We show that this method enables the analysis of cells during erythroid differentiation with a variety of techniques including FACS, cytospin and Western blotting.

Introduction

The maturation of erythroid progenitors is a complex process of which many aspect, such as gene expression, cell signalling and enucleation, are still unclear. Erythroid precursors differentiate *in vivo* in a structure called the erythroblastic island, a sponge-like structure composed of one or two central macrophages in close contact with erythroid progenitors in different stages of differentiation lying in the invaginations of the macrophage (Bessis et al. 1983). The adhesion between erythroid cells and the macrophage is thought to occur at the CFU-E stage of maturation (Breton-Gorius et al. 1991).

Erythroid precursors such as CFU-E, BFU-E and CFU-mix that are present fetal liver, are studied mainly in methyl cellulose-based (MeC) assays, that allow these precursors to form colonies (Wong et al. 1986; Metcalf 1989). Because these MeC assays are clonal assays, they can be used to determine the number of CFU-Es, BFU-Es or CFU-mixes by counting the number of colonies derived from these precursor cells. Disadvantages of these MeC assays are that they are labour-intensive, use only small numbers of cells because of the low cell densities that are required in these assays. Growing progenitors in suspension bags is an alternative method that allows the generation of large numbers of cells (Dolznig et al. 2001).

Here, we describe a method to culture primary erythroid precursor cells in hanging drops. Culturing cells in hanging drops has been described previously for a variety of different cell types. It is most commonly used in studies of T-cell development (Brenner et al. 1983; Sagara et al. 1997; Tokoro et al. 1998). Hanging drops are also used to study oocyte and embryo development (Sanders et al. 1978; Potter and Morris 1985; Spindler et al. 2000), ES cell differentiation (Prelle et al. 2000), mesenchymal cell aggregates and macrophages (Crowle and May 1978). So far, this culture system has not been applied to analyse terminal differentiation of erythroid precursor cells.

In this assay, ~$2.5x10^4$ fetal liver cells are placed in 20μl droplets of medium hanging from the lid of a Petri dish filled with PBS. Within two days, erythroid progenitors derived from the fetal liver differentiate quantitatively to mature erythrocytes. Major advantages of this method are that only small amounts of media and growth factors are needed, and that it requires limited numbers of cells. We describe a method for this purpose that uses a simple medium containing growth factors (erythropoietin (Epo) and insulin), fetal calf serum (FCS) and hemin. Erythroid cells derived form the fetal liver are grown for two days in a drop of medium suspended from the lid of a culture dish. By FACS analysis and cytospins, we show that the cells undergo terminal erythroid differentiation, including enucleation, during these two days. To validate the usefulness of this assay, we have used fetal liver cells of mice with perturbed expression of the transcription factor GATA-1, that have well-characterized defects in erythropoiesis.

Results

In order to have an assay in which we can mimic the erythroblastic island structure and differentiate erythroid progenitors in close proximity of each other we designed the erythroid hanging drop cultures. With this method the cells sink towards each other at the bottom of the droplet.

Maturation of erythroid precursors in hanging drop cultures

As erythrocytes mature they go through several morphological changes, including a pronounced decrease in size as they develop from proerythroblasts (14-19 μm in diameter) to basophilic erythroblasts (12-17 μm), polychromatic erythroblasts (12-15 μm), orthochromatic erythroblasts (8-12 μm) , reticulocytes and finally mature erythrocytes (7.8 μm maximum) (Dessypris EN 1998). We used this property to follow maturation of the primary murine fetal liver cells cultured in hanging drops.

After gating the late erythroid cells based on the TER119 marker, we used forward scatter (FSC) as a measurement for cell size and 7AAD staining as a measurement for cell death. At the start of the hanging drop culture (day 0), most cells are immature, and hence relatively large. During the two days of culture most alive cells had matured and decreased in cell size (Fig. 1).

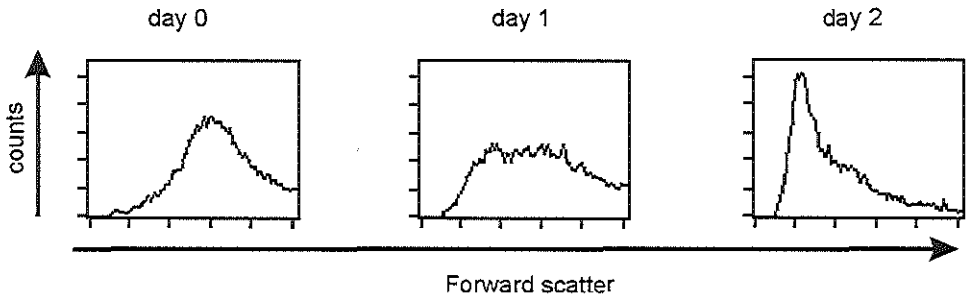

Figure 1 :Live erythroid cells (TER119$^+$ and 7AAD$^-$) analysed on size (forward scatter) : hanging drops day 0, day 1, day 2. Mouse erythroid cells were identified with the R-PE conjugated TER119 antibody, viability of the cells was measure by negative staining with stain 7-aminoactinomycin-D (7AAD). 10^5 events were analysed.

To confirm that the FACS analysis described above measures differentiation, cultured fetal liver cells were sorted in three populations: Ter119$^+$/FSC^{high}/7AAD$^-$, Ter119$^+$/FSC^{low}/7AAD$^-$ and Ter119$^+$/FSC^{low}/7AAD$^+$ cells. Cytospins were prepared from these sorted populations, and stained using a combination of neutral benzidine histological dyes. Hemoglobin has a peroxidase activity by which it can oxidize benzidine, or benzidine derivatives such as O-dianisidine, generating a brown chromophore which can be used as an estimate of hemoglobin concentration (Liem et al. 1979). This showed that FSC^{high}/7AAD$^-$ population contains the immature cells, since they display basophilic (blue) staining of their cytoplasm (Fig. 2A). In this population the majority of the cells still have a nucleus and only a minority stains weakly brown for hemoglobin. The FSC^{small}/7AAD$^-$ cells population contains mainly cells staining positive for hemoglobin; most of these cells have enucleated (Fig. 2B). The FSC^{small}/7AAD$^+$ appears to consist predominantly of expelled nuclei, occasionally still surrounded by a rim of cytoplasm (Fig. 2C).

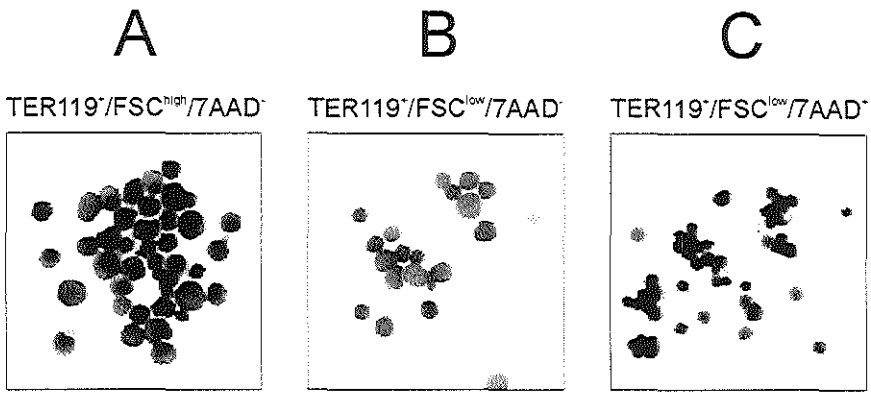

Figure 2: Cytospins of the sorted populations. (A) $TER119^{+}/FSC^{high}/7AAD^{-}$ cells, the immature erythroblasts, (B) $TER119^{+}/FSC^{low}/7AAD^{-}$ cells, the mature enucleated cells (C) $TER119^{+}/FSC^{low}/7AAD^{+}$, the expelled nuclei.

Composition of differentiation media

To examine which factors in the medium influence the differentiation and proliferation we compared several media and compared the hanging drop cultures with standard methyl-cellulose (MeC) CFU-E cultures. We followed differentiation by compared the number of small enucleated cells (M1), the intermediated sized cells (M2) and the big immature cells (M3) (figure 3). We varied the amount of fetal calf serum (FCS), Erythropoietin (EPO), insulin and hemin. The FCS is necessary for cell survival. The standard medium contains 20% FCS and a reduction to 10% FCS only resulted in a slight decrease in cell survival (figure 3, sample 1 and 2). Without FCS most cells did not survive (figure 3, sample 3). Hemin has a small but positive effect on differentiation and the viability of the $TER119^{+}$ cells during culture (compare sample 1, 7 and 8, figure 3) . Hemin is known to stimulate the hemoglobinisation of erythrocytes and stimulate erythropoiesis in vitro (Alter et al. 1989). The presence of insulin did not affect differentiation or viability (sample 1 and 6, figure 3) although it has been reported to be important for the final stages of erythropoiesis (Beug et al. 1995). Insulin is present in very low concentration in FCS and this might be sufficient for the maturing erythroblast. Surprisingly EPO did not appear to be a crucial factor. It is known to be important for the survival of the progenitors as this has been shown to be a role in vivo (Lacombe and Mayeux 1998) . This is reflected in the number of big cells (M3) in the culture with 0, 2 and 10 units of EPO (sample 5, 1 and 4 respectively, figure 3).

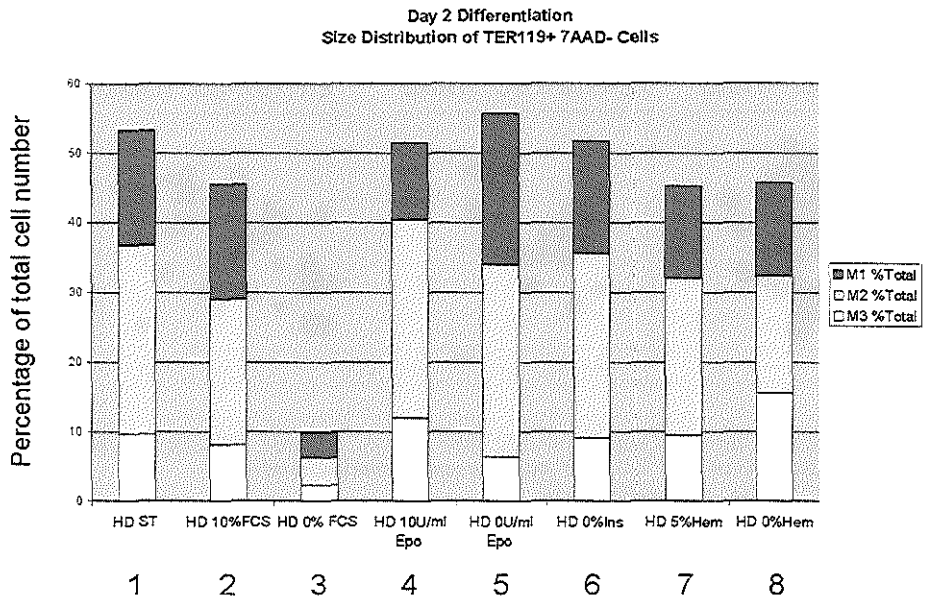

Figure 3: Differentiation of erythroid progenitor cells measured by cell size. M1 are the enucleated, small cells, M2 are the intermediate size cells, M3 are the immature, big cells. Percentages are of total number of cells in the culture. Cells cultured in 8 different media in hanging drops, $5x10^4$ events analysed. This is a representative of three experiments.

Genetically modified mice

GATA-1

The assay described above is particularly useful for the analysis of the late stages of erythroid differentiation. We examined several transgenic mouse lines with genetic modifications that influence the expression level of the erythroid-specific transcription factor GATA-1 in erythroid progenitor cells. GATA-1 is required for erythroid cells to mature beyond the proerythroblast stage (Pevny et al. 1991; Fujiwara et al. 1996). However, GATA-1 overexpression in erythroid progenitors also blocks differentiation (Whyatt et al. 2000). Furthermore, GATA binding sites are found in the promoters of almost all erythroid-specific genes suggesting a pivotal role for GATA-1 in erythropoiesis. We isolated fetal liver cells from embryos at 12.5 dpc, since this is the predominant site of erythropoiesis, and at this time point the embryos of the different genotypes used are still alive .

GATA-1 overexpression

A transgenic mouse line was used that overexpressed GATA-1 under the control of the β-globin locus control region and the β globin promoter. This GATA-1 overexpressing transgenic mouse line has the transgene integrated on the X chromosome (Whyatt et al. 2000). Therefore, female mice overexpress GATA-1 in ~50% of their cells, as a consequence of X-inactivation. Males hemizygous for this transgene overexpress GATA-1 in all their erythroid cells and die *in utero* owing to a late defect in erythroid differentiation (Whyatt et al. 2000). In contrast, the females survive and surprisingly, the overexpressing cells contribute normally to the erythroid cell population. It is thought that the rescue of GATA-1 overexpressing erythroid cells in female transgenic mice is brought about by a signal emanating from the non-overexpressing, normally differentiating, erythroid cells (i.e the cells that have X-inactivated the transgene) (Whyatt et al. 2000).

In our differentiation assay, cells derived from 12.5 day transgenic male fetal livers are indeed severely impaired in differentiation, with only a few cells undergoing enucleation. The female cells with the active transgene (the overexpressing cells) differentiate normally *in vivo* but are defective in culture since they are blocked in differentiation when they are removed from the fetal liver and cultured in hanging drops (Whyatt et al. 2000). This is demonstrated in the differentiation histogram plots of the $TER119^{+}/7AAD^{-}$ cells after 2 days of hanging drop culture. The GATA-1 tg female cells have an intermediate phenotype between the wt en GATA-1 tg male cells (figure 4).

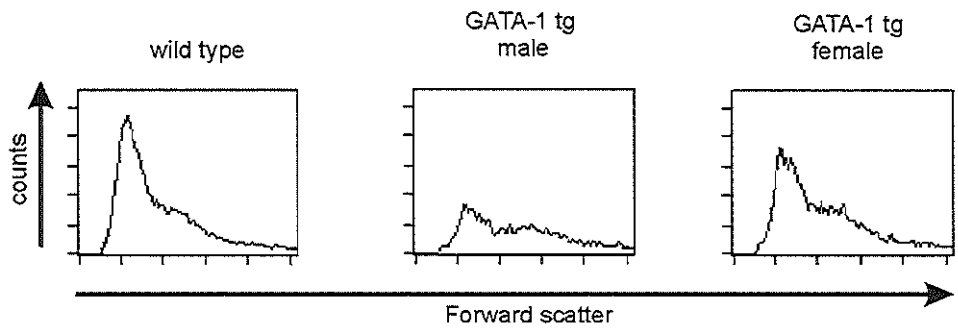

Figure 4. FACS analysis of wt and GATA-1 tg male and female overexpressing erythroid progenitors ($TER119^{+}/7AAD^{-}$) after 2 days of hanging drop culture.

Overexpression of a tamoxifen-inducible GATA-1-LBD fusion protein

To study the role of GATA-1 further we also generated transgenic mice with an inducible GATA-1 –Ligand Binding Domain (GATA-1-LBD) fusion protein. This fusion with a

mutated LBD from the estrogen receptor confers inducibility upon the presence of 4-hydroxy-tamoxifen (4OH-T) (Metzger et al. 1995). Fetal liver cells, isolated from the GATA-1-LBD transgenics, were cultured in hanging drops in medium with and without 4OH-T. Cells collected from 10 drops were isolated and nuclear proteins were extracted from these cells. The extracts were used for the Western blot analysis (Figure 5). This demonstrate that GATA-1-LBD can be clearly detected in the nuclear fraction after 4OH-T addition, possibly reflecting the nuclear translocation of the fusion protein that is thought to occur upon ligand binding. The cells cultured with 4OH-T showed a mild but significant reduction of $TER119^{+}/7AAD^{-}/FCS^{low}$cells (13%) (Whyatt et al. 2000). We conclude that the culture method can also be used for the analysis of the biological effects of inducible systems, as exemplified by LBD fusion proteins.

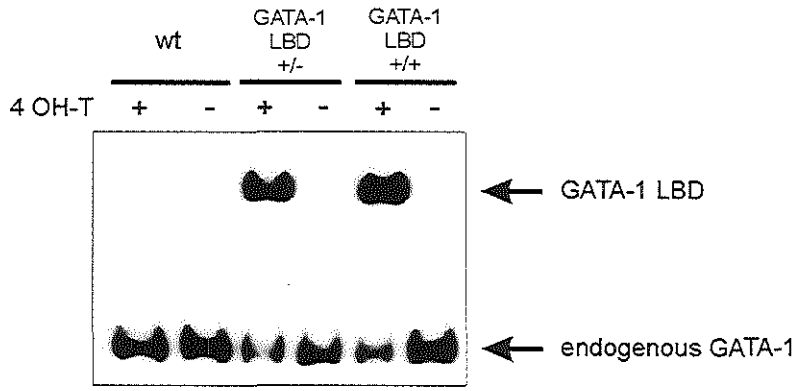

Figure 5. Western blot of nuclear extracts of fetal liver cells cultured in hanging drops with or without 4-hydroxy tamoxifen (4OH-T) after one day of hanging drop culturing.

GATA-1 null mutant

GATA-1 null mutant mice die between 10.5 and 11.5 dpc.(Pevny et al. 1991; Fujiwara et al. 1996). Fetal liver cells from heterozygous female mice express TER119 in approximately 50% of the cells compared to wild type fetal liver cells (Table 1). The variation between the number of $TER119^{+}$ cells is due to variable X-inactivation which is also thought to be the cause of prenatal death of one third of the heterozygous females (Tsai et al. 1998) (Fujiwara et al. 1996) (Chapter 3). GATA-1 null cells are arrested in the proerythroblast stage and never make it to the stage were they start expressing the TER119 antigen. During the cultures, of fetal liver cells from the heterozygous null mutant mice, there is a relative increase of

TER119+ cells, compared to the wild type cultures.(Table 1). This increase of TER119+ cells can not be explained by a more cell death of the TER119- cells as measure by 7AAD positive staining .

		% TER119$^+$ cells			% 7AAD$^+$ of TER119$^-$ cells
	n=	day 0	day 1	day 2	day 2
WT	10	70 (± 6)	81 (± 2)	76 (± 7)	57 (± 12)
GATA-1 +/-	6	36 (± 9)	56 (± 4)	62 (± 4)	57 (± 3)

Table 1: Percentage of TER119$^+$ fetal liver from wild type and GATA-1 +/- cells after 0, 1 and 2 day of hanging drop culture, and the percentage of 7AAD$^+$ (dead) cells of the TER119$^-$ cells. n is number of embryos.

The TER119$^+$ cells, in which the GATA-1 null allele is inactivated and the wild type allele is active, differentiated slightly less well than wild type cells did (Table 2). This could be caused by a negative influence of the dead TER119$^-$ GATA-1 +/- cells, with the wild type allele inactivated, in the culture.

	% differentiated cells			
	n=	day 0	day 1	day 2
WT	10	3 (± 2)	12 (± 2)	31 (± 2)
GATA-1 +/-	6	3 (± 1)	9 (± 1)	24 (± 3)

Table 2: Percentage of differentiated cells (FSC^{low}) of the TER119$^+$/7AAD$^-$/cells from wild type and GATA-1 +/- fetal liver cells after 0, 1 and 2 day of hanging drop culture. n is the number of embryos.

Discussion

Here, we describe a method that allows the quantitative analysis of differentiation of primary erythroid progenitors. In two days, freshly isolated fetal liver cells complete differentiation into mature erythrocytes. A major advantage of this method is that a small number of cells can be cultured and analysed. This is important when, for instance, a 11.5 dpc fetal liver is used as the source of cells. It is also useful when many different media have to be used to test

different components. We cultured fetal liver cells of several mouse lines defective or inducible defective in their erythroid differentiation process and found that this method is useful to analyse the defects found in these mice.

Surprisingly the cells in hanging drop assays do differentiate in the absence of exogenous Epo. Normally, Epo is produced in response to hypoxia in the kidneys during adult life or in the liver during fetal and neonatal life (Krantz 1991; Eckardt 1995). It is known that Epo is needed for survival, proliferation and differentiation of erythroid progenitors (Kelley et al. 1993; Kirby et al. 1996; Lacombe and Mayeux 1998). Epo is essential to form CFU-E colonies in methyl-cellulose, although Epo is not required for the formation of the progenitor from which this colony is formed (Wu et al. 1995; Wu et al. 1997). Small amounts of Epo are present in FCS but at levels insufficient for erythroid survival and differentiation. The ability to differentiate in the absence of Epo could be explained by the fact that the final steps of the erythroid differentiation process are thought to be Epo-independent, and/or the observation that erythroid progenitors are capable of producing Epo themselves (Stopka et al. 1998). Since the cells grow in close proximity of each other, this might result in a locally effective Epo concentration allowing erythroid differentiation.

This assay however does not mimic the cell-cell interactions that occur *in vivo* completely. GATA-1 overexpressing cells in a mixed cell environment (transgenic females or chimaeric mice) differentiate normally *in vivo* owing to the activity of the proposed REDS signal, but in hanging drops these overexpressing cells are blocked in differentiation even when they are mixed with wild type cells. It has been suggested that the 3D structure of the erythroblastic island is required for REDS signalling to operate (Whyatt et al. 2000); this structure is not maintained in the assay described here.

Further analysis of the behaviour of the heterozygous GATA-1 null mutant cells has to be done to explain the increase in TER119$^+$ cells and the lower number of TER119$^+$ cells that fully differentiate.

In conclusion, we have described a differentiation assay for primary erythroid cells that is complementary to the classical CFU-E assay. The advantage of the new assay is that allows the simultaneous analysis of large numbers of samples, and that it is well geared towards the screening of growth media, biological and pharmaceutical compounds. It thus is a useful new tool to study the terminal differentiation of erythroid progenitors.

Methods

Cells

Mouse embryos were collected at 12.5 or 13.5 days of gestation. Fetal livers were dissected and disrupted in DMEM with 20% FCS amd antibiotics.

Hanging drop cultures

The cell concentrations were measured with a Coulter counter or an electronic cell analyser (Casy1, Schärfe Systems, Reutlingen, Germany). The required number of cells was spun down and taken up in the medium, consisting of DMEM supplemented with Epo (1 unit/ml), insulin (5μg/ml), hemin (2mM), penicillin (100u/ml), streptomycin (100μg/ml), β-mercapto-ethanol (100μM) and 20 % fetal calf serum (FCS). The cells were taken up in the medium at a concentration of $1.25x10^6$cells/ml, but cell concentrations ranging from $5x10^5$ to $5x10^6$ cells/ml have been used successfully. 20_l drops, containing ~$2.5x10^4$ cells each, were pipetted on the inside of a culture dish lid, after which it was carefully placed back on the dish which was filled with PBS to avoid evaporation. Cultures were harvested after 1, 2 or 3 days in PBS with 10% FCS and washed once. Since we have found that differentiation did not proceed further after day 2 of incubation, so that cultures were collected after 2 days.

FACS analysis

Single cell suspensions were stained and analysed as described (Sawada et al. 1989). The R-PE conjugated TER119 antibody (Pharmingen) was used in a 1 in 50 dilution (Kina et al. 2000), to identify erythroid cells. 7-aminoactinomycin-D (7AAD, Molecular Probes BV) was used for determining cell viability at 1μg/ml. Measurements were done on the FACScan (Becton Dickinson).

Cytospins

Cells were prepared on slides by 5 minutes cyto-centrifugation at 500 rpm. The slides were dried and stained with 1% O-dianisidine (Sigma) in methanol and a modified Giemsa like stain (Diff.Quick Red and Blue, Dade Diagnostika) (Beug et al. 1982).

Western blotting

Nuclear extracts were prepared as described (Andrews and Faller 1991). Each sample was subjected to electrophoreses through a 10% SDS polyacrylamide gel, transferred onto nitrocellulose and probed with anti-GATA-1 antibody N6 (SantaCruz) and an appropriate secondary antibody before detection by chemiluminesence.

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Chapter 6

General Discussion

General discussion

The work described in this thesis is aimed at examining the function of the transcription factor GATA-1 in the proliferation and differentiation of erythroid progenitors and in the Sertoli cells of the testis. Experiments are preformed both *in vivo* and *in vitro*, in mice, using molecular methods, transgenics, gene targeting and newly developed hanging drops.

What is the role of GATA-1 during proliferation and differentiation of erythroid progenitors ?

The necessity of GATA-1 for erythroid differentiation was shown by the null mutant mice, that dies due to fatal anemia around 10.5-11.5 dpc (Pevny et al. 1991). Erythroid progenitor cells without GATA-1 do not progress beyond the proerythroblast stage. The differentiation of an erythroid GATA-1 null cell line, G1E, is rescued by transfecting it with GATA-1 (Weiss et al. 1997). Furthermore, the null mutant mouse phenotype could be partially alleviated by a knockin of the GATA-3 cDNA into the GATA-1 locus *in vivo* (Tsai et al. 1998). This partial rescue might be explained by insufficient GATA-3 levels. GATA-1 knockdown mice, that express 5% of the normal level of GATA-1 protein, die around 10.5 dpc of severe anemia, comparable to the null mutant. These mice can be rescued by GATA-1, -2 and -3 transgenes driven by a GATA-1 promoter (Takahashi et al. 2000). During adulthood the mutants rescued by GATA-2 and –3 develop anemia while the mice rescued by GATA-1 do not (Takahashi et al. 2000). Two explanations are possible, the first, and most probable, is that GATA-2 and –3 can not completely substitute for GATA-1 due to different biochemical properties or posttranslational regulation of GATA-2 and -3, especially during adult life. The second is that GATA-2 and –3 do not accumulate not enough protein and therefore are not able to completely replace GATA-1 in the GATA-1 knockdown mutant mice, but GATA-2 or GATA-3 are expressed at similar or higher levels than GATA-1 is expressed in the wt mice (Takahashi et al. 2000). Thus, the knockout and rescue experiments show that GATA-1 is essential for erythroid differentiation. The overall protein homology between GATA-1 and GATA-2 and –3 is 32 % in both cases. The highest homology, up to 90 %, is found in the Zn-finger domains. Outside those domains it is ~15%. Although the proteins are very homologous in the Zn-finger domains, it is still surprising that the family members 2 and 3 could rescue the lethal phenotype of the GATA-1 knockdown so well. It appears that the protein-protein interaction and DNA-binding activities of the Zn-finger domains comprise a large part of the biological function of these GATA factors.

It is suggested that GATA-1 drives cells into differentiation (Shivdasani and Orkin 1996). To test this hypothesis, we preformed experiments in which we manipulated the protein levels of GATA-1. The overexpression experiments demonstrated that artificially

elevated GATA-1 levels cause a block in erythroid differentiation at the stage of the basophilic proerythroblast (Chapter 2). These results lead us to propose a model in which GATA-1 regulates differentiation in two ways. One way is by regulating the expression of erythroid-specific genes like a classical transcription factor. All erythroid specific genes known to date have GATA binding sites in the regulatory elements. The second way would be that GATA-1 regulates differentiation by stimulating cell cycle progression.

Proliferation during differentiation

Overexpression experiments in MEL showed that high levels of GATA-1 block differentiation probably through an illegitimate acceleration of the cell cycle by stimulating S-phase entry (Whyatt et al. 1997). *In vivo* overexpression also results in a block in differentiation (Chapter 2). In our model, we propose that high levels of GATA-1 are needed to drive the rapid cell divisions characteristic for immature erythroid progenitors, as it is known that CFU-E stage progenitors divide more quickly than BFU-E progenitors and that beyond CFU-E stage the cells exhibit a progressive decrease in proliferative capacity (Iscove 1977; Gregory and Eaves 1978). Terminal differentiation is however associated with cell cycle arrest in the G1-phase. We therefore claim that as erythroid progenitors progress to terminal differentiation, they have to down-regulate GATA-1 activity in order to stop dividing (Figure 1). Thus, proliferation and differentiation are intertwined processes.

In the GATA-1 null mutant mice, erythroid-specific genes are expressed at lower levels and cells do not go through the proliferative phase but instead undergo apoptosis at the proerythroblast stage, possibly due to the reduced expression of the survival factor Bcl_{xL}, a GATA-1 target gene. In GATA-1 overexpressing cells, the cells are unable to exit the cell cycle, causing a differentiation block at the later developmental stage of the basophilic proerythroblast. The cells don't succumb to apoptosis, since they express GATA-1 target genes including Bcl_{xL} at normal levels.

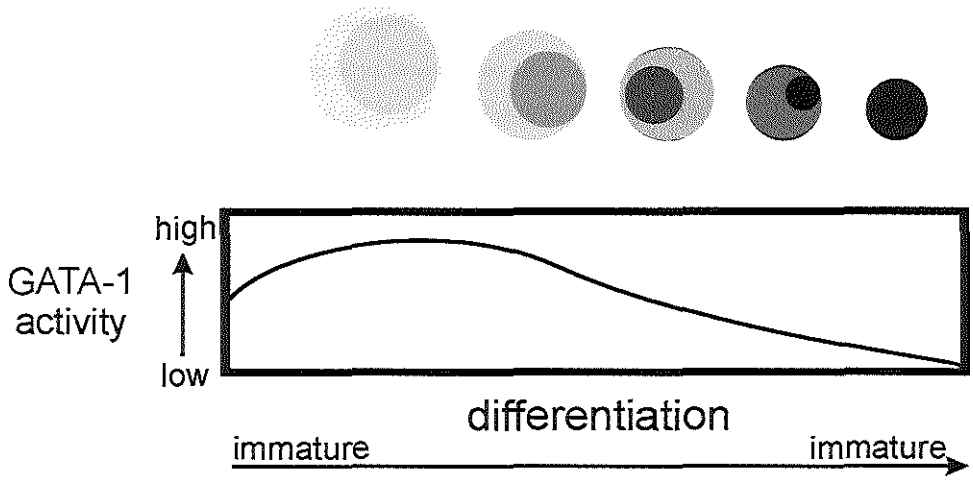

Figure 1: Dynamic regulation of GATA-1 activity during erythroid differentiation. As cells go through a proliferative phase the GATA-1 activity should be high. To undergo terminal differentiation and cell cycle arrest, GATA-1 activity has to be down-regulated.

GATA-1 and the cell cycle

How does GATA-1 influence the cell cycle? The first clues to answer this question came from an erythroid progenitor MEL cell line overexpressing GATA-1. When this cell line was induced to differentiate, it displayed an accelerated cell cycle and lack of expression of differentiation markers (Whyatt et al. 1997).

The expression of known players in cell cycle regulation was measured and it was found that the phosphorylation status of a pivotal player, the Retinoblastoma protein (Rb) was affected. In cells overexpressing GATA-1, Rb protein remained hyperphosphorylated after induction of differentiation (Whyatt et al. 1997).Rb is an important regulator of terminal differentiation of several cell types including erythrocytes (Jacks et al. 1992). The activity of Rb is regulated by phosphorylation (Sherr 1996) (Fig.2); hypophosphorylated (active) Rb is a repressor of G1 to S-phase progression. Hypophosphorylated Rb binds to the E2F transcription factors thus inhibiting the transcription of E2F target genes. Upon phosphorylation, Rb is released from the E2Fs, these can now activate their target genes and G1 to S-phase progression can occur. The question arises how GATA-1 influences Rb phosphorylation. GATA-1 can bind directly to Rb *in vitro* (Whyatt et al. 1997) and this interaction could influence phosphorylation status of Rb.

Other proteins involved in cell cycle regulation were also measured such as p27, p21 cyclin D2, cyclin D3, cyclin E, and one of the few proteins that changed before Rb phosphorylation was cyclin A (D.Whyatt, unpublished data). This cyclin can bind to cyclin

dependent kinase 2, and is needed at two points in the cell cycle, for S-phase entry S-phase entry and mitosis (Pagano et al. 1992). Attempts to overexpress cyclin A in MEL cells were unsuccessful, since higher cyclin A mRNA levels did not lead to higher cyclin A protein levels (D.Whyatt, unpublished data). Furthermore, although the promoter of the cyclin A gene contains a potential GATA binding site, it is probably not a direct GATA-1 target gene since the cyclin A levels are mainly regulated post-transcriptionally in MEL cells, and cyclin A mRNA levels are unaltered in MEL cells overexpressing GATA-1 (Minshull et al. 1989).

The observation that the erythroid phenotypes of mice overexpressing GATA-1 and of Rb *null* mutant mice are very similar suggests functional relationships between these factors. In both cases, the block in erythropoiesis occurs at a similar stage of differentiation of the progenitors. More surprising, both phenotypes appear to be cell nonautonomous defects (Williams et al. 1994; Whyatt et al. 2000). This means that in a homogeneous mutant cell population (mutant cells only) the mutant cells have a mutant phenotype, but that in a mixed cell population (mutant + wild type cells) mutant cells do not display the mutant phenotype. We have proposed that in the case of GATA-1 overexpressing mice, the mutant cells are rescued by a signal supplied by wild type erythroid cells. This postulated signal is called REDS for red cell differentiation signal. This signal must come from the wild type red blood cells as in the mixed population (e.g. a transgenic GATA-1 female with the transgene on one of the X chromosomes, or a chimaeric mouse) the only cell that is different compared to a uniform overexpressing cell population (e.g. in a transgenic GATA-1 male with the transgene on the X chromosome) is the wild type erythroid cell (Whyatt and Grosveld 2002). It is possible that a similar signal operates in the cell nonautonomy of the Rb *null* mutation.

Another potentially important observation is that the co-activator p300 can acetylate Rb, which inhibits phosphorylation by Cdks (Chan et al. 2001). The lysines that are acetylated might be part of the docking site for the Cdks (Adams et al. 1996). This mechanism might be the entry point for the regulation of Rb phosphorylation by GATA-1, since GATA-1 can interact directly with p300 and the closely related CBP protein (Blobel et al. 1998; Boyes et al. 1998). High levels of GATA-1 will bind more CBP/p300 thus preventing it from binding to Rb. This would result in reduced acetylation of Rb thus indirectly promoting Rb phosphorylation, allowing the cells to progress to S phase. Conversely, low GATA-1 levels would permit Rb acetylation, resulting in an abundance of hypophosphorylated Rb as required for the G1 arrest associated with terminal differentiation (see Fig. 2, D.Whyatt, personal communication).

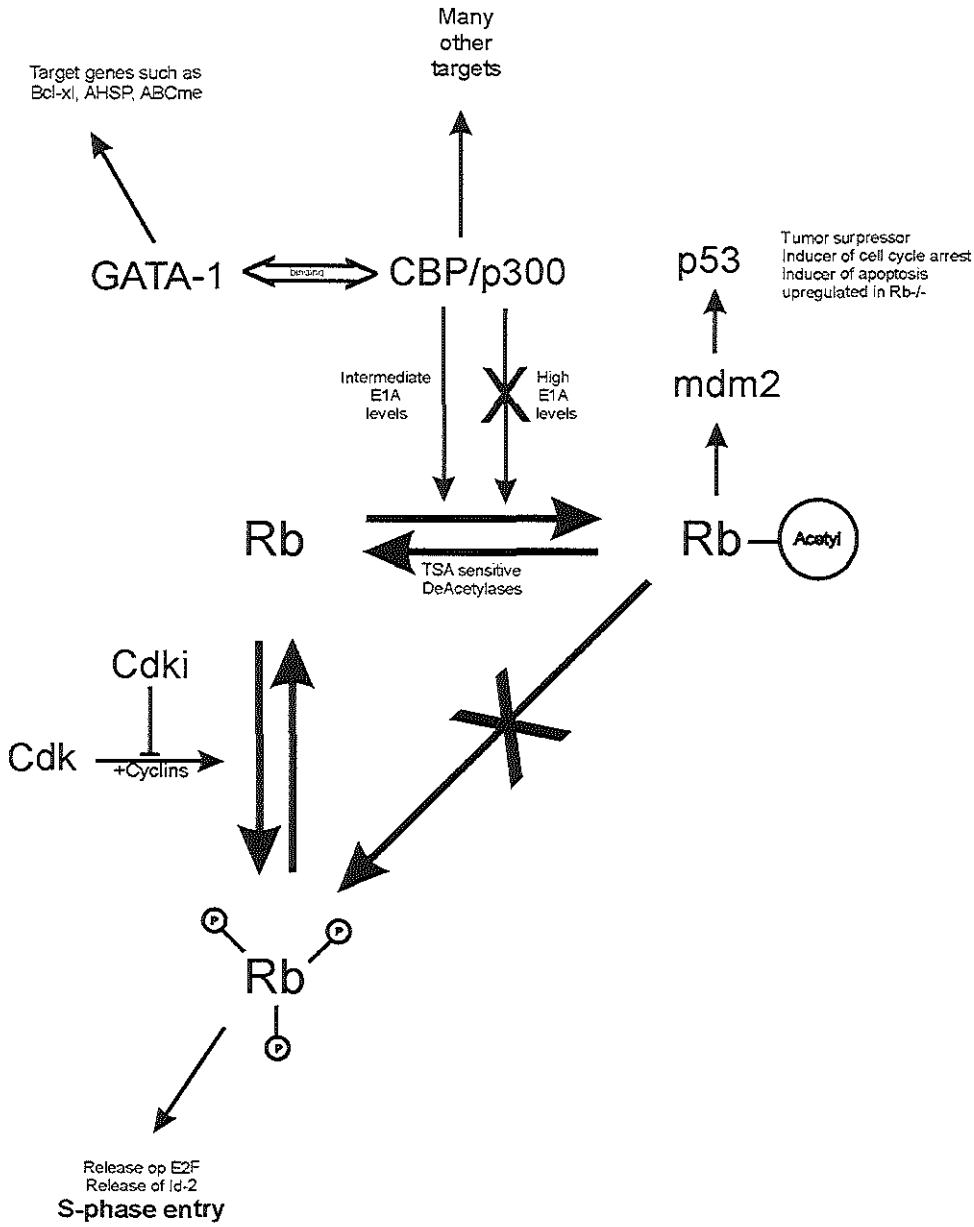

Figure 2: Possible mechanism for the regulation of the cell cycle by GATA-1 via CBP/p300 and Rb.

How does this fit with the wild type, GATA-1 overexpressing and GATA-1 knockout phenotype?

Wild type situation

In the wild type situation, GATA-1 is regulated such that GATA-1 peaks (Rb is more easily phosphorylated) when the progenitors have to divide rapidly. During terminal differentiation GATA-1 is downregulated, Rb is acetylated and the cells arrest in G1. GATA-1 target genes are expressed and the progenitors mature to erythrocytes.

GATA-1 overexpression

CBP/p300 is bound quantitatively by GATA-1, and therefore Rb is less acetylated leading to a shift in the balance of phosphorylation of Rb to hyper-phosphorylated Rb, leading to a progression from G1 into S-phase because of the release of E2F transcription factors. In a mixed cell population this mechanism is bypassed at some point by the signal REDS (Chapter 2). Most likely is the possibility that the signal causes a decrease of GATA-1 activity. One way this can be achieved is via protein degradation. It has been shown that late erythroid progenitors (the orthochromatic erythroblast) express the Fas ligand for the receptor protein Fas (CD95) that is expressed in the earlier cells (basophilic erythroblasts) (De Maria et al. 1999a). Signalling via these receptors can trigger a pathway that can activate Caspase 3 that is able to cleave the GATA-1 protein(De Maria et al. 1999b). It was suggested that this pathway regulates the rate of erythropoiesis as a negative feedback mechanism (Orkin and Weiss 1999). In our model, it is a mechanism to downregulate GATA-1 during differentiation and therefore a pathway that is always necessary for erythropoiesis. Our experiments have shown that GATA-1 activity must be downregulated during terminal differentiation (Chapter 2).

If the signal does not rescue via GATA-1 degradation it has to influence the cell cycle machinery via one of the other proteins like Rb, CBP/p300, E2Fs, CDKis etc. Maybe REDS functions as a regulator of GATA-1 and one or more of these proteins. That REDS influences more than one target is more likely, as cell signalling pathways often do not influence only one target at the time.

GATA-1 null mutant

In the GATA-1 null mutant, CBP/p300 is not squelched by GATA-1 at all. Rb can be acetylated more efficiently and is therefore less well phosphorylated. This blocks cell cycle progression of the erythroid progenitors prior to a proliferative phase. The cell cycle arrests

prematurely at the proerythroblast stage. The cell cycle arrest should occur at the terminal differentiation stage and not before. Obviously, GATA-1 target genes are less well transcribed in the GATA-1 null mutant.

In the absence of GATA-1, GATA-2 is upregulated (Weiss et al. 1994). The explanation for this effect is suggested to be the downregulation of GATA-2 by GATA-1. GATA-2 can not completely compensate for the loss of GATA-1. GATA-2 is able to bind to CBP/p300 like GATA-1, but maybe its effect is not the same. Also the upregulation of GATA-2 in GATA-1 null mice is probably not exactly the same as the normal level of GATA-1. The level of GATA-1 activity is one very important parameter. This can be concluded from the experiment in which the transgene with the GATA-2 cDNA does rescue the GATA-1 knockdown, as it is (Takahashi et al. 2000). Although the protein is different from GATA-1, GATA-2 only causes problems later in life. The rescue by GATA-2 expressed under the GATA-1 regulating sequences shows that the level of expression is very important. The drawback of this experiment, however, is that it was performed with GATA-1 knockdown mice instead of GATA-1 null mutants. This also leaves the possibility that the remaining 5% GATA-1 expression is actually supporting a minimal rescue by GATA-2, leading to the aberrant conclusion that GATA-2 can fully take over GATA-1's role in terminal erythroid differentiation.

CBP/p300 the pivotal protein?

P300 and CBP interact with a growing list of proteins including general transcription factors, tissue-specific transcription factors, and cell cycle regulating proteins. This suggests a pivotal role in transcription, cell growth, transformation and development (Goodman and Smolik 2000).

In the model suggested above, CBP/P300 has to be ratelimiting. This is in agreement with the phenotype of the CBP and the P300 heterozygous knockout. Mice heterozygous for the CBP gene develop multi-lineage defects in hematopoietic differentiation. Fewer heterozygous p300 mice were born than the expected Mendelian ratio (Yao et al. 1998). Mice homozygous for the null allele of either CBP or p300 are not viable and die around 10.5 dpc (Kung et al. 2000). p300/CBP compound heterozygous mice are also not viable. The embryos were severely stunted and exhibited similar defects as the p300 or CBP homozygous knockout (Yao et al. 1998). Thus although P300 and CBP are highly homologous and they can perform some of the same functions they are not able to compensate for the loss of the other gene. This demonstrates that the combined dose of p300 and CBP is critical for mouse embryonic development. Furthermore CBP/P300 has tumor suppressing activity.

Heterozygous CBP mice developed a high incidence of hematological malignancies. Bone marrow of CBP heterozygotes causes leukemia when transplanted in sublethally irradiated mice in almost 40% of the cases (Kung et al. 2000). It was shown that in these cancers the wild type allele was also lost. This is compatible with the notion that CBP is an important regulator of Rb. However, CBP also interacts with a wide variety of other proteins that can influence the cell cycle, including p53, mdm2 and c-myb (Goodman and Smolik 2000).

The regulation of GATA-1 activity by REDS

What is REDS? And how does it work? These questions still have to be answered. The first candidates are the so-called death ligands. FasL, TRAIL and TNFα are capable of activating caspase-3 which can degrade human GATA-1, and are produced by late erythroid progenitors (De Maria et al. 1999b). Experiments with caspase inhibitor have shown that caspase activity is required for terminal erythroid differentiation (Zermati et al. 2001), although the caspase-3 knockout does not have an obvious erythroid defect (Woo et al. 1998). Collectively, death ligands meet several of the requirements for REDS activity. However, death ligands added to *in vitro* cultures do not rescue the developmental defect of GATA-1 overexpressing progenitors (data not shown). Possibly, REDS signalling requires close contact between the mutant cells and the cells providing the REDS signal, and such close contact may not be easily reproduced *in vitro*. Furthermore, other as yet unidentified death ligands could be the REDS signal. This is suggested by the discovery of a novel death ligand receptor in zebrafish which is thought to stimulate erythropoiesis (Long et al. 2000). We are currently searching for the mammalian homologue of this receptor.

An additional mechanism for the regulation of GATA-1 activity would be via protein modifications such as phosphorylation and acetylation. Phosphorylation/dephosphorylation can regulate distinct aspects of transcription factor function including localization, protein stability, protein-protein interactions and DNA binding (Whitmarsh and Davis 2000). It might for instance interfere with the interaction with CBP. Acetylation can also regulate localisation, protein-DNA interactions and protein-protein interactions (Bannister and Miska 2000). Until now the roles of phosphorylation and acetylation of GATA-1 are not known, although there are reports indicating that these modifications influence the binding affinity of GATA-1 to DNA (Crossley and Orkin 1994; Boyes et al. 1998; Hung et al. 1999; Partington and Patient 1999).

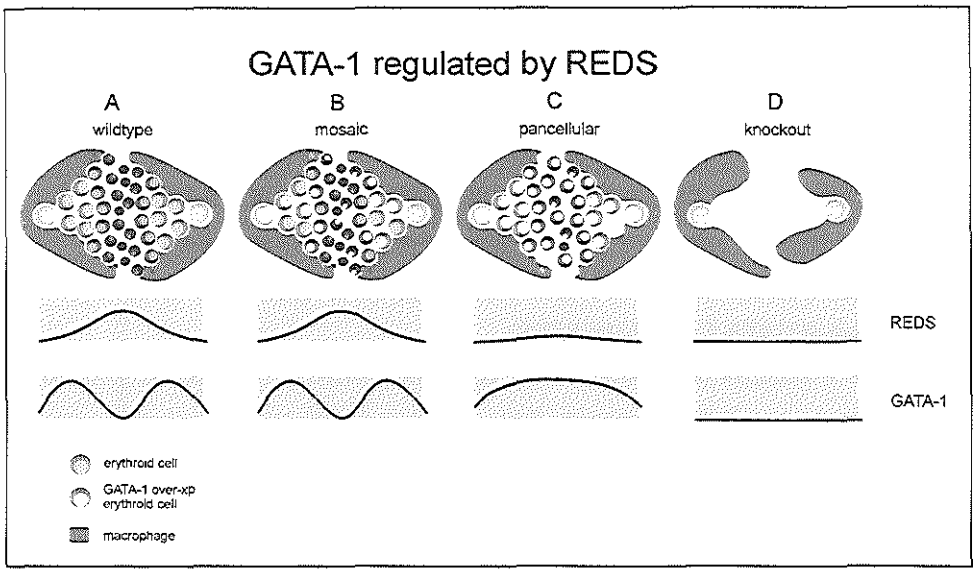

Figure 3 A, B, C and D: GATA-1 activity regulated by REDS in erythroblastic islands. (A) wild type, (B) mosaic GATA-1 overexpression, (C) pancellular GATA-1 overexpression and (D) GATA-1 knockout.

What is the role of GATA-1 in the testis?

The role of GATA-1 in the Sertoli cells has not been elucidated by our the testis-specific deletion of GATA-1. The results of our experiments show that GATA-1 is not essential for testis structure or function. It can not be excluded that GATA-1 is a transcriptional regulator in this tissue, since this function may be shared with other co-expressed GATA factors, GATA-2, -4 and -6. It is conceivable that functional redundancy serves as a back-up mechanism to assure Sertoli cell function. The generation of compound Sertoli cell-specific GATA null mutants would be required to address this issue. Humans with a mutation in GATA-1 that disrupts the interaction with the cofactor FOG-1, suffer from anemia, thrombocytopenia and cryptorchidism (Nichols et al. 2000). The cryptorchidism may be related to the GATA-1 mutation this could be tested by introducing the corresponding mutation in the mouse germline. In the testis a FOG-1 homologous protein, FOG-2 is highly expressed and a mutated GATA-1 protein may have a more pronounced defect than the tissue specific null mutant (Lu et al. 1999).

Future experiments

In the future, new experiments will shed more light on the role of GATA-1.

To investigate the role of acetylation and phosphorylation of GATA-1, knock-in alleles of GATA-1 mutants with alterations in the amino acids that are thought to be essential for these processes, have to be generated. Mutations in residues known to influence FOG-1 interaction could also be studied this way. A knock-in allele with a GATA-1 mutation that is resistant to caspase-degradation could show if GATA-1 regulation via degradation is an important pathway for inactivation. Knock-in mutant mice have the advantage over transgenic mice that the endogenous locus is used for expression, and that there is no influence of the endogenous wild type gene products.

Null mutant mice or transgenic mice from genes thought to play a role in the regulation of GATA-1, such as caspase-3 and CBP, could be crossed with GATA-1 overexpressing mice to see if the interactions between these gene products are important. These experiments have been started in our laboratory (D. Whyatt and L. Gutiérrez)

Further progress can be expected from the application of new technologies. Microarray target gene detection is becoming a common technique that enables the genome-wide detection of differences in gene expression between wild type, null mutant, knock-in and transgenic mice.

Human GATA-1 has been purified in an active state and this can be used for studying interactions with proteins or DNA (Doubeikovskaia et al. 2001). In our laboratory, biotinylated mouse GATA-1 is currently used to isolated protein complexes containing GATA-1. Small amounts of co-purifying proteins can now be identified with very sensitive mass spectrometry techniques; such analyses reveals which biochemical activities are associated with GATA-1-containing multi-protein complexes (J. Strouboulis unpublished data). The results of these experiments will provide important answers to the question how GATA-1 regulates red blood cell differentiation.

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Summary

In this thesis experiments are described, that were designed, to understand the process of red blood cell formation, better. In particular the final stages of the pathway that a stem cell has to follow to become a red blood cell. The experimental chapters are introduced by Chapter 1 of which a more simplified summary is given here, including some basic biology. The use of lab-slang has been tried to be avoided.

Introduction

Cells are the basic units of our body. Imagine them as little bags filled with water or better broth. These bags are not completely waterproof. The cells contain several smaller working units such as the nucleus (headquarters and control room) and the mitochondria (the power station). Many cells together form a tissue or an organ such as the skin, the liver, the heart or the blood.

Blood

The blood contains different type of blood cells that can be divided in; the platelets, the white blood cells and the red blood cells. The platelets are responsible for coagulation of the blood. The white blood cells are the army of our immune system. They protect us against bacteria, viruses and other unwanted intruders and clean up the mess afterwards. The red blood cells are responsible for the transport of oxygen and carbon dioxide. They distribute the oxygen from the lungs through the body and collect the carbon dioxide to deliver that to the lungs.

All types of blood cells are being produced continuously depending on the demands of the body. Every day, 200 billion (=200.000.000.000) new red blood cells are made, this comes down to 2,3 million red blood cells per second. All the different types of blood cells are descendants from the hematopoietic stem cell. These are relatively rare cells that are found in the blood producing organs, the AGM (a small structure in the embryo that forms the embryonic kidney and develops in aorta and genitals) and the fetal liver in the embryo, and bone marrow after birth. These cells have the ability to become every type of blood cells. This process is called differentiation and during this process the cells go through several intermediate stages. We then call the cells precursors. During the differentiation process the cells divides many time, thus one stem cell can have many descendants. Stem cell can also divide without changing into mature blood cells, this way the number of stem cells is maintained at the same level.

The differentiation is regulated via regulation mechanisms. Failure of a regulation mechanism can lead to diseases in which the formation of a certain blood cell type is affected. Anemia is the disease in which not enough blood cells or crippled red blood cells are made. In leukemia a precursors keep dividing and do not differentiate to a mature blood cell.

Differentiation

Differentiation of cells does not only occur in blood cells but in all tissues, and during development of the fertilized oocyte to an adult. The fertilized oocyte is the ultimate stem cell. Cells from all the tissues are very different. A neuron with its long dendrites has a totally different appearance than a muscle cell. And the functions liver and skin cells are very different. All these cells are however descendants of the fertilized oocyte. What a cell looks like and what it does depends mainly of the proteins in the cell. Protein are the largest group of molecules in the cell. The proteins are the building blocks of the components of the cell, perform the communication, repair broken parts, generate energy, collect and recycle garbage and catalyse many reactions. Proteins are molecules that are build out of a group of 20 amino acids. Thus, the differences between cells are mainly determined by the proteins they express. How does a cell know which proteins to make?

The genetic material: DNA

The information for the development of the body and the individual cells is written in the genetic material of the cell, the DNA, that found in the nucleus of the cell. The DNA can be envisioned as a manual (the genome) that consists of two sets of big books (the chromosomes, 46 in humans). These books have only one very long line, in which only four letters; A,T, G and CBP/p300 (the bases) appear. These letters form three letter words (codon) that form sentences (the genes). The total number of bases in the genome (all the books together) is about 6.000.000.000. If every letter would represent a base, it would come down to 20.000 copies of this thesis in which approximately 40.000 genes are written. In a gene the order of the codon determines the order of the amino acids of the protein. From genes, relatively instable copies are made (mRNA) that are read and translated outside the nucleus (in the cytoplasm. These two processes are called transcription and translation (Figure 1).

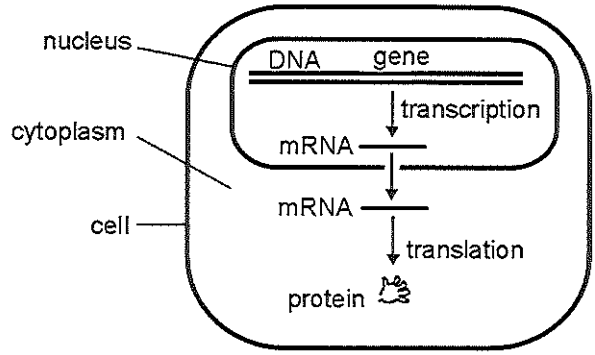


Figure 1: Schematic representation of gene expression; transcription from DNA to RNA, followed by transport of the mRNA and translation of the mRNA to protein.

Different cell types all have the same genome (the manual). The differences between the different cell types are determined by which genes of the manual are transcribed to mRNAs. There are genes that are used in basically all cells, the so-called house keeping genes. The differences are made by the cell type specific genes. Some part of the manual are not important for one cell type and very important for another cell type. For example, the genes for hemoglobins are transcribed in red blood cells and not in cell of the pancreas. The cells in the pancreas do have the hemoglobin gene but do not use it. They do make insulin that is not made by red blood cells. Which genes are used depends on the presence of proteins that are able to regulate transcription, the transcription factors.

Transcription factors

Transcription factors are protein that regulated transcription because they are able to bind to the DNA. Therefore, they are found in the nucleus of the cell. They can regulate transcription in several ways. The thin thread of DNA is wrapped around little spools (the nucleosomes), these spools form a compact structure called the 30nm fiber. This is a rather inaccessible structure. For DNA to be transcribed it has to be made accessible for proteins that make the RNA copies. Transcription factors can bind to DNA and unfold it or attract other proteins that can. An other mechanism is that transcription factors bind to the stretch of DNA just in front of the transcription start site called the promoter, that is very important for transcription. Transcription factors are thought to attract the protein that carry out the actual transcription, and thereby increasing the frequency of transcription. Transcription factors can also inhibit transcription, for instance by attracting proteins that can block transcription or block the unfolding of the DNA.

This Thesis

GATA-1 is very important for the differentiation of red blood cells, but what is the precise function of the protein is still not clear, as is way it is regulated. In this thesis, experiments that investigate the function and regulation of GATA-1 are described. GATA-1 is a red blood cell specific transcription factor that owns its name to the core of the DNA sequence it binds: G, A, T, A. These bind sites are found in the promoters and other regulatory sequences of all red blood cell specific genes among which the hemoglobin genes. In experiments with mice that made no GATA-1 in their red blood cell precursors, the precursors arrested during their maturation process, and the mice died due to anemia. GATA-1's importance for the differentiation process was shown. In experiments done in our laboratory red blood cell precursors were generated that made more GATA-1 than normal. These cells, that normally can be induced to differentiate, refused to differentiate and kept dividing unlike the control cells.

In **Chapter 2** experiments are described in which mice are generated that make more GATA-1 in their red blood cell precursors. Mice that make to much GATA-1 in all their red blood cells die by the time they are half way of their gestation. The process of red blood cell differentiation is blocked and is the cause of the lethal anemia. This was unexpected because it was assumed that GATA-1 would stimulate differentiation. The generation of these mice was only possible because in mice that produced more in only a part of their red blood cell precursors instead of in all cells. This was achieved in only one experiment in which the extra copy of the GATA-1 gene integrated in the X chromosome. In cells of female mice (with 2 X chromosomes), one of the X chromosomes is inactivated. This implies that in 50 % of the cells the extra copy is inactivated. Surprisingly the other 50 % of the cells, that over-produced GATA-1, were able to fully differentiate. We concluded from our experiments that the normal cells with the inactivated extra GATA-1 gene, can rescue the abnormal cells, that overproduced GATA-1 from their fatal block in differentiation. The abnormal cells are getting a message from the normal cells to proceed differentiation as if nothing happened. The defect of the abnormal cells in non-autonomous. This means that they do not behave as their genetic material tells them but that they are controlled by other, normal, cells. It is not known what this signal is that is responsible for this but we called it REDS, which stands for red blood cell differentiation signal.

In **Chapter 3** experiments are described in which mice should have been able to shut down their GATA-1 genes at any given time. Since a mouse without GATA-1 dies after 10 days of embryonic development, the lack of GATA-1 can not be studied at later timepoints.

To overcome this problem a mouse was generated with an adapted GATA-1 gene. The gene was adapted in such a way that in front and after the gene DNA sequences were placed, so called lox sites, that can be recognized by an enzyme (Cre recombinase) that can delete the DNA in between the two lox sites. Imagine a long rope (DNA) in with a knot (two lox sites combined) that creates a big loop (the DNA in between the two lox sites), after which scissors (the Cre recombinase) can cut the loop out of the rope. By breeding the mice with the adapted GATA-1 gene with mice that produce the Cre recombinase (the scissors) in their cells, the offspring can have both and these mice will have the GATA-1 gene deleted. After we added a biological on/off switch to Cre recombinase it should have been possible to delete the GATA-1 gene at any given time. This was possible but the efficiency was to low to remove the GATA-1 gene in all the cell we wanted. This system worked perfectly when we used the Cre recombinase without the biological on/off switch. The deletion of GATA-1 in a specific cells type was achieved by generating mice that produced the Cre recombinase in a specific cell type, and this also worked fine.

An example of the cell type specific deletion is given in **Chapter 4** in which experiments are described in which we generated mice that do not produce GATA-1 in their testes but do make GATA-1 in their red blood cells. Without this technique it would not have been possible to study the deficiency of GATA-1 in the testes because in the mice that do not make GATA-1 at all, die as a embryo. Others claimed that GATA-1 probably had an important role in the testes. Our experiments have shown that GATA-1 can be omitted with out inhibiting the function of the testis, and that if GATA-1 has any effect on the function or structure, it is very subtle and not detected by us (yet).

In **Chapter 5** a method is described that makes it possible to test red blood cell precursors on their ability to differentiate. It is a simple and small scale experiment and is used in Chapters 2 and 3.

In **Chapter 6** results from us and others are compared and we speculate about models that explain these results, a function and the regulation of GATA-1. First we speculate about the link between GATA-1 and the cell cycle. One of the characteristics of the final stage differentiation of the differentiation process is a deceleration of the cell cycle. And secondly we speculate about the regulation of GATA-1 by a signal called REDS. We think that the signal is produce by normal cell at the final stages of erythropoiesis. This signal has the ability to instructs abnormal cells to differentiate when otherwise the wouldn't. We suggest that this signal that this signalling system has a also a function under normal circumstances. Possible candidates are discussed. Finally new experiment are discussed that might further elucidate the role of GATA-1.

Samenvatting

In dit proefschrift zijn experimenten beschreven die tot doel hadden de vorming van rode bloedcellen beter te begrijpen. En wel in het bijzonder een klein maar belangrijk gedeelte van het traject dat een stamcel aflegt om uiteindelijk een rode bloedcel te worden. De feitelijke experimentele hoofdstukken zijn ingeleid door **Hoofdstuk 1** waarvan hier een vereenvoudigde samenvatting met wat basisbiologie waarin het gebruik van lab-slang (jargon) zoveel mogelijk is vermeden.

Inleiding

Cellen zijn de basiseenheden van ons lichaam. Cellen kunnen worden voorgesteld als kleine zakjes met water of beter nog bouillon. Deze zakjes zijn niet geheel waterdicht en bevatten zelf weer vele kleine onderdeeltjes die bepaalde functies kunnen uitvoeren zoals bijvoorbeeld de celkern (het hoofdkwartier en controlecentrum) of de mitochondriën (de energiecentrales). Vele cellen samen kunnen een weefsel of een orgaan vormen zoals de huid, de lever, het hart of het bloed.

Bloed

Het bloed bevat verschillende bloedcellen die in drie soorten zijn in te delen, de bloedplaatjes, de witte bloedcellen en de rode bloedcellen. De plaatjes zijn verantwoordelijk voor de stolling van het bloed. De witte bloedcellen vormen het leger van ons afweersysteem. Ze beschermen ons tegen bacteriën, virussen of andere indringers en ruimen de rommel op. De rode bloedcellen zijn verantwoordelijk voor het transport van zuurstof en koolstofdioxide. Ze verspreiden het zuurstof vanuit de longen door het hele lichaam en verzamelen onderweg het koolstofdioxide om het af te leveren in de longen. Alle soorten bloedcellen worden continu bijgemaakt naargelang de behoefte van het lichaam. Per dag maken wij bijvoorbeeld zo'n 200 miljard (200.000.000.000) nieuwe rode bloedcellen, wat neerkomt op ongeveer 2,3 miljoen rode bloedcellen per seconde. Alle verschillende bloedcellen stammen af van hematopoetische stamcellen (HSC). Dit zijn relatief zeldzame cellen die zich bevinden zich in de bloedvormende organen, de AGM (een onderdeel van een embryo de embryonale nieren gaat vormen en zich later ontwikkeld tot de aorta en de genitaliën) en de foetale lever in een embryo en vanaf de geboorte in het beenmerg. Deze cellen hebben het vermogen te veranderen in iedere soort bloedcel. Dit noemen we differentiëren en tijdens dit proces doorloopt een cel verschillende tussenstadia die we voorlopercellen noemen. Tijdens dit proces deelt de zo'n cel zich vele malen zodat van 1 stamcel vele cellen kunnen afstammen.

Daarnaast delen de stamcellen zich ook zonder te veranderen zodat hun aantal min of meer gelijk blijft. Voor een goed verloop van dit productieproces zijn er verschillende regelmechanismen. Als een of meer van deze "schakelaars" defect zijn kunnen bepaalde ziektes zich openbaren doordat bijvoorbeeld een bepaald celtype niet meer gevormd wordt. Zo is bloedarmoede het symptoom van te weinig of niet goed functionerende rode bloedcellen. Bij leukemie deelt een voorlopercel zich ongeremd en ontwikkelt zich niet volledig tot een volwassen cel. Die cel is daardoor niet in staat de functie te vervullen van een volwassen cel.

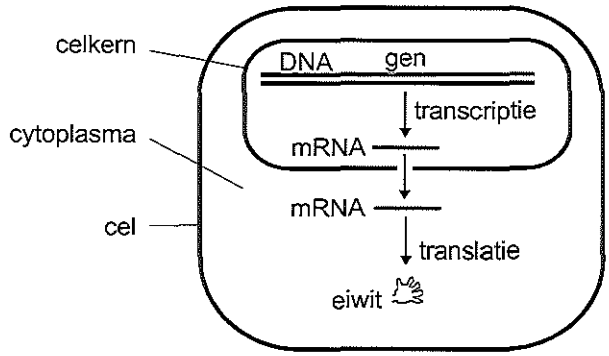
Differentiëren

Het differentiëren van cellen gebeurt niet alleen in het bloed maar in alle verschillende weefsels en gedurende de ontwikkeling van bevruchte eicel tot volgroeide mens. De eicel en de stamcel zijn in dat opzicht vergelijkbaar. De bevruchte eicel is eigenlijk de ultieme stamcel afgezien van het feit dat hij zich niet direct vernieuwt maar in zijn geheel differentieert. De cellen van de verschillende weefsels lijken over het algemeen totaal niet op elkaar. Een zenuwcel met zijn lange uitlopers heeft een ander uiterlijk dan een spiercel. En ook qua functionaliteit kunnen cellen van verschillende weefsels totaal verschillen. Zo heeft een levercel weer hele andere functies dan een huidcel. Toch zijn deze cellen allemaal afstammelingen van de eerste cel, de bevruchte eicel. Hoe een cel eruit ziet en wat een cel kan doen hangt af van de eiwitten in die cel. Eiwitten zijn de grootste groep functionele moleculen in een cel. Ze kunnen de bouwstenen vormen voor de cel en onderdelen van de cel, de interne en externe communicatie verzorgen, de energiecentrales vormen, reparaties uitvoeren, het vuilnis ophalen en recyclen en bepaalde celspecifieke reacties katalyseren. Eiwitten zijn moleculen die zijn opgebouwd uit 20 verschillende bouwstenen, de aminozuren. De verschillen tussen cellen worden dus bepaald door de verschillende eiwitten die in een cel worden gemaakt en gebruikt. Hoe weet een cel nu wat hij moet doen en worden?

Het genetische materiaal: DNA

Het gehele bouwplan voor het lichaam en de opdrachten voor de cellen zijn gecodeerd in het genetische materiaal van de cel dat zich in de celkern bevindt, het DNA. Het DNA kan worden voorgesteld als een gebruiksaanwijzing (het genoom) die bestaat uit twee verzamelingen dikke boeken (de chromosomen, 46 bij de mens) met in ieder boek één lange regel opgebouwd uit 4 verschillende letters ; A, T, G en C (de basen). Deze letters vormen drieletterwoorden (codons) die op hun beurt weer zinnen vormen (de genen). De totale hoeveelheid basen in het genoom (al deze boeken bij elkaar) bedraagt ongeveer

6.000.000.000. Als iedere letter een base zou vertegenwoordigen zou dit neerkomen op ongeveer 20.000 exemplaren van dit proefschrift. En zij beschrijven ongeveer 30 à 40.000 genen. In een gen bepaalt de volgorde van de codons de volgorde van de aminozuren van een eiwit. Van de genen worden namelijk relatief instabiele kopieën (mRNA) gemaakt die vervolgens buiten de kern (in het cytoplasma) worden gelezen en vertaald in eiwitten. Dit heet respectievelijk transcriptie en translatie (Figure 1).



Figuur 1: Schematische weergave van genexpressie: de transcriptie van het gen, het modificeren en het transport van het mRNA, gevolgd door de translatie van het mRNA naar eiwit.

Verschillende celtypen hebben echter allemaal hetzelfde genoom (de handleiding). Het verschil tussen deze celtypen zit hem dan ook in welke gedeeltes van het genoom wordt overgeschreven naar mRNA's. Er zijn genen die in alle cellen worden gebruikt (= tot expressie komen), de zogenaamde huishoudgenen. Maar het verschil wordt gemaakt door het gebruik van celspecifieke genen. Het is te vergelijken met een gebruiksaanwijzing voor verschillende modellen van een apparaat. Sommige hoofdstukken zijn niet van toepassing op het ene model en sommige niet op het andere model en andere hoofdstukken zijn weer op alle modellen van toepassing. Zo wordt het gen voor hemoglobine, het eiwit dat zuurstof en koolstofdioxide bindt en vervoert, alleen in rode bloedcellen overgeschreven en niet in cellen van de alvleesklier. Deze cellen hebben het gen wel maar gebruiken het niet. Zij maken wel weer insuline dat rode bloedcellen weer niet maken.Welke genen worden gebruikt wordt bepaald door eiwitten die de transcriptie van genen kunnen beïnvloeden, de transcriptiefactoren.

Transcriptiefactoren

Transcriptiefactoren zijn eiwitten die hun functie, het reguleren van transcriptie, kunnen uitoefenen door aan het DNA van een gen te binden. Zij bevinden zich dus altijd in de celkern. Ze kunnen transcriptie reguleren op verschillende manieren. DNA, dat je je voor kan stellen als een hele lange dunne draad, is normaal gesproken strak opgewonden om kleine klosjes (nucleosomen) die op hun beurt weer strak opgevouwen zijn (30 nm fiber). Dit is een ontoegankelijke structuur. Om DNA geschikt te laten zijn voor transcriptie moet het een relatief open structuur hebben. Transcriptiefactoren kunnen binden aan DNA en de compacte structuur opvouwen of andere eiwitten binden die dat kunnen. Een andere manier om transcriptie te stimuleren is door te binden aan de promoter van een gen. Dit is het stuk DNA dat zich vlak voor de transcriptiestartplaats bevindt en belangrijk is voor de transcriptie. Door vervolgens de eiwitten die het transcriptieapparaat vormen te binden, wordt de frequentie van transcriptie verhoogd. Transcriptiefactoren kunnen ook de transcriptie blokkeren door in de weg te gaan zitten of door eiwitten te binden die transcriptie blokkeren.
In hun functioneren kunnen transcriptiefactoren ook weer afhankelijk zijn van signalen van binnen of van buiten de cel. Door middel van deze signalen kan hun eigen expressie worden gereguleerd of kunnen er chemische modificatie aan het eiwitmolecuul worden aangebracht of weggehaald die de functie kunnen beïnvloeden.

Dit proefschrift

Voor de differentiatie van rode bloedcellen is de transcriptiefactor GATA-1 erg belangrijk maar de vraag wat nu de precieze functie van dit eiwit is en hoe het gereguleerd wordt is nog verre van opgehelderd. In dit proefschrift worden experimenten beschreven die de functie en regulatie van GATA-1 onderzoeken. GATA-1 is een voor de rode bloedcel specifieke transcriptiefactor die zijn naam te danken heeft aan het middelste gedeelte van de DNA base volgorde waar het kan binden: G, A, T, A. Deze bindingsplaatsen zijn gevonden in de promotoren en andere regulerende stukken DNA van alle rode bloedcel-specifieke genen tot nu toe, waaronder de hemoglobine genen. In experimenten waarbij muizen in hun rode bloedcel voorlopercellen geen GATA-1 aanmaakten en als embryo stierven door bloedarmoede, bleven de rode bloedcellen steken tijdens hun rijpingsproces. Dat GATA-1 belangrijk is voor het laatste gedeelte van het differentiatieproces werd hier mee als bewezen beschouwd. In eerdere experimenten gedaan in ons laboratorium werd in rode bloedcel voorlopercellen extra GATA-1 gemaakt. Deze cellen, die normaal gesproken onder bepaalde omstandigheden differentiëren, bleven onrijp en gingen, in tegenstelling tot de controlecellen, door met delen.

In **Hoofdstuk 2** worden experimenten beschreven met muizen die extra GATA-1 aanmaken in hun rode bloedcel voorlopercellen. Muizen die GATA-1 aanmaken in al hun rode bloedcel voorlopercellen sterven als embryo iets over de helft van de zwangerschap. De differentiatie van de rode bloedcellen is geblokkeerd en dit is de oorzaak van de fatale bloedarmoede. Dit was onverwacht omdat aangenomen werd dat GATA-1 differentiatie stimuleert en als gevolg daarvan de celdeling remt. Het genereren van deze muizen was slechts mogelijk als muizen in een gedeelte van hun cellen extra GATA-1 maakten in plaats van in alle cellen. Dit bleek gelukt te zijn doordat in één van de experimenten een extra kopie van het GATA-1 gen in het X-chromosoom terecht bleek te zijn gekomen. In een vrouwelijke muis (met 2 X-chromosomen) wordt altijd één van de twee X-chromosomen uitgeschakeld. Dit in tegenstelling tot de andere chromosomen. Dat betekent dat in 50% van de cellen de extra kopie ook was uitgeschakeld. Verrassend genoeg bleken de andere 50% van de cellen (de "abnormale" cellen) die extra GATA-1 maakten nu wel volledig te kunnen differentiëren. Hieruit blijkt dat normale cellen de abnormale cellen kunnen redden van hun ondergang. De abnormale cellen krijgen een signaal van de normale cellen om toch vooral maar normaal te differentiëren. De abnormale cellen hebben een niet-autonoom defect. Dat wil zeggen dat ze zich niet gedragen zoals hun genetisch materiaal ze eigenlijk vertelt maar dat ze gestuurd worden door de cellen in hun omgeving. Het is nog niet bekend wat het signaal is dat hiervoor

gebruikt wordt maar wij hebben het alvast een naam gegeven: REDS. Dat staat voor red blood cell differentiation signal, rode bloedcel differentiatie signaal.

In **Hoofdstuk 3** worden experimenten beschreven met muizen waarvan het GATA-1 gen op een willekeurig tijdstip zou moeten kunnen worden uitgeschakeld. Een muis zonder GATA-1 gen sterft al ongeveer halverwege de zwangerschap en zo kan het gemis aan GATA-1 niet op latere leeftijd worden bestudeerd. Hiervoor werd het GATA-1 gen zo aangepast dat er voor en er achter zgn. knipplaatsen (lox sites) werden aangebracht die kunnen worden herkend door een knipenzym (Cre recombinase) dat deze plaatsen herkent en het DNA tussen de knipplaatsen kan verwijderen. Dit is voor te stellen als lang touw waar je op een bepaalde plaats een knoop met een lus in legt en vervolgens de lus er met een schaar afknipt. Door nu muizen met het aangepaste GATA-1 gen te kruisen met muizen die het knipenzym (de schaar) in hun cellen maken kan je muizen krijgen die beide hebben en in hun cellen zal het GATA-1 gen (de lus) worden verwijderd. Door nu het knipenzym te voorzien van een biologische aan/uitschakelaar zou het mogelijk moeten zijn om op ieder willekeurig tijdstip het GATA-1 te verwijderen. Dit bleek vooral in theorie een mooi idee. Het systeem werkt wel maar is niet efficiënt genoeg om in alle cellen het GATA-1 gen te verwijderen. Het systeem met het knipenzym zonder aan/uitschakelaar werkt perfect en is ook bijzonder goed bruikbaar voor het verwijderen van het GATA-1 in een bepaalde groep cellen. Dit werd bereikt door muizen te genereren die het knipenzym alleen in een bepaalde groep cellen maakt. Alleen in deze cellen werd het GATA-1 gen uitgeschakeld.

Een voorbeeld hiervan wordt gegeven in **Hoofdstuk 4** waar muizen worden beschreven die geen GATA-1 gen meer in hun testis (testikels) hebben maar wel in hun rode bloedcellen. Zonder deze techniek was het niet mogelijk het gemis aan GATA-1 in de testis te bestuderen aangezien de muizen zonder GATA-1 in al hun cellen voortijdig sterven. Andere onderzoekers hadden door middel van andere experimenten afgeleid dat GATA-1 wellicht een belangrijke functie in de testis zou kunnen vervullen. Onze experimenten tonen aan dat GATA-1 kan worden weggelaten zonder dat dit enig effect heeft op de structuur en de functie van de testis. Wellicht heeft GATA-1 een functie, op andere tijdstippen van de ontwikkeling dan die nu zijn bestudeerd of in een stress situatie.

In **Hoofdstuk 5** wordt een techniek beschreven die het mogelijk maakt om rode bloedcellen in een eenvoudige en kleinschalige proef te testen op hun vermogen volledig te differentiëren tot een volwassen rode bloedcel. Dit is een nieuwe techniek die is gebruikt in Hoofdstukken 2 en 3.

In **Hoofdstuk 6** worden resultaten van ons en anderen vergeleken en wordt er gespeculeerd over mogelijke modellen voor de functie en de regulatie van GATA-1. Het

eerste model behelst een verband tussen celdeling en GATA-1. Een afnemende celdeling is namelijk een kenmerk van de eindfase van het differentiatieproces. In het tweede model wordt gespeculeerd over de regulatie van GATA-1 door REDS. Gedacht wordt aan een signaalmolecuul dat wordt uitgescheiden door een normale cel, die wel volledig differentieert, dat wordt opgevangen en herkend door een abnormale cel die dan vervolgens toch kan differentiëren. Dit zal een bestaand systeem zijn dat ook onder normale omstandigheden een functie heeft. Mogelijke kandidaten passeren het voetlicht. Als laatste worden mogelijke nieuwe proeven besproken die verdere opheldering kunnen geven.

Curriculum vitae

name:	Fokke Albert Lindeboom
Born:	27 August, Zwolle, The Netherlands
1981 - 1988	Carolus Clusius College,VWO, Zwolle
1988 - 1995	Rijks Universiteit Groningen, Netherlands Department of Chemistry, specialisation biochemistry. Practical training in the groups: Signal transduction (Prof van Haasterd) and Eukaryotic gene regulation (Dr. AB) Graduated in the Eukaryotic gene regulation group. Subject of graduation project:'The functional analysis of the single strand DNA binding protein.'
1995 -	Ph.D. student, Department of Cell Biology and Genetics, Erasmus University Rotterdam Title of thesis:'The devil is in the level, The function of the transcription factor GATA-1.' Promotor: Prof. dr. F.G. Grosveld Co-promotor: Dr J.N.J. Philipsen
1999	The Oxford examination in English as a foreign language.
2001-	Post doctoral researcher, Department of Hematology, Erasmus University Rotterdam Subject of the project:'The function and regulation of the NM23-M2 gene'. Dr R. Delwel, Dr. M. von Lindern, Prof. Dr B. Löwenberg

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Fokke