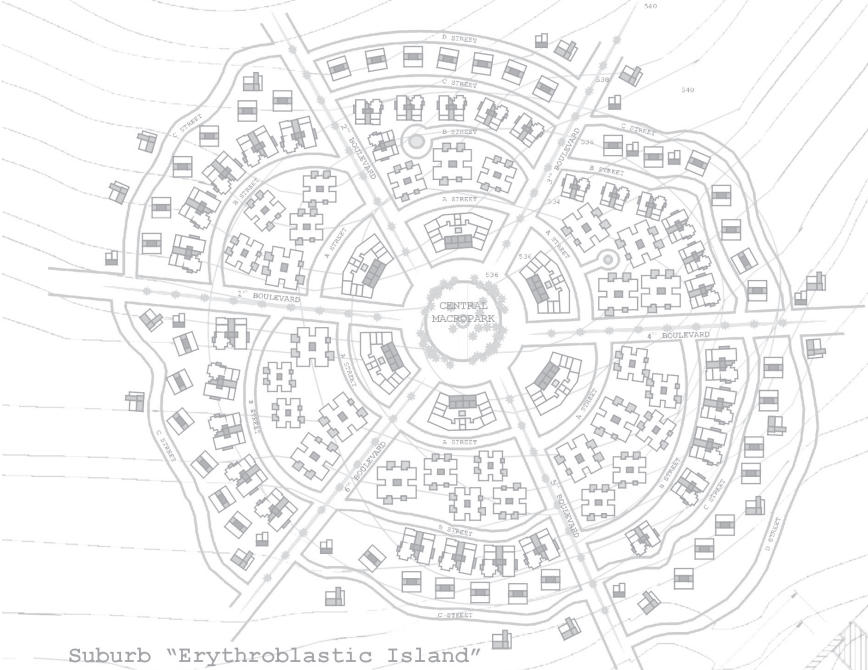
Studies on Gata1: Cell Cycle, Survival and Differentiation



Suburb "Erythroblastic Island"


PROERYTHROBLAST


BASOPHILIC ERYTHROBLAST


POLYCHROMATIC ERYTHROBLAST


ORTHOCHROMATIC ERYTHROBLAST


ERYTHOCYTE


PYCNOTIC NUCLEUS

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Cover:
Urbanistic project Suburb “Erythroblastic Island”, linking functionality with architecture. Idea by Manuel Gutiérrez Torralba. Design Andrés Arbiza Jiménez and Laura Gutiérrez Gutiérrez.

Studies on Gata1: Cell Cycle, Survival and Differentiation

De rol van Gata1 in de cel cyclus, overleving en differentiatie

Proefschrift

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Dr. J.N.J. Philipsen

To all my educators, specially to my parents, until now and thereafter.

A mis educadores, especialmente a mis padres, hasta y desde ahora.

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Selected colour pictures are displayed in the cover

CHAPTER 1

INTRODUCTION

INTRODUCTION

1.- An introduction to cell specialisation. Why is cell specialisation needed?

All living organisms are characterised by a common feature, maybe simplistic, or maybe too biased by a darwinian point of view: the need of perpetuating the genetic content (Darwin 1871). This requires energy, and all living organisms have developed different adaptive ways of obtaining energy. In other words, living organisms need to assure an energetic income, which will be metabolised and managed into movement, growth, or any other investment that will lead to the final aim. When the nutrient sources were exhausted the organisms needed to find other niches to exploit. The search for new niches and the selection of the most successful adaptive forms of live marked evolution. From the ecological point of view, we could consider the diversity of unicellular organisms as cases of cellular specialisation in order to adapt to certain environmental conditions. Sometimes the forms of adaptation to new conditions favoured the association of unicellular organisms into colonies. A colony assures a larger surface of exposure with the exterior and subsequently a more efficient nutrient intake. However, in this type of association basically all the cells are of the same type. Further advance came amongst multicellular organisms with cell specialisation into endoderm and ectoderm (Figure 1, see *Hydra*). From then on through evolution, the whole diversity of living organisms shows the maximum grade of cell specialisation in the sense that all constituent cells adopt a specialised form. How can this be achieved when all the cells of an organism have the same genetic content? The tight regulation of the transcription program of specialised cells makes this possible. Specialised cells, that *per se* do not propagate the genetic content of the species, contribute to the integrity and functionality of the organism, which ultimately will accomplish its aim. Figure 1 shows a very simplified evolutionary procession from unicellular organisms to mammals, highlighting cell specialisation (Futuyma 1998; Ridley 2003).

Cell specialisation has the disadvantage that specialised cells lose their plasticity and cannot adapt to drastic changes in their environment anymore, as compared to unicellular organisms. In order to maintain homeostasis, multicellular organisms must have a supply and waste disposal system and protect themselves from external challenges. This is why circulatory systems evolved: the blood reaches every cell in the organism to supply energy and collect waste, to transport signals or cell types that will access the target cells or tissues in case of need and to protect the system from invaders. Cells derived from the haematopoietic system populate the blood. This system is very dynamic and flexible in that its stem cells can differentiate into many different cell types depending on the requirements of the organism and/or a change in conditions (reviewed in (Zon 2001)).

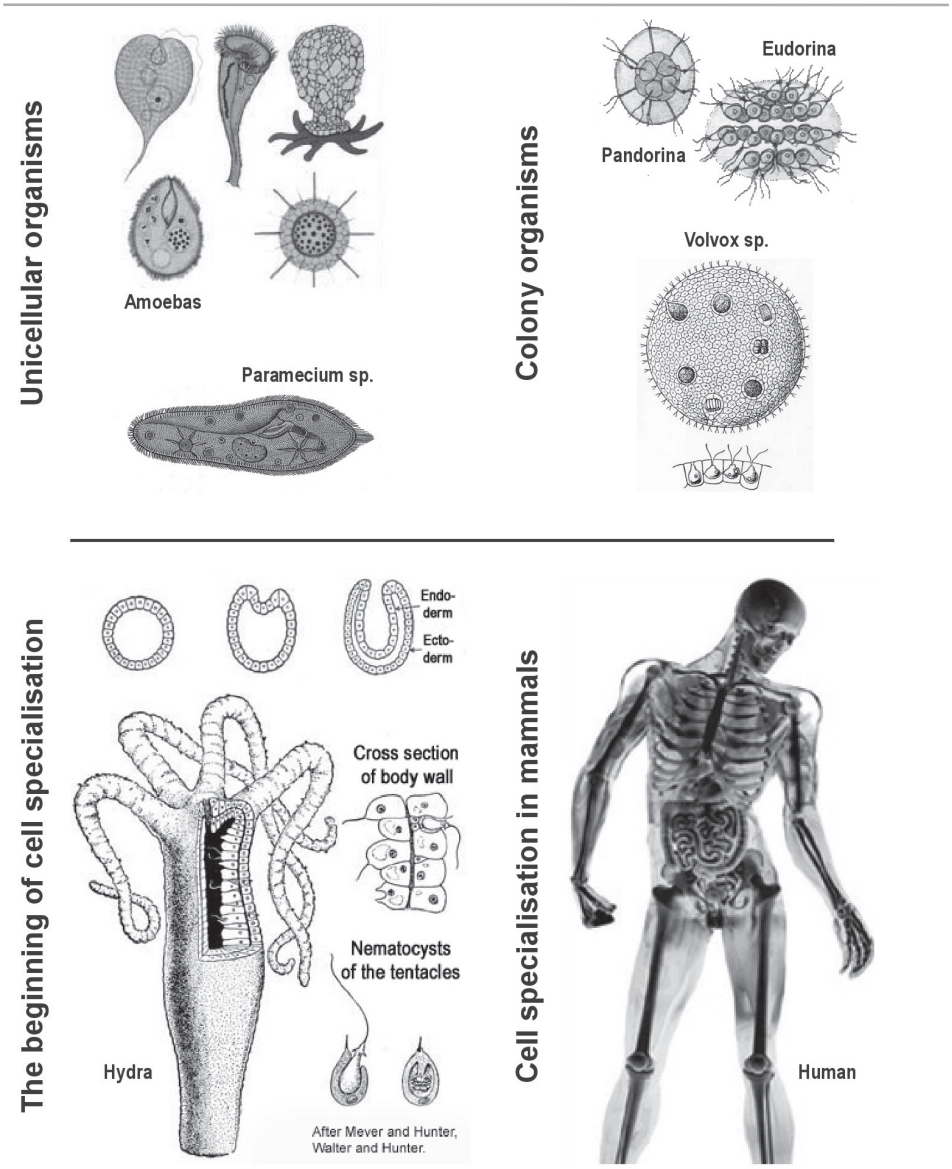

Figure 1. Cell specialisation through evolution.

Unicellular organisms: Drawings of unicellular organisms, such as *Amoeba* and *Paramecium* are shown in the left side of the panel. Note that the diversity of the unicellular organisms is enormous, as exemplified by the different types of amoebas on the top, due to the need to adapt to different ecosystems.

Colony organisms: Drawings of some colony organisms, such as *Pandorina*, *Eudorina* or *Volvox* algae, are depicted. The colonies are formed by the biflagellated single cells embedded in protoplasma.

The beginning of cell specialisation: The main body of *Hydra* is formed by two layers of cells: endoderm and ectoderm. The ectodermic cells in the tentacles have specialised into nematocysts, which release toxic substances upon mechanical activation. In the drawing nematocyst schemes before and after discharge are depicted.

Cell specialisation in mammals: Specialised cells contribute to the different organs and structures of a human, such as the skin, muscles, skeleton, stomach, heart and so on.

The following introduction of the haematopoietic system is not exhaustive but covers the relevant information needed to understand the background and scope of the thesis. As presented in this thesis, the main interest of my work is the regulation of the erythroid compartment and in particular the role of Gata1, a key transcription factor, in this process. However, it is artificial to completely separate the study of the erythroid compartment from the haematopoietic system as a whole and my studies include a broad new field: the dendritic cell compartment. Still, this introduction might be somewhat biased and presents the haematopoietic system from an erythroid and Gata1 angle.

2.- The haematopoietic system

The haematopoietic system in mammals is composed of a collection of mature differentiated cells that derive from a common progenitor named the haematopoietic stem cell (HSC) (Figure 2). Mature blood cells have a limited life span and they need to be replenished in

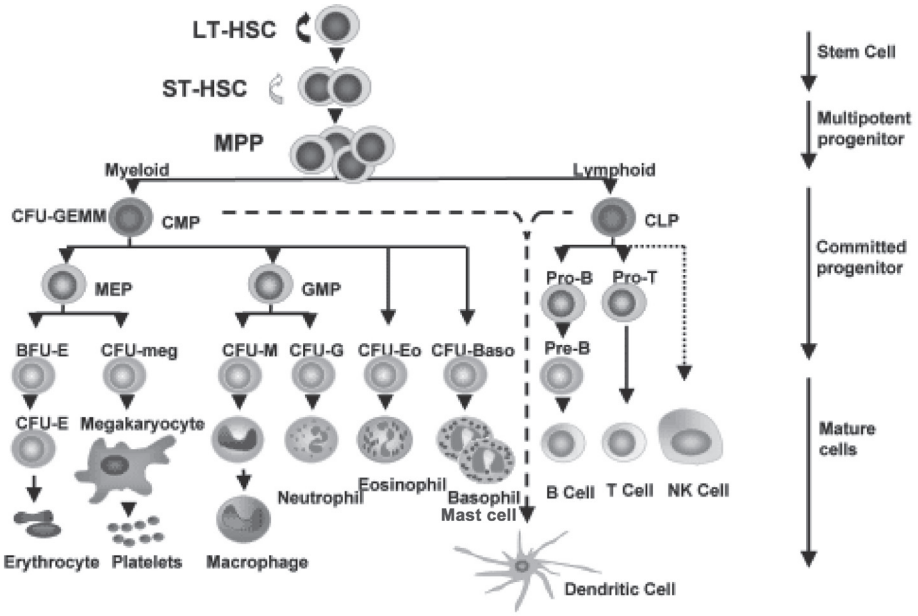

Figure 2. The haematopoietic system.

Representation of the haematopoietic system based on a figure obtained from the web (www.molmed.lu.se/images/webfig5_new.gif). LT, long term; ST, short term; HSC, haematopoietic stem cell; MPP, multipotent progenitor; CMP, common myeloid progenitor; CFU- GEMM, colony forming unit-granulocyte erythroid macrophage megakaryocyte; MEP, megakaryocyte erythroid progenitor; BFU-E, burst forming unit-erythroid; CFU-E, colony forming unit-erythroid; CFU-meg, colony forming unit-megakaryocyte; GMP, granulocyte macrophage progenitor; CFU-M, colony forming unit-macrophage; CFU-G, colony forming unit-granulocyte; CFU-Eo, colony forming unit-eosinophil; CFU-Baso, colony forming unit-basophil; CLP, common lymphoid progenitor; NK, natural killer.

a regulated manner in order to maintain the homeostasis of the organism and to protect it (reviewed in (Roitt *et al.,* 1993; Zon 2001)).

The protective function is accomplished by the innate and adaptive immune system. These systems are composed of different kinds of lymphocytes. For example, B and T cells specifically recognise pathogen antigens and trigger the adaptive immune response. They are formed in the bone marrow and complete their maturation in the spleen, thymus, lymph nodes or lymphoid tissue. Mature B and T cells are selected for tolerance to auto-antigens, to ensure they only recognise antigens from invading pathogens. Upon the recognition of an antigen, B cells are activated to produce antibodies against it. These antibodies are presented to other cells of the immune system that will eliminate the invading pathogen. Mature T cells can exert different functions. Helper T cells (Th) interact with activated B cells and nurse their differentiation and antibody production. They also cooperate with phagocytes in the elimination of pathogens or with dendritic cells in the antigen presenting process. Cytotoxic T cells (Tc) are involved in the destruction of host cells that were infected by viruses or other intracellular pathogens. Another type of cell related to T cells is the natural killer (NK) cell, whose origin is not very well understood. They are quite unspecifically reactive to tumour cells or virus-infected cells and induce cytotoxicity to the target cells.

The maturation and function of B and T cells is closely linked to that of mononuclear phagocytes, such as dendritic cells and macrophages. They are both originated in the bone marrow and become resident in different tissues where they perform their function. Dendritic cells endocytose antigens in the tissue of residency. They migrate to the lymph nodes or spleen, process the antigen and present it to B and Th cells. This process is crucial for the adaptive immune response, and for the maturation of lymphocytes to acquire tolerance against auto-antigens. Macrophages are basically professional phagocytic cells that manage the cell waste in the tissue of residency. In addition, they can be recruited to the infected area during immune or inflammatory response.

Other haematopoietic cell types, *i.e.* neutrophils and eosinophils, are also characterised by their phagocytic properties. These granulocytes are formed in the bone marrow and circulate in the blood. The neutrophils contain lysosomes that are very effective in the destruction of circulating bacteria. They also release their granules and cytotoxic substances upon activation by immunocomplexes. The eosinophils are also able to phagocytose and destroy the ingested microorganisms, although this is not their main function. Eosinophils are specialised in the response against parasites that are not phagocytosed by specialised cells due to their large size. Eosinophils are recruited by chemotaxis (mainly attracted to substances released by T, mast cells or basophils) to the surface of the parasite, where they release their granules that contain cytotoxic agents. At the same time they release substances that counteract the stimuli originating from mast cells and basophils.

Mast cells and basophils are also granulocytes. Mast cells are very similar to circulating basophils, but are never found in the circulation. Upon activation, they release mediator agents like histamines, which attract eosinophils. They are closely related to the immune response against parasites. When the immune response of the basophil-eosinophil team is not well balanced it may lead to an allergic reaction.

The remaining cell types derived from the haematopoietic system are the megakaryocytes and the erythroid cells. They are formed in the bone marrow, and the final products of their differentiation are released in the blood stream: platelets and red blood cells respectively. Platelets are important to heal wounds and vessel fissures, by clot formation, and also during inflammatory responses. The red blood cells are highly specialised cells that lose their nucleus in order to allow the maximum space for gas transportation.

The nomenclature of the different progenitors is based on *ex vivo* culture methodology or engraftment experiments. For example, long term HSCs (LT-HSCs) are the population of cells that give rise to different blood cell types after long term engraftment in irradiated mice (Medvinsky and Dzierzak 1998). Short term HSCs (ST-HSCs) are the cells that just after engraftment give rise to colonies in the spleen with granulocytic and erythroid characteristics. They are also named Colony Forming Unit-Spleen (CFU-S) cells (Lemischka 1992). The HSCs in the adult mammal are characterised by the following properties: they are quiescent, capable of self-renewal, they can replenish blood cells in engraftment experiments and are resistant to 5-fluorouracil (Palis and Yoder 2001). When culturing cells derived from the bone marrow in the presence of different growth factors in semi-solid medium, colonies of the different lineage restricted precursor cells form, and hence the nomenclature Colony Forming Unit is used. HSCs give rise to multipotent progenitors (MPPs). From these progenitors, two cell types originate depending on the stimuli of the environment: common myeloid and lymphoid progenitors (CMP and CLP respectively) that give rise to the different lineage restricted progenitors.

The CMP also known as colony forming unit - granulocyte erythroid monocyte megakaryocyte (CFU-GEMM) can give rise to different types of committed progenitors which can differentiate upon certain stimuli into mast cells, basophils, eosinophils and neutrophils (granulopoiesis), macrophages and dendritic cells (monopoiesis), megakaryocytes producing platelets (megakaryopoiesis) and erythrocytes (erythropoiesis). The CLP generates Pro-B and Pro-T cells that will migrate to the spleen and thymus respectively to mature into B and T cells, and natural killer cells (NK cells) (lymphopoiesis). Dendritic cells also originate from CLPs.

All these cells were characterised originally by recognisable cytological staining properties. An example of how cells within the erythroid lineage are classified based on their staining properties is shown in Figure 3. Later on, after the development of techniques that allowed the characterisation of cells based on the display of surface markers, the

Figure 3. Cytological analysis of differentiating erythroid cells.
Differentiating erythroid cells show different staining properties. From the left to the right, differentiating cells selected from cytospins stained with neutral benzidine and histological dyes are shown. The proerythroblast (PrE) has a large nucleus that stains lightly. The basophilic erythroid precursor (B) has reduced its size and the cytoplasm is basophilic. The polychromatic erythroid precursor (P) has a mixed cytoplasm in terms of baso or acidophilia. The orthochromatic erythroblast (O) has a high content of haemoglobin, hence the brown colour of the stain and the enucleated erythroblast (E) is brown as well. (See colour picture in the cover).

Table 1. Cell membrane markers in the murine haematopoietic system

Cell Type	Markers
HSCs	AA4.1, CD11c, CD34, c-Kit, Sca-1, Tie-2
CLP	Sca-1, CD127
B cells	B220, CD35, CR2, Igβ, IgMα, Ly-1 (CD5)
T cells	CD2, CD3, CD4, CD8, Ly-1 (CD5), TCR, Thy-1
CMP	Hle-1, Sca-1, CD33, CD34
Monocytes	CD11c, CD11b
Dendritic cells	B220, CD11b, CD11c, CD35, MHC-II
Macrophages	CD11b, CD11c, CD35, F4/80, Mac-1
Eosinophils	CD11b, CD35, Gr-1, CCR3
Basophils, Mast cells	CD11b, CD35, c-Kit
Neutrophils	CD11b, CD35
Erythroid cells	CD71, TER119
Megakaryocytes	CD11c, CD31

definition of cell stages during the differentiation of each committed haematopoietic cell lineage became more accurate. In Table 1 some of the cell markers that are present in murine HSCs and mature differentiated cell types are summarised. Note that frequently one unique marker is not enough to discriminate a specific cell type; the combination of surface markers is required for an accurate identification. This characteristic is crucial, as we assessed erythroid differentiation by analysing the surface marker display of erythroid cells by Fluorescence Activated Cell Sorting (FACS) analysis.

2.1.- The sites of erythropoiesis/haematopoiesis through evolution and ontogeny

The multipotent characteristic of haematopoietic stem cells made them a target of study for many years. The possibility to isolate HSCs and induce all the different kinds of blood cells *in vitro* opens a broad spectrum of clinical applications. In addition, this led to the investigation of the origin and location of haematopoietic stem cells during gestation.

The haematopoietic system took many forms during evolution. As explained above, HSCs have the following properties: quiescence, self-renewal and multipotency, although invertebrates such as worms do not need a population of self-renewing stem cells. Their life span is the same as the life span of their blood cells. The blood cells, which are produced in the coelomic cavity, function through the entire life of the worm. In some insects, functioning blood cells still experience a few divisions in the circulatory space. These divisions involve only the nucleus, while later in development they involve the nucleus and the cytoplasm (Tavassoli 1991).

The first form of erythroid architectonic factory through evolution, the blood islands, is seen in segmented worms. The blood islands are located in the lateral vascular channels of these worms. Arthropods display the blood islands in the stomach glands. These islands occur in the wall of the gastrointestinal tract particularly in the submucosa, probably as an evolutionary need to have close interaction with the tissues directly providing nutrients. From then on through evolution, the haematopoietic system has been located in the diffuse spleen, compact spleen, intestinal submucosa, kidney, gonads, meninges, liver, pancreas and bone marrow (Tavassoli 1991).

2.2.- Does ontogeny recapitulate phylogeny? What happens with the haematopoietic system?

The idea that the mammalian ontogeny is a replica of evolution (Haeckel's law), made haematologists think of the nomadic behaviour of the haematopoietic system within the mammalian embryo. However, mammalian haematopoiesis does not seem to completely recapitulate phylogeny.

The yolk sac produces the first blood cells in mice and men. In mice the yolk sac production starts at 7.5 days *post coitum* (dpc) and in human it happens at 3-6 weeks of gestation. The yolk sac haematopoiesis consists of erythropoiesis mainly. As the embryo develops, haematopoiesis abandons the yolk sac. These observations raised two important questions: Can other cells of the haematopoietic system be obtained from the yolk sac in *ex vivo* cultures in the presence of the appropriate growth factor conditions and, second, what is the origin of HSCs.

The yolk sac is the first factory of blood and vessels and it seemed logical that it provides the HSCs that migrate into the embryo proper later during development. Moore and Metcalf gave the first insights into this in 1970: an 8 dpc yolk sac could give rise to

erythroid/granulocyte/macrophage colonies *in vivo* and *ex vivo*, although only 11 dpc yolk sac cells were able to engraft irradiated animals (Moore and Metcalf 1970). Yolk sac hosts HSCs, which migrate later in ontogeny to the embryo proper depending on certain stimuli. One of the first experiments addressing this was performed by Cudennec and colleagues (Cudennec *et al.,* 1981). They showed that the yolk sac contained HSCs but its milieu is not permissive for migration or differentiation of HSCs. They require factors that are present in the foetal liver and in an undefined region within the embryo proper. This region was later identified as the Aorta Gonad Mesonephros (AGM) region, and was proved to contain the first HSCs in the embryo proper (Godin *et al.,* 1993; Medvinsky *et al.,* 1993). HSCs as such appear at 11 dpc in the yolk sac, and in the embryo proper at 10-10.5 dpc in the AGM and in the foetal liver at 11-12 dpc. Non-haematopoietic microglial precursors of the Central Nervous System also originate in the yolk sac. However, the origin of lymphoid cells is controversial as no evidence of lymphoid progenitors has been found in yolk sac *ex vivo* cultures to date (Palis and Segel 1998). From these experiments it was concluded that the progenitors of the yolk sac can give rise to other lineages that are not produced at that time of gestation *in vivo*, and that their potential is restricted to a certain time window. The changing environmental conditions during gestation and the different stages of development determine the requirements of the organism, and therefore haematopoietic production. As mentioned above, the yolk sac is mainly an erythroid factory. The foetal liver milieu allows granulopoiesis although erythropoiesis is favoured in a 5 to 1 ratio. Later in development, haematopoiesis moves to the bone marrow, whose milieu favours granulopoiesis versus erythropoiesis in a 5 to 1 ratio (Tavassoli 1991; Palis and Segel 1998).

3.- Primitive and Definitive erythropoiesis

The erythroid process in the yolk sac is defined as primitive erythropoiesis. The blood cells produced are nucleated and complete their maturation including enucleation in the blood stream (Palis and Yoder 2001; Kingsley *et al.,* 2004). In the mouse, primitive blood cells appear at day 8.5 of gestation and stop dividing at 13.5 dpc (Sasaki and Matsumura 1986). Enucleated primitive cells can still be detected in blood until day 15 of gestation (Wong *et al.,* 1985) (Palis and Yoder 2001). Primitive blood cells appear in human in the blood at 4-5 weeks of gestation and they remain in circulation until week 12 (Palis and Yoder 2001).

Primitive erythropoiesis occurs in blood islands. It is closely linked to vascular development and mesoderm/endoderm signals are required for blood island formation. The blood island originates from a so-called haemangioblast that gives rise to erythroid as well as endothelial cells, with endothelial cells in the exterior of the island, surrounding erythroid precursors (Palis and Yoder 2001). Looking back in evolution, blood cells in arthropods are nucleated and divide within the circulatory space. In birds blood cells are

also nucleated and, like primitive erythropoiesis, intravascular in origin (Tavassoli 1991).

Definitive erythropoiesis starts in the mouse embryo at 10.5 dpc in the foetal liver, and moves later during ontogeny to the spleen and bone marrow (15 dpc) (Wong *et al.,* 1985; Palis and Segel 1998). In human, the foetal liver becomes haematopoietic after 6 to 22 weeks of gestation, and the bone marrow from 12 weeks onwards (Palis and Segel 1998). Definitive erythrocytes appear in blood at 12.5 dpc in the mouse and at week 8 of gestation in human (Wong *et al.,* 1985; Palis and Segel 1998). Definitive erythropoiesis is extravascular and the cells are enucleated. Hence it was thought that the enucleation of definitive erythrocytes was dependent on crossing the endothelial wall into the bloodstream. However, enucleation of erythroid cells can occur in *ex vivo* cultures independently of crossing the endothelium (Tavassoli 1991).

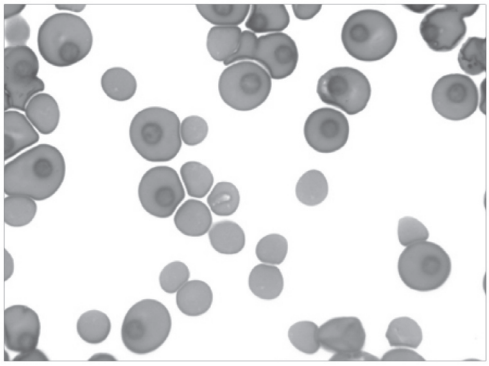

Figure 4. Murine foetal blood cytospin.
Cytospin of foetal blood collected at 13.5 dpc and stained with neutral benzidine and histological dyes is shown. The products of primitive (nucleated) and definitive (enucleated) erythropoiesis can be seen. (See colour picture in the cover).

In Figure 4, a cytospin of mouse foetal blood collected at 13.5 dpc is depicted. At this stage both primitive and definitive erythroid cells are circulating. The primitive erythroid cells are nucleated, while the definitive erythroid cells are not.

The different stages of definitive erythroid maturation are, from the first recognised committed erythroid precursor: burst forming unit-erythroid (BFU-E), colony forming unit-erythroid (CFU-E), proerythroblast, basophilic, polychromatic and orthochromatic erythroblasts, pycnosing erythroid cell and enucleated erythrocyte. In Figure 3, a sequence of differentiating erythroid cells from the proerythroblast stage is depicted. The differentiation process is characterised by a number of cell divisions, which are accompanied by a reduction in cell size and a fast and dramatic change in the protein content. The cell division number has been measured and it is known that a CFU-E cell divides once before becoming a proerythroblast, and 4 more cell divisions are required since then to reach the reticulocyte stage (Allen and Testa 1991). The last stages of erythroid differentiation include the most extreme example of cell specialisation: erythroid cells condense their nucleus and extrude it. The mature erythrocyte is able to pass through

the microvasculature due to its minimal size, and to efficiently use the intracellular space for gas transportation.

In the present work, and as mentioned above, we assessed erythroid differentiation based on surface-marker expression. In the mouse, the erythroid lineage has a specific cell surface marker that is recognised by TER119 (Ikuta *et al.,* 1990; Kina *et al.,* 2000) (Table 1). This marker is associated with Glycophorin A and is expressed from the proerythroblast stage onwards. TER119 is not detected in earlier progenitors, like CFU-E or BFU-E. However, CD71 is expressed at these early stages. TER119 is not expressed in the widely used murine erythroleukaemia (MEL) cells and therefore, another method must be used to assess the differentiation occurring in DMSO stimulated MEL cells.

In human another combination of markers is utilised to assess the erythroid differentiation stages. CD71 is expressed in BFU-Es and Glycophorin A is expressed beyond the CFU-E stage. If progenitors earlier than BFU-Es need to be identified, HLe-1 is a common leukocyte antigen (T200) expressed in early committed progenitors of the myeloid lineage (Loken *et al.,* 1987).

3.1.- The erythroblastic island

Definitive erythropoiesis occurs in the erythroblastic island (Bessis 1958). It consists of a macrophage surrounded by maturing erythroid cells, with the more immature erythroid cells in the centre of the island and the more mature cells at the periphery. The function of this macrophage is to nurse the differentiating erythroid cells and to phagocytose the expelled nuclei of mature erythroid cells before they enter the blood stream.

In Figure 5A we show a representative scheme of the erythroblastic island. In foetal liver sections immuno-stained with the macrophage marker F4/80, we detect erythroblastic island-like aggregates. Some high magnification pictures are shown in Figure 5B. Unfortunately, the three dimensional structure is difficult to analyse from sections, but the regular disposition of these cellular aggregates can be observed in the lower magnification picture (v). When looking at higher magnification, erythroid cells at different stages of differentiation as estimated by cell size can be seen (i-iv).

Bessis described the island in the late 1950s (Bessis 1958). He showed that erythroid cells bind to the macrophages in a calcium dependent manner and that fibronectin molecules are directly involved in the binding. They are no longer expressed in the reticulocyte membrane, when the erythrocytes enter the blood stream. He proposed that signalling mechanisms originate from the macrophage, which might be involved in the enucleation and release into the blood stream of the mature erythroid cell. However, these particular processes are still poorly understood (Bernard 1991). For example, erythroid cells in the final stages of maturation release matrix metalloproteinases (MMPs) and vascular endothelial growth factor (VEGF), which might be directly involved in the migration from the bone marrow into the blood stream (Ratajczak *et al.,* 2001). The erythroblastic island

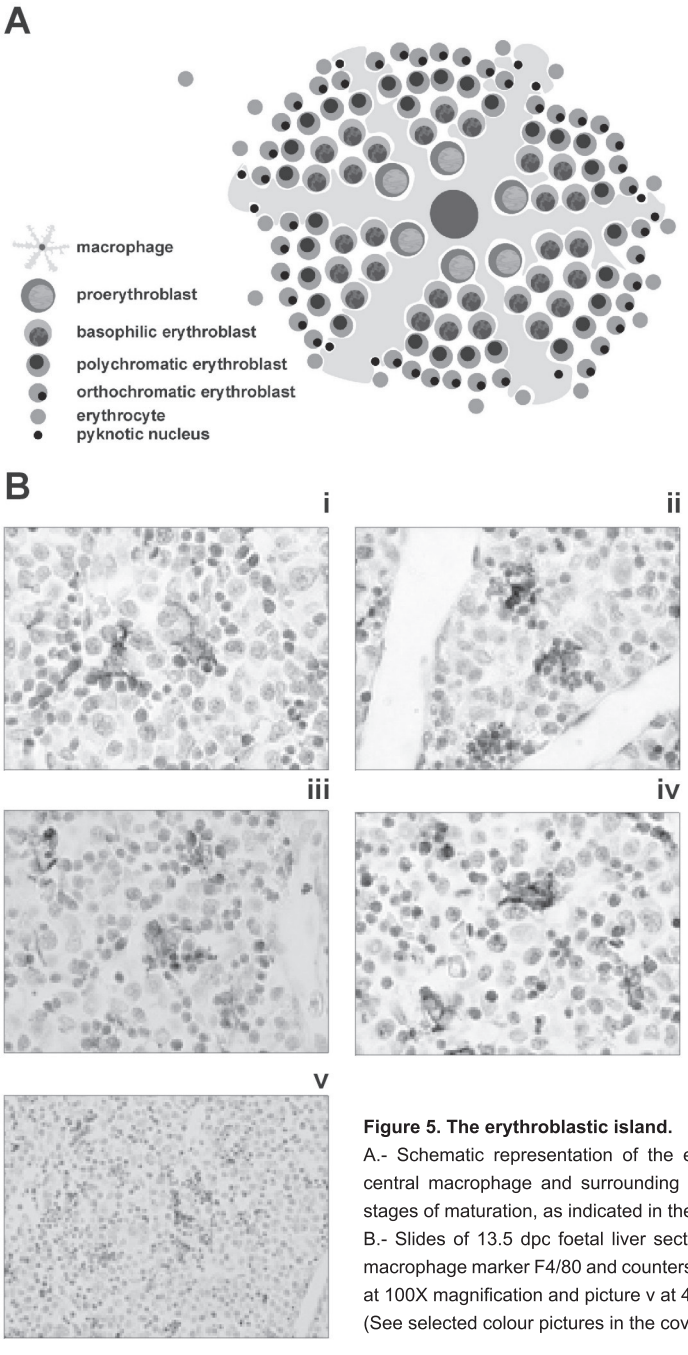

Figure 5. The erythroblastic island.
A.- Schematic representation of the erythroblastic island with the central macrophage and surrounding erythroid cells in successive stages of maturation, as indicated in the legend.
B.- Slides of 13.5 dpc foetal liver sections immunostained with the macrophage marker F4/80 and counterstained. Pictures i-iv are taken at 100X magnification and picture v at 40X magnification.
(See selected colour pictures in the cover).

itself is also not a static unit. The foetal liver is colonised at 10 dpc and the erythroid pool expands until reaching its maximum at 14 dpc. The erythroid pool of the foetal liver starts involution at 15 dpc. The erythroblastic island aspect changes through the duration of the foetal liver stage: islands are approximately 35-70x20-40 μM at 13-14 dpc and they have an ellipsoidal shape. The macrophages in the centre still divide at this stage. Later on, at 15 dpc, the islands no longer show an ellipsoid shape and they aggregate forming cords close to sinusoids. The islands are finally disrupted as mature cells are released into the blood stream (Sasaki and Sonoda 2000). It has been described that in rat bone marrow, erythroblastic islands migrate from the inner matrix of the bone marrow towards the sinusoids. Furthermore, the erythroblastic islands that are in close contact with sinusoids present a pool of erythroid cells that are in more advanced stage of differentiation than the pool of erythroid cells of islands that are far away from the sinusoids (Yokoyama *et al.,* 2003). Thus erythroblastic islands are mobile both in foetal and adult stages.

3.2.- Which are the environmental signals that condition the different types of erythropoiesis in the developing embryo?

During the development of the embryo, all the nutrients and oxygen are supplied by the mother through the placenta. The embryo develops in increasing hypoxic conditions as growth is accomplished. The first blood cells circulate in an embryo that needs to adapt to the changing conditions. Thus, the hypoxia levels may determine the switch from primitive to definitive erythropoiesis. The production of the hormone Erythropoietin (Epo) is proportional to the levels of hypoxia (Koury and Bondurant 1991). EpoR KO mice were generated and it was observed that primitive erythropoiesis was normal while definitive erythropoiesis beyond the CFU-E stage was blocked (Lin *et al.,* 1996). During primitive erythropoiesis the cells are produced in a very synchronised manner, as if the number of cells is predetermined to be enough to supply the required nutrients and oxygen to the embryo proper. Once the hypoxia levels have risen sufficiently, the switch to definitive erythropoiesis occurs. Definitive erythropoiesis determines its production levels based on the levels of Epo. From that moment on and after birth, this will be the major regulatory mechanism for red blood cell production. During the foetal stages, the level of hypoxia is high, as are the levels of Epo in blood. This is the reason why erythroid committed progenitors are highly proliferative in the embryo. Epo responsiveness, that is high in definitive erythropoiesis, affects a specific stage of precursors, the CFU-E, and the Epo receptor is downregulated later through erythroid differentiation (Palis and Segel 1998).

The transition from primitive to definitive erythropoiesis is accompanied by a switch in globin expression. Globins form the haemoglobin heterotetramer that binds oxygen or carbon dioxide. In the adult, the haemoglobin molecule is formed by two α and two β globins, but other types are used during ontogeny in the embryonic, foetal and adult stages (Figure 6). The different haemoglobins are characterised by different

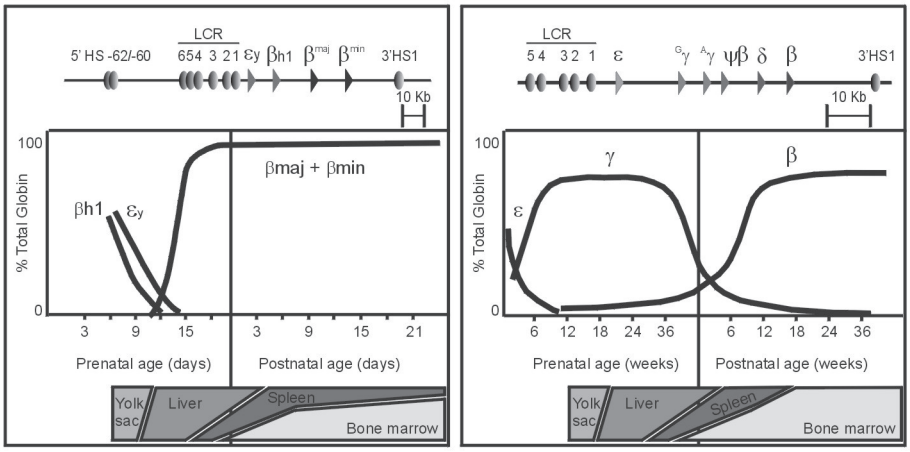

Figure 6. Globin Switching of mice and men.

Schemes of the β globin locus and the expression pattern of the different globins during mouse (left) and human (right) development are depicted. In the bottom of the panels the contribution of the haematopoietic organs through development is included. Note that in contrast with human, the spleen continues as a haematopoietic source during adulthood in the mouse. Design of the schemes based on a figure by Dr. R. Drissen (Drissen 2004).

affinities to the gas molecules. For example, foetal globins have greater affinity to O_2 compared to adult globins, and therefore the transfer of O_2 from the mother across the placental barrier is favoured. This fits with the idea that the increasing hypoxia level during development determines the transition from primitive to definitive erythropoiesis and globin switching. However, globin switching is not lineage dependent. It has been reported that co-expression of embryonic, foetal and adult haemoglobins occurs in human in erythroid cells of primitive and definitive origin (Peschle *et al.,* 1985; Stamatoyannopoulos *et al.,* 1987; Luo *et al.,* 1999). Alarming situations in the adult can induce the expression of foetal haemoglobins, as it happens during stress erythropoiesis in cases of acute or chronic anaemia (DeSimone *et al.,* 1978; Stamatoyannopoulos *et al.,* 1985; Bard *et al.,* 1999).

Altered expression of globins leads to different types of haemoglobinopathies (Forget 1998; Buchanan *et al.,* 2004; Cohen *et al.,* 2004). Partial or total deletion of the adult globin genes leads to thalassemia. α and β thalassemias cause a wide range of phenotypes depending on the mutation on the globin genes. Thalassemic individuals display anaemia, extramedullar haematopoiesis and, in severe cases, hepatotoxicity due to accumulation of iron. Sickle cell disease is caused by a single permutation of the aminoacid sequence in the β-globin chain resulting in the precipitation of β chains in anoxic conditions (typically encountered in the small vasculature) leading to a change in cell shape to a characteristic sickle shape. Such cells can block small vessels leading to local necrosis. Hereditary Persistence of Foetal Haemoglobin (HPFH) is characterised by the

continued expression of foetal globins during adulthood. HPFH can be accompanied by anaemia, but in the cases that the foetal globin expression does not affect the adult globin expression, the anaemia is very mild. In general, an efficient treatment of such pathologies has not been established yet.

4.- Model systems to study erythropoiesis/haematopoiesis

The study of a biological process can be approached directly in an animal model (*in vivo*), in cultured primary cells obtained from an animal (*ex vivo*) or in cell lines that mimic the *in vivo* situation (*in vitro*).

Adopting the mouse as animal model, erythropoiesis can be studied by isolating the haematopoietic organs during embryo development (yolk sac, AGM, foetal liver) or during adulthood (bone marrow, spleen) and determining the cell types present either by cytological histochemistry or by assessment of cell surface marker display either by immunocytochemistry or by FACS analysis. Anaemia can be induced in mice with haemolytic agents to generate stress erythropoiesis *in vivo*.

Ex vivo cultures of primary progenitors

The potential of progenitors can be studied *ex vivo* by culturing cells isolated from haematopoietic organs in semi-solid medium supplemented with the required growth factors. Depending on the growth factors added, certain types of cells will grow and form a colony (Bradley and Metcalf 1966; Metcalf 1991).

In addition primary progenitors can be differentiated *ex vivo* under specific supplemented conditions into the different cell types of interest. For example, IL-5 favours the differentiation of eosinophils from bone marrow progenitors (van Rijt *et al.,* 2002) and specific concentrations of erythropoietin can induce the proliferation or the differentiation of erythroid committed progenitors (Dolznig *et al.,* 2001).

The study of the erythroblastic islands is complicated in *ex vivo* cultures. Although the cultures are very artificial there are descriptions on how to isolate them. Zakharov and Prenant described the isolation and culture of erythroblastic islands from rat bone marrow (Zakharov and Prenant 1982). In 1988, Morris and colleagues described a method to culture erythroblastic islands from mouse foetal liver cells based on mechanical and enzymatic disruption. In these studies they showed that macrophages bind maturing erythroid cells via a non-phagocytic haemagglutinin and that mature erythroid cells lack the ligand that recognises it, resulting in their release from the island into the blood stream (Morris *et al.,* 1988). This culture method has been utilised lately by two different groups. Gloria Lee and Joal Ann Chasis reconstitute islands *in vitro* and study the role of integrins in the reconstitution process. They showed that decoy ICAM4 might be involved in releasing mature erythroid cells from the island (Lee *et al*., 2004). Iavarone *et al*, showed that pRb

null macrophages are defective and hence they are not interacting correctly with erythroid cells. This causes a defect in erythropoiesis that is non-autonomous to the erythroid cells. pRb *null* erythroid cells themselves are capable of reconstituting erythroblastic islands of wildtype macrophages *in vitro* (Iavarone *et al.,* 2004). However, it seems that pRb is intrinsically required in erythroid cells during stress erythropoiesis (Spike *et al.,* 2004). The defect in pRb *null* macrophages is caused by free proteins that are normally bound to pRb, such as Id2. In fact, loss of Id2 in the pRb *null* background rescues the macrophage defect (Iavarone *et al.,* 2004).

Embryonic Stem (ES) cells can be induced to develop into embryoid bodies and generate haematopoietic precursors under certain growth conditions. The appearance of lineage-committed progenitors follows a consistent sequence: first primitive erythroid progenitors appear, followed by macrophages and definitive precursors. Later during culture, other progenitors appear like neutrophil/macrophage and multilineage precursors, and finally mast cells (Keller *et al.,* 1993; Weiss and Orkin 1996).

Cell lines to study erythropoiesis in vitro

Murine erythroleukaemia (MEL) cells are Friend leukaemia virus-transformed spleen derived cells in the proerythroblast stage that start differentiating producing haemoglobins upon stimulation with DMSO (Antoniou 1991). Four days are necessary for MEL cells to be completely haemoglobinised. Exogenous expression of proteins at high levels can be achieved in this cell line in combination with an LCR expression vector (Needham *et al.,* 1992). However, after 4 days of culture in the presence of DMSO, a small percentage of cells enucleate and the rest rather experience catastrophe and die (Figure 7). MEL cells overexpress PU.1, which antagonises Gata1 function, and generates the leukaemia character of the cells. Gata1 forced expression in MEL cells overcomes the block to differentiation exerted by PU.1 and reduces their tumorigenicity when transplanted to mice (Choe *et al.,* 2003).

I11 cells are immortalised proerythroblasts obtained from p53 *null* mice that when induced with a certain concentration of Epo differentiate in a synchronous manner including enucleation (Dolznig *et al.,* 2001).

G1ER cells are Gata1 *null* proerythroblasts derived from embryoid bodies from ES cells that exogenously express two transgenes: human Bcl-2 that was used to protect Gata1 *null* proerythroblasts from apoptosis, and estrogen-inducible Gata1. Without induction of Gata1 the proerythroblasts remain proliferative. When activating the inducible Gata1 fusion protein, the cells differentiate (Weiss *et al.,* 1997).

Ex vivo culture of human progenitors

Human haematopoietic progenitors can be obtained from different sources. For example, CD34 positive cells can be isolated from umbilical cord blood and expanded or differentiated

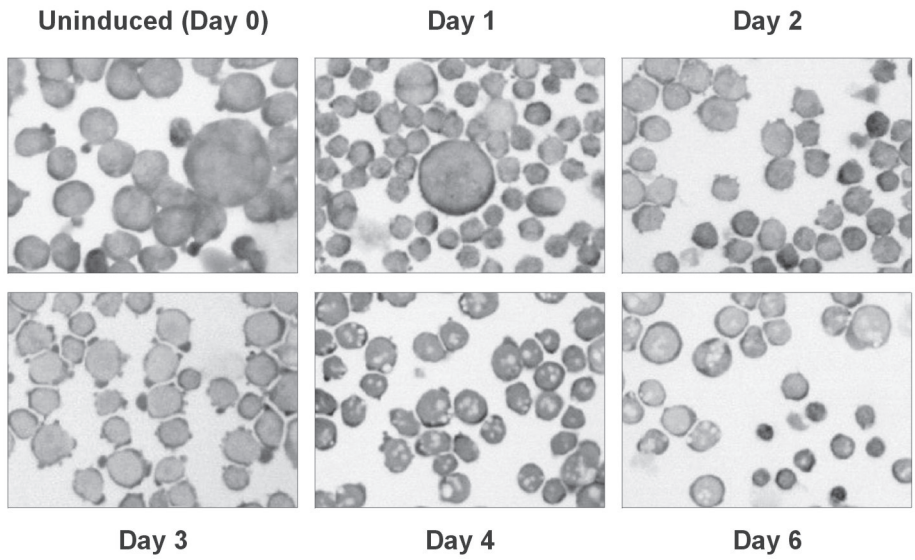

Figure 7. Differentiation of MEL cells.
Cytospins of MEL cells during differentiation were observed under the microscope after neutral benzidine and histological staining. Cells reduce slightly their volume after induction, although the major histological changes are related to the haemoglobinisation found at day 4 after DMSO differentiation induction. At day 6 there is a lot of cell catastrophe and heterogeneous differentiation of the population of cells. Some cells are dying and lyse, other appear to be enucleated, and the majority are nucleated haemoglobinised cells.

depending on the culturing conditions (Leberbauer *et al.,* 2005).

Recently, a new method involving co-culture with stromal cells has been described in which large-scale production of human red blood cells from haematopoietic stem cells of diverse origin is achieved (Giarratana *et al.,* 2005).

Human cell lines

K562 cells are an erythroleukaemia cell line derived from a chronic myeloid leukaemia patient in blast crisis (Lozzio and Lozzio 1975). Cells remain proliferative in proerythroblast stage and upon induction (with hemin) they start to produce globins (Benz *et al.,* 1980). However, globin levels are much lower than normal levels and similarly to what happens in MEL cells, K562 do not enucleate in a homogenous manner after induction of differentiation induction.

The culture systems to analyse the potential and the differentiation properties of erythroid progenitors have their own limitations and advantages and it is therefore important to exert caution when extrapolating the results obtained from cells in culture.

5.- Gata1

This thesis is focused on one of the most important erythroid transcription factors: Gata1. It is a zinc finger transcription factor and is the founding member of the GATA family of transcription factors (Patient and McGhee 2002). There are six family members: Gata1 is expressed in the haematopoietic system mainly; Gata2 and 3 are expressed in many tissues including the haematopoietic system while Gata4-6 are expressed in other mesodermal and endodermal tissues. Gata1, discovered by E. de Boer, was first cloned in 1989 (Tsai *et al.,* 1989) and located to the X chromosome soon after (Zon *et al.,* 1990; Chapman *et al.,* 1991). Many names were given to this crucial factor, such as Eryf-1, NF-E1, GF-1 (Martin and Orkin 1990). Like the other GATA family members, it recognises the WGATAR consensus sequence in the DNA (Evans *et al.,* 1988; Orkin 1992). This sequence is highly conserved through evolution and GATA factors are key regulators of different cell differentiation processes in fungi (Kudla *et al.,* 1990), plants (Teakle *et al.,* 2002; Zhao *et al.,* 2004), invertebrates (Spieth *et al.,* 1991) and vertebrates, such as zebrafish (Lyons *et al.,* 2002), *Xenopus* (Zon *et al.,* 1991), chicken and mammals (Evans *et al.,* 1988; Weiss and Orkin 1995; Lowrey and Atchley 2000; Patient and McGhee 2002; Morceau *et al.,* 2004). Gata1 is expressed at basal levels in haematopoietic progenitors (Sposi *et al.,* 1992; Leonard *et al.,* 1993), and also in erythroid (Yamamoto *et al.,* 1990), mast cells (Harigae *et al.,* 1998), megakaryocytes (Romeo *et al.,* 1990; Shivdasani *et al.,* 1997; Takahashi *et al.,* 1998), eosinophils (Zon *et al.,* 1993; Hirasawa *et al.,* 2002) and in Sertoli cells of the testis (Ito *et al.,* 1993). Gata1 is expressed in erythroid cells and in MEL cells in a punctuate pattern with a few characteristic foci in the nucleus (Elefanty *et al.,* 1996). However, Gata1 is also found in the cytoplasm of K562 cells (Zhang *et al.,* 2000). In the chicken, Gata1 is cytoplasmic in early progenitors and becomes nuclear in differentiating erythroid cells (Briegel *et al.,* 1996).

The promoter of the murine Gata1 gene has a CACC box and a double palindromic GATA site, which suggests that Gata1 regulates its own expression (Tsai *et al.,* 1991). However, whether this autoregulation is positive or negative remains controversial, as it might be dependent on the cell type and/or stage of differentiation (Orkin 1992; Yu *et al.,* 2002). For example, a targeted deletion of the palindromic GATA site causes loss of the eosinophil lineage only (Yu *et al.,* 2002) while overexpression of Gata1 in the erythroid lineage causes downregulation of the endogenous Gata1 (Whyatt *et al.,* 2000). In zebrafish, the promoter of Gata1 also has a double palindromic GATA motif, a CAAT box and a CACC box, indicating that the conservation of the regulatory sequences of Gata1 is also high (Meng *et al.,* 1999). Gata1, and also Gata2, have two tissue specific promoters: distal and proximal. In the Gata1 gene, the distal promoter contains the first exon, which is specifically transcribed in Sertoli cells. The proximal promoter encodes a transcript that is shorter than the one found in testis and is found in haematopoietic cells. Figure 8A shows

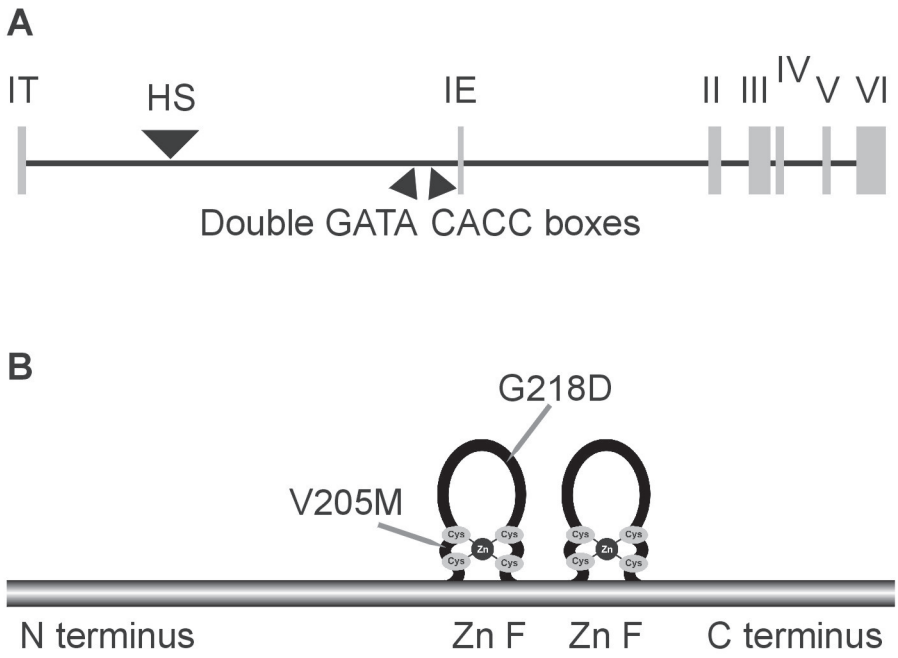

Figure 8. Gata1 gene and protein structure.

(A) The Gata1 mouse gene structure. IT, First exon testis; IE, first exon erythroid; HS, hypersensitive site that is disrupted in Gata1-low mouse (Shivdasani *et al.*, 1997). Double GATA palindromic site and CACC boxes are indicated. Deletion of the double palindromic GATA site generates mice with specific loss of the eosinophil lineage (Yu *et al.*, 2002).

(B) The Gata1 protein domain structure. The zinc fingers (Zn F) are pointed, as well as the amino (N) and carboxi (C) termini. The position of the mutations in the N terminus Zn finger described in human are indicated (Nichols *et al.*, 2000; Freson *et al.*, 2001). Note that the V205M mutation, located between the amino cystidines (Cys), resulted in the most severe phenotype.

a schematic representation of the murine Gata1 gene.

Gata1 protein has two zinc fingers, the amino and the carboxy terminal, encoded in exons 4 and 5 respectively (Figure 8B). Both fingers are implicated in DNA binding, although with different affinities (Whyatt *et al.,* 1993). The amino terminal finger is involved in protein-protein interactions, for example with FOG1 (Fox *et al.,* 1998; Cantor and Orkin 2001; Katz *et al.,* 2002). Other proteins such as PU.1 interact with the carboxy terminal Zinc finger directly blocking DNA binding of Gata1 (Zhang *et al.,* 2000; Cantor and Orkin 2001). Gata1 binds to itself (Elefanty *et al.,* 1996) and this self-association was described to enhance transcriptional activity in zebrafish (Nishikawa *et al.,* 2003). Gata1 has been identified as the DNA binding protein of pivotal activating and repressing transcription complexes during erythropoiesis (Osada *et al.,* 1995; Rodriguez *et al.,* 2005). All these

data suggest that protein interactions ultimately determine the function that a single protein has as a result of a higher protein association. The balance of transcription factor interactions and hence the role of Gata1 in haematopoiesis is therefore depending on the levels of Gata1 and the other proteins.

5.1.- Gata1 animal models

Gata1 is essential for erythroid differentiation. Gata1 *null* mice die due to severe anaemia at 10.5-11.5 dpc, coinciding with the time of onset of definitive erythropoiesis in the foetal liver (Fujiwara *et al.,* 1996). Although primitive erythropoiesis was quite normal, Fujiwara *et al* described a defective proliferation of primitive erythrocytes in blood. Gata1 *null* heterozygous females were born at expected rates, although with mild anaemia from which they recovered later during adulthood (Pevny *et al.,* 1991; Fujiwara *et al.,* 1996). Gata1 *null* erythroid precursors derived from embryoid bodies are blocked at the proerythroblast stage and undergo apoptosis (Weiss *et al.,* 1994; Pevny *et al.,* 1995; Weiss and Orkin 1995). Colony assays performed with Gata1 *null* cells showed that the macrophage and neutrophil lineages were not affected at all. Gata1 *null* megakaryocytes differentiate although their turnover is affected. Mast cells also differentiate bypassing the loss of Gata1. These last two lineages still express Gata2, and this might be the reason why in the absence of Gata1 they are still functional (Pevny *et al.,* 1995). In concordance with this, later studies showed that favouring the balance of Gata2 over Gata1 in K562 cells promotes megakaryocytic instead of erythroid differentiation (Ikonomi *et al.,* 2000).

The levels of Gata1 fluctuate during haematopoiesis and erythroid differentiation. Levels are basal in haematopoietic progenitors and upregulation takes place in erythroid committed cells (Sposi *et al.,* 1992; Dalyot *et al.,* 1993). During the terminal differentiation of erythroid cells, Gata1 levels are downregulated (Leonard *et al.,* 1993). Alteration of Gata1 levels causes different defects in haematopoiesis. Several animal models are available with an altered level of expression of Gata1.

Mouse models with downregulated levels of Gata1 were generated, with specific erythroid and megakaryopoietic defects. Gata1.05 mice express less than 5% of the Gata1 levels due to a disruption of the erythroid promoter in the Gata1 gene. The homozygous mice die at 12.5 dpc. Heterozygous mice live shorter and they display anaemia, thrombocytopenia and splenomegaly (Takahashi *et al.,* 1997; Suwabe *et al.,* 1998; Takahashi *et al.,* 1998), and they have a tendency to develop leukaemia with aging (Shimizu *et al.,* 2004). In contrast to Gata1 *null* derived cells, Gata1.05 derived cells do not apoptose at the proerythroblast stage, but remain proliferative (Suwabe *et al.,* 1998).

Gata1-low mice were generated by disrupting the hypersensitive site I (HS I) of the Gata1 promoter with a neo cassette (see Figure 8A) (Shivdasani *et al.,* 1997). Deletion of the cassette restored Gata1 levels. In both transgenics, either with or without the cassette the megakaryocytic lineage was affected (McDevitt *et al.,* 1997; Shivdasani

et al., 1997). The levels of Gata1 in Gata1-low mice are 4-5 fold lower than in wildtypes. Gata1-low embryos die at 10.5-11.5 dpc. However, 5% of the males are born. Of this 5% of born males, half of them die within the first two days after birth and the other half survive to adulthood with severe anaemia and aberrant platelet numbers (McDevitt *et al.,* 1997; Shivdasani *et al.,* 1997; Vyas *et al.,* 1999). The heterozygous females have thrombocytopenia, and although their haematocrit is normal, they show splenomegaly. Their spleens contain higher numbers of BFU-E and CFU-E compared to wildtypes, and their fertility is affected. They have accentuated response to induced haemolytic anaemia (Vannucchi *et al.,* 2001).

Gain of function of Gata1 is also causing defects in erythroid differentiation. Gata1 overexpressing mice from an X-linked transgene under the regulation of the LCR and β-globin promoter die at 13.5 dpc. However, the heterozygous females are born normal (Whyatt *et al.,* 2000).

5.2.- Regulation of and regulation by Gata1

Gata1 is regulated at the post-translational level both by phosphorylation and acetylation. For example, when Gata1 is nucleated in chicken erythroid cells upon differentiation as mentioned above, it is hyperphosphorylated (Briegel *et al.,* 1996). Gata1 is also acetylated by p300/CBP and this enhances the DNA affinity probably by favouring certain protein-protein interactions (Blobel *et al.,* 1998; Boyes *et al.,* 1998; Blobel 2000; Blobel 2002; Ohneda and Yamamoto 2002). The moment and the extent at which these modifications occur might have different effects. For example, CBP is required for self-renewal of haematopoietic progenitors and p300 for differentiation of committed precursors (Rebel *et al.,* 2002).

All the erythroid genes described, starting from globins to Gata1 itself contain GATA sites in their promoter sequence (Mantovani *et al.,* 1987; Wall *et al.,* 1988; Martin *et al.,* 1989; Mignotte *et al.,* 1989; Plumb *et al.,* 1989; Watt *et al.,* 1990; Whitelaw *et al.,* 1990; Gong *et al.,* 1991; Tsai *et al.,* 1991; Zon *et al.,* 1991; Orkin 1992). However, Gata1 interacts with different kind of proteins (SCL/TAL1, LMO2, pRb, PU.1, FOG-1 or CBP/p300) (Ferreira *et al.,* 2005) that might lead to a higher organisation involved in a positive or a negative transcriptional activation (Rodriguez *et al.,* 2005). Gata1 target genes, that are essential for erythropoiesis, have been found sequentially in a more functional manner. The Epo receptor (EpoR) gene has a GATA site at –30bp and two independent groups reported the transactivation of the EpoR by Gata1 (Zon *et al.,* 1991; Komatsu *et al.,* 1997). As mentioned before, Gata1 has been described to upregulate the transcription of globins (Whitelaw *et al.,* 1990). In addition, Gata1 appears to be crucial in the positive regulation of certain genes involved in cell cycle regulation such as Gfi-1B (Huang *et al.,* 2004) a gene essential in the development of the erythroid and megakaryocytic lineages (Saleque *et al.,* 2002), cdc6 (Vilaboa *et al.,* 2004) and cyclin D1 (Tanaka *et al.,* 2000). Gata1 also

transactivates genes important for cell survival, such as Bcl-2 (Tanaka *et al.,* 2000) and Bcl-xL (Gregory *et al.,* 1999). The function of Gata1 in the regulation of the cell cycle and cell survival is introduced in sections 5.4 and 5.5.

Gata1 is not only a positive transactivator. The Epo gene has been reported to be negatively regulated by Gata1 (Imagawa *et al.,* 1997). Other repression targets of Gata1 are Gata2, cMyb and cMyc (Briegel *et al.,* 1996).

The studies of Gata1 target genes by loss of function are complicated as other haematopoietic GATA proteins might take over the functions of Gata1. Gata2 is co-expressed with Gata1 in haematopoietic progenitors, and they seem to antagonise each other during lineage commitment (Ohneda and Yamamoto 2002). For example, overexpression of Gata2 in K562 cells favours megakaryocytic instead of erythroid differentiation (Ikonomi *et al.,* 2000). Weiss and colleagues observed that Gata1 target genes are still expressed, including the negatively regulated genes such as cMyb, in ES cells that are Gata1 *null* before they apoptose (Weiss *et al.,* 1994). The fact that Gata1 genes were upregulated suggests that Gata2 overlaps with the transcriptional activity of Gata1. In fact, rescue experiments of Gata1 *null* mice with Gata2 and Gata3 transgenes partially restores their phenotype (Takahashi *et al.,* 2000).

Very little is known about pathways interfering with Gata1 activity, while this is crucial information in order to understand Gata1 function completely. So far it is known, for example, that Gata1 transcriptional activity is repressed by the Notch signalling pathway when c-Jun upregulates the expression of HERP2 (Elagib *et al.,* 2004). Notch1 forced expression in K562 cells leads to the disruption of the p300-Gata1 interaction through HES1, resulting in inhibited Gata1 activity (Ishiko *et al.,* 2005).

5.3.- Clinical cases of Gata1 mutations

Previous studies of Gata1 loss or gain of function have clearly shown the importance of this factor and the consequences of deregulated Gata1 expression *in vivo*. Naturally occurring Gata1 mutations in human have been described, making the study of the regulation of Gata1 more relevant. For example, two types of mutation have been described affecting the amino terminal Zinc finger. The substitution of glycine for aspartate in the codon 218 (D218G) of the amino terminal Zinc finger leads to mild dyserythropoiesis and macrothrombocytopenia (Freson *et al.,* 2001). The other mutation described, a substitution of methionine for valine in the codon 205 (V205M) causes acute dyserythropoietic anaemia and thrombocytopenia (Nichols *et al.,* 2000). Both mutations affect the interaction with FOG1, although the second mutation is located between the two amino terminal cystidines and might generate a more significant conformational change in the Zinc finger, causing a more severe phenotype (see Figure 8B). In fact, the valine 205 residue has been reported to be directly recognised by FOG1 (Fox *et al.,* 1998).

Down syndrome children present a predisposition to develop myeloproliferative

disorders originated by clonal mutations in the megakaryoblastic lineage. Recently, somatic mutations of Gata1 have been associated with the leukaemic myeloproliferative disorder in Down syndrome patients. Mutated Gata1 generates a truncated protein. The extra 21 chromosome dose present in Down syndrome patients, and the number of haematopoietic key regulators located on that chromosome, causes abnormalities at different levels. For example, the levels of foetal haemoglobin at birth are lower in Down syndrome patients than in healthy newborns. Curiously, RUNX1 (AML-1) and Bach1 are genes located in the chromosome 21, and overexpression of these genes have been implicated in the development of leukaemias and myeloproliferative disorders (Qiu *et al.,* 2003; Cameron and Neil 2004). Overexpression of RUNX1 leads to the generation of several translated isoforms of the protein that can bind to the target promoters in an irreversible way and cause and aberrant replication of the DNA leading to the clonal mutation. Gata1 being a potential target gene of these haematopoietic factors becomes a probable subject of mutations, as irreversible binding of transcription factors to the Gata1 promoter/locus might cause mismatched replication and subsequent mutations.

5.4.- Gata1 and the Cell cycle

Exogenous expression of human Gata1 in fibroblasts leads to a slower proliferation rate. Interestingly, upon serum starvation, cells failed to accumulate in G1 (Dubart *et al.,* 1996). This was the first experiment indicating that Gata1 might interact with the regulation of the cell cycle. Cullen and colleagues observed that in MEL cells Gata1 mRNA and protein levels are upregulated in S phase. They correlated it with an increased DNA binding activity that is downregulated in late S phase (Cullen and Patient 1997). It not only seems that Gata1 might interfere with the cell cycle, but the levels of expression are regulated tightly within the cell cycle phases. The expression of a target gene of Gata1, EpoR, seems to be cell cycle dependent (Komatsu *et al.,* 1997). Overexpression of Gata1 in MEL cells revealed that the level of Gata1 interferes with the cell cycle machinery (Whyatt *et al.,* 1997). MEL cells overexpressing Gata1 showed altered patterns of expression of cyclins, and were reluctant to arrest cell cycle upon differentiation induction by DMSO. Furthermore, Gata1 binds to the retinoblastoma (pRb) protein (Whyatt *et al.,* 1997), which is an established key regulator of the cell cycle progression (Goodrich *et al.,* 1991). Uninduced MEL cells express high levels of the oncoproteins PU.1, cMyb and cMyc. PU.1 blocks Gata1 activity by binding to its carboxy-terminal Zinc finger (Zhang *et al.,* 2000) and PU.1 requires pRb to repress Gata1 activity in MEL cells (Rekhtman *et al.,* 2003). Forced expression of Gata1 in uninduced MEL cells overcomes the inhibition exerted by PU.1. As a result, Gata1 activates the expression of p21 and represses cMyc and cMyb, events that favour MEL cell differentiation over proliferation (Choe *et al.,* 2003). These observations confirm again that the level of Gata1 and the time of action determine the cellular fate.

In other cell types of the haematopoietic system, Gata1 has also been reported

to regulate the cell cycle. For example, in megakaryocytes, Gata1 might modulate the expression of cdc6, which is required during polyploidization of terminal maturating megakaryocytes (Vilaboa *et al.,* 2004). Furthermore, Gata1 overexpression in a murine myeloid cell line (M1) induces proliferation, which causes a block of macrophage induced differentiation (Tanaka *et al.,* 2000). It is known that Gata1 is present in multilineage progenitors and that these cells shift the gene expression and engraftment phenotype relative to the cell cycle transit (Lambert *et al.,* 2003). Therefore, we could hypothesise that factors such as Gata1 that influence the cell cycle in haematopoietic stem cells might play a role in the fate of progenitors.

5.5.- Gata1 and apoptosis

Lineage committed cells need to activate a number of anti-apoptotic pathways in order to counteract apoptotic signals which are intrinsic to cell lineage differentiation induction (see Figure 9) (Nagata 1997; Ashkenazi and Dixit 1998). The haematopoietic system needs to assure that the production of a certain cell type will not cause a surplus, and this is the reason why lineage differentiation is always a balance between survival and apoptosis (Testa 2004). Gata1, as an essential erythroid transcription factor, has been related to the survival of erythroid progenitors. Gata1 *null* ES cells undergo apoptosis, but in the presence of exogenously expressed human Bcl-2, an anti-apoptotic factor, they survive and remain proliferative (Weiss *et al.,* 1997). It was reported that Gata1 directly regulates the transcription of Bcl-2 and Bcl-xL, both members of the Bcl family of anti-apoptotic factors (Weiss *et al.,* 1997; Gregory *et al.,* 1999; Tanaka *et al.,* 2000). Bcl-xL is also regulated by EpoR activation (Dolznig *et al.,* 2002), and expression of a truncated EpoR fails to protect erythroid cells from induced cell death (Nakamura *et al.,* 1992). In a synergistic action, and as we mentioned above, Gata1 transactivates the EpoR (Zon *et al.,* 1991; Komatsu *et al.,* 1997). Bcl-x *null* ES cells do not terminate erythroid differentiation suggesting that counteracting the apoptotic processes is essential during erythroid differentiation (Motoyama *et al.,* 1999). Overexpression of Bcl-2 increases the viability and proliferation of HSCs in the embryo (Orelio *et al.,* 2004). Supporting this idea, progenitor cells expressing Bcl-2 and Bcl-xL showed high levels of certain cyclin-dependent kinase inhibitors, which might shift the balance of the cells towards self-renewal rather than differentiation (Marone *et al.,* 2000). Furthermore, the study of the recovery of bone marrow cells after paclitaxel treatment, a cancer chemotherapeutic agent that causes bone marrow cytotoxicity, revealed that surviving cells upregulated a number of factors that were crucial for the activation of anti-apoptotic pathways, amongst them Gata1, EpoR, Bcl-xL and NF-E2. The expression levels of these factors were downregulated to normal levels after bone marrow recovery (Romero-Benitez *et al.,* 2004). Therefore, Gata1 is a factor required to induce activation of anti-apoptotic pathways.

Deregulation of the apoptotic process leads to defects in the haematopoietic

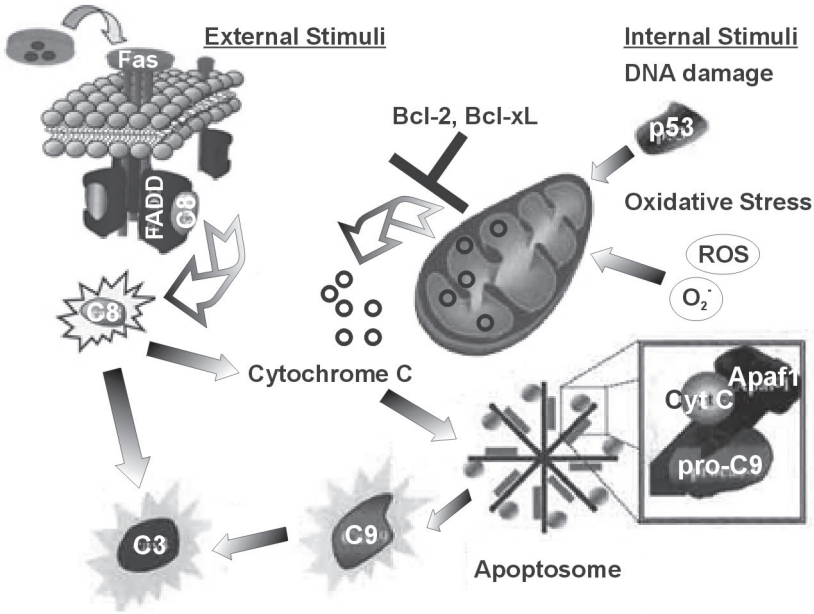

Figure 9. Apoptotic and anti-apoptotic pathways.

Schematic representation of the pathways involved in the regulation of apoptosis. Apoptotic pathways can be activated by the binding of a ligand to a death receptor (external stimuli). External stimuli induce a caspase cascade that subsequently causes protein and DNA degradation leading to apoptosis. Internal stimuli such as DNA damage or oxidative stress activate the cytochrome C pathway. Cytochrome C is released from mitochondria and couples to Apaf-1 and procaspase-9 forming the apoptosome. After cleavage of procaspase-9, a caspase cascade is induced leading to apoptosis. Activation of a death receptor can also generate apoptosis via the cytochrome C pathway. Anti-apoptotic proteins such as Bcl-2 and Bcl-xL block the release of cytochrome C from mitochondria, inhibiting the formation of the apoptosome and protecting the cell from apoptosis.

compartment, supporting the concept that correct regulation of apoptosis and differentiation is essential for production of mature cell types. A clinical case has been described in which MDS (myelodisplastic syndrome) is caused by high expression of Fas receptor (FasR) (Fontenay-Roupie *et al.,* 1999). Mutations in the FasR or Fas ligand in mice cause autoimmune diseases (Takahashi *et al.,* 1994) affecting the B and T cell compartment. Usually these mutations also lead to increased extramedullary haematopoiesis, which could be due to anaemia caused by progenitor death in the bone marrow (Schneider *et al.,* 1999). Several studies revealed that activation of death receptors like FasR or TNF-α leads to apoptosis of erythroid progenitors (Rusten and Jacobsen 1995; Barcena *et al.,* 1999; De Maria *et al.,* 1999; Otsuki *et al.,* 1999; Gibellini *et al.,* 2000; Zamai *et al.,* 2000; Silvestris *et al.,* 2002). Upregulation of FasR levels is induced by activated Interferon-γ and TNF-α, which suggests a synergistic action of apoptotic signals and that apoptosing cells

induce apoptosis to neighbouring cells (Maciejewski *et al.,* 1995; Dai *et al.,* 1998). At the stage when erythroid progenitors are susceptible to apoptosis induction, they are Epo and Stem Cell Factor (SCF) dependent. The interplay between apoptotic signals and Epo/SCF determines the survival or apoptosis of the erythroid progenitors (Kapur and Zhang 2001; Oda *et al.,* 2001).

De Maria *et al*, showed that human Gata1 contains caspase recognition sites and that it can be degraded upon caspase activation by FasR coupling (De Maria *et al.,* 1999; Fadeel *et al.,* 2000). Furthermore, Gata1 degradation by caspases upon activation of the FasR (De Maria *et al.,* 1999) causes downregulation of anti-apoptotic proteins, leading to apoptosis (Orkin and Weiss 1999).

However, death receptor coupling does not have apoptotic effects in every situation (Fadeel *et al.,* 2000; Wajant 2002). TRAIL (TNF-related apoptosis inducing ligand) prevents the cell cycle progression with no signs of apoptosis in lymphocytes (Song *et al.,* 2000). Activation of FasR in immature haematopoietic progenitors promotes cell survival (Josefsen *et al.,* 1999) while in more mature haematopoietic and erythroid committed progenitors it causes apoptosis (Maciejewski *et al.,* 1995). More recently it was shown that caspase activation is required for terminal erythroid differentiation (Zermati *et al.,* 2001) and that caspase 3 has a non-apoptotic role during erythropoiesis (Carlile *et al.,* 2004). Mice deficient for Raf-1 are unable to restrain caspases. Raf-1 *null* erythroid cells cannot be expanded in culture because they have accelerated differentiation (Kolbus *et al.,* 2002), indicating that non-restricted caspases act favouring differentiation instead of inducing apoptosis. When the outcome of death receptor/caspases activation is related to the levels of Gata1, it seems as if activation of death receptor pathways has non-apoptotic functions when Gata1 levels are basal (haematopoietic progenitors, terminally differentiating erythroid cells) but that it has an apoptotic function when Gata1 levels are high, in the erythroid lineage. Death receptors are activated by their ligands, but they are also inactivated by coupling to shedded ligands (Tanaka *et al.,* 1998). In addition, death ligands can be blocked by decoy receptors (Roth *et al.,* 2001). Therefore, the expression of a certain receptor or ligand does not necessarily mean that it will exert its function, making the understanding of cell-cell interactions more complex.

In summary, the outcome of an activated death pathway depends on the cell-stage when signals occur, as it depends on environmental situations that allow the activity of the signalling molecules (Testa 2004).

6.- REDS

Red Cell Differentiation Signal (REDS) was proposed by Whyatt *et al* in 2000 (Whyatt *et al.,* 2000). This signal was based on the results obtained after the analysis of Gata1 overexpression *in vivo*. Mice that overexpress Gata1 from an X-linked transgene under the

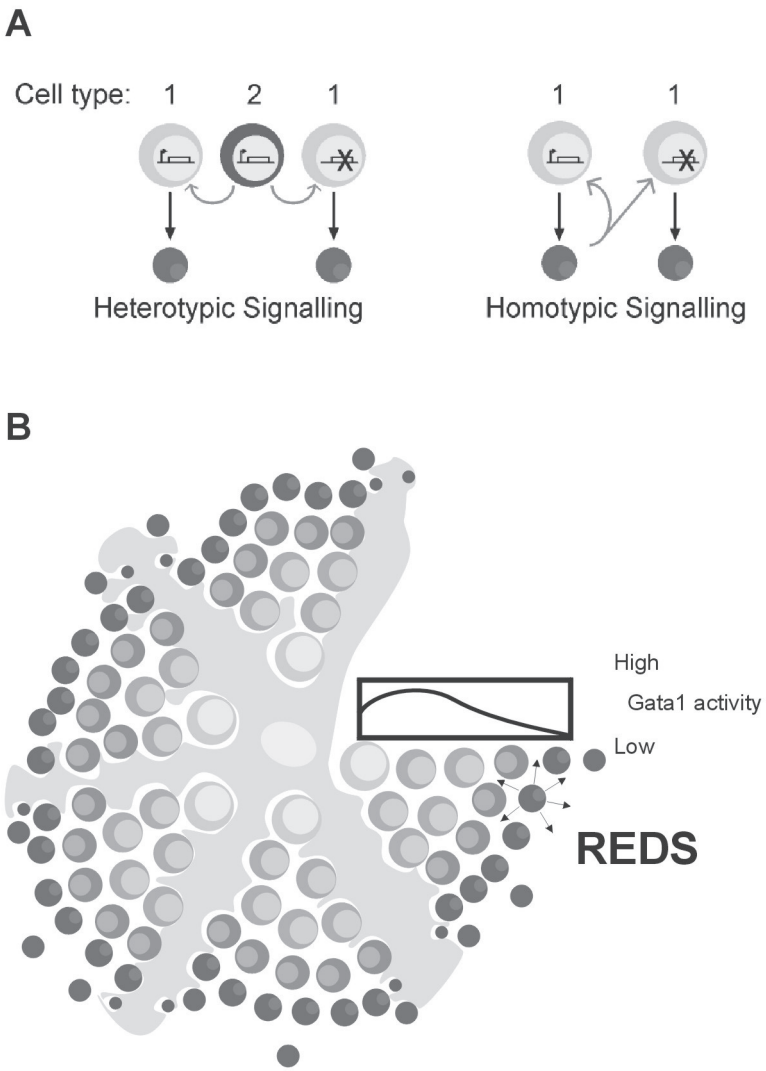

Figure 10. REDS model as a homotypic signalling.

(A) An scheme of homotypic and heterotypic signalling mechanisms is depicted. In a heterotypic signalling mechanism cells of different type communicate with each other, while in a homotypic signalling mechanism, the communication happens between cells of the same type.

(B) Model of REDS as a homotypic signalling mechanism within the erythroblastic island. Mature erythroid cells supply the REDS signal to less mature erythroid cells. Gata1 levels are downregulated in the last stages of differentiation and REDS might be involved in the regulation of Gata1 activity, thus favouring erythroid differentiation.

regulation of the human LCR and β-globin promoter die at midgestation (13.5 dpc) due to severe anaemia. Gata1 overexpressing cells fail to differentiate *in vitro* (Whyatt *et al.,* 1997) and *in vivo* (Whyatt *et al.,* 2000). They are unable to arrest the cell cycle, a requirement for terminal erythroid differentiation. Gata1 overexpressing cells remain proliferative at the proerythroblast-like stage and die eventually due to a failed differentiation program. Heterozygous females are born normal. Interestingly, Gata1 overexpressing cells, which are intrinsically defective in erythroid differentiation, are able to differentiate *in vivo* in the presence of wildtype cells, as happens in heterozygous Gata1 overexpressing females. This result led to the concept that there must be a signal originating from wildtype cells that overcomes the defect generated by Gata1 overexpression. This signal was named REDS. It was hypothesised that REDS might directly favour the terminal differentiation of erythroid cells. In the Gata1 overexpressing system where REDS was unmasked, this signal overcomes the defect generated by Gata1 overexpression. Most likely, REDS signal leads to downregulation of Gata1 activity in these mice. As mentioned above, human Gata1 has caspase recognition sites (De Maria *et al.,* 1999), and death receptors are expressed in erythroid cells (De Maria *et al.,* 1999). It was hypothesised that the REDS receptor is a death receptor-like protein(s) that upon activation triggers the activation of caspases and regulates the activity of factors such as Gata1. It is possible that the putative REDS receptor has already been described. For example, a novel death receptor was described in zebrafish involved in the negative regulation of erythropoiesis (Long *et al.,* 2000). In human, RCAS1, a receptor expressed in several types of carcinoma, was described to be a novel regulator of apoptosis in the erythroid compartment (Matsushima *et al.,* 2001).

In order to unravel the mechanisms of REDS it is necessary to identify the wildtype cell type supplying the signal. REDS could be supplied by a maturing erythroid cell, or by a stroma cell. Although a homotypic signalling mechanism, *i.e.* a signalling mechanism involving cells of the same type, was already hypothesised in the published work, this question was not answered (Figure 10).

We hypothesised that the REDS signal provides a mechanism assuring the control of the homeostasis by balancing the ratio between proliferation/differentiation, beyond the actions of Erythropoietin. The Gata1 overexpressing mice were the means to unmask such mechanism. Identification of the molecular nature of REDS and its mechanism of action is the main subject of my thesis project.

7.- Scope of the thesis project

The overall aim of the work described in this thesis is to characterise the REDS signal and identify its targets. The following specific questions were addressed:
1.- Is REDS a homotypic or a heterotypic signalling mechanism?
2.- Dissection of the molecular mechanisms of REDS.
3.- Study of candidate REDS counterparts and/or identification of new candidates.

In addition, we studied the role of Gata1 in other cells of the haematopoietic compartment, in particular in a novel compartment, the dendritic cells.

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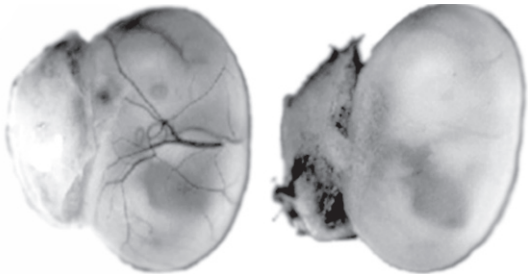
CHAPTER 2

ANALYSIS OF GATA1 OVEREXPRESSING MICE



Analysis of Gata1 overexpressing mice

Laura Gutiérrez, Frank Grosveld and Sjaak Philipsen.

ABSTRACT

Gata1 is a transcription factor essential for erythropoiesis. Overexpression of Gata1 in murine erythroleukaemic cells makes them reluctant to undergo G0 arrest upon induction of differentiation. Gata1 overexpressing mice have been generated which are lethal at midgestation showing a defect in definitive erythropoiesis. This deregulated erythropoietic phenotype is intrinsic to the cell overexpressing Gata1. However the defect can be overcome in the presence of wildtype erythroid cells as occurs in chimaeric mice or heterozygous transgenic females. Therefore the defect generated by Gata1 overexpression is cell-nonautonomous. This chapter describes the in depth analysis of the phenotype of Gata1 overexpressing mice that have been described previously and that became the most important tool in the present thesis work.

INTRODUCTION

The transcription factor Gata1 plays an essential role in erythropoiesis (Pevny *et al*., 1991; Simon 1993; Fujiwara *et al*., 1996). It is a member of the GATA family of transcription factors, which bind the consensus sequence WGATAR (Orkin 1992; Merika and Orkin 1993). To date all the erythroid genes, including Gata1 itself (Tsai *et al*., 1991), contain GATA sites in their promoter regions (Welch *et al*., 2004). Gata1 knockout mice were generated to reveal the role of Gata1 *in vivo* (Pevny *et al*., 1991; Fujiwara *et al*., 1996). They die at midgestation and display a severe anaemia at the time when definitive erythropoiesis starts in the foetal liver. Gata1 knockout erythroid progenitors derived from embryoid bodies undergo apoptosis at the proerythroblast stage (Simon *et al*., 1992; Weiss *et al*., 1994; Weiss and Orkin 1995). This and other data led to the concept that Gata1 is a survival factor. Furthermore, Bcl-2 and Bcl-xL, two anti-apoptotic proteins of the Bcl family of transcription factors, are targets of Gata1 (Gregory *et al*., 1999; Tanaka *et al*., 2000). Gata1 is not only required for erythropoiesis, its levels fluctuate through commitment to the different haematopoietic lineages. Gata1 levels are low in multilineage haematopoietic progenitors and they rise upon erythroid commitment (Leonard *et al*., 1993). However, proper terminal differentiation of erythroid cells requires downregulation of Gata1 (Leonard *et al*., 1993; Whyatt *et al*., 1997; Tang *et al*., 2001). This prompted a study of the effect of Gata1 gain of function (Whyatt *et al*., 1997; Whyatt *et al*., 2000). Gata1 overexpression in murine erythroleukaemia (MEL) cells alters the cell cycle machinery. They are reluctant to undergo G0 arrest upon differentiation induction and keep proliferating (Whyatt *et al*., 1997). The role assigned to Gata1 therefore became more complex: Gata1 is involved in transcription of erythroid genes, survival and regulation of the cell cycle. In order to study the outcome of Gata1 overexpression *in vivo*, Gata1 overexpressing mice were generated (Whyatt *et al*., 2000). These mice contain an X-linked *Gata1* transgene that is expressed under the regulation of the human Locus Control Region (LCR) and β globin promoter. The endogenous Gata1 gene is also X-linked (Zon *et al*., 1990). Pancellularly overexpressing Gata1 mice (transgenic males) die at midgestation of severe anaemia, although at a later stage than Gata1 knockout mice. Definitive erythropoiesis is defective in these mice and their foetal livers contain a higher proportion of basophilic and polychromatic erythroid progenitors compared to wildtype littermates. On the other hand, heterocellularly overexpressing Gata1 mice (heterozygous transgenic females) survive and are normal. In these females, due to X-inactivation, a proportion of erythroid cells overexpress Gata1 (overexpressing cells) and the rest express normal levels of Gata1 (wildtype cells). Based on the analysis of CFU-Es of adult mice it was shown that there is not a selective growth of wildtype progenitors. In summary, Gata1 OX females are normal and do not show anaemia at any developmental stage, Therefore, Gata1 overexpressing erythroid cells, which are intrinsically defective, must be able to differentiate in the presence of wildtype

cells in the heterozygous females. The explanation for this phenomenon was attributed to a signalling mechanism, originating in wildtype cells, that enables defective erythroid cells to differentiate properly. This signal was named Red Cell Differentiation Signal (REDS) (Whyatt *et al*., 2000).

The rationale of REDS mechanism is summarised as follows. At the moment when erythroid progenitors are induced to differentiate, the cells must undergo several ultra specialization processes. Terminal differentiation is characterised by an exit of the cell cycle and enucleation. This is like a long-lasting apoptotic process: erythroid cells are released into the blood stream to perform their ultimate function before eventually dying. Gata1 levels are downregulated during terminal differentiation, which is in concordance with the idea of Gata1 as a survival factor. Therefore, it is easy to understand that when a decontrolled high level of Gata1 is persistent in the differentiating erythroid cell, this cell will not be able to undergo the specialised "apoptosis". This cell will survive and proliferate until other mechanisms induce its necrosis due to a failed differentiation program. When overexpressing Gata1 cells are in a chimaeric context, in close contact with wildtype cells, the defect is overcome by REDS signalling. REDS acts through a signalling pathway that directly or indirectly downregulates Gata1 activity, allowing the cells to undergo terminal differentiation. Two questions were formulated in the published paper that described the Gata1 overexpression in mice and formed the basis of this thesis:

1.- What is the source of REDS? Is it an erythroid cell or is it a wildtype cell of another lineage present in the stroma? The answer to this question is the topic of Chapter 3.

2.- What is the receptor of REDS? Considering that human Gata1 has caspase recognition sites (De Maria *et al*., 1999), could they be death receptor like proteins? Experiments aimed to elucidate this question are presented in Chapter 4.

In this chapter, a more in depth study of the phenotype of Gata1 overexpressing mice is presented by analysing adult heterozygous overexpressing females, overexpressing embryos, and *ex vivo* cultures derived from foetal livers of Gata1 overexpressing embryos.

MATERIALS AND METHODS

Mice

Mice bearing an X-linked Gata1 transgene under the regulation of the human Locus Control Region (LCR) and β globin promoter were generated before (Whyatt *et al*., 2000) and are designated as Gata1 overexpressing (OX) mice. Btk-LacZ mice (Hendriks *et al*., 1996) have a disrupted X-linked Btk gene with a LacZ knockin construct and therefore cells that express Btk naturally can be detected by LacZ staining. Gata1 OX females were crossed with Btk-LacZ males to obtain Gata1 OX|Btk-LacZ females, Gata1 wildtype (WT)|Btk-LacZ females and males, in order to study the wildtype population after anaemia induction. To be able to detect the Gata1 overexpressing cells in different studies, Gata1 OX|Btk-LacZ females were bred with wildtype males until mice bearing both transgenes in the same chromosome were obtained, and we name these animals as Gata1 OX::Btk-LacZ.

Induction of anaemia

Mice were injected subcutaneously with 0.24ml/20g of body weight of a 0.4% phenylhydrazine solution in phosphate buffered saline (PBS) at day 1 and 2 and collected at day 5.

Fluorescence Activated Cell Sorter Analysis (FACS)

Single cell suspensions from adult blood, bone marrow, spleen and foetal liver were obtained and stained before analysing them by FACS. The following antibodies and stains were used: Propidium Iodide (PI, Molecular Probes BV), 7-Aminoactinomycin-C (7AAD, Molecular Probes BV), CD71-FITC (Molecular Probes BV), TER119-PE (Molecular Probes BV), biotin-conjugated ER-MP20, Tricolor-streptavidin secondary antibody (Calatag Laboratories), Anti-Gata1 N6 (Santa Cruz Biotechnology), Anti Rat-FITC (Molecular Probes BV) and fluoroscein-di-β-D-galactopyranoside (FDG, Molecular Probes BV).

Western Blot

Blood samples from adult females were obtained by eye puncture. Blood samples were spun at 1500rpm for 15 min. Buffy coat and plasma was removed. Cells were washed three times with 10 volumes of PBS, and finally lysed in 5mM NaHPO4 pH 8. Lysates were allowed to stand on ice for 30 min and ghost cells were spun at 1500rpm for 15 min at 4°C. The supernatant was run on a 12.5% SDS-PAGE gel, electroblotted and incubated with N6 antibody against Gata1 (Santa Cruz Biotechnology). Anti Rat-Horse Radish Peroxidase (HRP) antibody was used as secondary antibody (Roche). The blot was developed with Enhanced Chemiluminescence as detailed by the manufacturer (Amersham Pharmacia).

Generation of I11 cells overexpressing Gata1

I11 cells were electroporated in triplicate following the manufacturer's protocol (Amaxa) either with a pEV^{Puromycin}Gata1-Myc (Whyatt *et al*., 1997) or pEV^{Puromycin} construct and cultured under selection until cells reached normal culturing density (10^6 cells/ml) and proliferated normally. The presence of the transgene was analysed by Southern blot. The established transgenic lines and their controls were grown in proliferation and differentiation conditions as described (Dolznig *et al*., 2001).

Cell Culture of primary erythroid progenitors

Foetal liver cells were cultured to induce differentiation either by hanging drop method (Appendix I) or in suspension (Dolznig *et al*., 2001).

UV-light assay

Gata1 overexpressing females were mated and embryos collected at 12.5 dpc. Foetal liver cells were expanded in suspension for three days as described in order to get enough cells for the experiment (Dolznig *et al*., 2001). The following UV-light treatment programs were performed:

- Proliferation: Cells were plated at a density of $5x10^4$ cells/ml, treated at day 0 and collected at day 5.
- 24 hours before differentiation induction: Cells were plated at a density of $20x10^4$ cells/ml and treated at day 0. Differentiation was induced at day 1 and cells were collected at day 4, *i.e.* 72 hours after differentiation induction.
- 24 hours after differentiation induction: Cells were plated in differentiation conditions at a density of $40x10^4$ cells/ml. UV-light exposure was performed at day 1 and cells collected at day 3, *i.e.* 72 hours after differentiation induction.
- 48 hours after differentiation induction: Cells were plated in differentiation conditions at a density of $40x10^4$ cells/ml. UV-light exposure was performed at day 2 and cells collected at day 3, *i.e.* 72 hours after differentiation induction.

When UV-light exposure was performed, cells were collected from their plates and resuspended in PBS + 10% FCS + 1% penicillin/streptomycin (stock) in a volume that just covers the bottom of the plate. The UV-light exposure cannot be performed in the culture medium as it absorbs UV-light. The doses applied were 0, 2, 4, 8 and 10 J/m². Immediately after exposure, cells were resuspended in proliferation or differentiation medium as required and incubated until collection day.

RESULTS

Tracing Gata1 overexpressing cells through development and *ex vivo* differentiation

Gata1 OX females are indistinguishable from wildtype littermates. However we know that these females display a population of erythroid cells that is intrinsically defective towards differentiation, and yet is able to differentiate in the presence of wildtype cells. Our hypothesis is that REDS, a signal originating in wildtype cells, rescues the defective cells efficiently. However it could be that there is selective growth of healthy progenitors in the Gata1 OX females. Previously, the number of progenitors present in the bone marrows of adult wildtype and Gata1 OX females was studied in CFU-E cultures. By using Btk-LacZ crossed with Gata1 OX mice, the wildtype progenitors could be detected by LacZ staining. There were no differences between Gata1 OX and wildtype females in terms of numbers of wildtype progenitors. We wished to analyse the overexpressing Gata1 cells directly and follow their fate through differentiation and development. For this reason we generated females bearing both transgenes in the same X chromosome (Gata1 OX::Btk-LacZ). Gata1 OX::Btk-LacZ females were mated with wildtype males and, after confirming the plug date, embryos were collected at 13.5 dpc. The Gata1 transgene is located close to the centromere and the Btk locus is close to the telomere and recombination was expected to occur with a high probability. The progeny obtained contained Gata1 OX:: Btk-LacZ and also Gata1 WT::Btk-LacZ mice, allowing us to follow by LacZ staining cells with the Gata1 OX or the WT allele active in the same experiment. We stained the foetal liver cells from the embryos and the bone marrow cells from the mothers with FDG to detect LacZ activity, TER119 and 7AAD and analysed them by FACS. The percentage of LacZ positive cells is maintained from foetal stages until adulthood when comparing foetal liver to bone marrow in the case of the females. This is independent of which Gata1 allele (wildtype or overexpressing) is linked to the Btk-LacZ marker (Figure 1A). Note that the values of the females are approximately 2-fold lower than the male values, due to the fact that in the male LacZ expression is pancellular and in the females it is subjected to X-inactivation. The percentage of TER119 and LacZ positive cells was found to be equal between females either in the foetal liver or in the bone marrow (Figure 1B). There is higher variation in the percentage of TER119 and LacZ positive cells in the Gata1 OX male compared to the raw percentage of LacZ positive cells. We have to consider that the time of collection of the plugs (13.5 dpc) is close to the moment where these males die of anaemia, and this could affect the number of TER119 positive cells. Taking all these results together, we conclude that there are no major changes in the progenitor compartment of Gata1 OX and wildtype females and that the progenitor population balance is maintained through embryonic development and adulthood.

We know that there is no selection of wildtype progenitors in Gata1 overexpressing

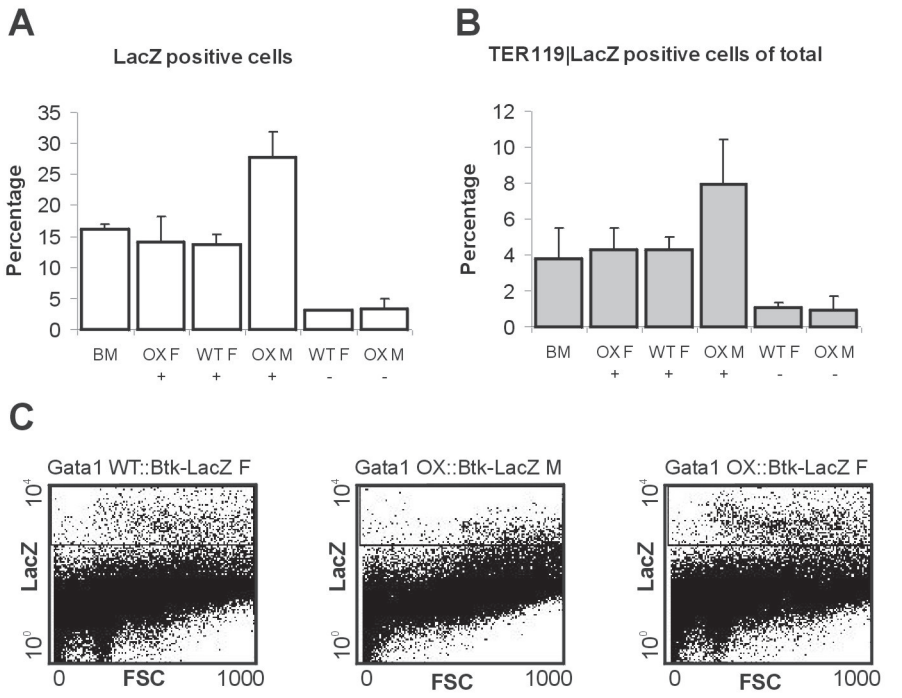


Figure 1. Analysis of Gata1 OX cells by linkage with the Btk-LacZ transgene.
(A) Percentage of LacZ positive cells. BM, bone marrow of the mother Gata1 OX::Btk-LacZ. OX F +, Gata1 OX::Btk-LacZ F; WT F +, Gata1 WT::Btk-LacZ F; OX M +, Gata1::Btk-LacZ M; WT F -, Gata1 WT F; OX M -, Gata1 OX M.
(B) The percentage of LacZ^{+}TER119^{+} cells in the different genotypes, as in section A.
(C) LacZ staining against the forward scatter (FSC) of cells from mice of different genotypes, when the Btk-LacZ transgene is linked to a Gata1 wildtype allele (Gata1 WT::Btk-LacZ F), or to a Gata OX allele either in the male (pancellular overexpression Gata1 OX::Btk-LacZ M) or to the female (heterocellular overexpression Gata1 OX::Btk-LacZ F).

females. It is assumed that a signal rescues Gata1 OX cells in heterozygous Gata1 OX females. However, analysing the progenitor pool is a very indirect way of confirming a signalling mechanism whose outcome should be detected in other cell populations and tissues. We cannot exclude the notion that the contribution of Gata1 OX cells in blood may be affected. We therefore analysed the presence of transgenic Gata1 in the blood and followed the fate of Gata1 overexpressing cells in differentiation culture.

We cultured the foetal liver cells from these transgenic embryos utilising the Hanging Drop culture method. After two days of culture, cells were collected and stained as explained before. The differentiation profiles were consistent with what was described previously. Cells derived from Gata1 overexpressing males fail to differentiate *ex vivo*, while Gata1 overexpressing female derived foetal liver cells differentiate almost as well as wildtype cells, although they show an intermediate phenotype. We then analysed the LacZ positive population in each embryo, which depending on the genotype, would correspond

either to wildtype Gata1 or to overexpressing Gata1 cells. In general, LacZ positive cells were reduced about 4-fold as compared to the time of collection (Table 1). Gata1 wildtype LacZ positive cells were found in cells of all sizes through differentiation, even in the smallest size where LacZ can be detected (Figure 1C). Gata1 OX LacZ positive cells from Gata1OX males were mainly larger cells, probably proerythroblasts, as it can be seen by comparing the mean size (Table 1). Surprisingly, Gata1 OX LacZ positive cells derived from Gata1 OX females, *i.e.* cells that differentiate within a mixed environment and in contact with wildtype cells, were found to be distributed evenly through all cell sizes, even in the smallest cell size where LacZ is detected (Figure 1C and Table 1). This is direct evidence that Gata1 overexpressing cells are able to differentiate when in the presence of wildtype cells. The explanation for this is that, although the erythroblastic island is disrupted, the Hanging Drop culture system is able to mimic to a considerable extent the *in vivo* situation, and REDS is able to rescue Gata1 OX cells *in vitro* if they happen to be in favouring contacts.

Table 1: Analysis of Gata1 OX primary cells after *ex vivo* differentiation.

	%LacZ$^+$ Day 0 / %LacZ$^+$ Day 2	Mean size of LacZ$^+$ cells Day 2
Gata1 OX::Btk-LacZ F	3.5	805.94
Gata1 WT::Btk-LacZ F	3.5	806.61
Gata1 OX::Btk-LacZ M	10	862.14

Next we aimed to analyse the blood of adult Gata1 OX females. We took advantage of the fact that the transgenic Gata1 protein is tagged by a Myc epitope. We obtained blood from adult mice by eye puncture, made protein extracts from total blood cells as explained in the Materials and Methods section and analysed them by Western blotting for Gata1 expression. A sample of these blots is shown in Figure 2. The endogenous Gata1 protein is detected in the three samples, being two wildtypes and one overexpressing female. Myc-Gata1 is detected only in the sample derived from Gata1 OX female. It is difficult to conclude from this picture the percentage of contribution, but it is clearly not a minor contribution. Therefore we concluded that REDS signalling is in fact rescuing Gata1 overexpressing cells and that these cells differentiate and are released into the blood stream in the heterocellular Gata1 OX background.

Gata1 expression profile *in vivo* and *ex vivo* by FACS analysis

Gata1 expression levels are dynamically regulated through differentiation and lineage commitment. We were able to detect Gata1 by FACS analysis and compare the Gata1 expression profiles between different tissues, genotypes and *ex vivo* differentiation. In

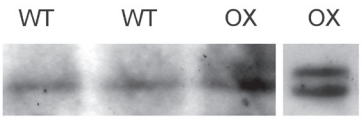


Figure 2. Western blot analysis of Gata1 in blood of adult mice.
WT, wildtype; OX, overexpressing female.

Figure 3A we show three graphs corresponding to foetal liver samples of wildtype, Gata1 OX female and Gata1 OX male. The foetal liver is mainly an erythroid organ. As it can be seen in the wildtype graph, the majority of cells are Gata1 positive, and the levels of Gata1 go down together with cell size. The arrow indicates the putative progression of cells through differentiation. In the overexpressing male, this pattern is lost completely, and yet recovered in the overexpressing females. In Figure 3B we depict two graphs from bone marrow analysis, the major haematopoietic compartment in the adult mice, one belonging to a wildtype female and the other to a Gata1 OX female. The bone marrow is mainly granulopoietic, and not uniquely erythroid (Palis and Segel 1998). The Gata1 positive cells follow a similar pattern as it occurs in the foetal liver: Gata1 levels go down together with cell size. In the bone marrow profile there are quite a lot of Gata1 negative or low cells. In the overexpressing females, the pattern of Gata1 positive cells is not lost, although reduced, and a population of Gata1 overexpressing cells is detected. In Figure 3C the spleen graphs are depicted. The spleen contains a high proportion of Gata1 low cells in general. In the overexpressing female graph there is a population of cells that is not present in the wildtype (indicated by a circle). This population suggests the presence of extramedullary erythropoiesis. In Figure 3D the blood of both genotypes is shown. No major changes are found with the exception of a population found in the overexpressing female that is FSC high and Gata1 low (indicated by a circle). This population might be composed of erythroid progenitors released to the blood stream due to anaemia. We then compared the profile of *ex vivo* differentiated foetal liver cells in hanging drops, and depicted them in Figure 3E. It is remarkable to see how comparable are the cell populations compared to the blood profiles, although they are displayed at different coordinates relative to the forward scatter (FSC) and Gata1 channel. In the *ex vivo* cultured cells we could also detect the presence of an extra population in cells derived from Gata1 overexpressing females (indicated by a circle). These cells are progenitors in ways of differentiation.

Gata1 staining for FACS analysis proved to be a very useful tool to demonstrate that Gata1 levels are downregulated through differentiation and that this pattern is altered completely in foetal liver cells from Gata1 OX males. Although Gata1 OX adult females were described to be normal, some differences are noted by this staining procedure.

Analysis of adult mice

The Gata1 overexpression transgene is maintained through heterozygous females, which are born with Mendelian ratios and are normal, as previously published (Whyatt *et al*.,

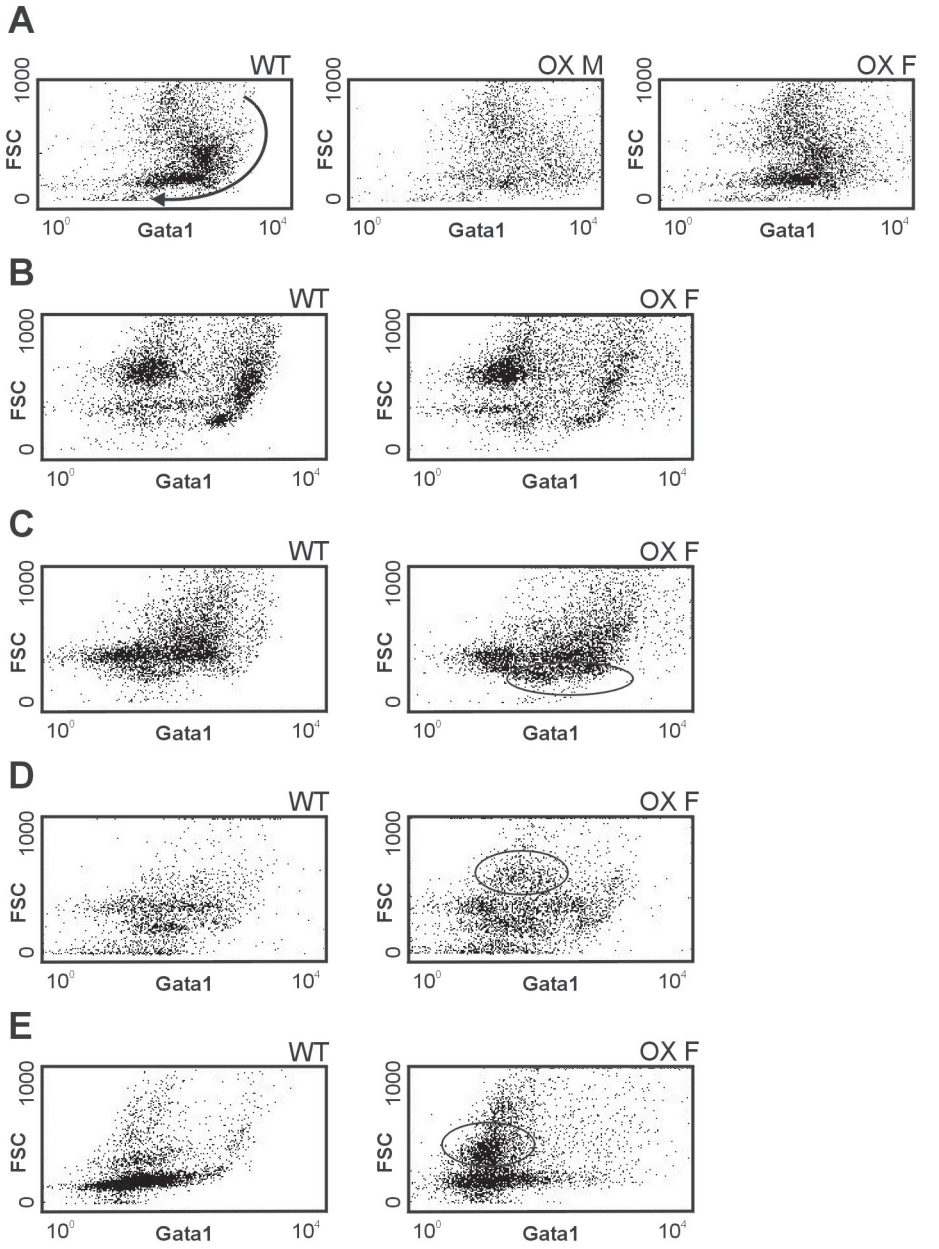

A
WT
OX M
OX F
B
WT
OX F
C
WT
OX F
D
WT
OX F
E
WT
OX F
FSC
1000
0
Gata1
10^0
10^4

Figure 3. FACS analysis of Gata1 expression in mice overexpressing Gata1 (previous page).
Gata1 staining is plotted against the forward scatter. WT, wildtype; OX F, Gata1 overexpressing female; OX M, Gata1 overexpressing male.
(A) Staining of Gata1 of foetal liver cells of WT, OX F and OX M. The arrow in the left plot indicates the direction that cells follow through differentiation.
(B) Staining of Gata1 of bone marrow cells of adult mice. The analysis was done in WT and OX F only, as OX M are never born.
(C) Gata1 staining of spleen cells of adult WT and OX F mice. The circle in the OX F graph indicates a population of cells that is more enriched than in the WT, and probably corresponds to differentiating erythroid cells.
(D) Gata1 staining of blood of adult WT and OX F mice. The circle in OX F graph indicates a population of cells that is more enriched than in the WT graph, probably corresponding to progenitors.
(E) Gata1 staining of foetal liver cells after hanging drop culture. The circle encloses a population of cells that is more enriched in the OX sample compared to the WT sample.

2000). We collected four Gata1 overexpressing and four wildtype females of 10 weeks of age in order to analyse their erythropoietic compartment. Single cell suspensions from blood, spleen and bone marrow were stained with CD71 (expressed in early erythroid progenitors), TER119 (expressed in late erythroid progenitors and until the last stages of differentiation), Gata1 and 7AAD (which stains non-viable cells). Cells were analysed by FACS and the obtained data is summarised in Table 2 and Figures 4 and 5. The bone marrow of the overexpressing females has a slightly higher percentage of dead cells (Table 2), which are not undergoing apoptosis (data not shown). If we look at the cell composition

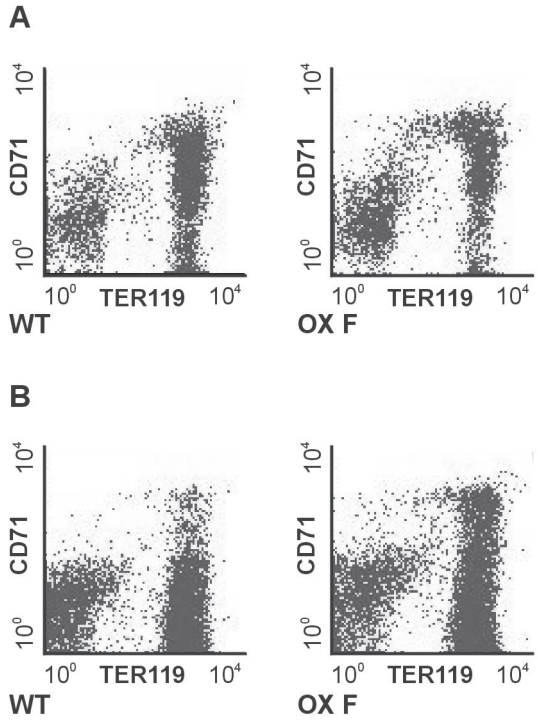


Figure 4. FACS analysis of the erythroid compartment in Gata1 overexpressing mice.
Profile of live cells against the CD71-FITC and Ter119-PE respective channels. WT, wildtype; OX F, Gata1 overexpressing female.
(A) Bone marrow cells of adult WT and OX F mice.
(B) Spleen cells of adult WT and OX F mice.

Table 2: FACS analysis of the erythropoietic compartment of adult Gata1 OX females.

Bone Marrow	**OX F**	**WT F**
% Dead	18.58±2.11	15.07±0.77
% Double negative	37.52±4.93	28.30±1.42
% $CD71^+$	2.74±0.77	1.96±0.77
% $CD71^+$|$TER119^+$	20.32±2.94	18.91±1.77
% $TER119^+$	39.43±5.14	50.83±2.83
Spleen	**OX F**	**WT F**
% Dead	23.18±1.52	17.49±3.70
% Double negative	21.42±10.60	32.76±6.02
% $CD71^+$	0.65±0.42	0.13±0.08
% $CD71^+$|$TER119^+$	8.14±2.22	1.6±0.38
% $TER119^+$	69.79±10.71	65.51±6.39
Blood	**OX F**	**WT F**
% Dead	0.08±0.04	0.03±0.00
% Double negative	3.24±5.61	8.88±9.58
% $CD71^+$	0.07±0.10	0.09±0.10
% $CD71^+$|$TER119^+$	4.84±1.47	3.72±0.35
% TER119+	91.80±6.92	87.31±9.66

of the live cells of the bone marrow, the overexpressing females have a 10% reduction of the TER119 single positive population, indicating that the last stages of differentiation are less frequent. Intermediate stages (double positive CD71/TER119, and single positive CD71) are not affected and are comparable in both genotypes (Table 2 and Figure 4A). The balance makes the percentage of double negative CD71 and TER119 precursors 10% higher compared to wildtype littermates. This implies that erythroid differentiation is slightly impaired at the last stages. If this impairment generates a slightly anaemic condition, this is compensated, as the blood of the Gata1 overexpressing females is phenotypically normal. We wondered whether extramedullar erythropoiesis was activated in the Gata1 overexpressing females by analysing their spleens. As found in the bone marrow, overexpressing females had a slight higher percentage of dead cells in the spleen (Table 2). Not surprisingly, the population of CD71/TER119 double positive progenitors was found to be 6% higher in transgenic females compared to wildtype (Table 2 and Figure 4B). This result indicates that the Gata1 overexpressing females have an increased rate of extramedullar erythropoiesis in comparison to wildtypes due to a slight impairment of marrow erythropoiesis. We also analysed the blood by using the same parameters described (Table 2). No significant difference was detected. The slight erythropoietic

impairment in the bone marrow is coped by extramedullar erythropoiesis leading to a normal phenotype in the blood.

We also stained the same samples to detect TER119 and Gata1 (Table 3). Concordant with the previous results, there were 10% fewer Gata1|TER119 double positive cells in Gata1 overexpressing females compared to wildtypes. When analysing the dead cells, we observed that the Gata1 overexpressing females had 4% more Gata1 positive cells than wildtype females. It could be that this population of cells is enriching the dead-cell fraction in Gata1 overexpressing females, as shown in Table 2. Also concordant with previous results, the spleen of Gata1 overexpressing females showed 10% more Gata1/TER119 double positive cells than wildtypes. Analysis of the Gata1 staining provided us a quantitative estimation of Gata1 levels in the different tissues and genotypes analysed (Figure 5). Gata1 levels are on average higher in the overexpressing females both in bone marrow and more significantly in spleen, although the standard deviations are higher than in wildtypes due to random X-inactivation. It is remarkable to see that differences in Gata1 levels are not detectable anymore in blood, when analysing Gata1 staining of total blood samples. Concordant with the idea that Gata1 levels must be downregulated in the last stages of differentiation, Gata1 levels are in blood the lowest for both genotypes (Figure 5).

From this experiment we conclude that, although the phenotype of overexpressing females is normal, and the overexpressing cells are rescued by REDS, this rescue is not complete, and the overexpressing females use extramedullar erythropoiesis.

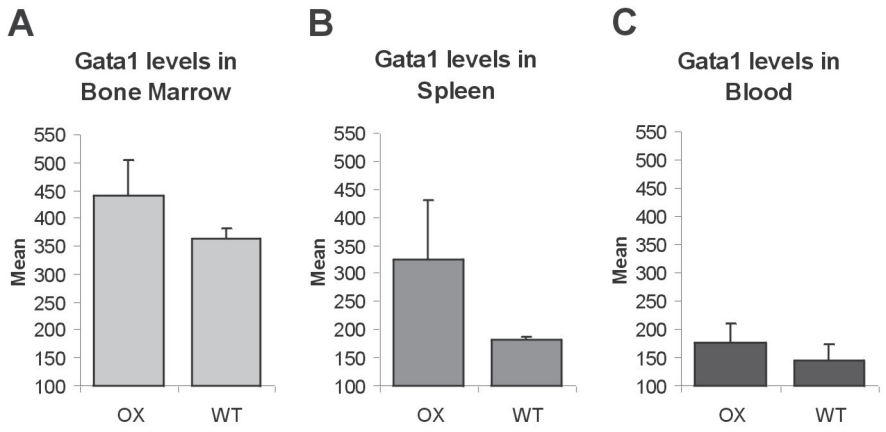


Figure 5. Analysis of Gata1 levels as measured by FACS in adult mice.
Average of the relative mean value of Gata1 level in the FACS analysis was pooled per genotype and tissue and plotted, including the standard deviations. Four samples were analysed per group.
(A) Gata1 levels in total bone marrow of wildtype (WT) and Gata1 overexpressing female (OX).
(B) Gata1 levels in total spleen.
(C) Gata1 levels in total blood.

Table 3: FACS analysis of the erythropoietic compartment of adult Gata1 OX females: Gata1 staining.

Bone Marrow	OX F	WT F
% Gata1$^+$ of dead	11.42±1.42	6.88±1.44
% Double negative	61.02±3.08	52.52±1.41
% Gata1$^+$	6.82±0.32	6.36±1.32
% Gata1$^+$|TER119$^+$	28.39±1.89	37.85±1.08
% TER119$^+$	3.78±1.20	3.28±0.80
Spleen	**OX F**	**WT F**
% Gata1$^+$ of dead	23.90±5.92	20.79±1.83
% Double negative	62.72±6.24	74.74±1.54
% Gata1$^+$	15.67±3.40	15.09±0.80
% Gata1$^+$|TER119$^+$	17.16±5.16	6.15±0.69
% TER119$^+$	4.45±2.62	4.04±0.71

Induction of anaemia of adult mice

Next, we studied the stress erythropoiesis response in the Gata1 overexpressing females. Due to the fact that some stress erythropoiesis was detected in transgenic females, we wished to analyse the response in these mice to induced anaemia by a haemolytic agent. To distinguish the wildtype from the Gata1 overexpressing population within overexpressing females, we crossed them with mice bearing an X-linked Btk-LacZ knocking modified gene. Two Btk-LacZ males, two Btk-LacZ/wildtype females and two Btk-LacZ/overexpressing females were used per treatment, and they were mock or phenylhydrazine treated as described in the Materials and Methods section. At day of collection, the spleens of both wildtype and overexpressing females had similar sizes (data not shown), which is the first indication that they respond to the treatment in a similar manner. The blood from treated and untreated mice was analysed haematologically and results are shown in Table 4. Both overexpressing Gata1 and wildtype females show no significant difference in the anaemic phenotype induced by the phenylhydrazine treatment. Spleen and bone marrow cells were analysed by FACS in order to see whether differences could be detected at the progenitor cell level. Not surprisingly, no significant difference was found, as the percentage of wildtype LacZ positive cells was similar between females (Figure 6). We conclude that Gata1 overexpressing females respond normally to stress erythropoiesis induction.

Analysis of the cell cycle of foetal liver cells

In order to verify the cell cycle deregulation that has been described in Gata1 overexpressing MEL cells we collected foetal liver cells of 12.5 dpc embryos derived from

Table 4: Hematological analysis of blood after anaemia induction.

Mock treated	Red Blood Cells (x1012/l)	Hemoglobin (mmol/l)	Hematocrit (l/l)
WT M	10.99±0.14	9.50±0.10	0.54±0.02
WT F	11.39±0.17	10.10±0.00	0.57±0.01
OX F	11.27±0.02	10.10±0.20	0.56±0.02
PHZ treated	**Red Blood Cells (x1012/l)**	**Hemoglobin (mmol/l)**	**Hematocrit (l/l)**
WT M	4.29±0.67	4.60±0.70	0.26±0.02
WT F	5.75±0.49	6.85±0.35	0.37±0.03
OX F	6.29±0.48	7,40±0.70	0.34±0.01

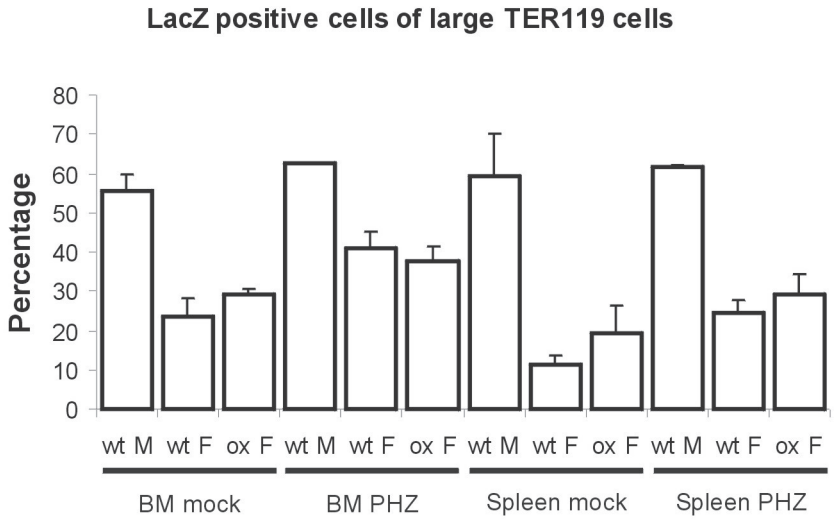


Figure 6. Anaemia induction in mice overexpressing Gata1.
The percentage of LacZ positive cells is compared in the spleen and bone marrow of mice mock-treated or treated with phenylhydracine (PHZ) to induce anaemia and stress erythropoiesis. When comparing stress erythropoiesis response, we found no difference between WT or Gata1 OX females.

Gata1 overexpressing mothers and performed a Propidium Iodide staining to analyse the cell cycle by Fluorescence Activated Cell Sorter (FACS).

In Figure 7A we depicted the percentage of cells in G1, normalised to the wildtype. There is a reduction of the cells in G1 in the Gata1 OX males, which might be a reduction in cells arresting the cell cycle prior to terminal differentiation. To assess the cell populations in G1 in each genotype, we plotted them against the forward scatter (Figure 7B). While 80% of the cells in G1 in the wildtype genotypes were small, indicating that they are probably in

the last division or arresting cell cycle before enucleation, only 70% were small cells in the overexpressing male G1 cells. This suggests that there is an increase in the proportion of proliferating progenitors in Gata1 overexpressing males. Gata1 overexpressing females always show an intermediate phenotype. This is concordant with the notion that Gata1 OX *in vivo* affects the cell cycle regulation as was shown *in vitro* in MEL cells.

Analysis of the cell cycle of Gata1 overexpressing I11 cells

I11 cells are immortalised foetal erythroid proerythroblasts derived from p53 knockout mice. These cells remain proliferative in medium containing SCF, dexamethasone and basal levels of Epo. Upon differentiation induction, I11 cells follow synchronised differentiation including enucleation. In order to complement studies from primary cells and MEL cells we generated I11 cells overexpressing Gata1 by transfecting them with a pEV^{Puromycin}Gata1-Myc construct as described in the Materials and Methods section. We used I11 cells transfected with the empty vector as control. After confirming the construct integration in the clones growing in selective medium by Southern blot (data not shown), we analysed the cell cycle profile of the cells either in proliferation or in differentiation medium. We found that the Gata1 OX I11 cells have a similar percentage of cells in G1 compared to the control cell lines in proliferative conditions (Figure 7C). However, we must consider that globin expression in proliferating I11 cells is much lower than in induced I11 cells, and this conditions the levels of the transgenic Gata1, which is under the regulation of the LCR and human β-globin promoter. When differentiation was induced, OX Gata1 cells were unable to accumulate in G1 contrary to what happened with the control cell lines (Figure 7C). The cell cycle deregulation generated by Gata1 overexpression is detected in I11 cells when differentiation is induced, and corroborates previous data.

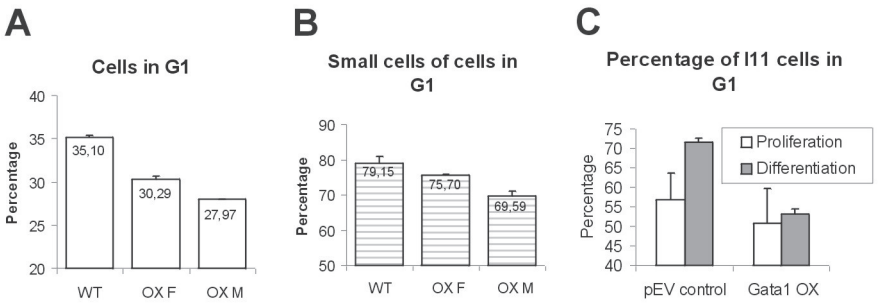


Figure 7. Cell cycle analysis of Gata1 overexpressing cells.
(A) Percentage of foetal liver cells in G1 phase, in wildtype (WT), overexpressing females (OX F) and overexpressing males (OX M).
(B) Percentage of small cells, *i.e.* terminally differentiating, of the cells in G1 plotted in A.
(C) Percentage of cells in G1 phase in I11 cells overexpressing Gata1 and control (transfected with empty vector -pEV control-) cells in proliferative and differentiating conditions.

Cell cycle arrest after UV-light induced DNA damage

One of the characteristics of the affected phenotype of Gata1 OX erythroid cells is their incapability to arrest cell cycle prior to terminal differentiation. Foetal liver cells directly isolated from 12.5 dpc from Gata1 OX mice show a significant reduction in G1 phase when compared to WT cells (Figure 7A-B). We wondered whether this inability to arrest cell cycle is specific to the erythroid differentiation process or if Gata1 OX cells are also unable to arrest the cell cycle upon DNA damage induction. With the aim to study the cell cycle checkpoint in Gata1 OX cells, we isolated foetal liver cells from 12.5 dpc WT and Gata1 OX foetuses. We expanded them in proliferative conditions and irradiated them with UV-light as described in the Materials and Methods section to study its impact on proliferation before and after differentiation induction of erythroid cells. We also included wildtype I11 cells as a control. I11 cells are p53 *null* and we expect that they will be hypersensitive to irradiation as showed for p53 *null* primary fibroblasts (Lackinger and Kaina 2000). At the day of collection we counted the cells and analysed cell death and the cell cycle by FACS. We calculated the efficiency of the cultures by dividing the cell number at the day of collection by the cell number at day 0. We observed a high percentage of dead cells in all the samples and treatments that was not correlating with the dose of UV-light applied. All the samples were either treated or mock treated, and this general cell death is most probably due to cell manipulation. For this reason, we excluded the comparison of cell death from the analysis.

The impact of UV-light irradiation on proliferation was studied by irradiating cells at day 0 and collecting them at day 5. The efficiency of the cultures in proliferative conditions is depicted in Figure 8A. We observed that WT primary erythroid progenitors are very sensitive to UV-light exposure. The efficiency is reduced drastically as the dose of UV-light increases. Interestingly, the reduction of efficiency in OX cells is not so acute as occurs in WT cultures when the UV-light dose increases. However, the efficiency of OX cells is lower compared to WT cells in mock-treated samples. We conclude therefore that Gata1 OX cells display a different sensitivity upon UV-light induced DNA damage compared to WT cells. In proliferative conditions, WT primary erythroid progenitors are sensitive to UV-light irradiation and arrest their cell cycle in order to repair the DNA damage. In contrast, OX cells are not sensitive to UV-light irradiation and continue proliferating to the same extent as mock-treated OX cells without arresting the cell cycle.

We irradiated cells 24hr before differentiation induction and collected cells at day 3 after differentiation induction, in order to study the outcome of DNA damage induction just before differentiation is induced. Similar to the previous observations, WT cells showed a significant sensitivity and the efficiency of cultures was drastically reduced with the increase of UV-light dose, while OX cells appeared to be less sensitive (Figure 8B). The cell cycle of WT differentiating cells is characterised by accumulation of cells in G1. Therefore the analysis of the cell cycle in differentiating cells is not as informative as

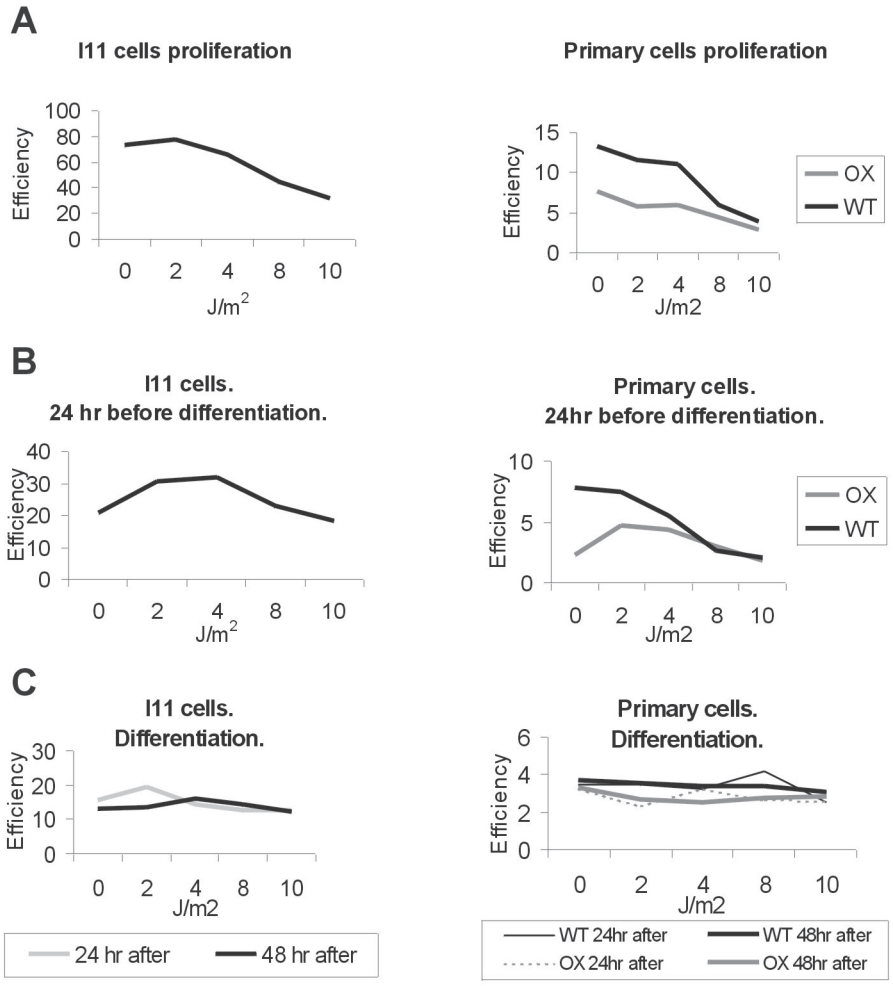


Figure 8. UV-light treatment of primary Gata1 overexpressing erythroid cells.
(A) Efficiency or survival of the cell cultures upon increasing doses of UV-light irradiation in proliferation conditions. WT I11 cells (left panel) and primary cells (right panel) either Gata1 overexpressing (OX) or wildtype (WT).
(B) Efficiency or survival of the cell cultures upon increasing doses of UV-light irradiation 24 hours before differentiation induction.
(C) Efficiency or survival of the cell cultures upon increasing doses of UV-light irradiation 24 or 48 hours after differentiation induction.

in proliferative conditions. Differentiation related cell cycle exit or DNA damage induced cell cycle arrest would be confused. In the same terms, OX cells that have been induced to differentiate are unable to arrest cell cycle and this inability would be mixed with the inability to arrest cell cycle upon DNA damage. However, by the efficiency parameter we could observe that UV-light irradiation before differentiation induction is harmful to WT cells while not so much for OX cells.

We wished to know whether, once differentiation is induced, cells show the same sensitivity as in the previous treatments. We treated cells 24 and 48 hours after differentiation induction. The efficiency of the cultures was not affected amongst WT or OX cells (Figure 8C). We could think that once differentiation is induced, DNA damage no longer challenges the cell functions.

We observed a different sensitivity to UV-light induced DNA damage in OX cells compared to WT cells. This result indicates that the cell cycle regulatory function of Gata1 is not only related to the differentiation process, but it can be extrapolated to general cell cycle regulation mechanisms.

DISCUSSION

The Gata1 overexpressing mice were generated and turned out to be the unmasking tool of Red Cell Differentiation Signal (REDS) (Whyatt *et al*., 2000). Therefore, they were meant to be the subject of research to reveal more features of such signalling mechanism, for example, which cell types are involved, who are the counterparts of it and how to manipulate REDS.

In previous studies Gata1 heterozygous females were described to be normal compared to wildtype littermates. In a CFU-E assay of bone marrow cells from adult Gata1 OX and wildtype mice the possibility of selective growth of wildtype progenitors in the Gata1 overexpressing females was discarded. However, it was not directly shown that the balance of Gata1 OX and Gata1 WT erythroid progenitors is not changed through development. Here we show by linking the Gata1 overexpression transgene and the Btk-LacZ modified gene in the same X chromosome that the overexpressing females have the same numbers of wildtype and overexpressing progenitors as observed in wildtypes females at foetal and adult stages. We compared the foetal livers with the bone marrows of the mothers, although it is not an entirely fair comparison. The foetal liver is an erythroid organ while the bone marrow is mainly granulopoietic. However, the percentage of LacZ positive cells was equal amongst females in the different organs and stages analysed, and the TER119 positive fraction of the LacZ positive population was also conserved.

Still this is not a direct prove that the REDS signalling is the explanation for the normal phenotype of Gata1 overexpressing females. One could argue that they survive with half of the progenitors as in the case of Gata1 heterozygous knockout females (Fujiwara *et al*., 1996). These females survive with approximately half of the erythroid progenitors compared to wildtype animals, as the other half of progenitors are Gata1 null and undergo apoptosis at the proerythroblast stage. This reduction in the progenitor pool causes transient anaemia during gestation. However, the females that are born are normal. So far, the only argument in favour of REDS is that the Gata1 OX females are normal throughout development and born at the expected rate despite half of their erythroid progenitors are overexpressing Gata1 (Whyatt *et al*., 2000), while the Gata1 heterozygous knockout females are anaemic during gestation and are born in low Mendelian ratios (Fujiwara *et al*., 1996). Due to the phenotype that Gata1 OX generates in the erythroid cells, one could argue that a percentage of the Gata1 OX cells are still able to differentiate *in vivo* spontaneously and this is the reason why the OX females are normal and healthier than the heterozygous Gata1 KO females. Moreover, Gata1 OX males are lethal in a later stage than Gata1 KO animals, as if they are able to cope with the defect for a longer time. In order to counter this argument and have direct evidence for REDS we analysed Gata1 OX::Btk-LacZ derived cells after differentiation induction in hanging drop cultures. We could detect a LacZ positive population linked either to a wildtype or a Gata1 OX allele, and the latter

either in a pancellular or a heterocellular context. We observed that the Gata1 OX cells are unable to differentiate in the pancellular context, but in the heterocellular context they differentiate to the same extent as wildtype cells. Moreover we show that REDS is not totally disrupted in the Hanging drop culture, suggesting that this culture method mimics physiological conditions more closely for a number of reasons: The Gata1 FACS pattern of differentiated cells resembles very much the pattern found in blood of adult mice; REDS is not totally disrupted; defective phenotypes can be detected consistently, for example, Gata1 OX cells are not differentiating properly, while in suspension cultures they do (data not shown).

The LacZ staining with FDG substrate is detected in the Fl-1 channel in the FACS sorter (green fluorescence). The reason why the blood of these mice was never analysed to sort the two populations directly is because the haemoglobin in mature cells absorbs the light beam needed by fluorescent proteins to be excited and detected in the FL-1 range of excitation. FDG is not detected in mature erythroid cells. The blood analysis was performed by Western blot. The transgenic Gata1 is tagged with a Myc epitope and this made it possible to distinguish transgenic versus endogenous Gata1 in the blood. We assume that the contribution of different cells is proportional to the intensities of respective bands on the Western blot.

Here we show that despite the fact that the blood parameters measured in a haematological analysis are within the normal range in the Gata1 OX females, they suffer slightly impaired erythropoiesis in the bone marrow resulting in enhanced spleen erythropoiesis. This indicates that rescue of the OX cells by REDS is not absolutely efficient, although the enhanced peripheral erythropoiesis copes with the inefficiency of REDS.

We wished to analyse the response of Gata1 OX females to induced haemolytic anaemia. It could be that these mice have a sensitised response compared to wildtypes, as it happens with Gata1^{low} mice (Vannucchi *et al*., 2001). However, upon haemolytic anaemia induction, these females responded equally to wildtype females.

Gata1 overexpressing cells are unable to arrest cell cycle (Whyatt *et al*., 1997), a requirement for terminal differentiation. We show that I11 cells that overexpress Gata1 show a cell cycle defect after differentiation induction as they have a reduced percentage of cells in G1 compared to control cell lines. This corroborates previous data and supports the idea that Gata1 interferes with the cell cycle machinery. This inability to arrest cell cycle occurred also under DNA damage induction by UV-light exposure, suggesting that the interference of Gata1 in the cell cycle regulation is not solely linked to erythroid differentiation. This is in agreement with previous data showing that exogenous expression of Gata1 in fibroblasts induces a slow proliferation rate (Dubart *et al*., 1996). Thus, the interference with the cell cycle is not uniquely linked to erythroid differentiation, nor to the cell type.

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CHAPTER 3

HOMOTYPIC SIGNALLING REGULATES GATA1 ACTIVITY IN THE ERYTHROBLASTIC ISLAND

Homotypic signalling regulates Gata1 activity in the erythroblastic island

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Summary
Gata1 is a transcription factor essential for erythropoiesis. Erythroid cells lacking Gata1 undergo apoptosis, while overexpression of Gata1 results in a block in erythroid differentiation. However, erythroid cells overexpressing Gata1 differentiate normally *in vivo* when in the presence of wild-type cells. We have proposed a model, whereby a signal generated by wild-type cells (red cell differentiation signal; REDS) overcomes the intrinsic defect in Gata1-overexpressing erythroid cells. The simplest interpretation of this model is that wild-type erythroid cells generate REDS. To substantiate this notion, we have exploited a tissue specific Cre/lox*P* system and the process of X-inactivation to generate mice that overexpress Gata1 in half the erythroid cells and are Gata1 null in the other half. The results show that the cells supplying REDS are erythroid cells. This study demonstrates the importance of intercellular signalling in regulating Gata1 activity and that this homotypic signalling between erythroid cells is crucial to normal differentiation.

Introduction

Erythropoiesis in mammals goes through two distinct stages, a primitive and a definitive stage. In the mouse, primitive erythropoiesis begins in the yolk sac at around 7 days post coitus (dpc) and produces nucleated erythrocytes. Shortly after 10 dpc, erythropoiesis switches from the primitive to the definitive stage, and at around 12 dpc enucleated erythrocytes begin to replace nucleated erythrocytes in the circulation (Rifkind *et al*., 1969; Russell, 1979; Wong *et al*., 1985). The primary site of definitive erythropoiesis is the foetal liver, followed by the spleen and bone marrow later in development (Medvinsky and Dzierzak, 1998; Moore and Metcalf, 1970). Definitive erythropoiesis takes place in erythroblastic islands, which consist of a central macrophage surrounded by erythroid precursors located further towards the periphery of the island in progressive stages of differentiation (Bessis *et al*., 1983).

Gata1 belongs to the GATA family of zinc-finger transcription factors (Evans and Felsenfeld, 1989; Patient and McGhee, 2002; Tsai *et al*., 1989; Yamamoto *et al*., 1990). It is mainly expressed in haematopoietic cells (erythroid cells, megakaryocytes, eosinophils and mast cells) (Hannon *et al*., 1991; Martin and Orkin, 1990; Patient and McGhee, 2002; Romeo *et al*., 1990; Weiss and Orkin, 1995a) but also in Sertoli cells of the testis (Ito *et al*., 1993; Yomogida *et al*., 1994). Gata1 recognises a consensus binding motif that is present in the regulatory elements of all erythroid-specific genes examined, including the *Gata1* gene itself (Ohneda and Yamamoto, 2002). Correct regulation of Gata1 levels appears crucial for normal primitive and definitive erythropoiesis. Erythroid cells null for Gata1 undergo apoptosis at the relatively immature proerythroblast stage (Pevny *et al*., 1995; Pevny *et al*., 1991; Weiss *et al*., 1994; Weiss and Orkin, 1995b) and *Gata1* al., 1996). The *Gata1* gene is X-linked (Zon *et al*., 1990) and, owing to X-inactivation, female mice heterozygous for a functional *Gata1* gene have two populations of erythroid cells with respect to Gata1 expression, one that is wild type and one that is *Gata1* null. These mice are transiently anaemic during gestation, but recover during the neonatal period, probably owing to the *in vivo* selection of progenitors able to express Gata1. Mutations resulting in reduced levels of Gata1 also inhibit erythroid differentiation (McDevitt *et al*., 1997; Takahashi *et al*., 1997).

Interestingly, overexpression of Gata1 in erythroid cells inhibits erythroid differentiation both *in vitro* and *in vivo* (Whyatt *et al*., 2000; Whyatt *et al*., 1997). In order to study overexpression *in vivo*, mice were generated that express Gata1 from an X-linked transgene under the control of the erythroid-specific β-globin gene promoter and locus control region. Transgenic males display pancellular Gata1 overexpression in the erythroid lineage and die of anaemia at around 13.5 dpc, because of a block in definitive erythroid differentiation (Whyatt *et al*., 2000). Furthermore, Gata1-overexpressing erythroid colonies grown from single precursors (colony forming units-erythroid, CFU-Es) fail to differentiate normally *in vitro.* By contrast, heterocellular overexpression of Gata1, as occurs in chimeric

mice or in the heterozygous transgenic females because of X-inactivation, results in live transgenic mice that are phenotypically normal. Remarkably, all erythroid cells, both wild type and overexpressing Gata1, contribute normally to the differentiated erythrocyte pool in these animals. This shows that the defect generated by overexpression of Gata1 is cell-nonautonomous (Whyatt and Grosveld, 2002). The explanation of this phenomenon is a signal, that we tentatively termed red cell differentiation signal (REDS), that is supplied by wild-type cells and directs Gata1-overexpressing cells to differentiate normally (Whyatt *et al*., 2000). However, Gata1-overexpressing CFU-Es isolated from the heterocellularly overexpressing mice fail to differentiate *in vitro*, even though their differentiation is normal *in vivo*. Thus, the defect generated by overexpression of Gata1 is intrinsic to the erythroid cells. This *in vitro* assay suggests that REDS cannot be mediated by a soluble factor and that cell-cell contact is required for REDS signalling. Thus, the erythroblastic island structure is an absolutely necessary context for REDS to act.

The fact that overexpressing males do not show erythroid differentiation suggests that the source of REDS must be a cell type that is reduced in numbers or absent in these males. In light of the highly organised structure of the erythroblastic island, the most likely source of REDS would be the wild-type erythroid cells in a late stage of differentiation. However, we could not exclude that a cell type other than erythroid is supplying REDS.

We therefore set out to distinguish whether REDS is a signalling mechanism involving cells of the same type (erythroid), defined as homotypic signalling, or a mechanism involving a different cell type other than erythroid, *i.e.* a heterotypic mechanism (Fig. 1) (Whyatt and Grosveld, 2002). In order to substantiate our model, we decided to ablate the wild-type erythroid cells in heterocellularly Gata1-overexpressing mice. As mentioned above, the survival of the earliest committed erythroid precursors is dependent on Gata1 function (Weiss and Orkin, 1995b) and, hence, deletion of the *Gata1* gene would result in loss of this population. As the *Gata1* gene itself is on the X-chromosome, we have exploited an erythroid specific Cre/lox*P* recombination system and the process of X-inactivation to generate such mice. Compound transgenic mice expressing the Cre recombinase under the control of the erythroid-specific β-globin gene promoter and locus control region (pEV-Cre), carrying a *Gata1* gene flanked by two lox*P* recombination sites on one X-chromosome and carrying the *Gata1* overexpression transgene on the other X-chromosome were generated. These transgenic females display two erythroid populations because of X-inactivation, one population overexpressing Gata1 and one population that is Gata1 null. If the cells supplying REDS are erythroid, which would be consistent with a homotypic mechanism for REDS, Gata1-overexpressing cells should no longer differentiate in such compound animals, which leads us to predict that these animals would die in utero because of anaemia caused by impaired differentiation. However, if the cells supplying REDS are not erythroid, which would be consistent with an heterotypic model for REDS, Gata1-overexpressing cells would still be able to differentiate in such compound animals. In this

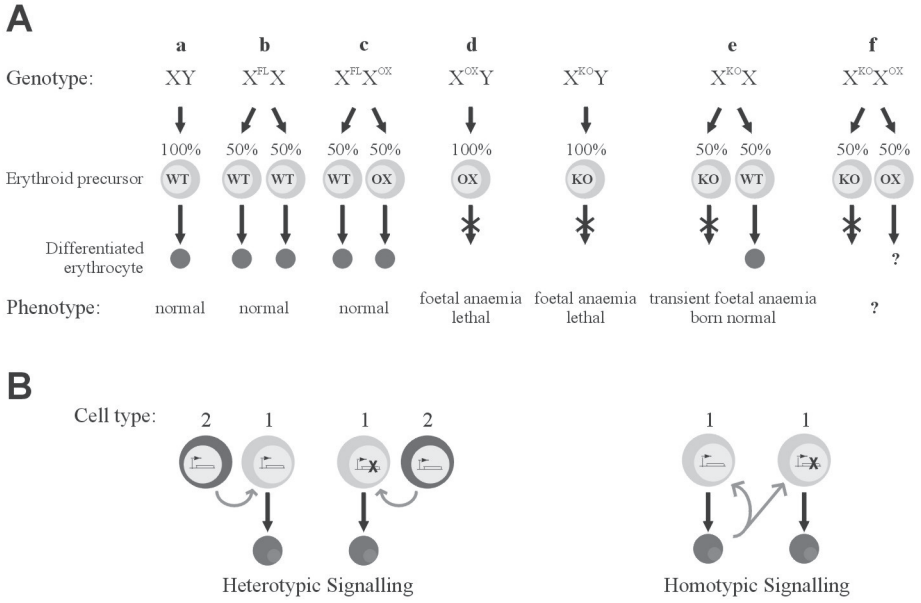

Figure 1.
(A) Summary of the different Gata1 mutants discussed and their outcome in terms of erythroid differentiation. FL, floxed; OX, overexpressing; KO, deleted Gata1 allele; WT, wild type. The progeny obtained in the crossing described in the present manuscript is indicated a-f, in accordance with Fig. 3A, Fig. 4C and Fig. 5A.
(B) REDS signalling and erythroid differentiation. Heterotypic and homotypic cell-cell signalling mechanisms. 1 and 2 are different type of cells.

case, these animals would develop with half of the erythroid progenitors, probably showing the same phenotype as heterozygous Gata1 knockout females, which are anaemic during gestation but survive normally to term with half of the erythroid progenitors (Fig. 1). In summary, if the compound females show a phenotype worse than heterozygous Gata1 knockout females, the mechanism would be homotypic. However, if the compound females show a phenotype equal to heterozygous *Gata1* knockout females, the mechanism would be heterotypic. In this way, we wished to identify the cell type supplying REDS.

Materials and Methods

Mice

Gata1 X^{FL} mice bear a modified *Gata1* gene flanked by *loxP* sites (Fig. 2A) (F.L., unpublished). Gata1 X^{OX} mice overexpress Gata1 from an X-linked transgene (Whyatt *et al*., 2000). pEV-Cre mice express the Cre recombinase under the control of the β-globin gene promoter and locus control region and this transgene is autosomal. Gata1 $X^{OX}X$: pEV-Cre (+/–) females were generated and crossed with Gata1 $X^{FL}Y$ males. Pregnant females were sacrificed at 13.5 and 14.0 dpc, or allowed to go to term, and progeny were analysed. CAG-Cre mice express the Cre recombinase ubiquitously and have been described previously (Sakai and Miyazaki, 1997). ROSA26-*lacZ* reporter mice express β-galactosidase upon lox*P* recombination and have been previously described (Soriano, 1999).

Genotyping and recombination analysis

The head or a tail snip was used in each case for determination of the genotype. The recombination efficiency was quantified in head and foetal liver by Southern blot analysis. The probe used was the murine engrailed 2 sequence located between the 3' lox*P* site and the *GFP* sequence of the vector (Fig. 2A). The restriction enzyme used was *Nco*I. In order to determine recombination efficiency by FACS analysis in different haematopoietic cell types, pEVCre and CAG-Cre mice were crossed with ROSA26-*lacZ* reporter mice. Fluorescein di-β-D-galactopyranoside (FDG) was used as galactosidase substrate and 7-aminoactinomycin-D (7AAD) as viability marker. Each cell type was assessed as follows: $TER119^+$ (erythroid), $CCR3^+$, FSC^{medium} (eosinophils), $Mac1^+$ (macrophages), $cKit^+$, SSC^{high} (mast cells), $CD31^+$, FSC^{high} (megakaryocytes). The antibodies used were phycoerythrin conjugated (R-PE). FSC stands for 'forward scattered', SSC for 'side scattered'.

Foetal blood analysis

Blood samples were collected by bleeding dissected foetuses in 5 ml of phosphate-buffered saline (PBS). Cell numbers were determined by counting in a hemocytometer. Blood samples were also analysed in an electronic cell counter (CASY-1, Schärfe Systems) to determine the proportion of primitive (nucleated) and definitive (enucleated) erythrocytes in blood.

Hanging drop culture

Half of the liver from each foetus was collected and placed in 0.5 ml of Dulbecco's modified Eagle's medium (DMEM) with 20% foetal calf serum (FCS). Foetal livers were disaggregated into single-cell suspension and cells counted. For hanging drop cultures, $5x10^4$ cells were resuspended in 20 μl hanging drop medium (DMEM supplemented with

20% FCS, 0.1% β-mercaptoethanol, $2x10^{-4}$ M hemin, 5 μg/ml penicillin/streptomycin, 2 U/ml erythropoietin, 5 μg/ml insulin) and cultured for 2 days (F.L., unpublished). Antimouse Fas antibody (Jo2) was purchased from BD Pharmingen (Catalogue number 554254) and used in hanging drop cultures at 20 μg/ml and 40 μg/ml concentrations.

Histological staining

Foetal blood and foetal liver single-cell suspension samples from each foetus were cytocentrifuged, and the preparations were stained with neutral benzidine and histological dyes as described (Beug *et al*., 1982). Cells cultured in hanging drops in the presence or absence of Jo2 were also collected after 2 days of culture, cytocentrifuged and stained. Images were acquired in an Olympus BX40 microscope. The lenses used were Plan 40X/0.65 and Olympus Plan 100X/1.25. The acquisition software used was Viewfinder Lite Version 1.0.125 and Studio Lite Version 1.0.124, Pixera Corporation. Image processing was done in Adobe Photoshop 5.5.

FACS analysis

FACS analysis was performed in every foetal liver with $5x10^4$ events taken per sample at day of collection and after two days of hanging drop culture. Single-cell suspensions were incubated with R-Peconjugated TER119 antibody and 7-aminoactinomycin-D (7AAD). Cell populations were divided as follows: non-viable ($7AAD^+$), erythroid ($TER119^+$), small erythroid ($TER119^+/FSC^{low}$). *Ex vivo* differentiation results were compared between the different genotype/phenotypes.

Western blot analysis

The same number of cells from 13.5 dpc foetal livers at day 0, 1 and 2 of hanging drop culture were lysed with 2xLaemmli buffer and these whole cell extracts were analysed by western blot. The N6 Gata1 rat monoclonal (sc-265) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibody against B23 nucleophosmin was a kind gift from Pui K. Chan (Baylor College of Medicine, Houston, TX). Secondary antibodies conjugated to horseradish peroxidase were purchased from Dako (DakoCytomation, Denmark). Enhanced chemoluminescence (ECL) was performed to develop the blots as described by the manufacturer (Amersham Pharmacia).

Results

Breeding strategy for the generation of Gata1 compound mutant mice and nomenclature

To determine whether the REDS signalling mechanism is homotypic or heterotypic, we generated compound mice that have one erythroid population that is overexpressing Gata1 and the other population that is *Gata1* null. It is feasible to obtain such mice because the *Gata1*-overexpressing transgene and the endogenous *Gata1* gene are X-linked and they would be subjected to X-inactivation. Because one of the X-chromosomes must pass through the male germline, and hemizygosity for the *Gata1* knockout allele and the *Gata1* overexpressing transgene are both lethal, we made use of a conditional knockout allele of the *Gata1* gene (Fig. 2A) and an erythroid specific Cre recombinase (pEV-Cre) to perform the experiment. The crossing strategy was designed to obtain the compound females together with the different genotypes that are needed as control to distinguish between homotypic or heterotypic signalling. The crossing strategy and the offspring obtained are depicted in Fig. 3A. A male carrying a *Gata1* modified gene surrounded by lox*P* sites (X^{FL}) is crossed with a female carrying one overexpressing X-linked *Gata1* transgene (X^{OX}) plus the endogenous *Gata1* gene on each chromosome and one autosomal *pEV-Cre* transgene.

From the progeny, males are scored as wild type when they carry no transgenes or carry only the *pEV-Cre* transgene and we refer to them as XY. Females positive only for the floxed *Gata1* allele are also considered wild type, and we refer to them as $X^{FL}X$. The heterozygous Gata1-overexpressing females are positive for the *Gata1*-overexpression transgene and the floxed *Gata1* allele and are indicated as $X^{FL}X^{OX}$. The Gata1-overexpressing males are positive for the overexpressing transgene or positive for both the overexpressing and *pEV-Cre* transgenes and are referred as $X^{OX}Y$. Females positive for the floxed *Gata1* allele and the *pEV-Cre* transgene are heterozygous *Gata1*-null females and are indicated as $X^{KO}X$. Females positive for the three transgenes, *i.e.* the X-linked *Gata1*-overexpression transgene, the X-linked floxed *Gata1* locus, and the autosomal *pEV-Cre* transgene, are named as compound females and indicated as $X^{KO}X^{OX}$. These compound females have one population of Gata1-null erythroid cells that undergo apoptosis very early during differentiation and one population of Gata1-overexpressing erythroid cells (see Fig. 1 for a summary of the expected outcomes of each genotype in terms of erythroid differentiation).

Analysis of the recombination driven by pEV-Cre

Southern blot analysis of foetal liver DNA from $X^{KO}X$ females demonstrates that the vast majority of floxed alleles have undergone recombination (Fig. 2B, lane 3). Although the foetal liver at this stage of development consists mainly of erythroid cells, other haematopoietic and non-haematopoietic cells are present. As we have found that pEV-

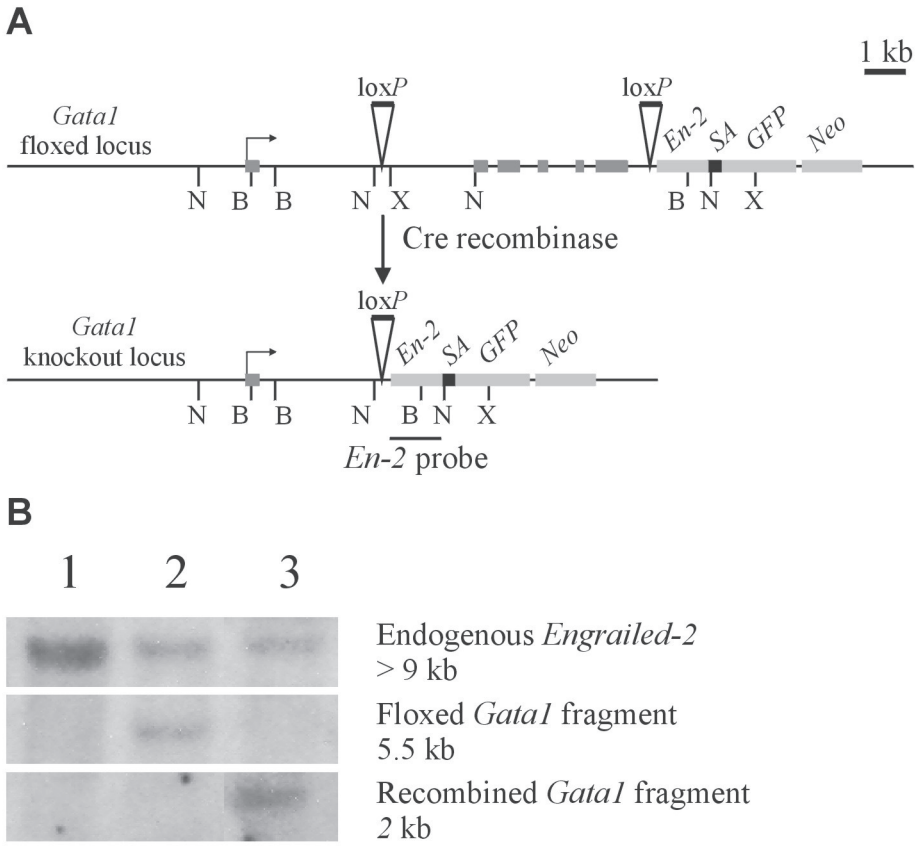

Figure 2. Recombination of the Gata1 floxed locus.
(A) Maps of the floxed and the knockout *Gata1* locus after recombination. N, *NcoI*; B, *BamHI*, X, *XbaI*; *En2*, murine engrailed 2 intronic sequence; *SA*, splice acceptor; *GFP*, green fluorescence protein. Lox*P* sites are indicated. (B) DNA samples of 13.5 dpc foetuses were digested with *NcoI* and blotted against the *En2* probe for quantifying recombination of the *Gata1* floxed locus. 1, XY wild-type male head DNA control; 2, $X^{FL}X$ female head DNA control; 3, $X^{KO}X$ female foetal liver DNA.

Cre is also expressed in other tissues (L.G., unpublished) it is important to assess recombination activity in other Gata1-dependent cells present in the foetal liver, as well as in macrophages (the central cells of the erythroblastic island), to avoid misinterpretation of the data. First we determined the percentage of erythroid and nonerythroid haematopoietic cells present in the foetal liver by sorting cell populations by fluorescence-activated cell sorting (FACS) on the basis of antigen expression specific for the different haematopoietic lineages. The results are presented in Table 1A and demonstrate that eosinophils, mast cells, megakaryocytes and macrophages represent only a small fraction of the haematopoietic cells present in the liver, while the vast majority of cells were classified as erythroid. In order to assess pEV-Cre transgene encoded recombination activity in these

Table 1A. Contribution of different haematopoietic cell lineages in the foetal liver

	Alive Cells (7AAD$^-$)				
	Eosinophils (CCR3$^+$)	Mast Cells (cKit$^+$ SSC^{high})	Megakaryocytes (CD31$^+$ FSC^{high})	Macrophages (Mac1$^+$)	Erythroid Cells (Ter119$^+$)
Contribution	1.4±0.3	1.8±0.5	3.5±0.9	1.8±0.3	71.0±4.0

Contribution is the percentage of positive cells of the total events analysed, in at least three foetuses (average ± s.d.).

Table 1B. Analysis of the recombination driven by pEV-Cre in the studied lineages

	Alive and LacZ Positive Cells (7AAD- FDG+)			
	Eosinophils	Mast Cells	Megakaryocytes	Macrophages
FDG-Unstained	0.8±0.2	0.4±0.3	8.0±0.5	7.0±2.0
Cre Negative	1.2±0.6	3.0±1.0	10.0±1.0	6.0±1.0
CAG-Cre*	48.2±7.0	95.0±1.0	72.0±0.5	80.0±7.0
PEV-Cre*	3.0±1.0	17.0±6.0	4.0±2.0	8.0±1.0

Percentage of alive and LacZ positive cells of each cell lineage (average ± s.d.). At least three foetuses were analysed per group. FDG-Unstained and Cre Negative are the negative controls and CAG-Cre is the positive control. Percentage of samples marked with * are values after subtraction of the background.

cells we crossed the pEV-Cre transgene and also the CAG-Cre (Sakai and Miyazaki, 1997) (as ubiquitous control) with ROSA26-*lacZ* reporter mice (Soriano, 1999). *lacZ* expression (from the recombined ROSA26 allele) in the different lineages was determined by staining with fluorescein di-β-D-galactopyranoside (FDG), which is hydrolysed into a fluorescent product by β-galactosidase, and analysed by FACS (Table 1B). From this analysis, we conclude that the pEV-Cre recombination activity, after subtraction of the background staining, is present only in 5-20% of the non-erythroid haematopoietic cells in the foetal liver. The floxed non-recombined allele present in non-erythroid cells does not show up in the Southern because the contribution of these cell types in the foetal liver to the total cell population is very low. As the recombined *Gata1* gene is X-linked, only half of the cells would have the recombined allele active. Thus, if 20% of the mast cells express pEV-Cre through a position effect, only 10% would be *Gata1*-null. Assuming that all of the cKit$^+$ SSC^{high} cells are mast cells (the real number is lower), 2% of the foetal liver cells would be mast cells. This brings us to the estimation that only 0.2% of the foetal liver cells would be *Gata1*-null mast cells. Even lower numbers are obtained for the other cell types. In the erythroid lineage, and as would be expected from the Southern blot analysis (Fig. 2), recombination driven by pEV-Cre was complete as was observed with CAG-Cre (data not shown). We therefore consider that the phenotypes observed are caused by the deletion of Gata1 in erythroid cells. Thus, pEV-Cre-mediated deletion is appropriate to assess the nature of REDS by analysing the phenotype of compound females.

Observed phenotype of Gata1-mutant mice

In total, 157 mice were analysed at mid-gestation and 60 were allowed to go to term (Tables

2, 3). Anaemia and death rate were scored at the day of collection. As indicated in Table 2, both wild-type foetuses and $X^{FL}X^{OX}$ females were normal in appearance at mid-gestation. As previously described (Whyatt *et al*., 2000), the majority of male $X^{OX}Y$ foetuses are anaemic or have died. As expected, a high proportion of the $X^{KO}X$ females are anaemic, though the rate of death is no higher than among wild-type foetuses. Most $X^{KO}X^{OX}$ females are anaemic and/or die similar to what is observed in $X^{OX}Y$ mice (Table 2). Representative foetuses at 13.5 dpc are shown in Fig. 3A. Wild-type genotypes and Gata1-overexpressing females are normal while the rest of genotypes are anaemic. $X^{KO}X$ mice appear anaemic, though their pallor is not as severe as in $X^{OX}Y$ and $X^{KO}X^{OX}$ mice and they display a greater variance in phenotype (see also Table 2). Foetal livers from all genotypes, which is the site of definitive erythropoiesis at this stage, were not significantly different in size. Mice born from an identical cross are depicted in Table 3. As expected, no $X^{OX}Y$ males were born (Whyatt *et al*., 2000). $X^{KO}X$ females recover from the anaemia observed during gestation and are born in the expected numbers for a viable phenotype. To our surprise, one $X^{KO}X^{OX}$ female out of 60 pups was born alive. Upon further analysis (including blood analysis, breeding, karyotyping and DNA-FISH; P. van Schalkwijk and A. Langeveld, unpublished), this female was found to have recombined the *Gata1*-overexpressing transgene onto an autosomal chromosome. Thus, we conclude that the genotype of the compound females is lethal during gestation (Table 3). The compound females die of anaemia around 13.5 dpc. This is strong evidence for a homotypic mechanism for REDS, as the phenotype displayed by $X^{KO}X^{OX}$ females is more severe than that found in $X^{FL}X^{OX}$ and $X^{KO}X$ females, and very similar to the phenotype of $X^{OX}Y$ males.

Table 2. Distribution of genotypes and phenotype of foetuses analysed at 13.5 and 14 dpc

			Alive					
Genotype	Obtained	Expected	Normal	(%)	Anaemic	(%)	Dead	(%)
XY	41	39	39	(95)	0	(0)	2	(5)
$X^{FL}X$	22	20	19	(86)	1	(5)	2	(9)
$X^{FL}X^{OX}$	20	20	18	(90)	1	(5)	1	(5)
$X^{OX}Y$	35	39	1	(3)	21	(60)	13	(37)
$X^{KO}X$	23	20	12	(52)	9	(39)	2	(9)
$X^{KO}X^{OX}$	16	20	1	(6)	8	(50)	7	(44)
Total	157							

Pallor is scored as anaemia and lack of heartbeat is scored as death

Table 3. Distribution of genotypes of progeny born alive

Genotype	Obtained[a]	Expected[b]	Expected[c]
XY	22	20	24
$X^{FL}X$	11	10	12
$X^{FL}X^{OX}$	14	10	12
$X^{OX}Y$	0	0	0
$X^{KO}X$	12	10	12
$X^{KO}X^{OX}$	1	10	0
Total	60		

a: All animals born were normal.
b: Assuming that $X^{OX}Y$ mice are not born.
c: Assuming that $X^{OX}Y$ and $X^{KO}X^{OX}$ mice are not born.

Analysis of the foetal blood of Gata1 mutant mice

In order to quantitatively measure the anaemic phenotypes, foetuses were bled into 5 ml of PBS and the numbers of red cells were counted (Fig. 3B). As expected, $X^{FL}X^{OX}$ females have red blood cell counts similar to wild-type foetuses. $X^{OX}Y$ males are clearly anaemic, having about sevenfold less cells in blood compared with wild-type males. $X^{KO}X$ females are also anaemic, though with greater variability than Gata1-overexpressing males probably owing to the random character of the X-inactivation process, which would lead to a gradient of phenotypes. $X^{KO}X^{OX}$ females are severely affected, consistently having very low red cell numbers. The low variability of the average of red cell counts in foetal blood in $X^{KO}X^{OX}$ females indicates that this value is not affected by a random X-inactivation process compared with what happens in $X^{KO}X$ females, demonstrating that the overexpressing cells are not being rescued at any level.

In order to quantify how the switch from primitive to definitive erythropoiesis was affected, blood samples were monitored for cell size in an electronic cell counter (CASY-1, Schärfe Systems). The large nucleated cells are mostly primitive erythroid cells (also non-erythroid cells and possible erythroid precursors), while the small enucleated cells are definitive erythrocytes. The ratio of definitive versus primitive cell counts was plotted to measure a shift in the balance amongst the two cell types (Fig. 4A). The ratio is just above one at 13.5 dpc and increases to above two at 14.0 dpc in wildtype animals, which illustrates the cessation of primitive erythropoiesis in the yolk sac and the beginning of definitive erythropoiesis in the foetal liver starting around 11 dpc. $X^{FL}X^{OX}$ females are delayed compared with wild-type animals with a lower ratio at both 13.5 and 14.0 dpc. The replacement of primitive erythrocytes in the circulation is significantly repressed in $X^{OX}Y$ males and $X^{KO}X$ females. $X^{KO}X^{OX}$ females are the most severely affected, displaying in blood at 14.0 dpc at least two times more primitive cells compared with definitive erythrocytes, suggesting a major block in definitive erythropoiesis.

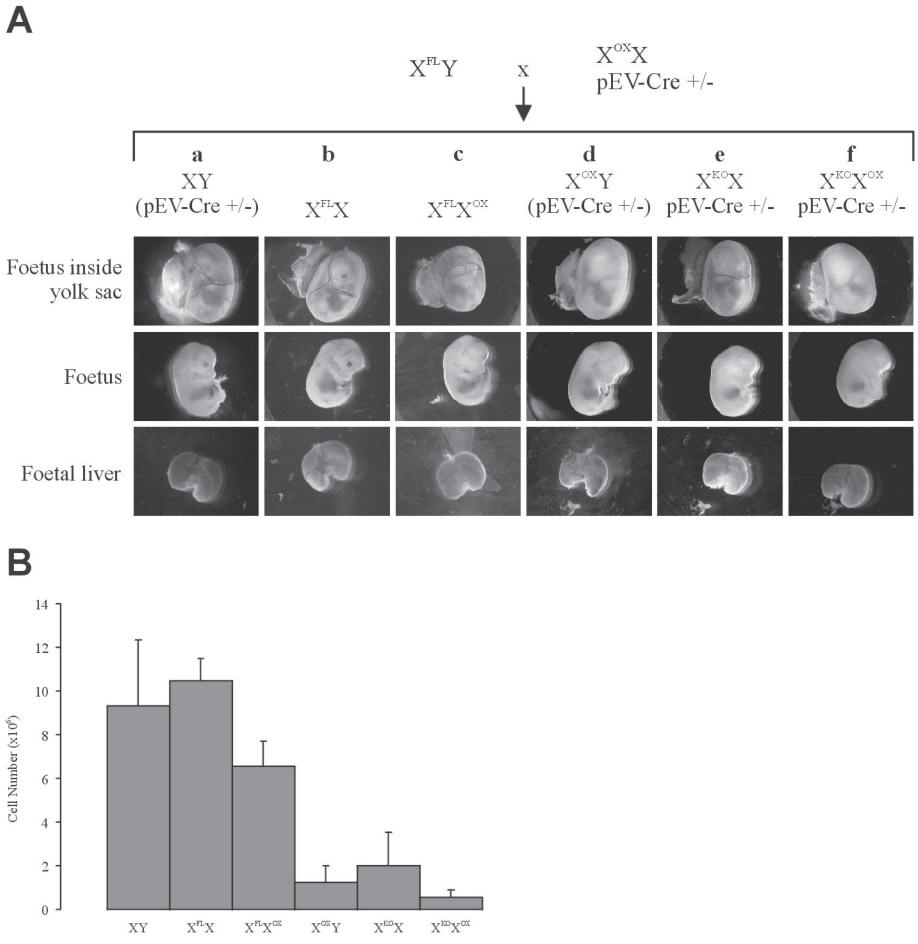

Figure 3.
(A) Crossing strategy and phenotype of the different Gata1 mutant foetuses obtained at 13.5 dpc. A photograph of the foetus and foetal liver is included for each genotype. Mice a and d are wild type or Gata1-overexpressing, respectively, regardless of expression of the pEV-Cre transgene (in brackets). a-f are as in Fig. 1A, Fig. 4C and Fig. 5A.
(B) Total cell number in foetal blood at 14.0 dpc. Average and s.d. are indicated. At least three foetuses were analysed per group.

With the CASY analysis, several other large cell types are detected, but they are not primitive erythroid cells. Thus, the percentage of definitive precursors in blood was determined on cytospins. A cytospin blood sample from each genotype is depicted in Fig. 4C. The blood of both wild-type foetuses and $X^{FL}X^{OX}$ females contain normal representations of primitive and definitive erythrocytes in the circulation at 13.5 dpc (in agreement with the CASY analysis). As previously reported (Whyatt *et al*., 2000), $X^{OX}Y$ male foetuses have proportionally fewer definitive cells, with an additional population (~2% of the total)

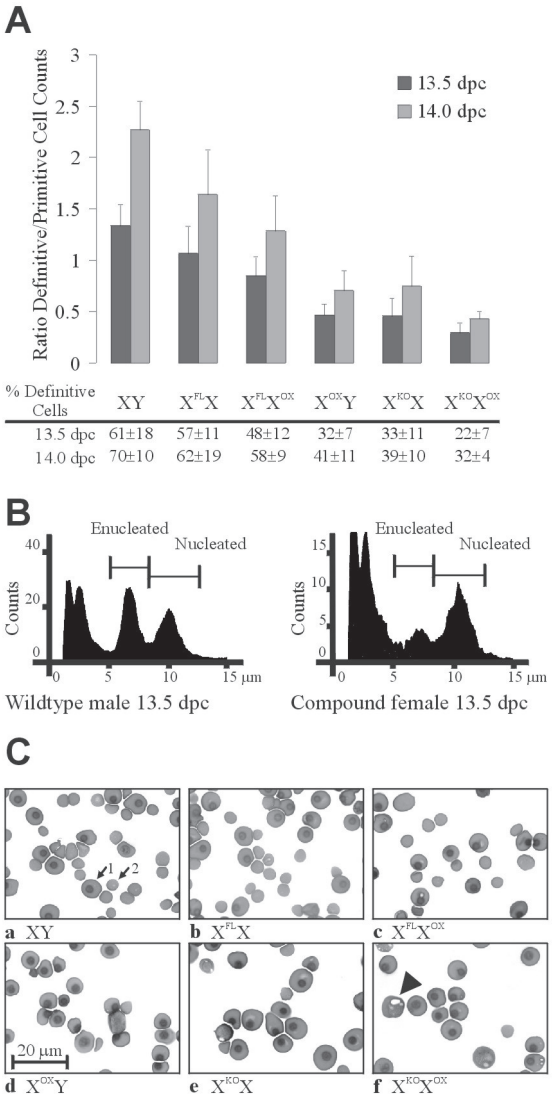

Figure 4.
(A) CASY analysis: the ratio of definitive versus primitive erythrocytes in foetal blood was compared at 13.5 and 14.0 dpc. The percentage of definitive cells in blood in each genotype at 13.5 dpc and 14.0 dpc is depicted below the graph. Average and s.d. are indicated. At least three foetuses were analysed per group.
(B) CASY analysis: example of CASY graphs from wild-type male and compound female at 13.5 dpc. Peaks corresponding to enucleated and nucleated cells are indicated. The peak below 5 mm corresponds to cell debris.
(C) Blood cytospins of each genotype at 13.5 dpc showing primitive nucleated erythrocyte (1), definitive enucleated erythrocyte (2), erythroid precursor (arrowhead). Pictures were taken at 100x magnification.

of immature precursors in the circulation. $X^{KO}X$ females and $X^{KO}X^{OX}$ females have fewer definitive cells than wild-type animals and ~4% of circulating cells are immature erythroid precursors. The presence of precursors in blood is indicative of a severe anaemia status. Thus $X^{OX}Y$ males, $X^{KO}X$ and $X^{KO}X^{OX}$ females are very anaemic at this stage, in agreement with the data described above.

Analysis of the foetal liver, the site of definitive erythropoiesis, of Gata1 mutant mice

To examine the process of differentiation at the source of definitive erythroid cells at this stage and confirm the anaemias, cytospins of disaggregated foetal livers were also stained and analysed. Foetal livers from all genotypes analysed have similar size, suggesting that colonisation of the liver by erythroid precursors is not affected in any genotype. An example cytospin for each genotype at 14.0 dpc is shown in Fig. 5A. Wild-type and $X^{FL}X^{OX}$ transgenic female foetal livers contain erythroid precursors at all stages of differentiation. As previously reported (Whyatt *et al*., 2000), $X^{OX}Y$ male foetal livers contain fewer cells late in the differentiation process, *i.e.* fewer orthochromatic erythroblasts and enucleated definitive cells. $X^{KO}X$ female foetal liver samples differ from wild-type samples in that they contain fewer cells beyond the polychromatic erythroblast stage. Similarly, $X^{KO}X^{OX}$ female foetal liver samples contain fewer benzidine-positive cells. In these three genotypes, there is a clear impairment in definitive erythropoiesis. The reason for this in the $X^{OX}Y$ male is that Gata1-overexpressing cells are intrinsically defective, as described previously. In the case of the $X^{KO}X$ female, the data suggest that owing to the loss of half of the precursors (*Gata1*-null cells), the wild-type population has an altered balance favouring proliferation versus differentiation in order to increase the numbers of progenitors to normal levels. In the case of the $X^{KO}X^{OX}$ females, the data show that the Gata1-overexpressing cells are not rescued by another cell type. Thus REDS appears to be blocked, favouring the notion of REDS acting through a homotypic signalling mechanism.

Of the three affected genotypes, $X^{KO}X^{OX}$ females and $X^{OX}Y$ males die at around 13.5 dpc. In order to confirm that these genotypes die from anaemia, we determined the viability and proportion of erythroid cells in the foetal liver compared with that seen in wild-type animals. This was assayed by FACS analysis using the erythroid marker TER119 and, as a marker for viability, 7AAD (Fig. 5B,C). The viable TER119-positive population makes up ~65-75% of the cells in the foetal liver at 13.5 and 14.0 dpc in wild-type foetuses and $X^{FL}X^{OX}$ female foetuses. At 13.5 dpc, this population is reduced to 50% in $X^{OX}Y$ male, $X^{KO}X$ female and $X^{KO}X^{OX}$ female foetuses (Fig. 5B) and, not surprisingly, the proportion of viable non-erythroid and dead cells (either erythroid or non-erythroid) has increased considerably. By 14.0 dpc (Fig. 5C), male $X^{OX}Y$ foetal livers contain less than 40% viable TER119-positive cells. At 14.0 dpc, the proportion of viable erythroid cells in $X^{KO}X$ females remains lower than in wild type and an increase in the proportion of viable non-erythroid

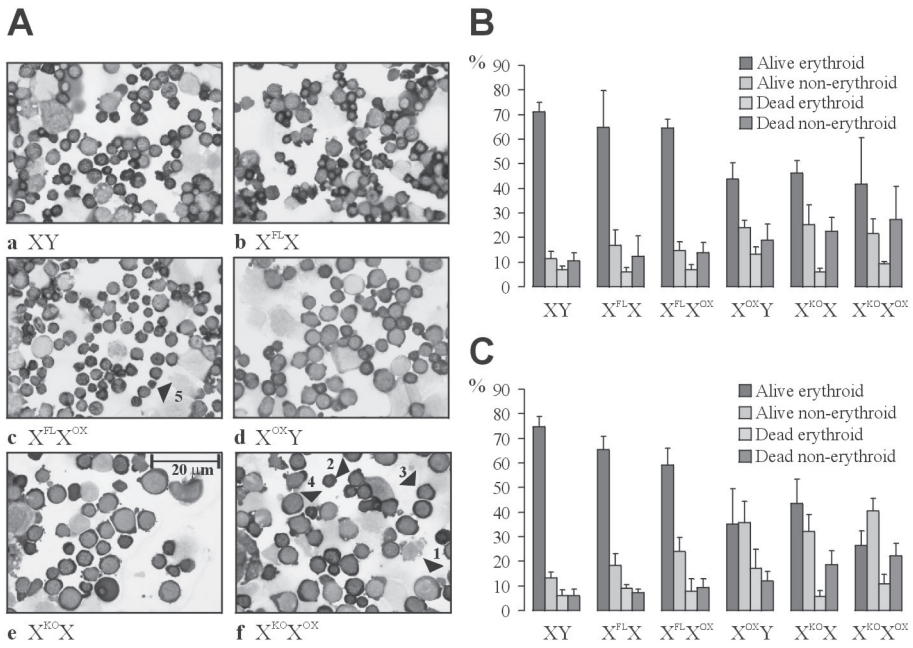

Figure 5.
(A) Foetal liver cytospins of each genotype at 14.0 dpc. Consecutive differentiation stages are indicated in photograph c and f: large proerythroblast (1), basophilic erythroblast (2), polychromatic erythroblast (3) (which exhibit both basophilia and benzidine positivity), orthochromatic erythroblast (4) (which are strongly benzidine positive) and enucleated definitive erythrocyte (5). Pictures were taken at 100x magnification.
(B,C) FACS analysis of (B) 13.5 and (C) 14.0 dpc foetal liver cells at the day of collection. Percentage of alive erythroid ($TER119^+/7AAD^-$), alive non-erythroid ($TER119^-/7AAD^-$), dead erythroid ($TER119^+/7AAD^+$) and dead non-erythroid ($TER119^-/7AAD^+$) cells are depicted. Average and s.d. are indicated. At least three foetuses were analysed per group.

cells is observed. However these foetuses are not as severely affected as $X^{OX}Y$ male foetuses. In 14.0 dpc $X^{KO}X^{OX}$ females the proportion of viable erythroid cells has been reduced to less than 30% of the total foetal liver cell population. In these foetuses, the proportion of viable non-erythroid cells was the highest (around 40%). Thus, the consistent and progressive deleterious status of the erythroid compartment in these in utero lethal genotypes suggests that in fact these mice die of anaemia.

In order to determine the *ex vivo* differentiation capability of erythroid progenitors, hanging drop assays were performed. In this assay, 14.0 dpc foetal livers were disaggregated to single cells and then cultured for 2 days in hanging drops. After culture, cells were analysed by FACS to measure differentiation (Whyatt *et al*., 2000). Differentiation is scored as the percentage of alive (7AAD negative), erythroid (TER119 positive) and low forward scattered cells (FSC^{low}), *i.e.* small cells corresponding to enucleated erythrocytes. Because the erythroblastic island is disrupted in this assay REDS signalling is lost (Whyatt *et al*., 2000), which means that the REDS-dependent cells will not differentiate.

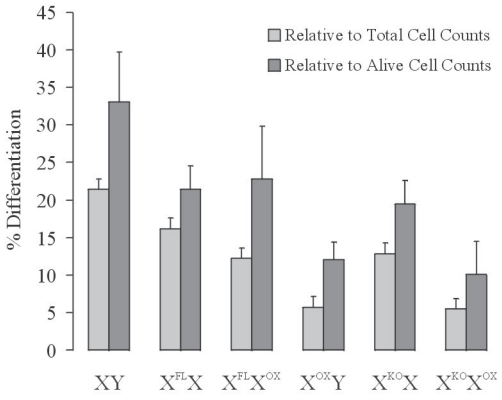

Figure 6.
FACS analysis of foetal liver cells at 14.0 dpc after 2 days of hanging drop culture to induce differentiation. Differentiation is estimated as the percentage of TER119$^+$/7AAD$^-$/FSC^{low} of the 50,000 events measured in total (light-grey bars) and as the percentage of TER119$^+$/FSC^{low} of the 7AAD$^-$ (alive) cells measured (dark-grey bars). Average and s.d. are indicated. At least three foetuses were analysed per group.

Differentiation rates in wild-type animals are between 15 and 23% of the total (Fig. 6, light-grey bars). As expected, male $X^{OX}Y$ foetal liver cells fail to differentiate. $X^{FL}X^{OX}$ female foetal liver cells display an intermediate phenotype relative to wild-type animals and $X^{OX}Y$ males, reflecting that on average half of the erythroid cells are wild type (the rest is Gata1 overexpressing) (Whyatt *et al*., 2000). Similarly, differentiation rates of $X^{KO}X$ female foetal liver cells are comparable with those of $X^{FL}X^{OX}$ mice, as half of these cells are wild type (the rest are Gata1 null). $X^{KO}X^{OX}$ female-derived cells differentiated as poorly as $X^{OX}Y$ males, in agreement with the fact that there are no wild-type cells, *i.e.* 50% of these cells are Gata1-null and the rest are overexpressing Gata1. The percentage of differentiation relative to the alive cell counts is depicted in Fig. 6 in dark grey bars.

Gata1 levels decrease during terminal erythroid differentiation

The most likely mechanism by which REDS acts, is by regulating the levels of Gata1 in the differentiating erythroid cell. The best candidates as signalling molecules are death receptors and their ligands, owing to the expression profile that has been described: differentiating erythroid cells express death receptors and, late in maturation, also their ligands (Barcena *et al*., 1999; Dai *et al*., 1998; De Maria *et al*., 1999a; De Maria *et al*., 1999b; Josefsen *et al*., 1999; Maciejewski *et al*., 1995; Oda *et al*., 2001; Silvestris *et al*., 2002; Zamai *et al*., 2000). We wanted to assess the kinetics of Gata1 protein levels during *ex vivo*-induced differentiation, and to study the involvement of a death receptor (Fas) expressed in erythroid cells in terminal differentiation. For this, 13.5 dpc foetal livers of wild-type and Gata1-overexpressing foetuses were collected and cultured in hanging drops. Whole-cell extracts of the same number of cells were prepared after 0, 1 and 2 days of culture. The levels of Gata1 in these extracts were analysed by western blot analysis. This shows that the levels of Gata1 decrease in wild-type erythroid cells during differentiation (Fig. 7A). After correction with the loading control, Gata1 levels decrease 17% by day 1 and 78% by day 2 compared with day 0. Male Gata1-overexpressing cells

have much higher levels of Gata1 at all stages, resulting in an impaired differentiation. Day 2 extracts from $X^{ox}X$ and $X^{ox}Y$ foetuses are shown in Fig. 7C.

Wild-type foetal liver cells treated with the Fas receptor activator Jo2 show even lower levels of Gata1 on day 2 of differentiation when compared with the standard cultures (Fig. 7B,C). After correction with nucleophosmin, the loading control, the reduction on Gata1 levels was measured to be 30%. The differentiation profile and the rate of cell death did not change even at the highest concentration of Jo2 used (L.G., unpublished). However the Jo2-treated cells showed a higher rate of enucleation on day 2 of culture. Cytospins of cultured cells were prepared and more than 400 cells were counted per treatment. The percentage of enucleated cells was found to be 19±5% in the standard cultures and 31±5% in the Jo2-treated cultures. Thus, the Fas pathway positively regulates the last stages of erythroid differentiation in wild-type cells. When Gata1-overexpressing cells from males are treated with Jo2, the Gata1 levels of both endogenous and transgenic protein are also reduced about 30%, after correction with the loading control. However, they are still higher than the level observed in wild-type cells on day 0 (Fig. 7C). As a result, there is no rescue of the impaired differentiation (data not shown). Thus, we conclude that a decrease of Gata1 levels is required for terminal erythroid differentiation and that a signalling pathway similar to the Fas pathway achieves this reduction.

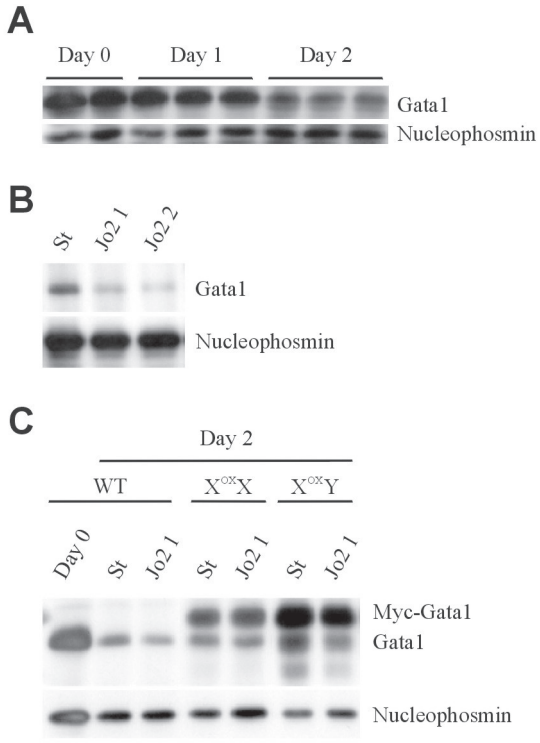

Figure 7. Gata1 levels decrease during terminal erythroid differentiation.
(A) Whole cell extracts from wild-type foetal liver cells, after day 0, 1 and 2 of hanging drop culture were analysed by western blotting using antibodies against Gata1 with nucleophosmin as loading control. Two samples at day 0 are shown and cultures were done in triplicate.
(B) Jo2 treatment of wild-type cells. Whole cell extracts at day 2 after standard (St) culture and culture with Jo2 1 (20 μg/ml) or Jo2 2 (40 μg/ml) were analysed by western blotting using antibodies against Gata1, and nucleophosmin as a loading control.
(C) Jo2 treatment of erythroid cells from Gata1-overexpressing foetuses. Whole cell extracts at day 0 and 2 after standard culture and culture with Jo2 1 (20 μg/ml) were analysed by western blotting using antibodies against Gata1 with nucleophosmin as a loading control. Extracts from wild-type, $X^{ox}X$ female and $X^{ox}Y$ male foetal liver cells are shown.

DISCUSSION

We have previously demonstrated that overexpression of Gata1 in the erythroid lineage inhibits erythroid differentiation, both *in vitro* (Whyatt *et al*., 1997) and *in vivo* (Whyatt *et al*., 2000). However, mice overexpressing Gata1 in a heterocellular manner display a wild-type phenotype, whereby the cells overexpressing Gata1 differentiate normally. This demonstrates that a signal (termed REDS) originating from wild-type cells causes Gata1-overexpressing cells to revert to a wild-type phenotype. As the erythroblastic island is a close and hierarchic microenvironment, we propose that wild-type erythroid cells would supply REDS, *i.e.* that a homotypic signalling model regulates the shift from Gata1-induced proliferation to terminal differentiation. Conversely, in a heterotypic signalling model, the cell type supplying REDS would be a non-erythroid cell type (Fig. 1B). To determine which model is appropriate, we ablated the wild-type erythroid lineage in heterocellularly Gata1-overexpressing mice by deleting the *Gata1* gene in the erythroid lineage. This was achieved by exploiting the Cre/lox*P* system and the process of X-inactivation.

The crossing strategy generated a set of transgenic mice that are either wild type, or overexpress Gata1 in all erythroid cells ($X^{OX}Y$ males), or overexpress Gata1 in 50% of cells ($X^{FL}X^{OX}$ females), or are null for Gata1 in 50% of cells ($X^{KO}X$ females), or overexpress Gata1 in 50% of cells and are null for Gata1 in the other 50% ($X^{KO}X^{OX}$ females) (Fig. 1A, Fig. 3A). We showed that recombination was efficient, and Gata1-null male foetuses generated using this Cre/lox*P* system are extremely anaemic by 12.5 dpc and die in utero (D.W. and F.L., unpublished). Interestingly, $X^{KO}X$ females display no reduction in survival, whereas classical Gata1-null heterozygous females are clearly affected (Fujiwara *et al*., 1996). This is expected as the $X^{KO}X$ females lose the *Gata1* gene during differentiation (as the Cre transgene is activated), which presumably results in residual levels of Gata1 and a less severe phenotype. Such a situation is similar to the mice where Gata1 levels have been reduced by a promoter knockout, males die and heterozygous females survive normally (Takahashi *et al*., 1997).

The different genotypes obtained from the breeding were essential controls for comparison. The compound female $X^{KO}X^{OX}$ has lost 50% of the erythroid precursors (Gata1-null cells that apoptose), while the remaining population is overexpressing Gata1. These females are therefore pancellularly overexpressing Gata1 in the remaining erythroid compartment. If a non-erythroid cell type supplied REDS, the remaining Gata1 overexpressing population (50%) would still be rescued by REDS in the compound female, as happens in the $X^{FL}X^{OX}$ female. The phenotype of the compound female would be comparable with that of $X^{KO}X$ females, *i.e.* transient anaemia, born normal. Conversely, if erythroid cells supply REDS, the source of the signal is ablated in the compound females and hence, the Gata1-overexpressing erythroid cells will not be rescued. The resulting phenotype would be expected to be more severe than that found in $X^{KO}X$ females and this

is what is observed.

We have found that $X^{KO}X$ females undergo a transient anaemia, but survive to birth. By contrast, $X^{KO}X^{OX}$ females are anaemic and die by 14.0 dpc, none survive to birth. In addition, the number of erythrocytes in blood in the $X^{KO}X^{OX}$ females was consistently lower than that found in $X^{KO}X$ females. The shift from primitive to definitive erythropoiesis was impaired in both $X^{KO}X^{OX}$ and $X^{KO}X$ females, though $X^{KO}X^{OX}$ females appeared to be consistently affected while $X^{KO}X$ females displayed some variability. This variability may be due to variation in the X-inactivation balance in the $X^{KO}X$ females. Consistent with the inhibition of definitive erythroid differentiation, $X^{KO}X^{OX}$ females had significantly fewer viable erythroid cells in the foetal liver than did $X^{KO}X$ females.

These results demonstrate that the cell type supplying REDS is the normally differentiating erythroid cell. In the compound female, the early cells are present (Gata1-overexpressing cells) and the only erythroid population missing is the more mature cells. Thus, the signal must be provided by erythroid cells in a late stage of differentiation.

These experiments do not address the identity of REDS itself. At present, the best candidates are the death receptor family of signalling molecules. Differentiating erythroid cells express death receptors and mature erythroid cells express their ligands (Barcena *et al*., 1999; Dai *et al*., 1998; De Maria *et al*., 1999a; De Maria *et al*., 1999b; Josefsen *et al*., 1999; Maciejewski *et al*., 1995; Oda *et al*., 2001; Silvestris *et al*., 2002; Zamai *et al*., 2000). Death receptors activate caspases and caspase activation is thought to be required for terminal erythroid differentiation (Kolbus *et al*., 2002; Zermati *et al*., 2001). It has been demonstrated that death receptor activation can induce the caspase-dependent degradation of Gata1 (De Maria *et al*., 1999b). Furthermore, in zebrafish, expression of a dominant-negative form of a haematopoietic death receptor dysregulates erythroid cell production (Long *et al*., 2000). Thus, death receptor-mediated activation of Gata1 degradation may be a component of REDS. As we have demonstrated that REDS is a homotypic signalling mechanism that takes place between erythroid cells, identification of the signalling molecules involved is focussed on molecules expressed by differentiating erythroid cells.

We induced one of the known death receptor pathways and showed that these can act at the last stages of differentiation of erythroid cells. At present, we do not know which of the pathways is used by REDS. Although erythroid cells can differentiate *ex vivo* under the appropriate conditions, they are arranged differently in hanging drops when compared with erythroblastic islands. The *ex vivo* differentiation of wild-type cells is improved by the Jo2-mediated induction of FasR, as shown by an increase in the number of enucleated cells. Gata1 levels decrease during differentiation and these levels decrease even more in FasR-activated cells. We therefore conclude that terminal differentiation of erythroid cells is enhanced by Jo2 mimicking the action of REDS, that occurs in the erythroblastic island *in vivo*. However, when the levels of Gata1 are very high, the Jo2 treatment *ex*

vivo cannot provide a sufficient decrease in the levels of Gata1 to rescue overexpressing cells, while the REDS pathway can rescue the same cells in the erythroblastic island (in $X^{ox}X$ females). Hence, we conclude that the required decrease of Gata1 levels *in vivo* is achieved by a pathway similar to that of FasR.

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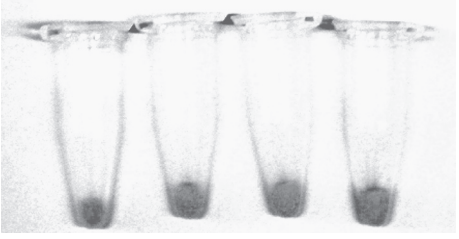
CHAPTER 4

IN VITRO STUDIES OF GATA1 OVEREXPRESSION TO ALLOW THE CHARACTERISATION OF REDS



In vitro studies of Gata1 overexpression to allow the characterisation of REDS

Laura Gutiérrez, Patrick Rodríguez, Frank Grosveld and Sjaak Philipsen.

ABSTRACT

The erythroblastic island is the entity where definitive erythropoiesis occurs. This hierarchical structure allows specific cell interactions amongst maturing erythroid cells. More mature erythroid cells communicate with less mature cells and favour their differentiation via a cell signalling process that was named Red Cell Differentiation Signal or REDS. In order to elucidate the nature of REDS, we biochemically blocked or induced candidate cellular pathways in primary erythroid cells cultured in hanging drops and studied the effects on differentiation. Furthermore, we performed initial studies on EEG1, a C1q related protein and a putative REDS receptor.

INTRODUCTION

Definitive erythropoiesis occurs in the erythroblastic island, an entity that allows a hierarchical organisation of the maturing erythroid cells (Bessis *et al*., 1983; Bernard 1991). We previously showed that maturing erythroid cells communicate with each other within the erythroblastic island in a signalling mechanism that was named Red Cell Differentiation Signal or REDS (Whyatt *et al*., 2000; Gutierrez *et al*., 2004). REDS favours differentiation of maturing erythroid cells and we have proposed that this regulatory process is important for the homeostasis of erythroid cell production. The signal was unmasked in Gata1 overexpressing mice (Whyatt *et al*., 2000), because their Gata1 overexpressing erythroid cells are defective in differentiation (Whyatt *et al*., 1997; Whyatt *et al*., 2000). They are incapable of arresting cell cycle prior to terminal differentiation. However, in the presence of mature wildtype erythroid cells, Gata1 overexpressing cells are rescued and differentiate normally (Whyatt *et al*., 2000). We hypothesise that the REDS receptor and ligand might be related to the death receptor family of proteins. It is known that death receptors are expressed throughout erythroid differentiation and their ligands are expressed at the final stages of maturation (De Maria *et al*., 1999; Zermati *et al*., 2001). Death receptors and caspases were first described to accomplish apoptotic events. However, caspase activation is also described for other cell regulatory events and hence the concept that death receptors are involved in many aspects of cell biology is broadly accepted (Carlile *et al*., 2004).

Several contradictory papers have been published regarding the function of caspases in terminal erythroid differentiation (De Maria *et al*., 1999; Carlile *et al*., 2004). The controversy is mainly generated by the fact that in each study, different stages/progenitors are utilised. It should be noted that terminal erythroid differentiation resembles apoptosis and that death receptors might play an essential role during terminal differentiation. Regulated protein degradation occurs in differentiating erythroid cells, which in the mature erythrocyte contain a protein pool that is enriched in globins. Activation of death receptors triggers the caspase cascade (Fadeel *et al*., 2000). Caspases are proteases that specifically digest proteins containing specific aminoacid sequences (Fadeel *et al*., 2000). Human Gata1 has caspase recognition sites (De Maria *et al*., 1999). It is possible that activation of caspases by death receptor coupling in erythroid cells generates the cleavage and degradation of a specific pool of proteins, including Gata1. Hence, Gata1 overexpressing cells are rescued due to targeted degradation of Gata1. There is a broad spectrum of death receptors present in erythroid cells, such as TNF or Fas (Rusten and Jacobsen 1995; De Maria *et al*., 1999). We previously performed experiments in which we showed that activation of Fas receptor with Jo2 antibody in *ex vivo* culture enhances differentiation of primary erythroid progenitors, as they present a higher percentage of enucleated cells (Gutierrez *et al*., 2004). We also showed that Gata1 levels were reduced

in treated samples. However, we never saw a complete rescue of Gata1 overexpressing cells in this system, suggesting that the Fas receptor is not the unique molecule involved in REDS *in vivo*. In the present chapter we performed additional experiments to elucidate whether caspases are part of the REDS pathway.

The caspase cascade can be activated also through a death receptor independent mechanism (Gupta 2003). Oxidative stress might lead to release of cytochrome C from mitochondria, which induces a caspase cascade. We took advantage of this and generated oxidative stress in primary erythroid cells and murine erythroleukaemia (MEL) cells by adding different concentrations of peroxide to the cultures. We observed apoptosis specifically in immature stages upon peroxide treatment and enhanced differentiation of surviving cells.

The effect of caspase inhibition in erythroid differentiation was also studied by utilising caspase inhibitors. As expected, cells were displaying poorer differentiation upon treatment and survival was not affected.

Inhibition of the proteasome caused massive cell death, indicating that protein degradation is essential for erythroid functionality during differentiation.

The studies mentioned above supported the idea that death receptor-like proteins might be crucial in REDS signalling. However, they were not informative about the transmembrane counterparts themselves.

Recently a new gene, EEG1, has been characterised which is expressed in erythroid cells and whose levels rise upon differentiation (Aerbajinai *et al*., 2004). It is cytoplasmic and transmembrane, and the extracellular motif has a C1q domain. The C1q domain can di- or trimerise upon ligand binding and it has a TNF-like structure (Eggleton *et al*., 2000). Furthermore, when exogenously expressed in Chinese hamster ovary (CHO) cells, it generated a slow proliferation rate and apoptosis. Taking all these characteristics together, EEG1 is a promising candidate for the REDS receptor. In the present chapter, some preliminary studies on this protein are described, as part of ongoing work.

MATERIALS AND METHODS

Mice

Mice overexpressing Gata1 were generated previously (Whyatt *et al*., 2000). Gata1 OX females were plugged and embryos collected at 12.5 or 13.5 dpc. The head was used to isolate DNA for genotyping and foetal liver cells were disaggregated and cultured in suspension as described previously.

Cell lines

MEL cells overexpressing Gata1 were generated previously (Whyatt *et al*., 1997). I11 cells are immortalised p53 null foetal erythroid cells (Dolznig *et al*., 2001).

Cell cultures

Cells isolated from foetal livers of wildtype or Gata1 overexpressing females were cultured in hanging drops as described (Appendix I). The following drugs and concentrations were used in respective experiments: peroxide H_2O_2 30% w/v stock in concentrations from 1μM to 100mM. Caspase inhibitors Z-VAD-fmk (BD Pharmingen) at 100 or 200μM and Z-DEVD-fmk (BD Pharmingen) at 100 or 150μM. Proteasome inhibitor MG132 at 5μM (Sigma) was provided by S. Bergink. Green fluorescent cell tracker (Molecular Probes) was used to track cells as described by the manufacturers.

Suspension cultures of I11 cells and primary cells were performed as described previously either in proliferation or in differentiation medium (Dolznig *et al*., 2001).

MEL cells were grown in DMEM+10%FCS+1%penicillin/streptomycin and induced to differentiate with 2% DMSO as described previously (Antoniou 1991).

Western blot analysis

Western blot analysis of Gata1 was performed as previously described (Gutierrez *et al*., 2004).

FACS analysis

Cells at day 0 or at each required time of culture were collected and stained prior to FACS analysis. 50000 cells were counted per sample. The following stains and antibodies were used: TER119-PE (BD Biosciences), 7AAD (Molecular probes), AnnexinV (BD Biosciences).

RNA isolation and Quantitative PCR (Q-PCR)

Gata1 overexpressing females were plugged and collected at 12.5 dpc. Foetal livers of the different genotypes were collected. Half of the foetal liver was used to isolate RNA at day 0. The other half was differentiated in suspension. The heads of the embryos were used for genotyping them. Cells in culture were collected at day 1, 2 and 3 and after pooling

cells from the same genotype, RNA was isolated by using Tri reagent (Sigma). cDNA was synthesised following the manufacturer's protocol (Invitrogen) and Real Time-PCR was performed to detect Gata1, cMyc, cMyb, Bcl-X and Bcl-2 transcripts, using HPRT primers as control. The primer pairs used were the following:

- Gata1: 5'-cggcctctatttcaagctcc-3'; 5'-ttcctcgtctggattccatc-3'
- EEG1: 5'-gcttctgaactcccacttgc-3'; 5'-gctcaaatgcttgcttggtga-3'
- cMyc: 5'-tctccactcaccagcacaa-3'; 5'-gctcgtctgcttgaatgga-3'
- cMyb: 5'-tacagagccggaactgcctc-3'; 5'-gcaaatgctctcaaggcagaa-3'
- HPRT 90bp: 5'-agcctaagatgagcgcaagt-3'; 5'-atggccacaggactagaaca-3'
- HPRT 200bp: 5'-cacaggactagaacacctgc-3'; 5'-gctggtgaaaaggacctctc-3'

The annealing temperature was 58ºC. We performed the reactions in BioRad equipment, using Cyber Green (Europrim).

Chromatin immunoprecipitation

Chromatin derived either from induced MEL cells or wildtype and Gata1 overexpressing primary erythroid cells in proliferating conditions was crosslinked as described (Rodriguez *et al*., 2005). Immunoprecipitations were performed with antibodies against Gata1 (N6), YY1 and IgG controls as described (Anguita *et al*., 2004; Rodriguez *et al*., 2005). The primers used for the EEG1 5'UTR and Exon I GATA sites are the following:

- EEG1 5'UTR: 5'-ggtgagggttgcagccacaa-3'; 5'-tccagggggatgcacttggt-3'
- EEG1 Exon I: 5'-accttcttcttctcgatgttc-3'; 5'-gcagtctactctcagttctg-3'

Other primer pairs used (Myc, Myb, MBP, GP91) have been previously described (Rodriguez *et al*., 2005).

RESULTS

REDS signalling occurs between erythroid cells in the erythroblastic island, favouring erythroid differentiation. We hypothesise that upon ligand binding the REDS receptor activates a cascade of caspases that induces the cleavage and degradation of specific proteins, including Gata1. We previously showed that activation of Fas receptor by Jo2 antibody enhances terminal differentiation in *ex vivo* cultures of primary erythroid cells (Gutierrez *et al*., 2004). However, we never detected a rescue of Gata1 overexpressing cells as is observed *in vivo*. In order to investigate if the candidate REDS intracellular pathways exert the hypothesised actions, we decided to induce or block caspase function directly.

Activation of the cytochrome C pathway

There are a number of death receptors present in the erythroid membrane, but it is plausible that the REDS receptor is not amongst those described. To confirm that REDS acts via a death receptor type pathway, an alternative and independent way to activate caspases was used in an attempt to stimulate differentiation. Death receptor triggering is not the only way to activate the caspase cascade. Oxidative stress can also initiate the release of cytochrome C from mitochondria, which leads to caspase activation (Gupta 2003). We took advantage of this physiological event and induced oxidative stress to cultured cells by adding peroxide at different concentrations.

We cultured wildtype primary erythroid cells in hanging drop cultures and treated these with a range of peroxide concentrations (100μM-100mM). A high percentage of cell death was observed with 10 and 100mM peroxide (Figure 1A). The surviving erythroid live cells displayed a consistently smaller volume in all the concentrations used compared to untreated cells (Figure 1A) indicating better differentiation. We performed Western blot analysis of Gata1 on hanging drop cultures treated with peroxide and observed a reduction of Gata1 levels when compared to standard cultures (Figure 1B). We analysed the effects of peroxide treatment on Gata1 OX and WT primary erythroid cells. In Figure 1C we show the fold change of the percentage of live erythroid cells and the mean volume of samples treated with 100mM peroxide normalised to respective untreated samples. This analysis was done in order to see the relative changes within each genotype. The treatment induces considerable cell death and the mean volume of surviving cells is reduced. We observed that both genotypes respond to the same extent.

We next studied apoptosis in the different peroxide treatments by staining the cells with AnnexinV. Although the size reduction reached its maximum at 100mM peroxide treatment, all the cells that we considered erythroid and live ($TER119^+$ $7AAD^-$) stained positive for Annexin V, indicating that they are early apoptotic cells (data not shown). The rest of the cells in these samples were late apoptotic cells, staining positive for AnnexinV

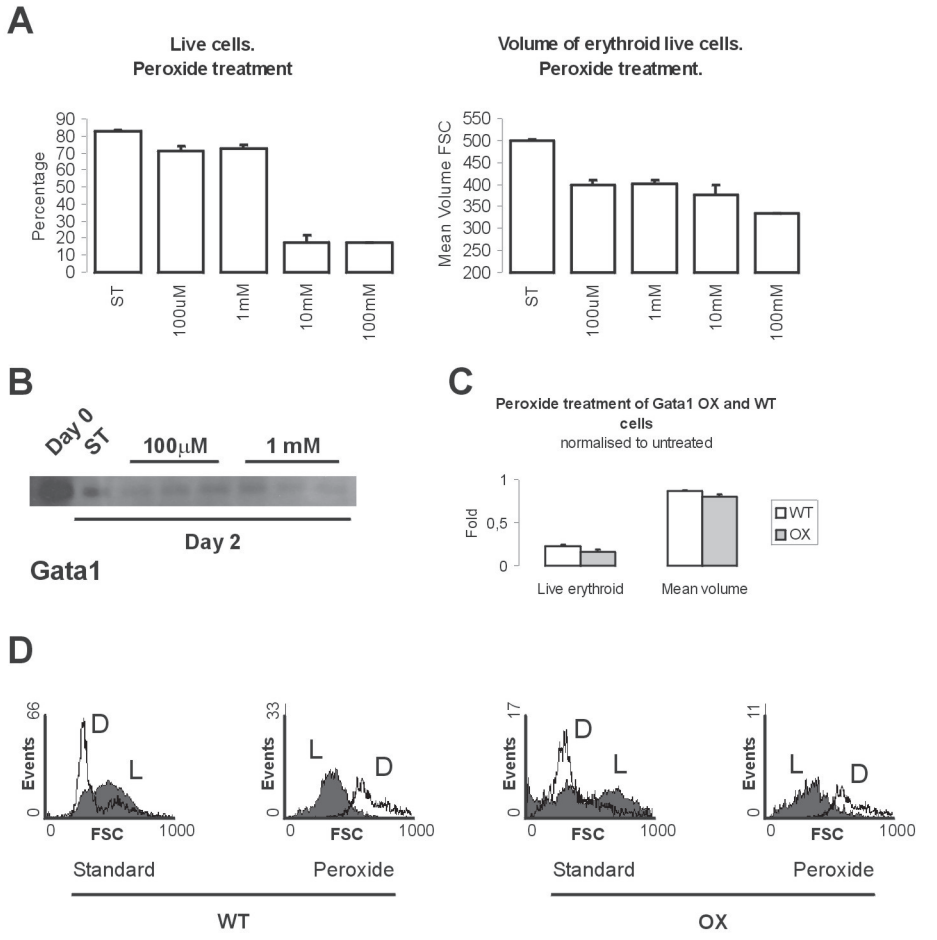


Figure 1. Activation of the cytochrome C pathway by oxidative stress.
(A) Wildtype primary erythroid progenitors were cultured in hanging drop in the presence of peroxide. The percentage of live cells is depicted in the graph on the left. The average mean volume of the live fraction as measured by FACS is depicted in the graph on the right.
(B) Western blot analysis of Gata1 of primary cells cultured in hanging drop mock-treated or treated with 100μM or 1mM peroxide. The protein corresponding to the same number of cells is loaded. Day 0 corresponds to extracts of the foetal liver cells at day of collection. ST corresponds to extracts of primary cells after hanging drop culture in standard conditions. Peroxide treatments were performed in triplo. Gata1 levels are reduced upon peroxide treatment compared to the standard culture.
(C) Peroxide treatment of Gata1 overexpressing (OX) and wildtype (WT) primary erythroid progenitors in hanging drop culture. The fold change normalised to untreated is depicted. The parameters showed are the percentage of live cells and the average mean volume. Both genotypes respond to the same extent to peroxide treatment.
(D) Histograms of Gata1 overexpressing (OX) and wildtype (WT) hanging drop cultures untreated or treated with peroxide. Filled histogram corresponds to the live fraction of cells (L) towards the forward scatter (FSC). Hollow histogram (overlay) corresponds to the dead fraction (D), also towards the forward scatter. Peroxide treatment induces cell death of immature (larger) cells.

and 7AAD. In the samples treated with a concentration of peroxide of 10mM or lower, we did not find AnnexinV positive cells. Caspase activation by oxidative stress might exert different actions depending on the level of oxidative stress induced. Doses of peroxide up to 10mM favoured differentiation, and doses up to 1mM did not induce cell death at all. 100mM peroxide induced apoptosis of erythroid cells.

Further analysis showed that the late apoptotic cells in the samples treated with 100mM peroxide were at the more immature stages during differentiation, *i.e.* larger cells. On the contrary, more mature cells survived longer and continued with the differentiation process before undergoing apoptosis (Figure 1D). These cells were early apoptotic cells at the day of collection, which suggests that indeed they survive longer than more immature cells. We plotted the erythroid live (early apoptotic) cells against the forward scatter and overlayed them with the erythroid dead (late apoptotic) cells in WT and OX cells treated or untreated. Concordant with what we observed before, the response is equal amongst genotypes. As it can be seen in untreated samples, the majority of dead cells after two days of culture corresponds to exhausted differentiated cells, and cells of all the sizes that eventually die within the culture. In treated samples, the dead cells are exclusively large, and surviving cells are the smaller ones. This shows that more immature cells are sensitive to peroxide and more mature cells are able to cope with oxidative stress, and they continue differentiation in a selective synchronised manner. This is the reason why the mean volume is reduced, as there is no presence of intermediate stages due to the synchronisation of later stages induced by peroxide.

Next we performed the experiment in murine eyrthroleukaemia (MEL) cells, in order to see whether peroxide could induce differentiation similar to DMSO. We used MEL cells transfected either with a Gata1 overexpression construct (Gata1 OX) or with the empty vector. MEL cells overexpressing Gata1 do not differentiate properly (Whyatt *et al*., 1997). After differentiation induction they proliferate at a higher rate than control cells and they do not complete haemoglobinisation. We treated these cells with a range of peroxide concentrations (1μM-1mM) in the presence or absence of DMSO and compared the differentiation induction. These lower doses were used because MEL cells were more sensitive than primary cells to the peroxide treatment. At the day of collection, we counted the cells, washed them and centrifuged them for comparison of these preliminary experiments used the redness of the pellets as a measure of haemoglobinisation, *i.e.* differentiation (real haemoglobin content still has to be measured in these experiments). Cell numbers were reduced 75% at 1mM peroxide in Gata1 OX and control MEL cells indistinctively. A picture example of the pellets is shown in Figure 2. Uninduced cells have whitish pellets and they become fully haemoglobinised after four days in the presence of DMSO. The redness of the pellet of induced Gata1 OX MEL cells is lighter than the control cells. Cells treated with peroxide reached almost the same intensity of red colour as DMSO treated cells, both in Gata1 OX and control MEL cells. Treatment of DMSO

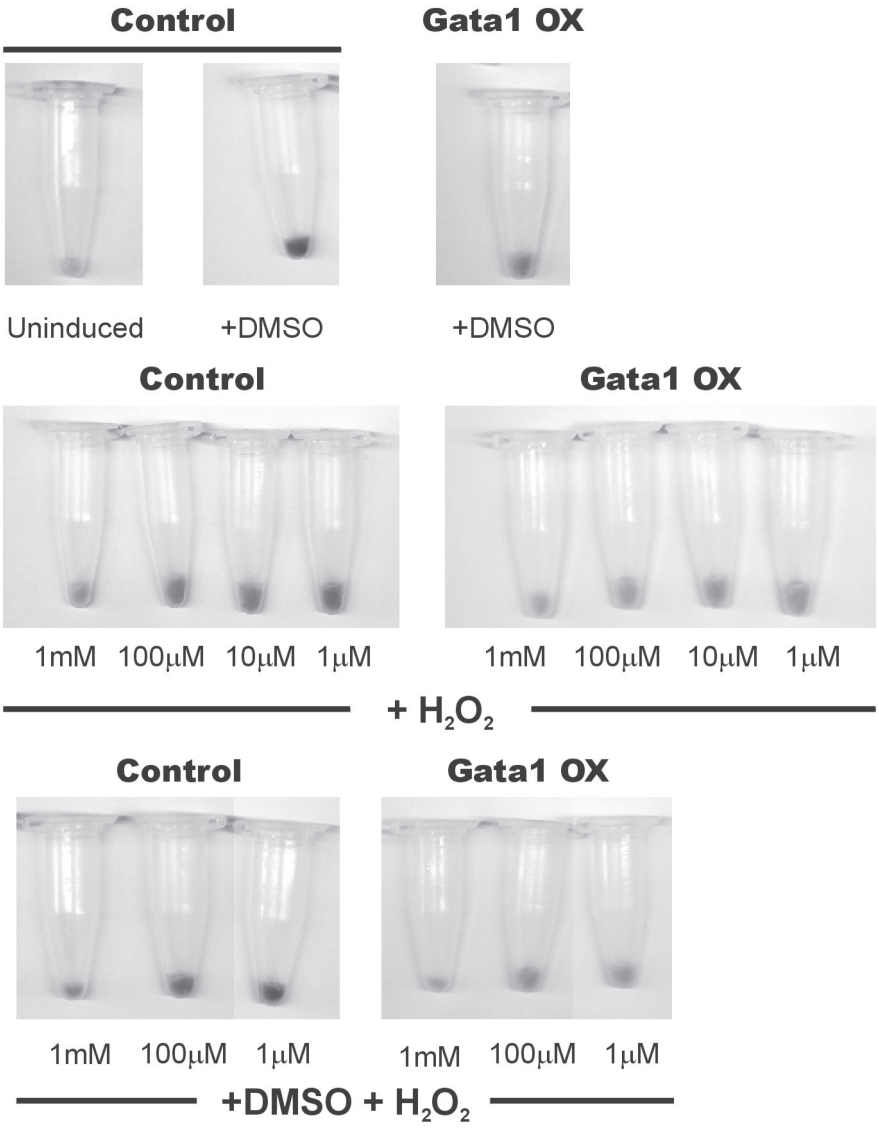


Figure 2. Peroxide treatment of MEL cells.
MEL cells overexpressing Gata1 (Gata1 OX) or transfected with an empty vector (control) were treated with DMSO, peroxide, or DMSO in combination with peroxide at different concentrations. All cells were collected at day 4 after treatment and pelleted. Peroxide alone is able to induce differentiation of MEL cells. Redness of the cell pellets is taken as a measure of haemoglobinisation and hence terminal differentiation. (See colour picture in the cover).

and peroxide did not improve the results obtained with peroxide alone or with DMSO alone, indicating that both agents do not cooperate in a synergistic manner to induce differentiation of MEL cells.

Inhibition of caspases

Next we aimed to block caspase activity with caspase inhibitors. We used two different inhibitors: Z-VAD-fmk and Z-DEVD-fmk. Z-VAD-fmk is an irreversible pancaspase inhibitor and was the initial selected caspase inhibitor to use. Paradoxically to its broad action spectrum, the effects obtained with this drug were not so obvious (Figure 3A). Z-DEVD-fmk is an irreversible caspase3 inhibitor, which also inhibits caspase6, 7 and 9. This specific caspase inhibitor showed much clearer effects (Figure 3A-C). We treated differentiating cells with 100μm and 200μM of Z-VAD-fmk. On the day of collection we measured cell differentiation by FACS analysis. Z-VAD-fmk treatment did not cause a significant change in the percentage of erythroid live cells when compared to untreated samples (Figure 3A). Changes in cell size are also not significant upon Z-VAD-fmk treatment (Figure 3A). In the graph depicting the mean volume of erythroid live cells we included 100μM Z-DEVD-fmk treatments for comparison of the effects of the different drugs, as Z-DEVD-fmk treatment results in significant changes in the parameters observed. We therefore decided to continue the experiments with 150μM Z-DEVD-fmk (Figure 3B). As a first outcome of caspase inhibition we can observe a higher percentage of live erythroid cells, probably due to protection to apoptotic general events. The mean volume of treated cells was considerably higher than the standard treatments, showing a delay or block in differentiation (Figure 3B).

We also treated Gata1 OX and WT primary erythroid cells (Figure 3C). We observed that caspase inhibition affected OX cells to a larger extent than WT cells. In all samples the mean volume of live erythroid cells of treated samples was larger than the volume of untreated ones, but the proportional increase of cell volume was higher in OX M cells than in WTs. The samples derived from Gata1 OX Fs consistently show an intermediate phenotype.

We also followed treated and untreated cells with a fluorescent cell tracker (Figure 3D). The intensity of the tracker after culture is an indicator of cell division and differentiation progression, although we cannot make an estimate of the number of cell divisions due to detection limitation and absorption of fluorescence by haemoglobin. We examined the tracker intensity of the erythroid live cells comparing the OX Ms to WTs. We observed that in OX Ms there is a much lower percentage of cells with low intensity of tracker, *i.e.* dividing/differentiating cells, compared to WTs. Concordant with this, the percentage of high intensity cells, *i.e.* cells that do not divide/differentiate, was much higher in OX M cells compared to WTs. The pattern of WT cells treated with Z-DEVD-fmk was similar to the untreated OX M cells, *i.e.* a high percentage of high intensity tracked cells corresponding to non-dividing/differentiating, and a low percentage of low

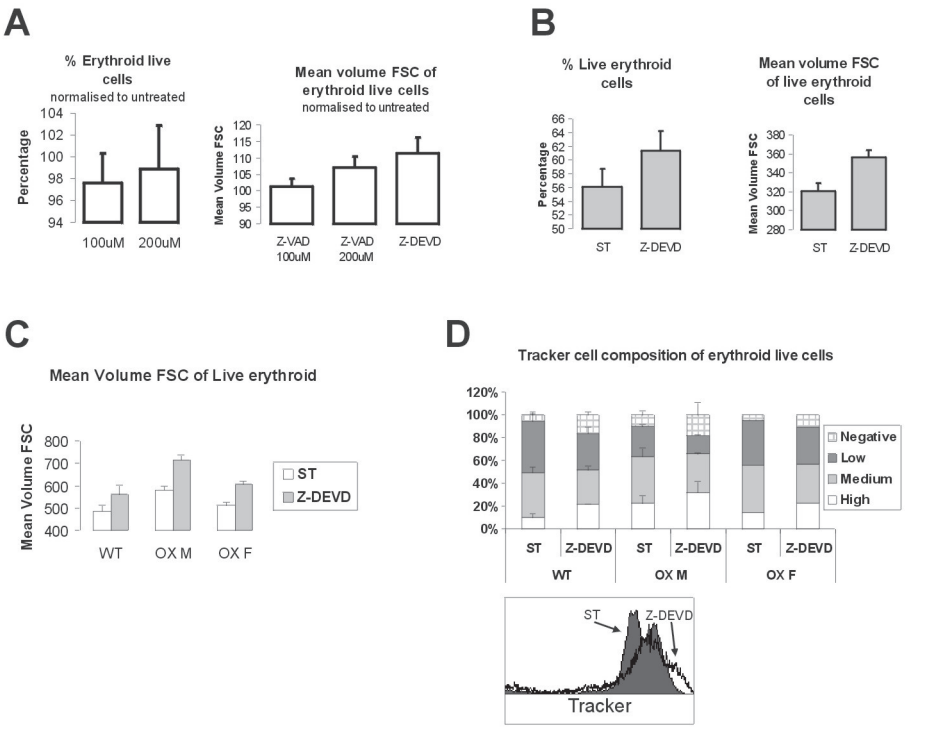


Figure 3. Caspase inhibitors treatment.
(A) Treatment of wildtype primary cells with Z-VAD-fmk. Percentage of live cells (left) and mean volume of live cells (right) is depicted. In the right panel, treatment with Z-DEVD-fmk is included to observe the more pronounced effect of the latter caspase inhibitor.
(B) Treatment of wildtype primary cells with Z-DEVD-fmk. Percentage of live cells (left) and mean volume of live cells (right) is depicted.
(C) Z-DEVD-fmk treatment of wildtype (WT), Gata1 overexpressing female (OX F) and Gata1 overexpressing male (OX M) primary cells. The mean volume of the live fraction is depicted.
(D) Z-DEVD-fmk treatment of Gata1 overexpressing (OX F, OX M) and wildtype (WT) primary cells that were labelled with a cell tracker. The cell composition relative to the cell tracker intensity is depicted in the graph for treated and untreated samples. A histogram of the distribution of the intensity of the cell tracker in untreated (ST) and treated (Z-DEVD) WT cells is included.

intensity tracked cells, corresponding to dividing/differentiating cells. Figure 3D shows a FACS histogram of WT cells untreated and treated (overlay) to see the distribution of cells based on tracker intensity. The tested cells still have a considerable percentage of high intensity cells, while untreated and differentiating normal cells distribute in lower intensities of the tracker. This suggests that caspase inhibition leads to a block during differentiation. The defective phenotype of OX cells was more accentuated upon Z-DEVD-fmk treatment, with an even higher percentage of high intensity tracked cells and lower percentage of low intensity tracked cells compared to untreated OX M cells. OX female cells always showed an intermediate phenotype, due to X-inactivation and heterozygosity of the Gata1

OX allele. As expected, treatment with caspase inhibitors affected differentiation and the cells were much larger than in untreated samples, as if blocked in an intermediate stage between proerythroblast and orthochromatic erythroblast.

Proteasome inhibitor treatments

Protein degradation is a major event occurring in differentiating erythroid cells. Caspases are very specific proteases whose products either become activated upon cleavage, or end up in a more general protein degradation machinery known as the proteasome. Therefore, we decided to inhibit the proteasome machinery and study its effects on cell survival and differentiation. We treated the cells at day 0 and at day 1 of hanging drop culture with 5μM of MG15 proteasome inhibitor and collected them at day 1 and 2. We observed that inhibition of the proteasome caused high levels of cell death in cells treated at day 0 (Figure 4A). The proteasome pathway is essential for cell survival, and due to the fact that erythroid differentiating cells must reduce their protein content, inhibition of this general pathway is causing the death of erythroid cells, as they are not able to continue the differentiation program correctly. The effect was not so prominent when cells were treated at day 1 (Figure 4A). This can be explained by the fact that differentiating cells have already reduced their protein content and inhibition of the proteasome machinery does not affect the cells to the same extent. However, when the percentage of live erythroid cells is plotted day by day, we observe that the reduction of viable cells is the same after one day of treatment, whether treatment was at day 0 or at day 1 (Figure 4B).

We next treated Gata1 OX and WT primary erythroid cells at day 0 and at day 1 of hanging drop culture. We observed the same effect as described before and no differences were found in the response amongst all genotypes, after normalisation to untreated samples (Figure 4C). Consistently with what we described before, WT cells seemed to be relatively less affected when treated at day 1 than when treated at day 0.

Preliminary studies on a candidate molecule involved in REDS

Two isoforms of EEG1 are expressed in human erythroid cells (Aerbajinai *et al*., 2004). EEG1L contains a C1q domain, and is located in the cytoplasm and cell membrane. EEG1S does not contain the C1q domain and is located in mitochondrial aggregates. The C1q domain folds in a TNF domain-like structure and triggers cytoplasmic pathways upon binding with its ligand. Miller and colleagues expressed EEG1 in Chinese hamster ovary cells and observed cell cycle arrest and apoptosis. EEG1 is upregulated at the last stages of differentiation in human erythroid cells. For these reasons, EEG1 is a very good candidate for REDS receptor.

By homology we identified the murine EEG1L isoform, which has a 75% identity with the human sequence, and designed primers to measure mRNA levels in different erythroid cell lines by quantitative PCR (Q-PCR). EEG1 levels rise upon differentiation

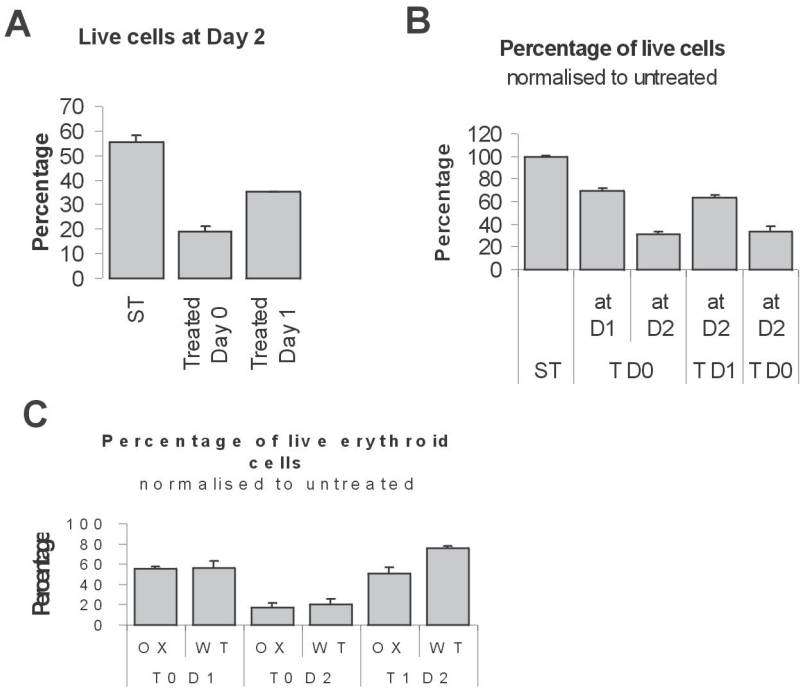


Figure 4. Proteasome inhibitor treatment in hanging drop cultures.
(A) Wildtype primary cells. The percentage of live cells at day 2 after treatment with proteasome inhibitor at day 0 or day 1 is depicted. ST, standard.
(B) Wildtype primary cells. The percentage of live cells collected at day 1 (D1) or 2 (D2), and treated at day 0 (T D0) or 1 (T D1) is depicted.
(C) Treatment of wildtype (WT) and Gata1 overexpressing (OX) primary cells. The percentage of live cells collected at day 1 (D1) or day 2 (D2), and treated at day 0 (T0) or day 1 (T1) is depicted.

induction of I11 cells (Figure 5A). I11 cell differentiation is synchronous and includes enucleation of the cells. We confirmed that the changes in level of murine EEG1 are similar to those described in human erythroid cells. We analysed EEG1 expression levels in MEL cells transfected with a Gata1 OX construct or with the empty vector, before and after differentiation induction with DMSO. EEG1 levels were higher in induced WT cells compared to uninduced cells. In OX Gata1 MEL cells, the levels were raised 4-fold more than in WT cells upon induction (Figure 5B). This suggests that EEG1 expression is driven by Gata1.

We therefore analysed the promoter sequence of the murine EEG1 gene and found two GATA sites: one in the 5' untranslated region, and another one in the first exon. We designed primers to analyse the enrichment of these GATA sites in Gata1 immunoprecipitated crosslinked chromatin from induced MEL cells by Q-PCR. We observed specific enrichment of the two GATA sites of the EEG1 gene in chromatin

immunoprecipitated with anti-Gata1 antibody as compared to YY1 immunoprecipitations of the same MEL cell crosslinked material (Figure 5C) and compared to GATA sites in other known gene promoter regions.

We then studied the expression levels of EEG1 in primary erythroid cells derived from Gata1 OX and WT embryos. We isolated RNA from foetal liver cells of embryos of 12.5 dpc. We analysed expression levels of Gata1, EEG1, cMyc and cMyb. The levels of these genes in the freshly isolated foetal livers are shown in Figure 6A. Gata1 levels are higher in the OX M, while cMyb levels are lower. There is not much difference in the levels of cMyc, and EEG1 seems to be slightly upregulated in the OX cells compared to WTs. Foetal liver cells of these embryos were also cultured in suspension as described to analysed gene expression levels in cells differentiating in a synchronous manner. We collected cells at day 1, as day 2 cultures already contain a lot of enucleated and dying cells in the WT cultures. cMyc and Gata1 levels are significantly upregulated in the OX cells compared to WTs (Figure 6B). While cMyb is slightly downregulated in OX cells, EEG1 has higher expression levels in the OX cells compared to WTs. In order to analyse the changes within each genotype and after differentiation induction, we plotted the data of both days in the same graph (Figure 6C). This shows that EEG1 levels are upregulated in the WT cells after differentiation induction, and that this upregulation is more accentuated in the OX cells.

We expanded primary Gata1 OX and WT cells in suspension, isolated and crosslinked the chromatin and performed Gata1 chromatin immunoprecipitation in order to see whether EEG1 GATA sites are enriched differentially amongst the two genotypes. Q-PCR on the immunoprecipitated material was done using primers designed to amplify the GATA sequences in the EEG1 and cMyc promoters. Interestingly, we detected a decrease of the enrichment of the GATA site in the cMyc promoter on OX cells compared to WT cells. The OX F cells showed an intermediate level of enrichment (Figure 6D). The GATA sites on the EEG1 promoter are enriched significantly in the OX compared to WT cells. This is in concordance with what is seen in MEL cells. Curiously, in OX females, the enrichment is similar to that found in WT cells, and the enrichment levels are not intermediate, as found with GATA cMyc site.

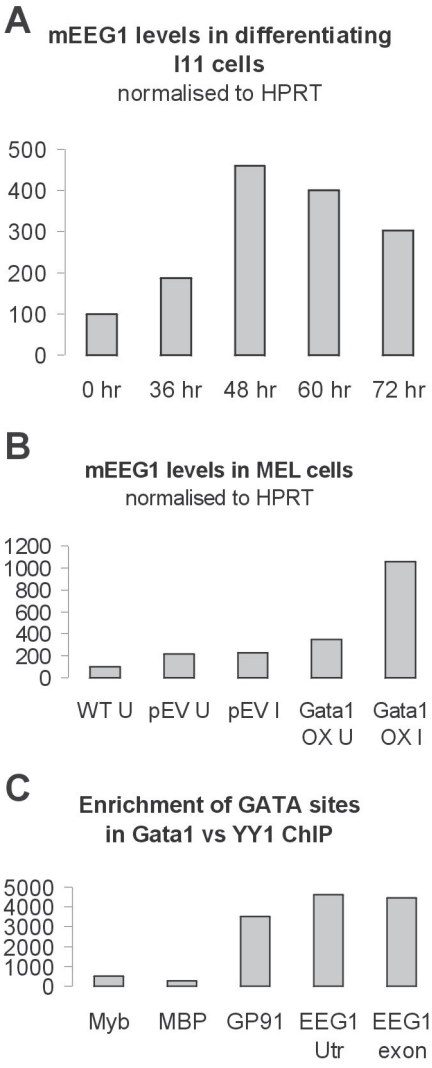


Figure 5. Preliminary studies on EEG1 in cell lines.
(A) Expression levels of EEG1 during differentiation of I11 cells, as measured by real-time PCR, and normalised to HPRT.
(B) Expression levels of EEG1 during differentiation of MEL cells. WT U, wildtype uninduced; pEV U, empty vector control uninduced; pEV I, empty vector control induced; Myc U, Gata1-Myc expressing cells uninduced; Myc I, Gata1-Myc expressing cells induced.
(C) EEG1 GATA sites enrichment as measured by real-time PCR in N6 (anti-Gata1) immunoprecipitated chromatin derived from uninduced MEL cells normalised to the enrichment measured in YY1 chromatin immunoprecipitation of the same material.

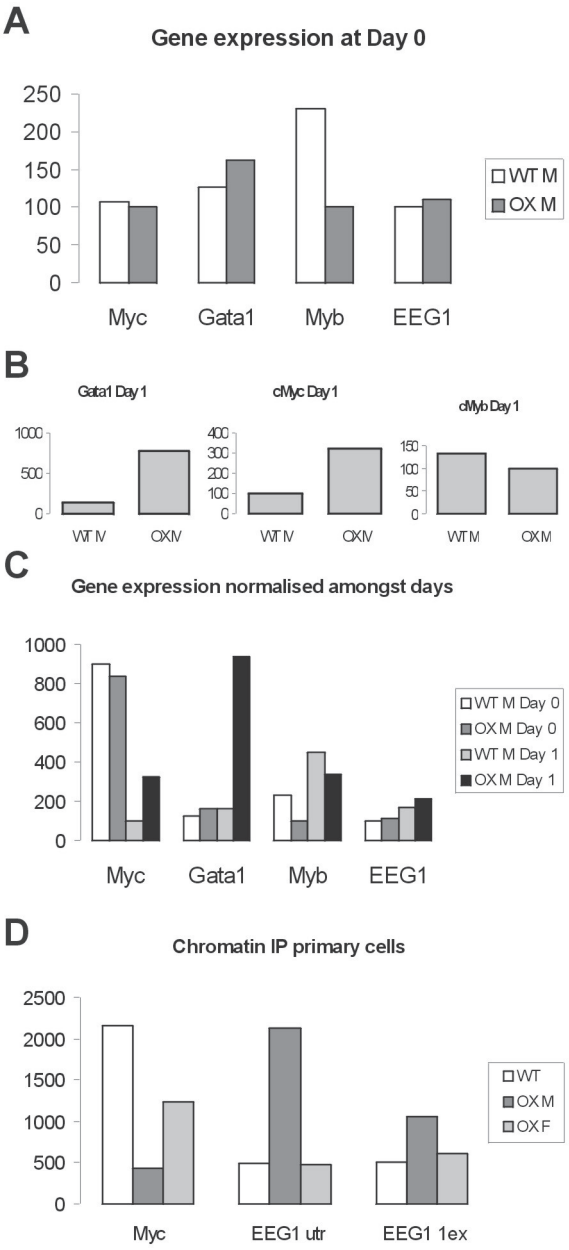


Figure 6. Preliminary studies on EEG1 in primary cells.
(A) Expression levels at 12 dpc in foetal livers of wildtype (WT) and Gata1 overexpressing (OX) mice. Levels of cMyc, cMyb, Gata1 and EEG1 are depicted.
(B) Expression levels of cMyc, cMyb, Gata1 and EEG1 in WT and OX primary cells after 1 day in differentiation conditions.
(C) Compilation of the data presented in sections A and B. Expression levels at day of collection and after 1 day in differentiation conditions of cMyc, cMyb, Gata1 and EEG1, in WT and Gata1 overexpressing primary cells.
(D) Gata1 overexpressing (OX) and wildtype (WT) cells were expanded in suspension culture, and chromatin was immunoprecipitated with N6 (anti-Gata1). Enrichment of the two GATA sites in the EEG1 promoter (EEG1 utr, EEG1 1Ex) as measured by real-time PCR is depicted. Also the enrichment of the GATA site in the cMyc promoter is presented, as a control.

DISCUSSION

The REDS signalling mechanism mediates interaction between maturing erythroid cells favouring differentiation (Whyatt *et al*., 2000; Gutierrez *et al*., 2004) and we are interested in unravelling which proteins are involved in this signalling. The study of individual candidate receptors is difficult due to protein expression complexity in the differentiating erythroid cell. Approaches towards the identification of the REDS molecule are indicated in Chapter 6 of this thesis. However, we performed the experiments described in this chapter to verify that the hypothesised pathways act in erythroid differentiation as we originally hypothesized: REDS is a death receptor-like mediated signalling mechanism and that caspases are involved in the cleavage of specific proteins whose levels need to be downregulated for proper terminal differentiation. This is the case for Gata1. We aimed to intercept or induce the caspase pathway by using inhibitors or by inducing oxidative stress respectively. We could observe a differentiation block upon caspase inhibition, which was clearer in treatments with the caspase3 inhibitor Z-DEVD-fmk. The pancaspase inhibitor Z-VAD-fmk did not cause significant inhibition of differentiation. However they have been reported previously to exert paradoxical actions (Liu *et al*., 2003). Addition of Z-VAD-fmk did not protect against apoptosis induction in treated cells and induced increased cell death compared to mock-treated samples. Caspases belong to a complex network that is regulated in a tight manner to allow distinct actions amongst the different caspases that might even have opposite effects. This makes the study of this network at the cellular level difficult, and it may explain why the pancaspase inhibitor did not show much of an effect, but in presence of the caspase3 inhibitor, a significant defect in differentiation occurred. Interestingly, studies by Carlile and colleagues revealed a non-apoptotic function of Caspase3 in erythropoiesis by RNAi technology (Carlile *et al*., 2004).

Assuming that Gata1 overexpressing cells are rescued by REDS *in vivo*, it should be possible to show the same rescue *ex vivo*, by activating the appropriate candidate pathways. Although we previously showed that Gata1 levels are downregulated in cultures where the Fas receptor is activated in comparison to standard cultures, and that the percentage of enucleated cells was increased, we did not observed a rescue of the differentiation profile of OX cells *ex vivo* (Gutierrez *et al*., 2004). The levels of Gata1 were still higher than in wildtype cells and many cells remained in the proerythroblast-like stage. That approach was rather limiting as only one death receptor was activated, whereas the signal that rescues OX cells *in vivo* might be composed of different interacting molecules, or it is simply not mediated by Fas receptor. We therefore decided to activate the caspase cascade by inducing oxidative stress in primary erythroid cells and also in MEL cells overexpressing Gata1 to test whether caspase activation by an alternative route would give rescue. The concentration of peroxide utilised determined the response of the treated cells: low doses induce better differentiation of cultured cells in general, while higher doses

induce apoptosis. We detected lower levels of Gata1 in Western blots in the lower dose treatments compared to the standard treatments. However, Gata1 OX cells show improved differentiation, but not a rescue of the normal differentiation profile as measured by FACS. Higher peroxide treatments showed a selective apoptosis induction in the more immature cells. Cells in intermediate stages survive longer and continue the differentiation program synchronously thereby improving the average mean volume of the cells as measured by the forward scatter parameter of the FACS sorter. Caspase activation by oxidative stress favoured differentiation of primary cells and also of MEL cells as observed by the redness of the pellet. However, higher doses generated apoptosis, particularly in the more immature erythroid precursors.

We also tested the effect of inhibiting the proteasome machinery. Protein degradation is a requisite to differentiate and inhibition of the proteasome machinery caused general cell death in both OX and WT cells. Although dependent on the litter used, it seemed that treatment after one day of differentiation induction is less deleterious than the treatment at day 0. Moreover cells differentiate in a non-synchronous manner in hanging drop cultures. It would be worthy to repeat these experiments in primary cells cultures in suspension, where a more precise treatment could be applied and measured. However it appears that the proteasome machinery is more important in the early than in the latter stages of differentiation. This may explain why inhibition of the proteasome after one day of hanging drop culture has less effect as inhibition of the proteasome at the beginning of the culture.

Taken all these results together we conclude that the caspase pathway fits the hypothesized actions of REDS. However, these experiments do not give a clue of which transmembrane proteins are involved.

The recent characterisation of EEG1 as a growth-related factor expressed in erythroid cells made it a good candidate (Aerbajinai *et al*., 2004). It contains a C1q domain that folds in a TNF-like domain. It is not clear what the ligand of this C1q domain could be, although for other C1q related proteins, interactions with shedded C1q domains, integrins and several surface markers have been described (Eggleton *et al*., 2000). There are two isoforms of EEG1: L, located in the cytoplasm and cell membrane, and S, which lacks the C1q domain and accumulates in mitochondria aggregates. It is possible that EEG1S is involved in cytochrome C release mechanisms, either promoting it or inhibiting it, like Bcl-2 or Bcl-x. Its exogenous overexpression in CHO cells generates apoptosis and cell cycle arrest. Cell cycle arrest is required for terminal differentiation. Not surprisingly, EEG1 is upregulated at the last stages of differentiation in human cells, and we confirmed this in cell lines such as I11 and MEL cells, as well as in primary erythroid cells. We observed upregulation in Gata1 OX cells and showed that the promoter of EEG1contains two GATA sites that were enriched in Chromatin IP experiments of MEL cells and in proliferating primary erythroid OX cells when compared to wildtype cells. It suggests that Gata1, which

is upregulated upon erythroid commitment, starts the transcription of EEG1, to increase its level towards terminal differentiation as part of the REDS pathway. Whether EEG1 exerts a negative feedback loop over Gata1 (protein levels) is an issue that still needs to be addressed.

We also observed that in Gata1 OX proliferating cells, Gata1 is not located at the Myc promoter. Interestingly, Gata1 represses Myc transcription. Gata1 OX cells have higher levels of cMyc after one day of differentiation induction, and their phenotype is proliferative. They are unable to undergo cell cycle arrest and therefore, proteins such as Myc might be uncontrolled in these cells. Gata1 overexpression might lead to unwanted protein-protein interactions, which will generate aberrant transcription of some products, or inhibition of the functions of other proteins. In overexpressing Gata1 cells, EEG1 appears to also be overexpressed, but in this case (contrary to what was shown in CHO cells) cells do not arrest cell cycle. There are obviously many unanswered questions about EEG1 in these preliminary experiments that may give a clue about its function, *e.g.* is the overexpressed EEG1 located in the cytoplasm, is it transmembrane, is it shedded? These can only be answered after the development of the appropriate reagents such as antibodies against EEG1.

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CHAPTER 5

GATA1 IS EXPRESSED IN DENDRITIC CELLS AND IS REQUIRED FOR THEIR SURVIVAL

Gata1 is expressed in dendritic cells and is required for their survival

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ABSTRACT

Gata1 is a transcription factor expressed in haematopoietic progenitors and in a number of committed lineages of the haematopoietic compartment, amongst those mast cells, megakaryocytes, eosinophils, and erythroid cells but also in Sertoli cells of the testis. Gata1 is essential for distinctive features required during cell lineage differentiation, *i.e.* cell proliferation and cell survival. In the present work we show that Gata1 is also expressed in cell types of the antigen-presenting cell (APC) family. We detected Gata1 expression in myeloid dendritic cells (MDC) and plasmacytoid dendritic cells (PDC). Using a Gata1 floxed gene and a tamoxifen inducible Cre recombinase, we show that both MDC and PDC require Gata1 for survival at different stages through dendritic cell maturation in *ex vivo* bone marrow cultures. The novel observation that Gata1 is expressed and required in both types of APCs questions the current models of lineage bifurcation and origin/plasticity of dendritic cells.

INTRODUCTION

The haematopoietic compartment is composed of multipotent cells that are able to differentiate into very distinct functional cell types (Rifkind *et al*., 1969; Moore and Metcalf 1970; Medvinsky and Dzierzak 1998). The gene expression pool must be uptight regulated and yet it must be plastic to respond to the different stimuli that indicate the requirements of the organism for a certain cell type. At the same time there must be mechanisms to avoid a surplus of production. This is why, in addition to the renewal of progenitors, two other cellular events are always detected in the haematopoietic compartment: cell lineage commitment and apoptosis. Precursor cells and cells that enter the differentiation program are by default at the risk of undergoing apoptosis and, therefore, must activate anti-apoptotic mechanisms. This suggests that when cells are lineage-committed, they are continuously in a balance between undergoing apoptosis or differentiation.

Tissue specific transcription factors play an essential role in the maintenance and cell lineage commitment of the haematopoietic progenitors. Gata1 is a transcription factor that is expressed at basal levels in haematopoietic multipotent progenitor cells (Sposi *et al*., 1992; Leonard *et al*., 1993) and at different levels in committed lineages as eosinophils (Zon *et al*., 1993), megakaryocytes (Martin *et al*., 1990; Romeo *et al*., 1990), mast cells (Martin *et al*., 1990) and erythroid cells (Yamamoto *et al*., 1990). Gata1 is also expressed in Sertoli cells of the testis (Ito *et al*., 1993; Yomogida *et al*., 1994). In all the haematopoietic Gata1 expressing lineages studied, Gata1 plays an essential role. Erythroid cells that lack Gata1 apoptose at the proerythroblast stage and Gata1 *null* mice die at midgestation (Pevny *et al*., 1995; Fujiwara *et al*., 1996). Gata1 is essential for eosinophil development and a targeted deletion of the palindromic GATA site in the Gata1 promoter leads to selective loss of the eosinophil lineage (Hirasawa *et al*., 2002; Yu *et al*., 2002). Mutations in the amino-terminal zinc finger of Gata1 lead to anaemia and macrothrombocytopenia (Nichols *et al*., 2000; Freson *et al*., 2001). Disruption of a hypersensitive site in the Gata1 promoter leads to selective loss of Gata1 in the megakaryocytic lineage, which generates deregulated megakaryocyte proliferation and production of low numbers of platelets (Shivdasani *et al*., 1997). Gata1 has been linked functionally to the regulation of the cell cycle, differentiation and survival of cells that express it (Blobel and Orkin 1996; Dubart *et al*., 1996; Yoshino *et al*., 1996; Cullen and Patient 1997; Whyatt *et al*., 1997; Yamada *et al*., 1998; Tanaka *et al*., 2000; Yu *et al*., 2002; Choe *et al*., 2003; Rylski *et al*., 2003; Romero-Benitez *et al*., 2004; Vilaboa *et al*., 2004; Ezoe *et al*., 2005). In particular, Gata1 has been described to activate the transcription of two anti-apoptotic proteins, Bcl-xL and Bcl-2, which belong to the Bcl family of proteins (Weiss and Orkin 1995; Gregory *et al*., 1999). Bcl proteins are involved in counteracting apoptosis in different cell types, including cells of the immune system.

Dendritic cells (DC) are leukocytes that belong to the antigen presenting cell

(APC) system (Abbas and Lichtman 2003). A proper immune response depends directly on the function and survival of DC, which require a life span of sufficient length in order to pick up antigens, process them and at the same time migrate from the peripheral tissues to lymph nodes, where they present antigens long enough to efficiently mount the acquired immune response (Steinman 1991). It has been described that Bcl-xL expression is required for APC to survive and perform their function (Pirtskhalaishvili *et al*., 2000; Lundqvist *et al*., 2002; Hon *et al*., 2004). Bcl-2 appears to be required as a timer that controls the life span and immunogenicity of APC (Nopora and Brocker 2002; Hou and Van Parijs 2004). Due to the fact that Gata1 transactivates the expression of Bcl-2 and Bcl-xL genes at least in the erythroid lineage, we asked whether these proteins are also regulated by Gata1 in dendritic cells, and if so, whether Gata1 would be a pivotal factor involved in the regulation of DC life-span.

We indeed detected Gata1 expression in both myeloid dendritic cells (MDC) and plasmacytoid dendritic cells (PDC). We further show that Gata1 transcription factor is expressed at constant levels through differentiation of bone marrow cells in Flt3-ligand cultures. By means of a floxed Gata1 transgene and a tamoxifen inducible Cre recombinase we also show that DC require Gata1 for survival during *ex vivo* cultures of bone marrow cells either in the presence of GM-CSF or Flt-3 ligand. Lastly we show that Gata1 is also expressed in human DC.

MATERIALS AND METHODS

Mice

Mice carrying a modified Gata1 allele flanked with loxP sites (Gata1-lox mice) (Lindeboom *et al*., 2003; Gutierrez *et al*., 2004) were crossed with mice expressing a tamoxifen inducible Cre recombinase under the ROSA 26 promoter (ER-Cre mice) (Vooijs *et al*., 2001). The progeny obtained is summarised as follows: ER-Cre males (Cre M), ER-Cre|Gata1-lox + males (KO M), ER-Cre|Gata1-lox +/- (Hz KO F), ER-Cre|Gata1-lox +/+ females (KO F) and Gata1-lox mice (WT) that were considered as wildtype siblings. Note that to simplify the nomenclature we used the term "KO" to refer to the floxed allele, which is subject to recombination upon tamoxifen treatment.

Cell cultures

Eosinophil cultures were done with unsorted bone marrow cells in the presence of IL-5 as described previously (van Rijt *et al*., 2002), supplementing the standard medium with 30%FCS and 24ng/ml IL-5 (Pharmingen).

Macrophage cultures were started with $2x10^5$ cells/ml unsorted bone marrow cells. Standard culture medium consisted of RPMI-1640 + glutamax-I, 10% foetal calf serum (FCS), 50 μg/ml Gentamycine (Gibco) and $5x10^{-5}$ M β-mercaptoethanol (Sigma). For macrophage cultures standard medium was supplemented with 50μg/ml M-CSF (R&D).

Myeloid dendritic cells (MDC) were cultured using the protocol described by Lutz *et al* (Lutz *et al*., 1999). GM-CSF (final concentration 20ng/ml) was a gift of Prof. K. Thielemans, Vrije Universiteit Brussels, Belgium. GM-CSF-generated mDC were finally matured at day 10 by adding 500 ng/ml lipopolysaccharide (LPS) (Sigma) to the cultures.

Flt3-ligand stimulated cultures were performed using modified protocol previously described by Gilliet *et al* (Gilliet *et al*., 2002). Prior to cultivation, lineage negative bone marrow progenitors were selected using antibodies against MHC-II (purified from hybridoma supernatant, M4/115), CD8 (Ly2), CD4 (L3T4), CD45 (B220), GR.1 (Ly-6C/G) (all from BD Biosciences) as previously described by Inaba *et al* (Inaba *et al*., 1992). Progenitor-enriched sorted cells were cultured in a density of $2x10^5$ cells/ml in standard medium and in the presence of 200ng/ml recombinant human Flt3-ligand. At day 11, cultured cells were sorted on the basis of CD11b expression to continue the experiment with separated $CD11b^+B220^-$ and $CD11b^-B220^+$ DC. The sorting was performed in LS columns (MidiMACS) as described by the manufacturer using rat-anti mouse CD11b antibody followed by anti-rat MACS beads.

Cells derived from spleen were sorted before culture using MACS columns to enrich the macrophage and DC populations with the following antibodies (of rat origin): CD19 (BD Biosciences), CD3 (home-made hybridoma supernatant, KT3 (ATCC)), TER119

(home made hybridoma supernatant) and followed by anti-rat MACS beads, as explained above. Sorted cells were further kept in standard medium supplemented with 2ng/ml GM-CSF and 20ng/ml Flt-3 ligand.

Tamoxifen was added at concentrations of 100nm or 500nm at day 0, day 5-6, day 10-12 of culture, depending on the cell culture type.

The efficiency of the cultures was calculated with the following formula:

$$\text{Efficiency} = \frac{\text{cell number day of collection}}{\text{cell number at start of culture}} \times 100$$

The normalisation of parameters of treated to untreated samples was calculated with the following formula:

$$\text{Normalised value} = \frac{\text{Value treated sample}}{\text{Value untreated sample}} \times 100$$

Human DC cultures

Human peripheral blood monocytes (PBMC) were isolated by Ficoll and Percoll gradient and cultured with GM-CSF and IL-4 for 14 days as described (Hammad *et al*., 2000) in order to generate mature DC.

MTT-bioassay

Cell proliferation and viability was tested with the MTT-bioassay in which the activity of mitochondrial dehydrogenases is measured by a colorimetric enzymatic reaction. This assay was performed in Flt3-ligand cultures after sorting the $CD11b^{-}B220^{+}$ from the $CD11b^{+}B220^{-}$ as explained above. 10^4 cells were cultured in 100μl/well in 96-well plates and incubated one day before addition of MTT (Sigma). MTT solution was prepared in phosphate buffer saline (PBS) at a concentration of 5mg/ml and filtrated before use. 1/10 volumes of MTT solution were added to each well. Cells were incubated 4hr. The supernatant was collected and dark blue crystals were solved by adding 100μl 0.04 N HCl in isopropanol pipetting vigorously. The cell death is proportional to the colorimetric measurement at 570nm. A 630nm reading was used to compensate for background.

Recombination PCR

DNA of cultured cells was isolated and recombination PCR was performed by using the following primers. Forward IV-S 5'-cgccgagctctgtctagtaa-3', reverse ExonIB 5'-ttcctctttctcctcctccg-3' (wildtype allele product) and reverse GFP 5'- ggtgctcaggtagtggtt-3'

(recombined allele product). The annealing temperature used was 60°C.

Gata1 expression by PCR

RNA from cultured cells was isolated using Trizma reagent (Sigma) as described by the manufacturer's protocol. cDNA was synthesised with 1μg starting RNA and as described by the manufacturer (Invitrogen). The Gata1 primers used were the following: forward 5'-ggcaagacggcactctacc-3' and reverse 5'-caagaacgtgttgctcttc-3'. We amplified HPRT as control with the following primers forward 5'-gctggtgaaaaggacctctc-3' and reverse 5'-cacaggactagaacacctgc-3'. The annealing temperature used was 58°C.

Western Blot

The same number of cells from Flt3-ligand cultures at day 5, 9 and 15 were lysated with 2XLaemmli buffer and analysed by Western blotting. Filters were incubated with anti-Gata1 antibody (N6, Santa Cruz Biotechnology) or B23 nucleophosmin (kind gift from Pui K. Chan, Baylor College of Medicine, Houston, TX). Respective secondary antibodies conjugated to horseradish peroxidase were purchased from Dako (DakoCytomation, Denmark). Enhanced Chemoluminescence (ECL) was performed as indicated by the manufacturer (Amersham Pharmacia) to develop the filters.

FACS Analysis

Cells were analysed by FACS sorting and the following stains and antibodies were used: 7-actinomycin-C (7AAD, BD Biosciences), propidium iodide (PI, Molecular probes), Annexin V, and directly labelled antibodies CD11c-APC, CD11b-PE, B220-FITC, MHCII-PE, CCR3-PE, F4/80-FITC (all BD Biosciences).

Gata1 intracellular staining was performed using the Perm/Wash Kit (BD Pharmingen) as indicated by the manufacturer, and utilising Gata1 (N6, Santa Cruz Biotechnologies) and anti-rat-FITC as secondary antibody.

Immunofluorescent staining

Cells from IL-5, M-CSF, GM-CSF and Flt-3 ligand cultures were collected and allowed to spread on polylysine slides (Sigma). Cells were fixed and stored in 70% ethanol at –20°C. Prior to immunostaining, cells were permeabilised with sodium citrate, blocked 2 hours at 4°C in 1% bovine serum albumin (BSA) in PBS and incubated overnight at 4°C with anti Gata1 (N6) (1:50 dilution in blocking solution). The next day, slides were washed with 0.05%Tween-20 in PBS and incubated for 1 hour at 4°C with anti-rat-Alexa^{596} (1:200). After washing, cells were incubated with FITC labelled antibodies for 1 hour at 4°C: M-CSF cultured cells with F4/80 (1:200), GM-CSF cultured cells with MHC-II (1:200) and Flt-3 ligand cultured cells with B220 (1:200). Eosinophils, as polymorphonucleated cells, are easily recognised by observing their nucleus, therefore a fluorescent marker for

their identification was not used. After washing the cells, slides were mounted with DAPI/ Vectashield and observed under DMRBE fluorescence microscope using the appropriate filters. Pictures were coloured and overlayed using Adobe Photoshop 5.5.

Immunofluorescent staining of human DC was performed as described for Gata1 detection. Mouse anti-human CD11b antibody followed by donkey anti-mouse-FITC was used to mark DC in the culture.

Immunostaining of cryosections of mouse NALT region

6μm cryosections were obtained from frozen lung/NALT (nose associated lymphoid tissue) tissue. Immunostaining was performed as described above. Gata1 N6 antibody was used followed by goat anti-rat-AP (Sigma) as secondary antibody. Detection was performed using New Fuchsine as a substrate.

Cytospins and cell preparations

Cells after IL-5 culture in the presence or absence of tamoxifen were collected and cytocentrifuged and stained as described previously (Beug *et al*., 1982). Pictures were taken with an Olympus BX40 microscope. The lenses used were Plan 40X/0.65 and Olympus Plan 100X/1.25. The acquisition software used was Viewfinder Lite Version 1.0.125 and Studio Lite Version 1.0.124, Pixera Corporation. Image processing was done in Adobe Photoshop 5.5.

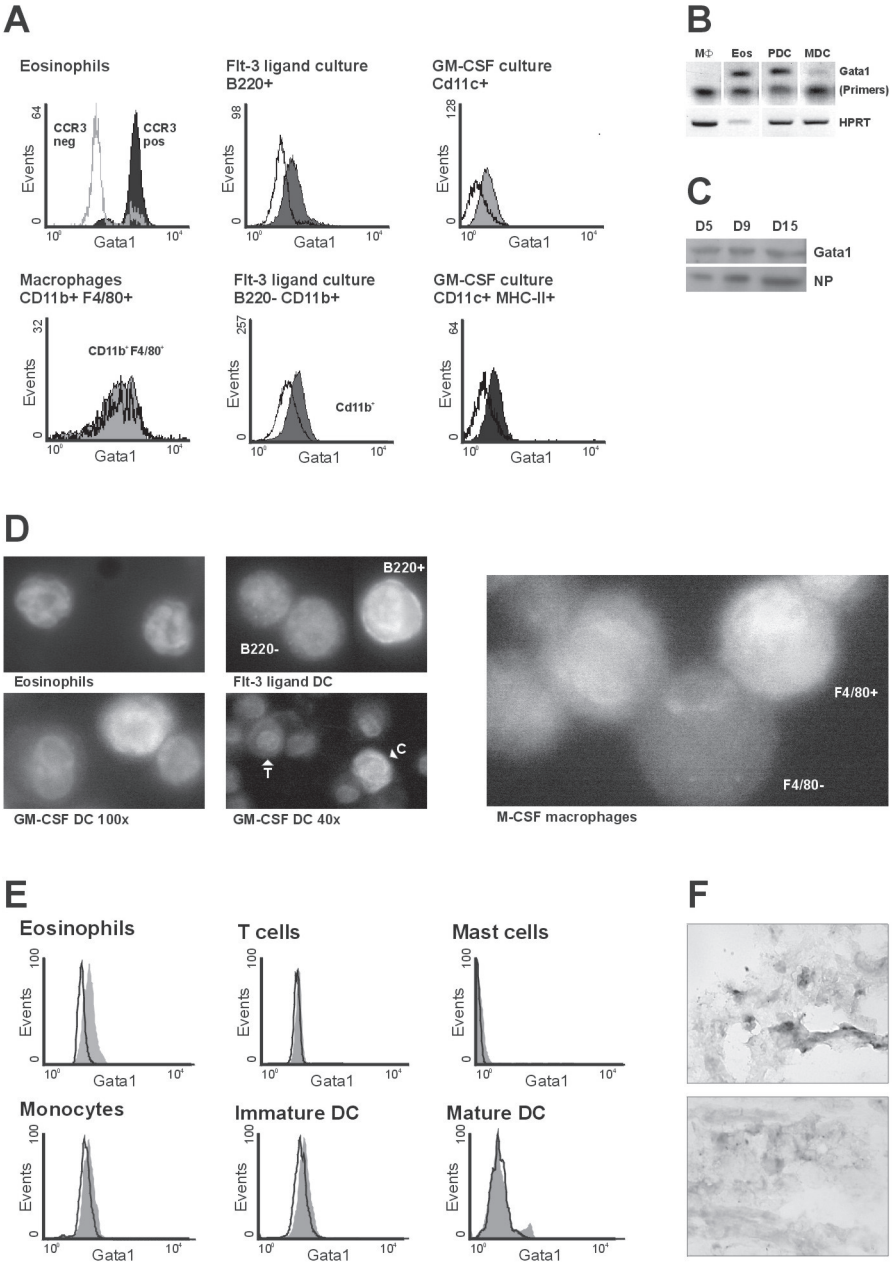
RESULTS

1.- Gata1 expression

Gata1 is expressed in ex vivo cultured DC

We cultured bone marrow cells in the presence of GM-CSF (MDC), Flt-3 ligand ($B220^-$ and $B220^+$ DC), IL-5 (eosinophils) as positive control and M-CSF (macrophages) as negative control and analysed Gata1 expression by FACS analysis. An example histogram of the Gata1 expression pattern is shown for every cell type in Figure 1A. We defined the cell types in each culture by co-staining identifying cell surface markers. The eosinophils, our positive control for Gata1 expression, were gated as $CCR3^+$ and they are clearly Gata1 positive compared to a $CCR3^-$ population. Macrophages are our negative control for Gata1 expression. We gated the $CD11b^+F4/80^+$ cells as mature macrophages. We performed two sets of staining procedures in all the samples: Gata1 staining (N6 and anti-rat), and background staining (anti-rat only), in combination with the surface marker stains. We analysed the Gata1 expression with respect to the background staining in the same gated population. Macrophages are negative for Gata1. We then analysed Gata1 expression levels in DC derived from Flt-3 ligand cultures, gated them based on the exclusive expression of CD11b or B220 and compared with their respective background staining. This reveals that Gata1 is expressed in both cell types (Figure 1A). Finally we performed the same analysis in DC derived from GM-CSF cultures, gating single $CD11c^+$ cells (immature MDC) and $CD11c^+MHCII^+$ cells (mature MDC). We confirmed that DC obtained from GM-CSF cultures also express Gata1 after comparing the gated populations with their respective background staining.

Figure 1. Gata1 expression in dendritic cells in vivo and in ex vivo culture (next page).
(A) FACS analysis histograms of Gata1 staining in *ex vivo* cultures of bone marrow cells. The cells stained were eosinophils (IL-5 culture), macrophages (M-CSF cultures), DCs from GM-CSF culture and MDCs and PDCs from Flt-3 ligand culture. Filled histogram is Gata1 staining, hollow histogram is background staining, with the exception of eosinophils, in which hollow histogram is Gata1 staining of non-eosinophil cells.
(B) PCR analysis of synthesised cDNA from M-CSF culture (macrophages, Mf), IL-5 culture (eosinophil, Eos), GM-CSF cultures (MDC) and Flt-3 ligand cultures (PDC). Pictures of the gels with Gata1 and HPRT amplification products are shown.
(C) Western blot analysis of Gata1 in DCs during Flt-3 ligand culture. Nucleophosmin (NP) was the loading control. D stands for day.
(D) Immunofluorescent staining of cells after IL-5 (eosinophils), GM-CSF, Flt-3 ligand and M-CSF (macrophages) culture is shown. Gata1 is the red $Alexa^{589}$ punctuate nuclear and cytoplasmic staining. Respective cell markers, depending on the cell culture, are seen in green. Nuclei are stained with DAPI. $B220^+$ and $B220^-$ cells are indicated in the picture from Flt-3 ligand cultured cells, and they are both Gata1 positive. The picture at 40X magnification from GM-CSF cultured cells shows immature (cytoplasmic MHC-II -C-) and mature (transmembrane MHC-II -T-) DC, and they are both Gata1 positive. In M-CSF cultures, Gata1 positive cells are $F4/80^-$ (they are either progenitor or other lineage-committed cells). Macrophages are $F4/80^+$ and in these cells Gata1 is not detected.
(E) FACS analysis histograms of Gata1 staining in different cells of the haematopoietic lineage obtained from peritoneal wash of wildtype mice. Filled histogram is Gata1 staining, and hollow histogram is background staining.
(F) Immunostaining of cryosections of the palatal region of wildtype mice. The region showed corresponds to the nose associated lymphoid tissue (NALT). Pink staining indicates Gata1 positive cells. These cells are dendritic or macrophage-like cells as deduced form their morphology.
(Selected colour pictures are depicted in the cover).


A
Eosinophils
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Flt-3 ligand culture
B220+
GM-CSF culture
Cd11c+
Macrophages
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CD11b+ F4/80-
Flt-3 ligand culture
B220- CD11b+
Cd11b+
GM-CSF culture
CD11c+ MHC-II+
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Gata1
(Primers)
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D5
D9
D15
Gata1
NP
D
Eosinophils
B220+
B220-
Flt-3 ligand DC
GM-CSF DC 100x
GM-CSF DC 40x
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C
F4/80+
F4/80-
M-CSF macrophages
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Eosinophils
T cells
Mast cells
Monocytes
Immature DC
Mature DC
F

We analysed Gata1 expression by PCR on synthesised cDNA material from the four cultures and we used HPRT as control (Figure 1B). Gata1 can be detected in eosinophils and DC from GM-CSF and Flt-3 ligand cultures, but not in macrophages.

GM-CSF DC are myeloid in origin, but Flt-3 ligand induces DC differentiation in both lymphoid and myeloid Flt-3 expressing cells. Flt-3 ligand cultures give rise to two types of DC, which exclusively express CD11b or B220, a lymphoid marker. The expression of Gata1 in these cells, always described as a myeloid transcription factor, seems to be paradoxical. We therefore studied the Flt-3 ligand cultures more carefully and collected cells at day 5, 9 and 15 of culture. Whole cell extracts were obtained and the protein corresponding to the same number of cells was loaded on a 10% SDS-PAGE and immunoblotted against Gata1 and nucleophosmin as loading control. Relative to the levels of nucleophosmin, Gata1 levels were maintained through the culture (Figure 1C). We therefore conclude that Gata1 is expressed at constant levels through dendritic cell maturation in Flt-3 ligand cultures. It should be noted however that DC are never challenged *ex vivo* as occurs *in vivo*.

We also performed immunostaining of matured cells in IL-5, M-CSF, GM-CSF and Flt-3 ligand cultures in order to confirm Gata1 expression and in particular, to analyse Gata1 expression in each DC type derived from Flt-3 ligand cultures and determine its location in the cell (Figure 1D). Eosinophils, as positive control, express Gata1. In the case of M-CSF cultures, Gata1 is detected in progenitors and cells expressing F4/80, *i.e.* mature macrophages, seem to have almost undetectable levels of Gata1, although the background in these cells was very high. GM-CSF derived DC are Gata1 positive and Gata1 is present regardless of the maturation stage of the DC based on MHC-II location (MHC-II is cytoplasmic in immature DC and becomes transmembrane upon maturation). We detected Gata1 expression in both $B220^-$ and $B220^+$ cells derived from Flt3-L stimulated DC cultures. In all samples, Gata1 staining shows a punctuate pattern, as described previously for erythroid precursors (Elefanty *et al*., 1996) and is located in the nucleus and in the cytoplasm.

Gata1 is expressed in DC in wildtype mice

Gata1 expression was detected in *ex vivo* derived DC, and we wished to confirm Gata1 expression in DC *in vivo*. We performed a Gata1 staining in cells derived from peritoneal wash from wildtype mice in order to analyse Gata1 expression in different haematopoietic cell lineages by FACS analysis. A histogram of Gata1 levels in the different gated cell types compared to their respective background stainings is shown in Figure 1E. We found that it was more difficult to distinguish the Gata1 staining from its background in the *in vivo* situation, due to the complexity of the staining procedure. However, eosinophils are clearly Gata1 positive, and T-cells are Gata1 negative. Mast cells, which have been described to express Gata1, showed a slight positive shift compared to the background staining.

Monocytes, which are the precursors of potential DC and macrophages, are positive for Gata1 staining. We couldn't make a distinction between PDC and MDC in the staining, but we could confirm Gata1 expression in immature DC. Interestingly, when analysing mature DC, we observed that only a small fraction still expressed Gata1. In conclusion, DC are Gata1 positive compared to macrophages (negative control) and their expression levels are lower than the level found in eosinophils (positive control) *in vivo*. The results show that Gata1 levels appear to be downregulated upon DC maturation *in vivo*.

In order to complete the study of Gata1 expression *in vivo* we performed immunostaining of cryosections of a known dendritic-cell enriched area: the nose associated lymphoid tissue (NALT), which is located in the nasal epithelium of the palate. In Figure 1F two pictures of Gata1 immunostaining of the NALT are shown. The dendritic cell aggregates can be identified by their branched morphology, and these cells are clearly expressing Gata1.

2.- *Ex vivo* recombination of Gata1: design of the experiment

We next aimed to know whether Gata1 plays an essential role in the differentiation or survival of dendritic cells. To this end, we crossed mice bearing a Gata1-lox allele, which is X-linked, with mice expressing a tamoxifen inducible Cre recombinase under the ROSA26 promoter. The progeny obtained was classified as follows: Gata1-lox animals which were considered as wildtypes (WT), Gata1-lox|ER-Cre males (KO M), which are pancellularly Gata1 null after tamoxifen treatment, Gata1-lox +/-|ER-Cre females (Hz KO F), which are heterocellularly Gata1 null after tamoxifen treatment, and Gata1-lox +/+|ER-Cre females (KO F), which are pancellularly Gata1 null after tamoxifen treatment.

First, we optimised the concentration of tamoxifen to be used in our experiments by setting Flt-3 ligand cultures with bone marrow cells in the presence of 100nM or 500nM 4-OH-Tamoxifen. Treated WT and Cre expressing samples were compared to untreated cultures and after FACS analysis of CD11c, CD11b and B220 expression, we concluded that none of the concentrations of tamoxifen used affected dendritic cell differentiation (data not shown). We next treated with 100nM or 500nM 4-OH-Tamoxifen Flt-3 ligand cultured bone marrow cells derived from each genotype and isolated DNA at day of collection to analyse the recombination frequency. We observed that 500nM 4-OH-Tamoxifen induced the most efficient recombination (80 to 90%, data not shown) and used that concentration for further experiments.

Parallel bone marrow cultures in four different conditions were studied: Flt-3 ligand cultures that generate $B220^-$ and $B220^+$ DC (test culture), GM-CSF cultures that generate MDC (test culture), IL-5 cultures to induce eosinophil differentiation (Gata1 dependent control culture) and M-CSF cultures to induce macrophage differentiation (Gata1 independent control culture). We collected the bone marrow cells from each

genotype and cultured them in the presence or absence of 500nM 4-OH-Tamoxifen and in the four different conditions described above. We collected the cells at day 7-10 and isolated DNA to analyse the recombination efficiency by PCR. Figure 2 shows an example of the recombination PCR products. WT samples showed a band corresponding to the wildtype and unrecombined allele, and no recombination product could be detected. The recombination product has a similar intensity to the unrecombined product in Hz KO Fs as expected for a very efficient recombination event. In the KO M samples, the unrecombined product was very faint and the recombined product was approximately 90%. This was observed in all the cell cultures tested. We therefore conclude that the recombination induced by 500nM 4-OH-Tamoxifen is sufficiently efficient to study the effect of a GATA1

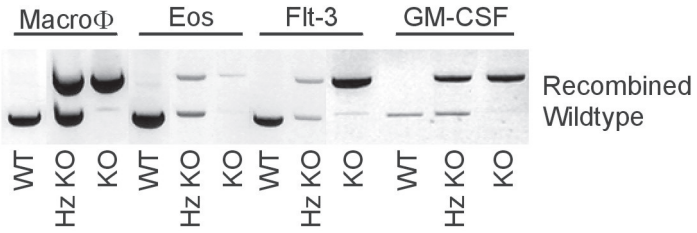

Figure 2. Recombination PCR of tamoxifen treated bone marrow cells in culture.
DNA from cultured bone marrow cells of wildtype (WT), heterozygous Gata1 inducible knockout (Hz KO) and homozygous Gata1 inducible knockout (KO) mice was extracted after tamoxifen treatment. The recombination efficiency was analysed by PCR. Cells from the different cultures were analysed, including macrophages (Macroφ), eosinophils (Eos), and DCs from GM-CSF and Flt-3 ligand culture. Recombination (recombined) and unrecombined (wildtype) products are indicated.

deletion in the different cell types.

FACS analysis was performed in order to evaluate the maturation of dendritic cells through the culture either with Flt3-ligand or with GM-CSF (Table 1) in order to set crucial time points of tamoxifen addition. Flt-3 ligand cultures already show full maturation at day 9 of culture. Subsequent changes in maturation affect only the ratio $B220^-/B220^+$ DC, which at day 9 is 1-2 fold and at day 15 it increases to 2-3 fold (Table 1). In GM-CSF cultures there is 30% of cell death by day 7. In further stages of the culture, the remaining cells survive better and the percentage of dead cells is reduced to 20%. The percentage of DC expressing CD11c increases from day 7 to day 12, but declines at day 13. After this day the culture starts to decline. The percentage of MHC-II expressing CD11c positive cells also increases until day 12. Compared to the Flt-3 ligand cultures, the maturation process is longer in GM-CSF cultures. Note that we term MDC the DC derived from GM-CSF cultures but the DC derived from Flt-3 ligand cultures are termed $B220^-$ and $B220^+$ DC, as the myeloid or lymphoid origin of the cells matured in this culture system is not certain.

Table 1: FACS analysis of Flt-3 ligand and GM-CSF cultures.

Flt-3 ligand culture	Day 5	Day 9	Day 15
% Live $CD11c^+$	19.10±0.52	76.81±1.58	74.98±2.49
% $B220^+$ (of live $CD11c^+$)	27.20±1.50	31.83±1.22	17.00±6.16
% $B220^-$ (of live $CD11c^+$)	37.48±0.55	40.28±0.67	52.20±8.18
GM-CSF culture	**Day 7**	**Day 12**	**Day 13**
% Live	77.17±0.47	92.64±4.80	69.05±6.02
% Live $CD11c^+$ of total	39.65±1.04	57.10±3.55	41.42±4.85
% Live $CD11c^+$ $MHCII^{high}$	4.99±1.67	6.72±0.98	5.12±0.56

We chose the following time points of treatment and collection based on maturity of the cultured cells:

- Eosinophils: treatment day 0, day 5-6. Collection day 5, day 7-8.
- Macrophages: treatment day 0, day 5-6. Collection day 5, day 7-8.
- Flt-3 ligand cultures: treatment day 0, day 5-6, day 9-10. Collection day 5, day 10-13.
- GM-CSF cultures: treatment day 0, day 5-6, day 9-10, day 12-13 (in the case of lipopolysaccharide -LPS- stimulated cultures). Collection day 7, day 12-13.

3.- Analysis of Gata1 loss in eosinophil and macrophage cultures

Eosinophils

We considered the eosinophil culture as a positive control of our experiment due to the known Gata1 requirement of this cell type. We cultured bone marrow unsorted cells from WT, Hz KO females and KO males in the presence of IL-5 and mock-treated or treated them with 500nM 4-OH-tamoxifen either at day 0 or at day 6 of culture and collected cells at day 7. As shown in Figure 3A, cell numbers did not change significantly between genotypes and treatments. We measured the live fraction of the collected cells by FACS analysis and we observed that the percentage of living cells in the Hz KO F and KO M cultures treated at day 0 was reduced compared to mock treated samples (Figure 3B). At time of collection we did not detect changes in the live cell fraction of cultures treated at day 6 compared to mock treated cultures (Figure 3B).

We next determined by FACS analysis the differentiation profile of the cultures and we observed that (most evident in the treatments performed at day 0) the eosinophil lineage ($CCR3^+$) was lost in Hz KO F and KO M cultures (Figure 3C). Sample FACS graphs from untreated and day 0 treated KO M cells are shown in Figure 3C. Note the loss of the $CCR3^+$ population in the treated sample.

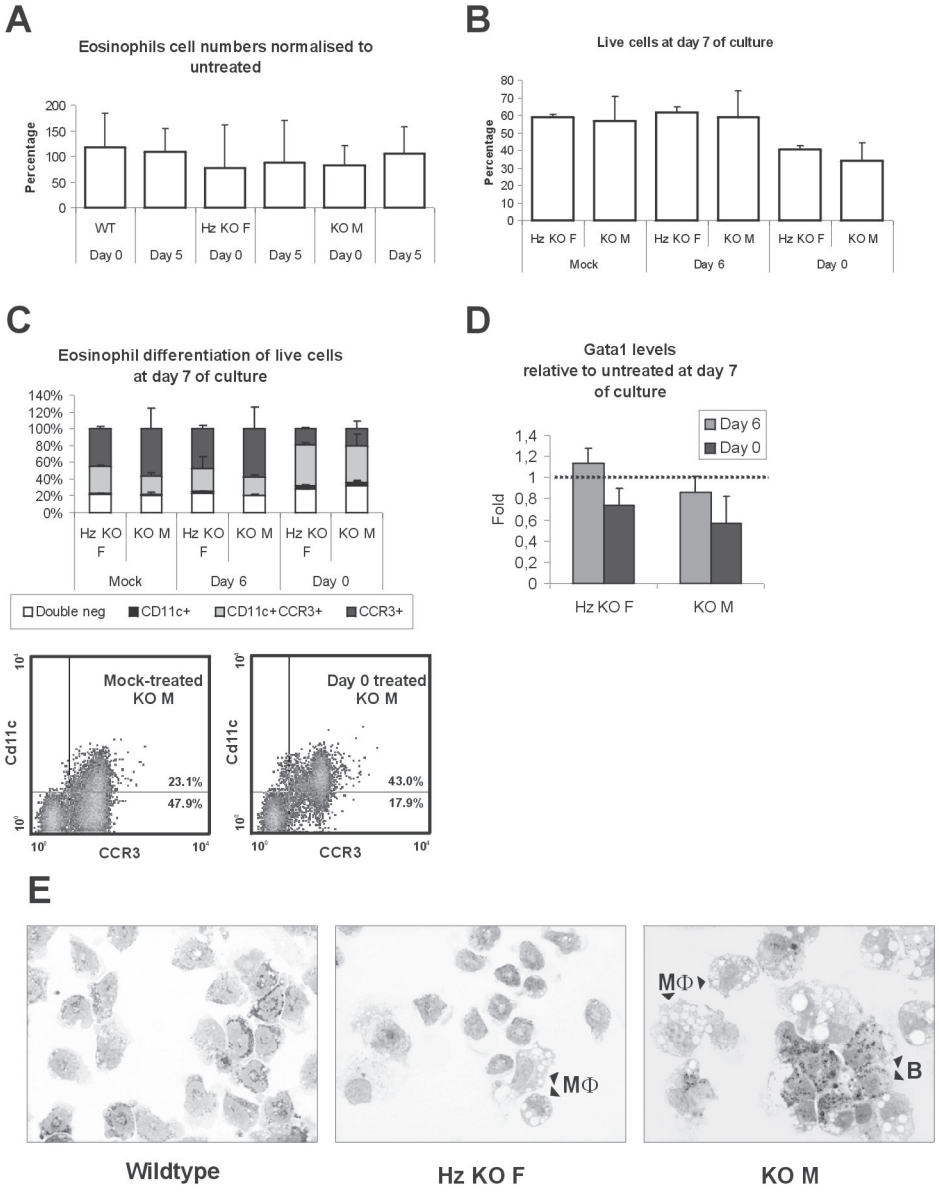
A
Eosinophils cell numbers normalised to untreated
Percentage
200
150
100
50
0
WT
Hz KO F
KO M
Day 0
Day 5
Day 0
Day 5
Day 0
Day 5
B
Live cells at day 7 of culture
Percentage
80
70
60
50
40
30
20
10
0
Hz KO F
KO M
Hz KO F
KO M
Hz KO F
KO M
Mock
Day 6
Day 0
C
Eosinophil differentiation of live cells at day 7 of culture
140%
120%
100%
80%
60%
40%
20%
0%
Hz KO F
KO M
Hz KO F
KO M
Hz KO F
KO M
Mock
Day 6
Day 0
Double neg
CD11c+
CD11c+CCR3+
CCR3+
Mock-treated KO M
23.1%
47.9%
Cd11c
CCR3
Day 0 treated KO M
43.0%
17.9%
Cd11c
CCR3
D
Gata1 levels relative to untreated at day 7 of culture
Day 6
Day 0
Fold
1,4
1,2
1
0,8
0,6
0,4
0,2
0
Hz KO F
KO M
E
MΦ
MΦ
B
Wildtype
Hz KO F
KO M

Figure 3. Induction of recombination of Gata1 in the eosinophil lineage in ex vivo cultures (previous page).
(A) Cell numbers at day 7 of cells treated with tamoxifen at day 0 or day 5 of culture and normalised to untreated samples (treated/untreated*100). The average and standard deviations are indicated. At least three samples are analysed together.
(B) The percentage of live cells as measured by FACS ($7AAD^-$ cells) at day 7 of cultures mock-treated or treated with tamoxifen at day 0 or at day 6. The average and standard deviations are indicated. At least three samples are analysed together.
(C) Eosinophil differentiation profile of live cells stained with CD11c and CCR3 as measured by FACS at day 7. Cells were treated with tamoxifen either at day 0 or at day 6, or mock treated. The average and standard deviations are indicated. At least three samples are analysed together. Two example CD11c/CCR3 dot blots corresponding to mock treated and day 0 treated KO M gated live cells are also shown.
(D) Gata1 levels as measured by FACS of cells treated with tamoxifen at day 0 or at day 6, normalised to untreated (treated/untreated*100). The average and standard deviations are indicated. At least three samples are analysed together.
(E) Cytospins of eosinophil cultures from wildtype, Hz KO F and KO M at day 7 after tamoxifen treatment at day 0. (Colour pictures depicted in the cover).

We analysed the reduction of Gata1 levels by FACS staining, and normalised the relative FACS mean values of each sample to its respective untreated sample. The relative values are plotted in Figure 3D. The levels of Gata1 are decreased in the Hz KO F and KO M cultures treated at day 0. However, in the samples treated at day 6 a slight decrease of Gata1 levels is observed in the KO M and the change is not significant in the case of the Hz KO F cultures. Samples treated at day 6 did not show a complete recombination of Gata1 when collected at day 7, and this explains the results obtained. Only samples treated at day 0, which display overall reduced levels of Gata1, show significant differences at the live fraction and differentiation profile levels.

Although the eosinophil lineage was lost in samples treated at day 0, the cell numbers did not change significantly. This suggests that other cell types are developing in the absence of the eosinophil lineage, and that these cell types are Gata1 independent. We prepared cytospins of treated cultures of each genotype and stained them prior to microscope study. In the WT cultures the majority of the cells found are eosinophils, although other cell types can be found such as macrophages, polymorphonucleated and other types of granulocytes. In the Hz KO F, there is still a considerable percentage of eosinophils, although the other cell types are more abundant than in wildtype samples. When we analysed the cytospins from KO M, we observed a very low percentage of eosinophils, while the majority of cells present in these samples are macrophages and basophils (Figure 3E).

Macrophages

We also analysed the effect of recombination of Gata1 in macrophage cultures. We treated M-CSF cultures with tamoxifen at day 0 and 5 and analysed the cells after collecting them at day 8. We compared the cell numbers obtained and plotted them in Figure 4A. The cell numbers are reduced in the samples treated with tamoxifen at day 0, but not at day 5. Although the percentage of live cells is decreased upon tamoxifen treatment at day 0 (Figure 4B), the differentiation of the live fraction is not perturbed (Figure 4B).

The reduced number of cells and reduced percentage of live cells from cultures treated at day 0 is probably due to cell death of progenitors present in the bone marrow that still require Gata1 and are not committed to the macrophage lineage yet. We observed that the percentage of mature macrophages of live cells is not affected by Gata1 ablation upon tamoxifen treatment (Figure 4B).

We conclude that the Gata1 recombination system is suitable to perform experiments *ex vivo*, and that Gata1 ablation in culture affects cells requiring Gata1 specifically.

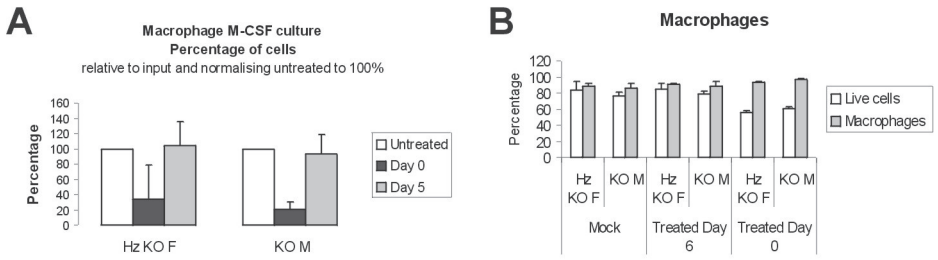

Figure 4. Induction of recombination of Gata1 in the macrophage lineage in *ex vivo* cultures.
(A) Efficiency of the cultures at day of collection of cells cultured in absence or presence of tamoxifen since day 0 or day 5 of culture. Untreated was considered 100% efficiency and all values were normalised to untreated. The average and standard deviations are indicated. At least three samples are analysed together.
(B) Percentage of live cells as measured by FACS ($7AAD^-$ cells) at day of collection of cultures in the absence or presence of tamoxifen since day 0 or day 6 (white bars). The percentage of differentiated macrophages of live cells in all the cultures is also depicted (grey bars). The average and standard deviations are indicated. At least three samples are analysed per group.

4.- Flt-3 ligand cultures

Two types of DC are developing in the presence of Flt-3 ligand, $CD11b^-B220^+$ and $CD11b^+B220^-$, and as shown above, both types of DC express Gata1. All cell cultures were started with the same cell density, and cell numbers were calculated per mouse and per treatment at each time point of collection. Results were pooled based on the genotype for statistical analysis. We observed a decrease in the cell numbers in both Hz KO Fs and in KO Ms and KO Fs compared to WTs, after normalisation with the respective untreated samples (Figure 5A). The dramatic reduction in cell numbers made it difficult to analyse the differentiation profile of the remaining cells. We could not detect differences in the percentages of $B220^-$ and $B220^+$ DC in the remaining cells of the treated samples, either by gating live or dead cells, suggesting that surviving cells develop equally into both cell types (data not shown). In order to study the effects of Gata1 ablation on each type of DC, we sorted $B220^-$ and $B220^+$ DC at day 11 of culture and started the tamoxifen treatment separately in the different populations of DC. Interestingly, we observed a higher impact

on the cell number reduction of sorted B220^{+} DC. It looked as if Flt-3 derived B220^{-} DC are less sensitive to Gata1 loss (Figure 5B).

When analysing the Gata1 levels by FACS analysis on treated samples, they appeared to be reduced in the surviving cells, but Gata1 was not completely abolished from them (Figure 5C). This observation suggests that the remaining cells still contain sufficient levels of Gata1 to survive.

Since we found that Gata1 is required for survival of dendritic cells in Flt-3 ligand cultures, we wished to know whether the defect generated by Gata1 loss affects the cell cycle, and if cells die of apoptosis.

Cell cycle analysis

Gata1 has been linked to the regulation of the cell cycle. Dendritic cells arrest the cell cycle upon differentiation and reduce their proliferation rate completely. We wished to see whether Gata1 loss in Flt-3 ligand cultures would generate any defect or deregulation in their cell cycle profile. For that purpose we added tamoxifen to cultures of different genotypes at day 0 and 5 and analysed their cell cycle 2 days later. Figure 6A shows the normal cell cycle profile of maturing dendritic cells collected at day 2 and day 7. Cells collected at day 7 accumulate in G1 compared to day 2, and the percentage of cells in S or G2/M phases is subsequently reduced, which is an indication of cell cycle arrest upon dendritic cell maturation. When we analysed Hz KO F and KO F samples and compared them to the wildtypes after normalisation with respective untreated samples we observed that the expected profile was altered (Figure 6B). Values in Figure 6B below 1 indicate the fold decrease compared the untreated sample, while values above 1 indicate the fold increase compared to the untreated samples. In the treated wildtype samples, either at day 0 or day 5, there is no change upon tamoxifen treatment. All values corresponding to each cell cycle phase are equal to the untreated samples, as all the values are maintained around 1. In the case of the Hz KO F and KO F cells, a deregulation of the cell cycle can be observed as not all the cell cycle phases are as the untreated (values different than 1). There is a slight deregulation of the cell cycle occurring upon recombination of Gata1. However, due to the fact that cell numbers were highly reduced in the Gata1 null samples and the measurements were taken with fewer cells, we cannot exclude that the variation found in the cell cycle profile is a secondary effect of the induced cell death.

Analysis of apoptosis

Cells were mock-treated or treated either at day 0, day 5 and collected at day 8. Collected cells were counted, stained with Annexin V and 7AAD and analysed by FACS to measure apoptotic events. In Figure 6C the obtained values are represented, also after normalising to the untreated samples by calculating the ratio treated/untreated. The tamoxifen treatment at day 0 caused a significant impact on Hz KO and KO cells compared to WTs. The

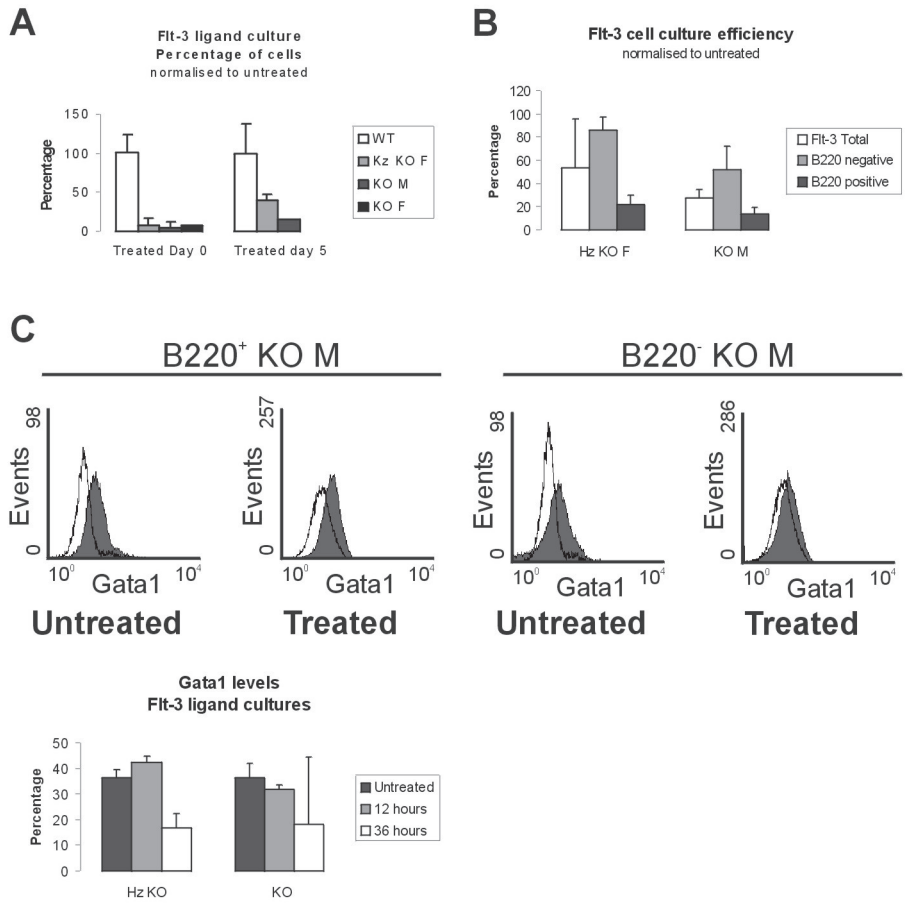

Figure 5. Induction of Gata1 recombination in Flt-3 ligand cultures.
(A) Efficiency of the cell cultures after normalisation with untreated samples. Flt-3 ligand derived dendritic cells die in the absence of Gata1. The average and standard deviations are indicated. At least three samples are analysed together.
(B) Efficiency of cultures of tamoxifen treated sorted MDCs and PDCs from Flt-3 ligand cultures. PDCs are more sensitive to Gata1 loss than MDCs. The average and standard deviations are indicated. At least three samples are analysed together.
(C) Histograms corresponding to gated MDCs and PDCs of the KO M treated or untreated samples are shown. Gata1 is ablated in both cell types. Gata1 levels after 12 or 36 hours of tamoxifen treatment in Flt-3 ligand cultures. The average and standard deviations are indicated. At least three samples are analysed together.

number of dead cells is much higher than in wildtypes, as well as the number of Annexin V positive cells, corresponding to late apoptotic cells. There is not much difference in the numbers of early apoptotic cells (Annexin V^+ $7AAD^-$) but this is expected, as the addition of tamoxifen was done at day 0 and cells were collected at day 8, which is probably too late a time point to detect early apoptotic cells. Also in Figure 6D we can see the data obtained

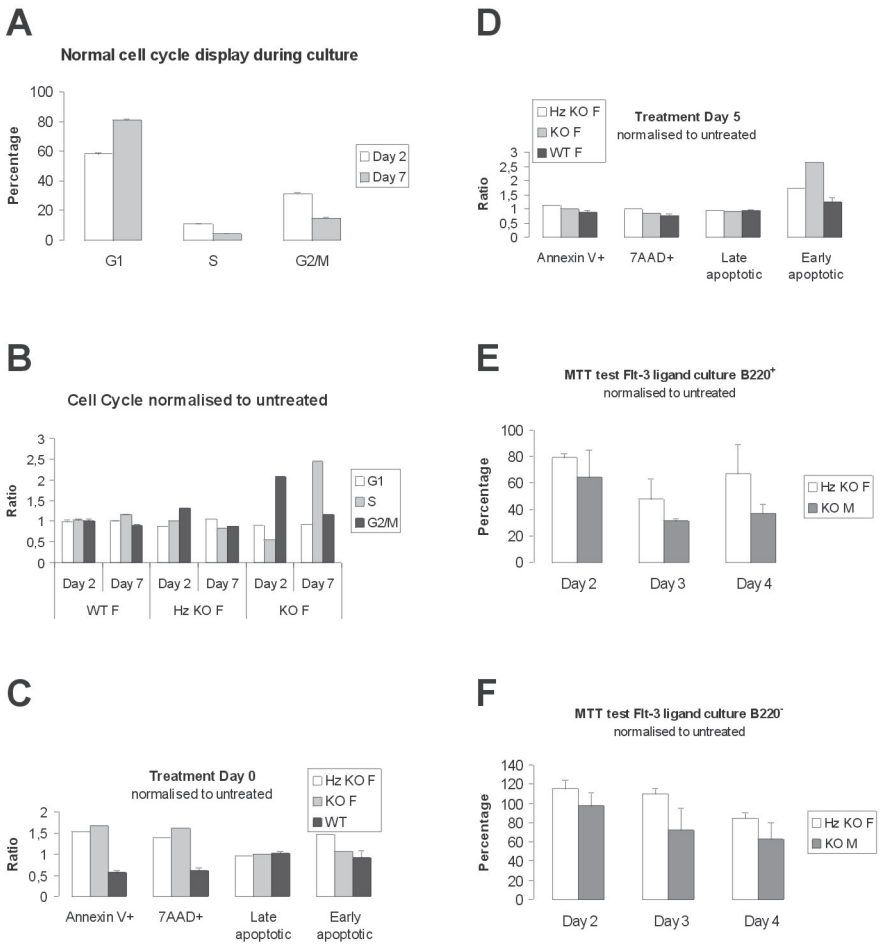

Figure 6. Induced recombination of Gata1 in Flt-3 ligand cultures. Cell cycle, apoptosis and MTT analysis.
(A) Normal cell cycle progression profile of wildtype cells at day 2 and 7 of culture. The average and standard deviations are indicated. At least three samples are analysed together.
(B) Cell cycle analysis of tamoxifen treated cells at day 2 and 7. The average and standard deviations are indicated. At least three samples are analysed together.
(C) Apoptosis analysis by FACS staining with Annexin V and 7AAD. Results from cultures treated with tamoxifen at day 0 and collected at day 7 are depicted. Late apoptotic are Annexin V^+ $7AAD^+$, and early apoptotic are Annexin V^+ $7AAD^-$. The average and standard deviations are indicated. At least three samples are analysed together.
(D) Apoptosis analysis by FACS staining with Annexin V and 7AAD. Results from cultures treated with tamoxifen at day 5 and collected at day 7 are depicted. Late apoptotic are Annexin V^+ $7AAD^+$, and early apoptotic are Annexin V^+ $7AAD^-$. The average and standard deviations are indicated. At least three samples are analysed together.
(E) MTT test of sorted PDCs, treated with tamoxifen at day of sorting and collected at day 2, 3 and 4 after tamoxifen treatment. The average and standard deviations of samples normalised to untreated are indicated. At least three samples are analysed together. Reduction of the colorimetric absorbance is indication of reduced viability. The relative reduction compared to the untreated samples can be visualised in the graphs.
(F) MTT test of sorted MDCs, treated with tamoxifen at day of sorting and collected at day 2, 3 and 4 after tamoxifen treatment. The average and standard deviations of samples normalised to untreated are indicated. At least three samples are analysed together. Reduction of the colorimetric absorbance is indication of reduced viability. The relative reduction compared to the untreated samples can be visualised in the graphs.

from the treatments at day 5. The changes are not so obvious, also because the analysis is done only three days after the treatment. However, we can detect an increase of early apoptotic cells.

Viability analysis of sorted B220$^-$ and B220$^+$ DC

As we detected a differential response to Gata1 loss amongst B220$^-$ and B220$^+$ DC derived from Flt-3 ligand cultures, we decided to measure viability separately in sorted cells at day 7 of culture. Tamoxifen was added after the sorting of B220$^-$ and B220$^+$ DC, and we performed and MTT test on the cultures as described in the Materials and Methods section. Cells were collected and analysed at day 2, 3 and 4 after tamoxifen treatment, and the data are shown in Figure 6E-D. The respiratory mitochondrial dehydrogenase activity was significantly reduced in sorted B220$^+$ DC three days after tamoxifen treatment in the KO M samples. This is an indication of cell death, as the cultures contain the same number of cells. The Hz KO F samples showed in general a higher standard deviation, which is expected due to X inactivation. The respiratory activity in sorted B220$^-$ DC is not as severely reduced as observed in sorted B220$^+$ DC. This is in concordance with the observation from the analysis of the efficiency of the cultures shown in Figure 5, namely that B220$^+$ DC are more sensitive to Gata1 loss than B220$^-$ DC derived from Flt-3 ligand cultures.

5- GM-CSF cultures

GM-CSF cultures generate an enriched population of myeloid dendritic cells that express CD11c, CD11b and upon later maturation stages they also express MHC-II. A follow up of bone marrow cells in GM-CSF culture after FACS analysis is depicted in Table 1. We analysed the cell culture efficiencies and plotted them in Figure 7A. A significant reduction of cell efficiency was observed with cells treated with tamoxifen at day 0 in Hz KO F and KO M samples, probably due to general loss of progenitors present in the bone marrow. Hz KO F and KO M cultures treated with tamoxifen at day 5 showed a significant reduction of efficiency, pointing to the requirement of Gata1 in MDC derived from GM-CSF cultures as well. Tamoxifen treatment at later time points of culture had a lesser effect on the reduction of culture efficiency. This could be either due to the maturation stage of DC (*i.e.* mature DC are less sensitive to Gata1 loss) or to the time point of collection (*i.e.* certain time is needed to detect an effect on the efficiency of the cultures after tamoxifen treatment).

In the next experiments, we added tamoxifen at different time points during the culture in order to assess the effects of Gata1 loss during dendritic cell differentiation, but we avoided treating the cultures at day 0 to exclude misinterpretation of data due to general cell death of progenitors. By day 5 of culture there is a high percentage of CD11c positive cells, committed towards dendritic cell differentiation. We added tamoxifen at this early

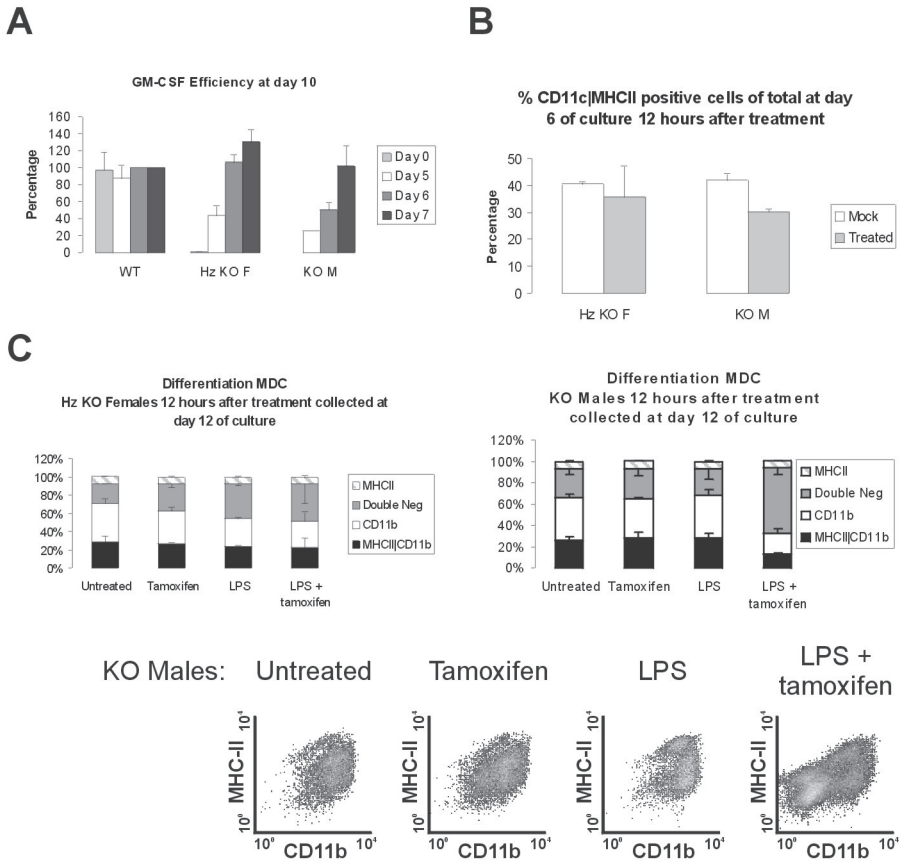

Figure 7: Induction of Gata1 recombination in GM-CSF cultures.
(A) Efficiency of cell cultures collected at day 10 and treated with tamoxifen at day 5, 6 or 7. Average and standard deviation of values after normalisation to untreated samples is shown. At least three samples were analysed per group.
(B) Differentiation of treated cultures at day 6, after 12 hours of tamoxifen addition. Average and standard deviation is shown. At least three samples were analysed per group.
(C) Differentiation of treated cultures in the presence or absence of LPS challenge in Hz KO F and KO M samples in respective graphs. Average and standard deviation is shown. At least three samples were analysed per group. Example density plots of all the treatments in KO M samples are included.

differentiation time point and analysed cells after 12 and 36 hours post treatment. We took day 10 as our next time point in order to analyse the effect in mature DC, and we mock-treated and treated with tamoxifen cultures in the presence or absence of LPS. As shown in Figure 7B, at day 6 of culture, and 12 hours after tamoxifen treatment, the percentage of differentiated MDC is reduced in the KO males compared to untreated samples. The Hz KO females present a higher standard deviation due to random X-inactivation, but the

average has a tendency to reduction. We also collected cells at day 12, after tamoxifen treatment combined with LPS challenge. The effects of tamoxifen are not so clear in both genotypes in the absence of LPS. Interestingly, in the presence of LPS, the tamoxifen treated samples were significantly affected and a percentage of cells started to die which were double negative for CD11c and MHC-II. In Figure 7C graphs corresponding to Hz KO F and KO M samples are displayed. The effect is stronger in KO M samples challenged with LPS. Sample density plots corresponding to the different treatments in KO M samples are presented. Note the difference in cell distribution towards FACS channels detecting CD11b and MHC-II surface markers. A histogram of the LPS + tamoxifen treatment in one KO M sample is depicted showing the Gata1 level compared to the background staining. Gata1 is completely absent in this sample (Figure 7C). Therefore, mature DC in *ex vivo* cultures appear to be still Gata1 dependent for their survival.

6.- The effect of Gata1 loss on sorted mature DC from spleen in *ex vivo* culture

At the moment and due to the limitations of our inducible system, which does not allow a cell specific study of Gata1 loss *in vivo*, we decided to isolate mature DC from spleen, and treat them with tamoxifen to detect cell death in *ex vivo* culture. We observed a reduction of cell number in treated samples, although it is difficult to conclude anything about specific lineage cell loss from the FACS analysis performed (data not shown) (Figure 8).

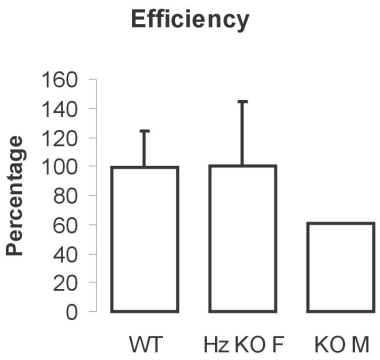

Figure 8. Ex vivo culture of sorted DCs from spleen. Sorted spleen cells were cultured in the presence of tamoxifen. The efficiency of the cultures after three days of treatment is depicted.

7.- Gata1 is expressed in human DC

We observed that Gata1 is expressed in DC *in vivo* and *ex vivo* in mice and we showed that ablation of Gata1 affects the survival of DC in culture. We therefore wanted to see whether we could detect Gata1 expression in human DC. We obtained *in vitro* generated DC and cells were analysed by FACS staining. In Figure 9A we show histograms corresponding to the Gata1 levels of human immature (day 14) and mature (day 21) DC. When compared to the background staining, human DC are positive for Gata1. As observed in mice, Gata1 levels are downregulated in human mature DC when compared to human immature DC.

We also performed immunohistochemistry on cultured human DC and example pictures are shown in Figure 9B. All the cells are Gata1 positive, and these cells express CD11b.

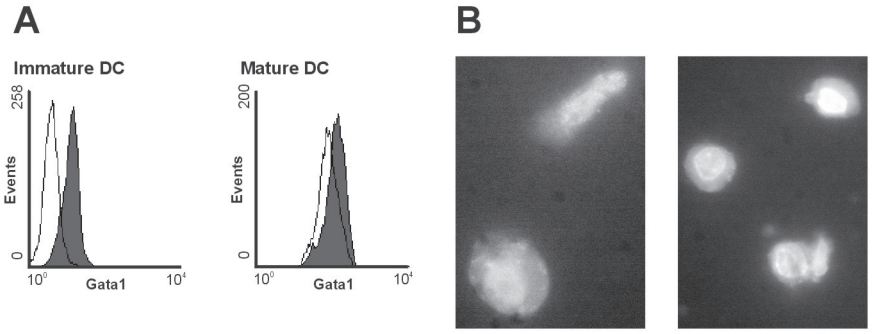

Figure 9. Gata1 expression in human DCs.
(A) Gata1 levels as measured by FACS in human DCs. Filled curve is the Gata1 staining, while unfilled curve corresponds to the background staining. Levels of Gata1 are reduced upon DC maturation.
(B) Gata1 immunostaining of cultured human DC. Gata1 shows an intracellular punctuate pattern (red) while CD11b is a transmembrane protein (green, best visualised in the left panel). (Selected colour picture is depicted in the cover).

DISCUSSION

In this work we show that Gata1 is expressed in murine dendritic cells (DC) *in vivo* and in *ex vivo* culture, as well as in human DC. In Flt-3 ligand cultures Gata1 expression was found in both types of DC, *i.e.* $B220^-$ and $B220^+$ DC. Furthermore, ablation of the Gata1 gene by using a floxed Gata1 allele and an inducible Cre recombinase under the ROSA26 promoter affects the survival of DC in Flt-3 ligand and GM-CSF cultures at early and late stages of DC maturation.

We observed that Gata1 levels are downregulated in mature DC isolated from fresh tissues in wildtype mice. It is complicated to relate the maturation of DC in *ex vivo* cultures with the maturation that occurs *in vivo*. The regulation and development of the immune system in the living animal is complex and the challenges that influence the maturation of DC are difficult to mimic in culture. We treated mature DC from GM-CSF cultures with lipopolysaccharide (LPS) in order to force the terminal maturation of DC in culture. LPS challenge has contradictory ways of action. Depending on the concentration and the phase in the DC development at which it is applied, stimulation with LPS can lead to different outcomes by inducing either pro- or anti- apoptotic mechanisms (Lutz *et al*., 2000). In general, transient LPS stimulation induces expression of higher levels of MHC-II molecules, as an indication of full DC maturation. Consequently, the majority of LPS stimulated DC are $CD11c^+MHC\text{-}II^{high}$ while cultures before the LPS challenge contain a mixture of DC at different maturation points. In our system, LPS stimulated mature DC appear to react to Gata1 ablation to a higher extent than unchallenged cells. The change in the composition of the LPS matured DC culture treated with tamoxifen, compared to the untreated one, was prominent. We can however only speculate about the cause for this profound change. One explanation could be the loss of CD11b marker expression due to the advanced maturation stage as a result of Gata1 downregulation (Martinez del Hoyo *et al*., 2002). However, we think the following explanation is more likely. We have to consider that LPS challenged DC have activated a series of anti-apoptotic pathways in order to expand their lifespan to perform their antigen presenting function longer and hence better. DC that are not able to activate these anti-apoptotic pathways apoptose. This means that LPS challenge creates a DC selection that generates a homogeneous population of DC in terms of maturation. Loss of Gata1, in this case, might cause a deregulation of the anti-apoptotic machinery and the phenotypic changes that we found might be an early sign of apoptosis (Rescigno *et al*., 1998; Wong *et al*., 2004). The reason why we cannot detect such an effect in LPS non-stimulated cultures is because LPS challenged surviving cells are a mature homogeneous population, while LPS non-stimulated cells are not. Therefore, LPS challenged cells might react in a more homogeneous way to Gata1 ablation, in comparison to the population of DC that were not challenged. Despite Gata1 levels might be downregulated upon maturation of DC as observed *in vivo*, ablation of Gata1 in LPS

challenged DC causes a deregulation of the anti-apoptotic machinery. Therefore, mature DC in *ex vivo* cultures are still Gata1 dependent.

The nomenclature of the type of DC that can be obtained in Flt-3 ligand cultures is still obscure. Progenitors either from myeloid or lymphoid origin that express Flt-3 receptor can be induced to develop into DC. Whether they are respectively the $CD11b^+B220^-$ or $CD11b^-B220^+$ is not clear, as maybe the expression of this markers is merely dependent on the maturation of DC in culture. We sorted both types of DC and added tamoxifen to respective cultures to see the effects of Gata1 ablation separately. This experiment was done in order to investigate whether Gata1 loss affects the survival of both DC types directly and equally or whether direct induction of apoptosis in one DC type indirectly causes the death of the other. In this experiment we observed that, although $B220^-$ DC were directly affected by loss of Gata1 in the absence of $B220^+$ DC, they were less sensitive to Gata1 loss than $B220^+$ DC. We saw, as explained above, that Gata1 levels are downregulated upon spontaneous maturation of DC but also that terminally differentiated DC by LPS in GM-CSF culture are still Gata1 dependent. Along these lines, the differential effect on survival of Flt-3 derived DC could argue that $B220^-$ DC are indeed a further maturation step compared to $B220^+$ DC. The ratio $B220^-/B220^+$ DC increases as culture advances. It could be that Flt-3 stimulated differentiation of DC follows the following surface marker pattern: $B220^+$, $B220^+CD11b^+$, $CD11b^+$.

We have to consider that the effects observed here are not completely informative of the single-cell effect, but of the population. Gata1 recombination induction leads to downregulation of the protein until it is completely extinct in the cell. In a way, this mimics what could happen in maturing DC. This can explain why $B220^-$ DC are not as sensitive as $B220^+$ DC upon Gata1 ablation. $B220^+$ DC are found in a stage where the Gata1 reduction is not happening at the proper moment and they are affected in a more homogeneous way. Whether this argument is correct or not, has to be confirmed. However, we question the nomenclature of DC, as we think that the surface marker display of DC might not be an indication of their progenitor origin, but an indication of the inductive pathway and an adaptation to the functions that the specific DC is going to perform and where it is going to perform them. Gata1 is expressed in several myeloid committed lineages but it has never been described to be expressed in lymphoid committed lineages. However, due to the fact that Gata1 is expressed in all the DC in Flt-3 ligand cultures, if these cells are originated from Flt-3 receptor expressing myeloid and lymphoid progenitors, there are two possibilities: that Gata1 is still expressed at basal levels in lymphoid progenitors and it is shut down when lymphoid lineage commitment occurs, with the exception of DC differentiation. Alternatively, the other possibility is that Gata1 transcription is activated upon Flt-3 ligand activation of lymphoid and myeloid progenitors. Both notions need to be further investigated.

Gata1 is expressed at basal levels in haematopoietic progenitors and in several

myeloid committed lineages and this is the reason why bone marrow cells treated with tamoxifen at day 0 showed the lowest efficiency rates in all culture types. However, macrophage committed progenitors were not sensitive to Gata1 ablation, as expected, and they gave rise to mature macrophages at the same extent as untreated samples. The eosinophil lineage proved to be Gata1 dependent at all stages analysed, as expected.

All the data presented here are mainly derived from *ex vivo* experiments due to the limitations of our inducible system to study recombination of Gata1 in the living organism. The Cre recombinase is ubiquitously expressed and it would be not possible to study the tissue specific effect of Gata1 loss in dendritic cells. Ablation of Gata1 would lead to lethal anaemia and to a major depletion of progenitors in the bone marrow, which would make impossible to withdraw conclusions of a lineage committed cell type as dendritic cells. To have an idea of the effects of Gata1 loss *in vivo*, we sorted DC from spleen and cultured them for three days in the presence of tamoxifen. We could detect a reduction of cell numbers, although it was difficult to assess a specific cell type loss. At the time that we were studying Gata1 requirements for dendritic cell differentiation *ex vivo*, I was inducing Gata1 recombination *in vivo* in mice belonging to another crossing strategy that was designed to answer the question whether REDS is an homotypic signalling mechanism or not (Chapter 3, Appendix II), and we analysed the presence and contribution of different haematopoietic cell types in different tissues. We observed a decrease in MDC/PDC and monocytic progenitors in addition to the expected erythroid and eosinophil lineages loss, in Gata1 OX/KO compound mice. This is indicative that Gata1 ablation, at least in an heterozygous background, leads to a reduced number of committed progenitors (monocytes) and subsequently, of differentiated DC.

Recombination of Gata1 in a cell-specific manner, *i.e.* by using a CD11c promoter driven Cre recombinase, would constitute a model system that would be useful to answer many questions about lineage origin of DC and to study pathologies where DC are involved, like allergy responses.

The origin of DC has been a very cryptic matter. It is accepted that DC can derive from either myeloid (MDC) or lymphoid (PDC) progenitors (Canque and Gluckman 2001; Liu *et al*., 2001; Manz *et al*., 2001; Gilliet *et al*., 2002; Karsunky *et al*., 2003). The fact that Gata1 is expressed in both types of DC questions this classification. We propose one origin and that the surface marker display of these cells is a versatile and plastic characteristic that is determined by the tissue were the APC is directed to perform its antigen presenting function.

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CHAPTER 6

DISCUSSION

Discussion and future directions

Erythropoiesis is the process by which blood cells are produced (Rifkind *et al*., 1974). Gata1 is a transcription factor essential for erythropoiesis (Pevny *et al*., 1995; Fujiwara *et al*., 1996). It has been related to the differentiation of several lineage committed progenitors (Shivdasani *et al*., 1997; Harigae *et al*., 1998; Hirasawa *et al*., 2002; Yu *et al*., 2002), to the regulation of the cell cycle (Dubart *et al*., 1996; Whyatt *et al*., 1997) and to survival (Gregory *et al*., 1999; Tanaka *et al*., 2000). Mutations in Gata1 or an alteration of its levels lead to different diseases affecting the differentiation of the different lineages requiring Gata1, *i.e.* anaemia (McDevitt *et al*., 1997; Whyatt *et al*., 2000), macrothrombocytopenia (Nichols *et al*., 2000; Freson *et al*., 2001) or leukaemia (Shimizu *et al*., 2004; Crispino 2005). All the erythroid genes studied, including the Gata1 gene itself, contain GATA sites, and this is the reason why Gata1 is thought to play a pivotal role in the regulation of those genes (Martin and Orkin 1990; Cantor and Orkin 2002). Importantly, Gata1 levels must be dynamically regulated, as the kinetics of the different interactions with other proteins and/or DNA might be dependent on that. How is Gata1 regulated?

At the transcriptional level, EpoR activation induces upregulation of Gata1 (Dalyot *et al*., 1993; Leonard *et al*., 1993). This occurs in the erythroid lineage around the CFU-E stage, the most Epo dependent erythroid progenitor. Gata1 is required through differentiation and downregulation must occur during terminal differentiation. Several post-translational modifications have been described for Gata1, such as phosphorylation and acetylation (Briegel *et al*., 1996; Boyes *et al*., 1998). In chicken, for example, Gata1 is cytoplasmic in early progenitors and becomes nuclear in late progenitors, and this nuclear Gata1 is hyperphosphorylated (Briegel *et al*., 1996). Acetylation of Gata1 enhances the DNA binding affinity and might play a role in directing the protein-protein interactions of Gata1 as well. Gata1 is often found in aggregates in the cells (Elefanty *et al*., 1996), and self-self interaction of Gata1 has been described in zebrafish, enhancing DNA binding activity to its own promoter (Nishikawa *et al*., 2003). How does the downregulation of Gata1 occur during terminal differentiation?

In apoptotic processes in early progenitors, caspases degrade human Gata1 and they undergo apoptosis (De Maria *et al*., 1999). During terminal differentiation, specific pathways might be activated to downregulate the levels of a team of factors, and Gata1 is amongst those.

The expression levels of Gata1 and the time frame of action of this protein make its analysis more complex. Gata1 not only binds to the DNA at GATA sites, it also interacts with a myriad of proteins and these protein interactions can lead to antagonistic actions. Whether Gata1 is inhibiting or activating the expression of a gene might be dependent on the protein content of the cell, the levels of Gata1, the stage of the cell cycle and the signals coming from the environment.

Gata1 overexpression in the erythroid lineage intrinsically arrests cells at the proerythroblast stage (Whyatt *et al*., 1997; Whyatt *et al*., 2000). The cell cycle is affected and they are unable to arrest in G1 prior to enucleation. Gata1 binds the retinoblastoma (pRb) protein, and regulates the expression of other cell cycle related proteins such as p21, cdc6, cMyc, cMyb and Gfi-1B (Bartunek *et al*., 2003; Rylski *et al*., 2003; Huang *et al*., 2004). Our group suggested previously that Gata1 activity might be regulated by a signalling mechanism named REDS (Whyatt *et al*., 2000). It was not clear whether REDS originated from a stromal cell or from an erythroid cell.

One of the goals of the thesis was to elucidate which cell type supplies REDS and which mechanisms and potential molecules are involved in the signal.

Summary of the thesis contents

The cell cycle defect found in MEL cells overexpressing Gata1 is present *in vivo*. Furthermore, Gata1 overexpressing I11 cells in proliferative conditions do not seem to be affected and expand normally, while in differentiation conditions they are unable to arrest cell cycle. We wondered whether this inability is linked to the erythroid differentiation process or whether it is a general cell cycle checkpoint failure that we could also detect in other situations, *i.e.* after DNA damage by UV-irradiation. In Chapter 2 we show that cells overexpressing Gata1 are less sensitive to UV-irradiation than wildtype cells, suggesting a bypass of the cell cycle checkpoint due to inability to arrest cell cycle upon DNA damage induction.

REDS is a signalling mechanism that occurs amongst erythroid cells, *i.e.* it is a homotypic signalling mechanism. Overexpressing Gata1 cells differentiate to the blood pool in adult mice and in hanging drop cultures they differentiate when in the presence of wildtype erythroid cells.

Pathway candidates for REDS were studied and the results support the idea that they are involved in the regulation of erythropoiesis, and that Gata1 might be a target for protein degradation upon activation of these pathways. Inhibition of caspases leads to a block in erythropoiesis, while activation of FasR favours differentiation. Activation of the cytochrome C pathway by oxidative stress also causes induction of differentiation in primary and MEL cells, and reduces the levels of Gata1 compared to untreated samples.

EEG1 is a novel protein and putative REDS receptor: we found that EEG1 is upregulated in Gata1 overexpressing cells, earlier than it occurs naturally in wildtype cells. This is probably one of the target genes with deregulated expression due to overexpression of Gata1. Considering the growth influencing functions of EEG1, we consider that it could be part of the mechanism that we want to unravel.

A novel expression site of Gata1 has been identified in dendritic cells (DC). By means of an inducible loxP/Cre recombination system Gata1 was ablated from DC in *ex vivo* cultures, which revealed a pivotal role of Gata1 in the survival of DC.

GENERAL DISCUSSION

Gata1 plays a crucial role in the haematopoietic system. In this tissue the regulation of the balance between proliferation, self-renewal, differentiation and apoptosis must be tight. Gata1 is expressed at basal levels in multipotent progenitors. In these cells, Gata1 might play a survival role. Gata1 positively regulates Bcl-2 and Bcl-xL expression. Expression of these proteins promotes proliferation of the cells in addition to the anti-apoptotic main function for what they are known (Marone *et al*., 2000). This proliferation-favouring situation inhibits differentiation of these progenitor cells and assures the self-renewal of the progenitor pool. Upon certain signalling mechanisms, Gata1 levels are upregulated, for example in the situations when Epo levels are physiologically high inducing erythroid differentiation (Dalyot *et al*., 1993). At this moment, the progenitors commit to the erythroid lineage. This higher Gata1 level induces the proliferation of the erythroid progenitors needed for differentiation. Probably the proliferation rate is higher than in other cell lineages. For example, Gata1 transformation of M1 cells inhibits macrophage-induced differentiation due to induction of proliferation and inhibition of apoptosis (Tanaka *et al*., 2000). Erythroid cells need to divide a certain number of times to reach the enucleated erythroid cell (Allen and Testa 1991). The number of divisions is fixed and this is very important, because it will also ensure a certain accumulation of globins in their cytoplasm. Terminal differentiation of the erythroid cell is accompanied by downregulation of Gata1, and probably downregulation of survival factors such as Bcl-2 and Bcl-xL. This supports the idea that since that moment, erythroid cells will experience a slow apoptosis event.

Erythroid cells communicate with each other at late differentiation stages within the erythroblastic island (Whyatt *et al*., 2000; Gutierrez *et al*., 2004). This signalling mechanism is thought to favour the differentiation process that is ultimately induced by high levels of Epo (Figure 1). Recent studies in cultured erythroblastic islands showed that the crown cells of the islands displayed different proliferation rates depending on the addition of erythroid cells at different stages of maturation to the culture, supporting the idea that erythroid cells provide signals to each other (Zackharov 2004).

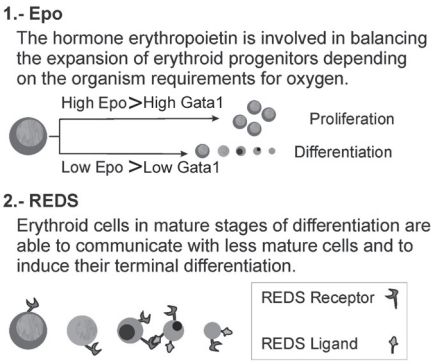

Figure 1. REDS is a homotypic signalling mechanism.
1.- The levels of Epo determine the balance between proliferation and differentiation of erythroid committed progenitors. The levels of Epo are proportional to the level of hypoxia.
2.- REDS is a homotypic signalling mechanism which favours erythroid differentiation in addition to the regulatory actions of Epo.

Which molecules are involved in REDS? We have preliminary results supporting that the REDS receptor(s) might be related to the family of death receptors. Activation of FasR led to improved erythroid differentiation and lower levels of Gata1 compared to untreated samples (Gutierrez *et al*., 2004). Caspase activation by oxidative stress also improves differentiation and results in lower levels of Gata1 (Chapter 4). A molecular dissection of surface proteins of maturing erythroid cells is needed in order to elucidate how REDS works.

Recently a novel erythroid protein was described (Aerbajinai *et al*., 2004), which displays many features that candidate it to the receptorship of REDS. EEG1 (epithelial embryonic gene 1) has a C1q domain that folds in a TNF-like domain. It can be cytoplasmic and transmembrane depending on the kinetics of the protein, and its levels are upregulated during terminal differentiation. Furthermore, when exogenously expressed in Chinese hamster ovary (CHO) cells, it induces cell cycle arrest and apoptosis (Aerbajinai *et al*., 2004). This would fit the fact that erythroid cells need to arrest their cell cycle prior to pycnosis. We confirm that the levels of EEG1 in mouse erythropoiesis follows the pattern described in human erythropoiesis (Chapter 4). We showed that its promoter has two GATA sites that are enriched specifically in chromatin immunoprecipitation assays and that overexpression of Gata1 leads to higher levels of EEG1 (Chapter 4). We propose a model of cooperation between Gata1 and EEG1 in the last stages of erythroid differentiation.

During erythroid commitment, Gata1 levels are upregulated. This event stimulates the expression of positively regulated Gata1 targets, like EEG1. During differentiation, EEG1 levels will accumulate in the cell until a transmembrane location of the protein is favoured. Accumulation of EEG1 in the cell by itself will generate the required cell cycle arrest needed prior to enucleation. EEG1 is possibly involved in direct or indirect mechanisms downregulating Gata1 and other factors, favouring differentiation and closing a negative feedback loop towards Gata1. Curiously, EEG1 has two isoforms. The EEG1L, that contains the C1q domain and is cytoplasmic or transmembrane, and the EEG1S, which lacks the C1q domain and is located in mitochondria aggregates. We suggest that the short version of EEG1 might be involved in the regulation of the cytochrome C pathway, as has been shown for other members of the C1q family (Kishore and Reid 1999; Eggleton *et al*., 2000; Kishore and Reid 2000; Kishore *et al*., 2004). We have evidence that activation of the cytochrome C pathway by oxidative stress induces differentiation of erythroid cells including downregulation of Gata1 levels (Chapter 4), suggesting that activation of the pathway might occur indeed during the last stages of erythroid differentiation. The balance between L and S forms, that might change through differentiation, will subsequently determine activation or de-block of the cytochrome C pathway that will lead ultimately to caspase activation and degradation of Gata1 amongst other factors. So far, we have described that this chain of events can sustain the differentiation of the cell without communicating with others. How do we link this negative feedback loop with REDS?

In Figure 2 we have summarised our new model involving Gata1 and EEG1 in REDS signalling mechanism. We have explained what occurs within a single cell. But how do cells communicate with each other? Accumulation of EEG1 in the surface of erythroid cells is proportional to the differentiation stage. Saturation of the surface by EEG1 molecules might lead to shedding of the C1q domain, as has been described for other members of the C1q family (Kishore and Reid 1999; Eggleton *et al*., 2000; Kishore and Reid 2000; Kishore *et al*., 2004). The shedded molecule might act as a ligand that activates transmembrane EEG1 triggering the caspase cascade either via the cytochrome C pathway or not. This way, mature erythroid cells that leave the erythroblastic island accelerate the differentiation process of neighbouring differentiating cells.

Haematopoiesis and Gata1

We described that dendritic cells express Gata1 and that recombination of the Gata1 allele by means of a loxP/Cre inducible system causes apoptosis in *ex vivo* cultures. This result suggests that Gata1 is required for survival of the DC population. Also the cell type specificity of the factor should be reconsidered. Apparently, Gata1 is a transcription factor that is expressed at basal levels in multipotent progenitors, and remains active in more lineages than previously described.

Taken together these results with the established knowledge related to Gata1 in the haematopoietic system we propose a model of the regulation of the cell fate in this tissue. We summarise the model in Figure 3. Multipotent progenitors express Gata1 at basal levels. They are characterised by quiescence, *i.e.* they are capable of exiting the cell cycle until adequate signals induce the self-renewal or differentiation of the cells. Both events contemplate the proliferation of cells. In other words, we can find the proliferation event either linked to self-renewal or linked to cell differentiation. The signals driving the different responses can be multiple, and some of them have been described. For example, activation of death receptors in this population of quiescent cells leads to proliferation linked to self-renewal, and inhibits proliferation linked to differentiation (Marone *et al*., 2000). In the case of the erythroid lineage, Epo stimulation induces proliferation linked to differentiation and the multipotent progenitors become committed progenitors. Committed progenitors are characterised by a high (although controlled) proliferation rate (Allen and Testa 1991). In the erythroid lineage or other Gata1 expressing haematopoietic cells, Gata1 levels are higher compared to multilineage progenitors. These cells expand the numbers of committed progenitors enormously. Several signals can change the differentiation fate of these cells. For example, when a surplus of the induced lineage is sensed, the expansion of committed progenitors must be stopped or reduced. In this case, activation of death receptor pathways leads to apoptosis. In the erythroid lineage, these cells express high levels of Gata1, which upregulates the expression of Bcl-2 and Bcl-xL anti-apoptotic proteins. Apparently, these cells are susceptible to apoptosis induction and this may be

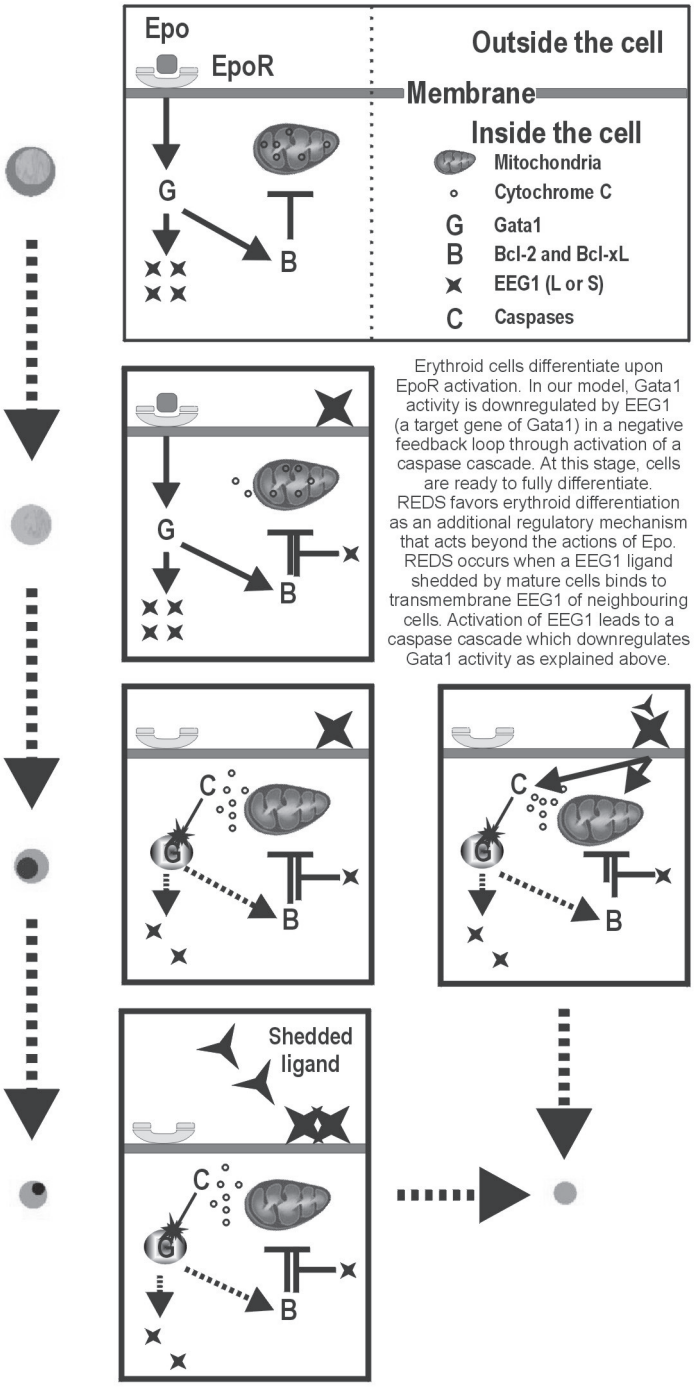

Figure 2. Model of EEG1 as a putative REDS receptor.

why they have activated anti-apoptotic pathways by default. However, this protection is just an extra regulatory pathway to maintain the homeostasis of erythroid cell production, but it is not absolute (Huang *et al*., 1999). Terminal differentiation of committed progenitors into mature cells is accompanied by downregulation of Gata1. This has been described in the erythroid lineage and we observed *in vivo* in mature DC. Curiously, this downregulation of Gata1 leads to a peculiar cell cycle status depending on the cell type. Megakaryocytes experience a series of nuclear divisions and become polykaryons. Erythroid cells arrest the cell cycle and undergo pycnosis. Dendritic cells arrest the cell cycle. Probably eosinophils and mast cells (basophils) are characterised by a terminal quiescent phenotype as well. External signals can only accelerate the differentiation process, as these cells are destined to die eventually, because they have a fixed life span within the organism. In fact apoptosis is not occurring in mature cells upon activation of death receptors. In contrast, and similar to what happens in multilineage progenitors, it leads to proliferation. Particularly, this proliferation is linked to terminal differentiation, in contrast with the proliferation linked to expansion characteristic of multilineage progenitors.

All the myeloid cells involved in allergy responses express Gata1. This myeloid contribution to the lymphoid derived immune system response supports the idea of plasticity and adaptation that characterises the haematopoietic system. We would like to name these cell types as the Gata1 haematopoietic team (Figure 3).

Can we learn from other GATA proteins?

GATA factors are involved in survival, differentiation and proliferation of the cells they are expressed in. The study of Gata1 revealed that depending on the cell type, the level of Gata1 and the signals coming from the environment, the cell responses can be very different, and Gata1 might be either a positive or a negative regulator of a number of cellular processes depending on the parameters mentioned above.

Other members of the GATA family of transcription factors have also been described as crucial factors in the survival, differentiation and proliferation of the cell types where they are expressed.

The remaining haematopoietic GATA factors are very interesting because they coexpress in certain cell types and/or exert overlapping functions. Gata2 is involved in the regulation of early haematopoietic progenitors (Tsai *et al*., 1994) and is closely linked to Gata1 function (Leonard *et al*., 1993; Dame *et al*., 2002; Anguita *et al*., 2004). In rescuing experiments of Gata1 null cells or mice with other GATA factors, Gata2 showed the most overlapping actions (Takahashi *et al*., 2000). Gata2 is expressed preceding Gata1 in mast cells and in the erythroid lineage (Harigae *et al*., 1998; Ohneda and Yamamoto 2002) and is required for survival and proliferation of progenitors (Tsai and Orkin 1997). Overexpression of Gata2 inhibits erythroid differentiation and induces megakaryocyte differentiation, a cell line that coexpresses Gata1 and Gata2 (Ikonomi *et al*., 2000).

Gata3 is required in T cells (Ting *et al*., 1996) at the stages that require cell

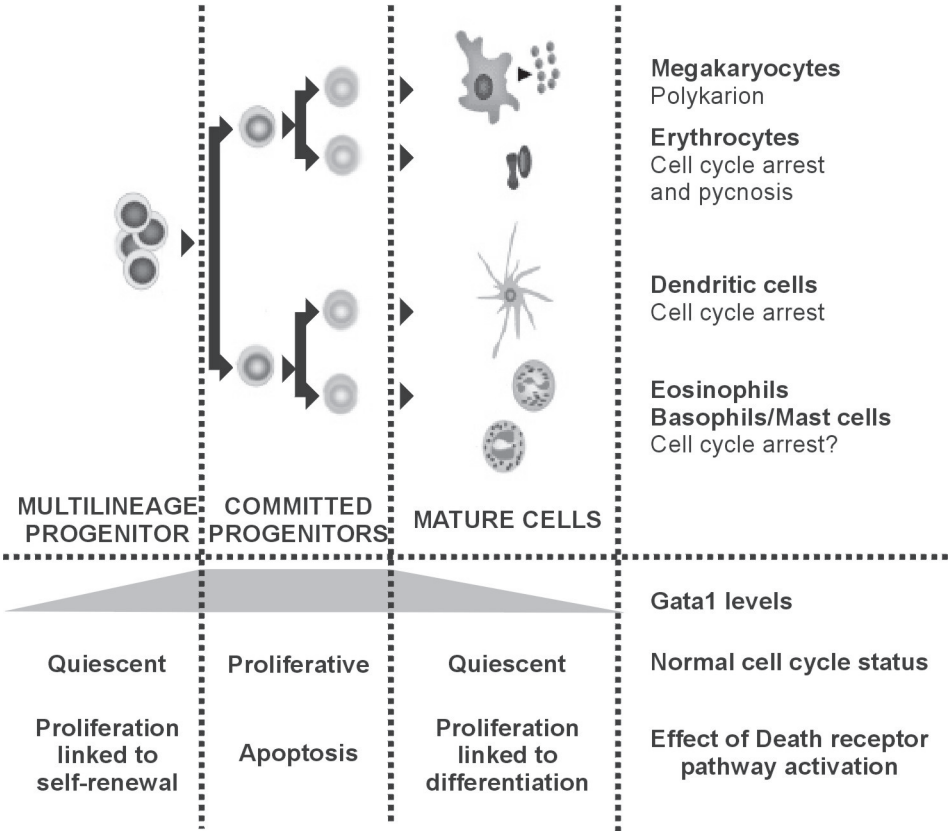

Figure 3. Haematopoiesis and Gata1.

proliferation prior to cell differentiation (Hendriks *et al*., 1999), and post-translational regulation of Gata3 seems to condition the survival and migratory capacities of T cells to lymphoid organs (Yamagata *et al*., 2000). Gata3 null mice die at 10-11 dpc with severe aberrations in the nervous and haematopoietic system (Pandolfi *et al*., 1995). In the lab, Dorota Kurek *et al* are studying skin-specific Gata3 null mice. This mouse model revealed an essential role of Gata3 in hair cycle regulation. Null mice did not renew their fur and displayed a nude-like phenotype with excess of skin production. Gata3 haploinsufficiency in human causes HDR syndrome, which is characterised by hypoparathyroidism, renal dysplasia and sensorineural deafness. Interestingly, a patient with Gata3 haploinsufficiency has been reported recently who presented generalized psoriasis in addition to the described symptoms (Aksoylar *et al*., 2004).

The non-haematopoietic GATA factors are not less interesting. Gata4, 5 and 6 are expressed in cardiac tissue (Evans 1997). Gata4 is described as a survival factor in

the heart (Kitta *et al*., 2003; Suzuki and Evans 2004), and it is essential for the survival of cardiocytes upon induced cardiotoxicity (Aries *et al*., 2004). Furthermore, overexpression of Gata4 leads to enhanced cardiogenesis (Grepin *et al*., 1997). On the other hand, overexpression of Gata6 in *Xenopus laevis* blocks cardiogenesis (Gove *et al*., 1997). Gata4 is also protecting granulosa cells from apoptosis in the ovarian follicle (Vaskivuo *et al*., 2001). Curiously, and connecting the function to the erythroid system, Gata4 positively regulates the transcription of Erythropoietin in hepatocytes (Dame *et al*., 2004). Differentiation of intestinal epithelial cells seems to be regulated by the cooperation between Gata4, 5 and 6. Gata6 exerts its function in proliferative precursors. Gata4 and Gata5 play an essential role in terminal differentiation and they transactivate the expression of Intestinal Fatty Acid Binding Protein (IFABP), which is a marker of mature enterocytes (Gao *et al*., 1998).

Not only in the mammalian or vertebrate world are GATA factors crucial in differentiation, proliferation and survival. In the plant *Arabidopsis thaliana*, GATA factors are implicated in light-responsive transcription (Teakle *et al*., 2002). HAN, a homologue of Gata3 is involved in controlling cell proliferation and differentiation, and ectopic expression of HAN generates growth retardation, aberrant cell division patterns, and loss of meristem activity, which is the stem cell factory of plants (Zhao *et al*., 2004).

FUTURE DIRECTIONS

REDS

A high priority is the continuation of the analysis of EEG1 function during erythroid terminal differentiation. For this purpose it is required to develop antibodies that recognise both isoforms of EEG1. Oligonucleotides for RNAi experiments in primary cell cultures have already been designed with the aim to unravel the function of EEG1 during terminal differentiation. Interfered cells will be assayed for cell cycle, differentiation and survival.

However, it is possible that other molecules are involved in REDS. We aim to enlarge the candidate list either by mass spectrometry analysis of surface protein at different stages of differentiation or by blocking REDS in culture with a library of single chain antibodies. We will knockdown the candidate genes by using morpholinos and study the outcome in terms of definitive erythropoiesis in the zebrafish model. After screening for the best candidates in zebrafish, we will proceed with studies in the mouse model. We have Gata1 overexpressing murine erythroid cell lines and transgenic mice. RNA interference (RNAi) of candidates in cell lines or primary cell cultures will be performed through lentiviral transduction. In this manner we can assess whether the absence of a candidate affects terminal differentiation in any way and if Gata1 levels are downregulated or not. Once the candidates are sorted out, we will perform immunohistochemistry or RNA-FISH of in cryosections of fetal livers in order to locate the expression of the candidate molecules within the erythroblastic island.

ChIP studies on Gata1 overexpressing cells

In collaboration with Patrick Rodriguez we will continue the analysis of Gata1 overexpressing primary cells by ChIP.

Dendritic cells

In collaboration with Dr. T. Nikolic, we will continue the study of Gata1 function in dendritic cells. We already have RNA material from two experiments of Gata1 ablation in *ex vivo* cultures. Analysis of the expression of genes involved in survival and dendritic cell differentiation will be performed.

We also have Gata1 RNAi oligonucleotides in order to ablate Gata1 expression by other means in *ex vivo* cultures and confirm the results obtained so far.

In vivo studies were not plausible with the system currently used. With the aim of specifically ablate Gata1 in DC *in vivo*, we are in the process of obtaining CD11c driven and inducible Cre expressing mice. Activation of the Cre recombinase driven by the CD11c promoter will lead to recombination of Gata1 in macrophages and DC. Macrophages do not require Gata1. We expect that recombination of Gata1 will lead to DC loss. This model system will be very useful in the determination of the myeloid and/or lymphoid origin of DC, and to study allergy responses in the absence of DC. In addition it would be interesting to determine which regulatory elements in the GATA1 locus direct its expression in DC.

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APPENDIX I

A HANGING DROP CULTURE METHOD TO STUDY TERMINAL ERYTHROID DIFFERENTIATION

A hanging drop culture method to study terminal erythroid differentiation

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ABSTRACT

Objective: To design a culture method allowing the quantitative and qualitative analysis of terminal erythroid differentiation.
Methods: Primary erythroid progenitors derived either from mouse tissues or human umbilical cord blood were differentiated using hanging drop cultures and compared to methylcellulose cultures. Cultured cells were analysed by FACS to assess differentiation.

Results: We describe a practical culture method by adapting the previously described hanging drop culture system to conditions allowing terminal differentiation of primary erythroid progenitors. Using minimal volumes of media and small numbers of cells, we obtained quantitative terminal erythroid differentiation within two days of culture in the case of murine cells and 4 days in the case of human cells.

Conclusions: The established methods for *ex vivo* culture of primary erythroid progenitors, such as methylcellulose-based Burst Forming Unit-Erythroid (BFU-E) and Colony Forming Unit-Erythroid (CFU-E) assays, allow the detection of committed erythroid progenitors but are of limited value to study terminal erythroid differentiation. We show that the application of hanging drop cultures is a practical alternative that, in combination with clonogenic assays, enables a comprehensive assessment of the behaviour of primary erythroid cells *ex vivo* in the context of genetic and drug-induced perturbations.

INTRODUCTION

The first haematopoietic culture methods for bone marrow and spleen cells were established in the mid sixties of the previous century. These methods, including Burst Forming Unit-Erythroid (BFU-E) and Colony Forming Unit-Erythroid (CFU-E) assays, rely on culturing cells in semi-solid media (Bradley and Metcalf 1966; Ichikawa *et al*., 1966) and are still widely used to assess the numbers and differentiation potential of haematopoietic progenitors. These cultures have been instrumental in determining the conditions and growth factors required for survival and differentiation of haematopoietic progenitors, such us interleukin 3 (IL-3), Stem Cell Factor (SCF) and Erythropoietin (Epo).

After multipotent haematopoietic cells become committed to the erythroid lineage, the cells remain proliferating until they are triggered to terminally differentiate. This process is mainly, but not exclusively, regulated by the paracrine action of Epo. The numbers of committed erythroid progenitors in a haematopoietic tissue or organ can be measured by *ex vivo* methylcellulose (MC)-based cultures. For example, reduced numbers of BFU-Es and CFU-Es are found in the bone marrow of patients with myelodysplastic syndromes, compared with healthy individuals (Backx *et al*., 1993). These cultures assay the clonogenic growth of progenitor cells. However, terminal erythroid differentiation occurs inefficiently in these cultures. By the time of harvesting, terminally differentiated cells are infrequently present and this is not improved by delaying harvesting time as differentiating cells eventually die. The colonies obtained are not homogeneous; they vary in size and shape indicating that lineage-committed cells can vary considerably in their proliferative potential (Metcalf 1986) (Metcalf 1992). This also affects the presence of terminally differentiated erythroid cells in each colony. Therefore, BFU-E and CFU-E assays are of limited value for the study of terminal erythroid differentiation. Furthermore, solid-state cultures are labour-intensive, making it difficult to study the effects of multiple genotypes and growth conditions.

Recently, a new culture method has been described in which sorted TER119-positive erythroid cells are seeded in fibronectin-coated wells and allowed to differentiate (Zhang *et al*., 2003). Differentiation was scored by Fluorescent Activated Cell Sorter (FACS) analysis, which is a very accurate tool. However, the seeding of cells in fibronectin-coated wells still does not solve the cell-isolation characteristic of methylcellulose-based cultures, and enucleation does not occur in a high percentage of cells.

It is possible to massively terminally differentiate erythroid cells in suspension cultures (Fibach *et al*., 1989; Dolznig *et al*., 2001). However, in many experimental settings it would be ideal to have a small-scale system in which erythroid differentiation can be induced consistently.

We are interested in studying the molecular basis of definitive erythropoiesis, which starts in the fetal liver at mid-gestation. EKLF and Gata1 are transcription factors

essential for erythroid differentiation. EKLF knockout fetal livers contain the same numbers of CFU-Es as wildtype fetal livers (Nuez *et al*., 1995; Perkins *et al*., 1995) but terminal differentiation is defective owing to a transcriptional failure that affects the expression of essential genes such as β-globin. Similarly, overexpression of Gata1 by β-globin regulatory elements specifically affects terminal differentiation of definitive cells (Whyatt *et al*., 2000). However, the fetal livers of these mice contain normal numbers of BFU-Es and CFU-Es.

We wished to develop a culture system to study terminal differentiation in detail. As a solution we turned to the hanging drop culture method, which was described previously for a variety of systems. It is most widely used to study T-cell development (Brenner *et al*., 1983) (Sagara *et al*., 1997; Tokoro *et al*., 1998) in so-called fetal thymic organ cultures (F-TOC). Hanging drop cultures are also used to study oocyte and embryo development (Sanders *et al*., 1978; Potter and Morris 1985; Spindler *et al*., 2000), Embryonic Stem (ES) cell differentiation (Prelle *et al*., 2000), and to test mesenchymal cell aggregation and macrophage functionality (Crowle and May 1978). So far, this culture system has not been applied to analyse terminal differentiation of erythroid precursor cells. Here, we describe the adaptation of this system to the study of terminal erythroid differentiation of fetal liver- and bone marrow cells from the mouse, and human cord blood cells.

The erythroid hanging drop (HD) cultures start with the seeding of 2.5 to $10x10^4$ cells in 20μl droplets of medium, which are suspended from the lid of a Petri dish filled with PBS. The standard medium contains Epo, hemin, insulin and Fetal Calf Serum (FCS), but completely synthetic media can also be used. Within two days, erythroid progenitors derived from mouse fetal liver or bone marrow differentiate quantitatively to mature erythrocytes. By FACS analysis and cytospin preparations we show that the cells undergo terminal erythroid differentiation, including enucleation. To demonstrate the usefulness of this assay, we have used HD cultures to study fetal erythroid progenitors from mice with perturbed expression of the transcription factors Gata1 and EKLF. The results show that the HD culture is an economical and convenient method to assess terminal erythroid differentiation, including enucleation. Hence we suggest the utilisation of HD cultures in combination with clonogenic assays for a better comprehension of erythropoiesis in the context of genetic or drug-induced perturbations and we foresee its application in diagnosis and drug testing in clinical trials.

MATERIALS AND METHODS

Mice

Gata1-overexpressing (Gata1-OX) mice express Gata1 from an X-linked transgene (Whyatt *et al*., 2000). Gata1-Ligand Binding Domain (Gata1-LBD) mice (R. F. and Nynke Gillemans, unpublished data, 1999, and Whyatt *et al* (Whyatt *et al*., 2000)) bear an autosomal Gata1 transgene that is fused to the mutant ligand binding domain (LBD™) of the estrogen receptor (Littlewood *et al*., 1995). Activation of the fusion protein is dependent on the addition of 4-hydroxy-tamoxifen (4-OH-T) (Picard 1994; Metzger *et al*., 1995). Gata1 knockout (Gata1-KO) mice were obtained by breeding mice bearing a modified *Gata1* gene flanked by lox*P* sites (Lindeboom *et al*., 2003; Gutierrez *et al*., 2004) with transgenic mice expressing Cre protein under the control of the zona pellucida 3 (Zp3) gene promoter (Lewandoski *et al*., 1997). The KO allele was maintained through breeding with heterozygous females (the *Gata1* gene is X-linked). EKLF knockout (EKLF-KO) mice were generated previously (Nuez *et al*., 1995).

Cells

Mouse fetuses were collected at 12.5 or 13.5 *days post coitum* (dpc). Fetal livers were dissected and disrupted to single cell suspensions by pipetting in Dulbecco Modified Eagle medium (DMEM) supplemented with 20% FCS and 1% penicillin/streptomycin (10000 U/mL penicillin/10000 mg/mL streptomycin stock solution). The cells were counted using an electronic cell counter (CASY-1, Schärfe Systems). Bone marrow cells were collected from adult mice. The femurs and tibias were isolated, cleaned of adjacent tissue and crushed in a porcelain mortar containing phosphate-buffered saline (PBS). The released bone marrow cells were further washed with PBS and collected in a clean tube through a cell strainer (Falcon) in order to get a single cell suspension. Human umbilical cord blood (UCB) cells were obtained from full-term pregnancies after obtaining informed consent according to the legal regulations in The Netherlands. Mononucleated cells were isolated by Ficoll density gradient centrifugation (1·077 g/cm2; Lymphoprep™, AXIS-SHIELD, Norway). Progenitors were initially expanded as described (Leberbauer *et al*., 2005).

Hanging Drop Cultures

Cells were spun down and resuspended in HD medium (DMEM supplemented with 20% FCS, 0.1% β-mercaptoethanol, $2x10^{-4}$ M hemin, 1% penicillin/streptomycin (stock), 2 U/mL Epo, 5 μg/mL insulin) at a density of $1.25x10^6$ cells/mL (cell concentrations up to $5x10^6$ cells/mL have been used successfully). 20μL drops containing approximately $2.5x10^4$ cells each were pipetted on the inner side of the lid of a tissue culture Petri dish. After distribution of the drops, the lid was gently inverted and placed on the Petri dish, which contained PBS to humidify the culture chamber (Figure 2D). Cultures were harvested after 1, 2 or 3 days

by washing the drops with PBS supplemented with 10% heat inactivated FCS. Five drops were sufficient for FACS analysis, and 30 drops for the preparation of protein extracts for Western blotting. Human erythroid progenitors derived from UCB were cultured with the standard HD medium and with synthetic media described previously (Leberbauer *et al*., 2005) supplemented either with 2 U/mL or 10 U/mL of Epo.

Methylcellulose cultures

Cells were harvested from fetal liver or bone marrow. After counting, $6x10^5$ cells were resuspended in 150 μL Alpha Modified Eagle medium (α-MEM). Cells were added then to 1.2 mL methylcellulose (Methocult, Stem Cell Technologies) supplemented with 2 U/mL Epo, 5 μg/mL insulin, $2x10^{-4}$ M hemin, and 1% penicillin/streptomycin (stock solution). After mixing thoroughly, cultures were plated in 35 x 10 mm Petri dishes. Cells were harvested after 2 or 3 days of culture by rinsing the plates 4 to 5 times with 2 mL PBS. The recovered cell suspensions were collected in a 15 mL Falcon tube and the final volume was adjusted to 15 mL with PBS. Cells were collected by centrifugation, counted and analysed by FACS.

Suspension cultures

Liquid suspension cultures to induce differentiation of primary erythroid cells derived from 13.5 and 12.5 dpc fetal livers were done as described previously (Dolznig *et al*., 2001).

FACS Analysis

FACS analysis was performed with $5x10^4$ events taken per sample. Single-cell suspensions were incubated with phycoerythrin (PE)-conjugated TER119 antibody (Pharmingen) to identify committed erythroid cells (Ikuta *et al*., 1990; Kina *et al*., 2000) and 7-aminoactinomycin-D (7AAD, Molecular Probes) to assess cell viability. Cell populations were divided as follows: live ($7AAD^-$), erythroid ($TER119^+$) and small (FSC^{low}).

Histological staining

Single cell suspensions were cytocentrifuged onto slides and the preparations were stained with neutral benzidine and histological dyes as described (Beug *et al*., 1982). Images were acquired with an Olympus BX40 microscope. The lenses used were Plan 40X/0.65 and Olympus Plan 100X/1.25. The acquisition software used was Viewfinder Lite Version 1.0.125 and Studio Lite Version 1.0.124, Pixera Corporation. Image processing was done in Adobe Photoshop 5.5.

Western Blotting

Nuclear extracts were prepared as described (Andrews and Faller 1991). The antibody

against Gata1 N6 (sc-265) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary anti-rat antibody conjugated to horseradish peroxidase was purchased from Dako (Dako Cytomation, Denmark). Enhanced Chemiluminescence detection was performed to develop the blots as described by the manufacturer (Amersham).

RESULTS

Analysis of erythroid terminal differentiation of hanging drop versus methylcellulose cultures

Erythroid cells are subject to several changes during terminal differentiation, both at the molecular and at the morphological level. The first recognisable erythroid precursor is the proerythroblast, followed by sequentially smaller cell types that have been classified by their staining properties as basophilic, polychromatic and orthochromatic erythroblasts. These cells enucleate to become reticulocytes, which undergo further maturation to erythrocytes. In the mouse, the proerythroblasts have a diameter between 11-13 μm. This size gradually decreases during erythroid differentiation, and reticulocytes have a diameter of 4-5 μm (Rifkind *et al*., 1974). A sequence of differentiating erythroid cells selected from bone marrow cytospins is depicted in Figure 1.

Figure 1. Definitive erythroid differentiation.
Sequence of erythroid cells, selected from bone marrow cytospins, in successive stages of differentiation from proerythroblast stage ("PrE", left) to reticulocyte ("R", right) passing through dividing proerythroblast, basophilic ("B", plus dividing basophilic cell), polychromatic ("P") and orthochromatic ("O") stages. Slides were stained as described in the Materials and Methods section. Original magnification 100X. (See colour picture in the cover).

We have taken advantage of this gradual size reduction to assess erythroid differentiation. We used the forward scatter (FSC) parameter of FACS analysis to determine the cell volume. In addition, we used the surface marker TER119, recognising all the cells beyond the proerythroblast stage, and 7AAD, which intercalates with the DNA of permeabilised dying cells. We have combined these three FACS parameters and analysed the cell volume of live (7AAD negative) and erythroid (TER119 positive) cells in order to gauge terminal differentiation.

Fetal liver cells from 13.5 dpc embryos were cultured *ex vivo* to induce erythroid differentiation using the HD culture method (Figure 2D). Cells were collected after 1 and 2 days of culture, counted, stained with TER119 and 7AAD, and analysed by FACS. Figure 2A shows sequential FACS graphs during two days of HD culture, demonstrating that the cells in the erythroid live ($TER119^{+}$ $7AAD^{-}$) fraction become smaller upon differentiation induction. A shoulder of smaller cells representing late polychromatic, orthochromatic and enucleated erythroid cells is already present in the graph corresponding to day 1 of culture. This is most likely due to the fact that fetal liver cells are not a synchronised

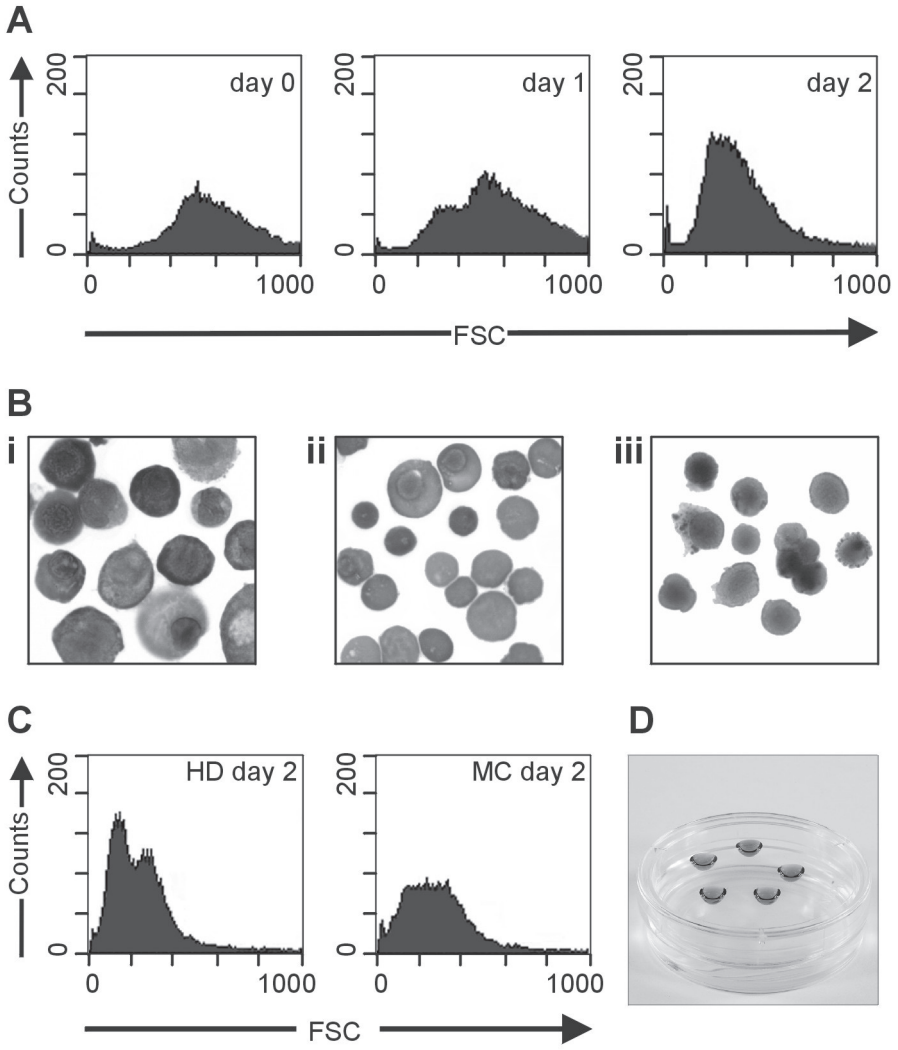

Figure 2. Terminal differentiation obtained in hanging drop cultures.
(A) Erythroid live (TER119^{+} 7AAD^{-}) cells plotted against the forward scatter (FSC) at day of collection (day 0), day 1 and day 2 of HD culture.
(B) Cytospins of sorted populations after HD culture, stained as described in the Materials and Methods section. Original magnification x100. (i) Erythroid large live (TER119^{+} FSC^{high} 7AAD^{-}), (ii) erythroid small live (TER119^{+} FSC^{low} 7AAD^{-}) and (iii) erythroid small dead cells (TER119^{+} FSC^{low} 7AAD^{+}).
(C) Erythroid live (TER119^{+} 7AAD^{-}) cells plotted against the forward scatter at day 2 after HD (left) and MC culture (right).
(D) Picture of hanging drop cultures.

erythroid progenitor pool. The cells at an intermediate differentiation stage at day 0 are therefore already terminally differentiating after 24 hours of culture.

In order to validate our FACS parameters, we performed cytological analysis of cells after 2 days of HD culture. We sorted the cultured cells in three populations: Erythroid live large cells ($TER119^+ 7AAD^- FSC^{high}$), erythroid live small cells ($TER119^+ 7AAD^- FSC^{low}$) and erythroid dead small cells ($TER119^+ 7AAD^+ FSC^{low}$). Cytospin pictures are shown in Figure 2B. The population of large erythroid and live cells ($TER119^+ 7AAD^- FSC^{high}$) contains the more immature cells of the culture, and is mainly composed of basophilic erythroblasts (blue cytoplasm, Figure 2Bi). In this population, the majority of the cells still have a nucleus and only a minority stains weakly for haemoglobin (brown cytoplasm). The population of small erythroid live cells ($TER119^+ 7AAD^- FSC^{low}$) contains mainly mature erythroid cells, beyond the orthochromatic stage, and positive for haemoglobin staining (Figure 2Bii). Most of these cells have enucleated. The dead erythroid small ($TER119^+ 7AAD^+ FSC^{low}$) population consists predominantly of expelled nuclei, occasionally still surrounded by a rim of cytoplasm (Figure 2Biii). This shows that terminal differentiation, including enucleation, is achieved efficiently in the HD cultures, and can be measured accurately with the FACS parameters used.

We then cultured 13.5 dpc fetal liver cells and compared the differentiation profile between HD and MC culture methods. By comparison, cells cultured in MC for two days lack the smallest cell size peak (Figure 2C). The proliferation rate, calculated as the ratio of the cell number at day 2 to the cell number at day 0, is 4- to 6-fold higher in the HD compared to the MC cultures (Table 1A). Since the concentration of Epo is equal in both cultures, this suggests that the forced cell proximity occurring due to gravity in the HD system, versus the cell isolation that characterises the MC culture, results in a growth advantage. The FACS analysis at day 2 (Table 1B) revealed that the percentage of dead cells in the MC cultures was 10% higher than in the HD cultures. The mean FSC value of dead cells in MC cultures is higher than in HD cultures (Table 1B). In HD cultures, a significant percentage of the dead population corresponds to expelled nuclei (FSC^{low}). In contrast, in MC cultures the dead population consists predominantly of cells that are nucleated (FSC^{medium}, FSC^{high}). This suggests that cell proximity plays an important role in cell survival during differentiation. The TER119 positive and live population was 15% higher in the HD culture. When this population was plotted against the forward scatter, the smallest cells (enucleated erythroid cells) were more frequent in the HD than in the MC culture (Figure 2B and Table 1B). After 2 days of culture, 35% of the total are enucleated cells in the HD culture while in the MC culture enucleated cells represent 20% of the total. The mean FSC value of erythroid and live cells is 18% higher in the MC cultures than that observed in the HD cultures (Table 1B). This shows that terminal differentiation is favourable in the HD system in comparison to the MC system.

Table 1A. Hanging drop versus methylcellulose. Cell numbers

	Hanging Drop	Methylcellulose
Cell Number day 0 (x10^5)	5	6
Cell Number day 2 (x10^5)	6.55±0.35	1.7±0.28
Ratio Cell Number day 2/day 0	1.31±0.07	0.28±0.03

Table 1B. Hanging drop versus methylcellulose. FACS analysis at day 2

	Hanging Drop	Methylcellulose
% Dead (7AAD$^+$)	25.1±0.3[a]	35.7±0.3[a]
Mean FSC value of Dead	286.3±6.4	356.9±2.4
% Non-erythroid Live (Ter119$^-$ 7AAD$^-$)	9.9±1.3	12.1±0.3
% Erythroid Live (Ter119$^+$ 7AAD$^-$)	61.2±1.2	45.2±0.2
% Enucleated of Total (Ter119$^+$ 7AAD$^-$ FSC^{low})	35.1±1.7	19.1±0.3
% Small nucleated of Total (Ter119$^+$ 7AAD$^-$ FSC^{medium})	24.4±2.1	23.7±0.2
% Enucleated of Erythroid Live (FSC^{low})	57.5±3.6	42.3±0.8
% Small nucleated of Erythroid Live (FSC^{medium})	39.9±2.9	52.4±0.3
Mean FSC value of Erythroid Live	251.9±8.3	296.7±2.4

Mean and standard deviation depicted, n=6. a) P<0.001.

Analysis of erythroid terminal differentiation of hanging drop- versus liquid cultures

Terminal differentiation of murine or human erythroid progenitors can be obtained in suspension in a variety of liquid culture systems that have been described previously (Dolznig *et al*., 2001; Leberbauer *et al*., 2005). In the HD culture system, cultured erythroid progenitors are in forced proximity due to the action of gravity. We wished to compare HD cultures with a well established liquid culture method to differentiate erythroid progenitors *ex vivo* (Dolznig *et al*., 2001).

We isolated fetal liver cells at 13.5 dpc and cultured them in HD or in suspension under differentiation conditions as described (Dolznig *et al*., 2001). We collected the cells after two days of culture, and analysed them by FACS. We could not detect any significant difference in the cell volume reduction, suggesting that erythroid differentiation was induced efficiently in both culture systems (data not shown). However, when analysing the display of CD71 and TER119 surface markers, we observed a more coordinated expression in the HD- compared to the liquid cultures. CD71 is expressed in early erythroid precursors and is downregulated beyond the proerythroblast stage. TER119 is expressed at the proerythroblast stage and is upregulated towards the last stages of differentiation. We observed the expected CD71- and TER119-based expression profiles in the HD cultures. However, in the liquid cultures the cell size reduction was not accompanied by downregulation of CD71, or upregulation of TER119 (Figure 3A). Consequently,

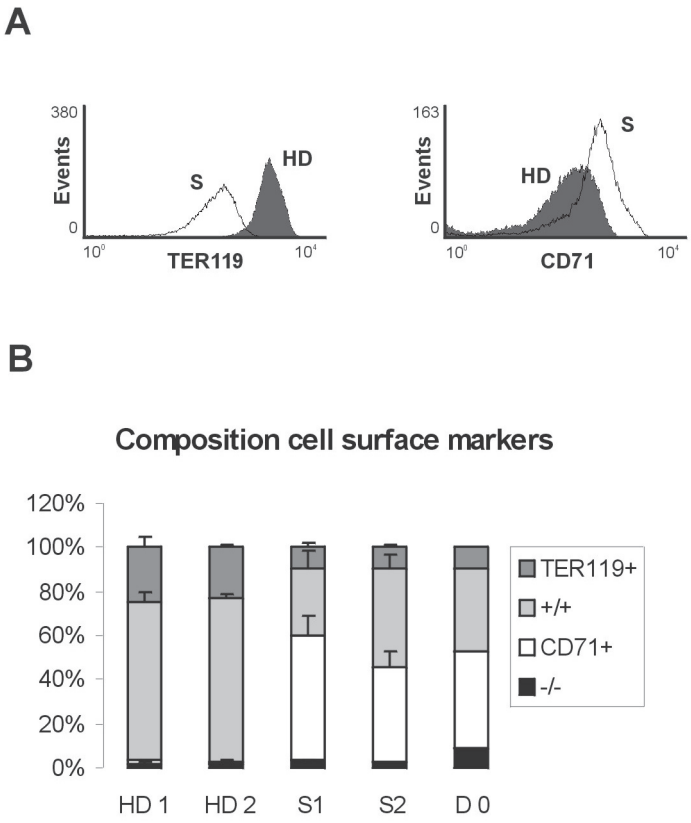

Figure 3. HD versus liquid suspension cultures.
(A) Histograms showing TER119 (left) or CD71 (right) expression levels in HD (hanging drop) and S (suspension) cultures. Live cells are plotted (7AAD$^-$).
(B) Cell composition of live cells based on CD71 and TER119 expression. Live cells (7AAD$^-$) were sorted and subdivided in the following populations: -/- (CD71$^-$ TER119$^-$), CD71$^+$, +/+ (CD71$^+$ TER119$^+$) and TER119$^+$. Average and standard deviations are depicted. At least three samples are analysed per group. HD, hanging drop; S, suspension culture. D0 is the day of collection of the fetal liver cells.

cells expressing only CD71 were more frequent in the liquid cultures compared to HD cultures (Figure 3B). Taken together, these data show that erythroid differentiation occurs in a more coordinated manner in the HD cultures indicating that, compared to liquid cultures, differentiation of erythroid cells in HD cultures mimics the *in vivo* situation more accurately.

Application of hanging drop cultures to study Gata1 mutants

Next, we determined whether the defect in definitive erythropoiesis found in Gata1 overexpressing (Gata1-OX), and EKLF knockout (EFLF-KO) mice could be studied with HD cultures.

Gata1-OX mice die of anaemia at 13.5 dpc; cells overexpressing Gata1 are unable to undergo cell cycle arrest and are blocked in terminal differentiation (Whyatt *et al*., 2000). We cultured cells from wildtype, Gata1-OX males (which have pancellular overexpression of Gata1) and heterozygous Gata1-OX females (which have heterocellular overexpression of Gata1, owing to X-inactivation). We compared HD versus MC cultures and assessed the phenotypic features that can be detected with these culture systems (Table 2A). The defect in differentiation of Gata1-OX cells is best appreciated by comparing the Gata1-OX males with the wildtypes. Compared to wildtypes, Gata1-OX cultures displayed a higher percentage of dead cells both in HD- and in MC cultures (Table 2A). The percentage of differentiated cells ($Ter119^{+}$ $7AAD^{-}$ FSC^{low}) reveals the defective phenotype of Gata1-OX male cultures. The cells derived from Gata1-OX females show an intermediate phenotype in both cultures in terms of differentiation due to the heterocellularity of Gata1 overexpression. However, this intermediate phenotype of cells derived from Gata1-OX females is more significant in HD cultures (15% difference compared to WT in HD cultures versus a 5% difference compared to WT in MC cultures) (Table 2A).

Table 2A. Differentiation of Gata1-OX primary cells after HD and MC culture.

	Hanging Drop		Methylcellulose	
	% Differentiation	% Dead cells	% Differentiation	% Dead cells
WT	100±1.6	22±1.5a	100±1.4	31.7±3.1a
Gata1-OX F	83.1±3.1	25.6±3.5b	93.6±2.0	34.4±1.2b
Gata1-OX M	46.6±1	41.1±1.3c	53.5±0.4	51.5±3.0c

% Differentiation: the percentage of Ter119+/7AAD-/FSClow cells, normalised to the wildtype value. % Dead cells: the percentage of dead cells of total. The mean and standard deviation are depicted. WT, wildtype, OX, overexpressing, F, female, M, male. At least three samples were analysed per genotype. a, b, c) P<0.001.

Table 2B. FACS analysis of HD and MC cultures of EKLF mutant cells.

	Hanging Drop		Methylcellulose	
	Mean FSC	% Dead cells	Mean FSC	% Dead cells
WT	300 ± 5	13.6 ± 1.1a	364 ± 11	23.1 ± 1.3a
EKLF-KO	423 ± 6	17.4 ± 4.2b	489 ± 5	29.1 ± 0.8b

The percentage of dead cells (7AAD+) and the mean FSC value obtained after FACS analysis of gated live cells (7AAD-) are depicted in the table. Mean and standard deviation of six samples per genotype were calculated. WT, wildtype; KO, knockout. a, b) P<0.001.

EKLF knockout (EKLF-KO) mice die of anaemia at mid-gestation (Nuez *et al*., 1995; Perkins *et al*., 1995). Surprisingly, CFU-E cultures show the same colony numbers when comparing KO mice with wildtype littermates. This suggests that the erythroid defect in EKLF-KO cells occurs during terminal differentiation. To further investigate this, we grew 12.5 dpc EKLF-KO and wildtype fetal liver cells in HD cultures, and compared these results

to those obtained with MC cultures (Table 2B). EKLF-KO definitive erythroid cells barely express TER119 (R. D., unpublished data, 2001). These cells also fail to express TER119 in HD and MC cultures (data not shown). We therefore displayed the differentiation profiles by plotting the live ($7AAD^-$) cells against the forward scatter. Consistent with previous data, the percentage of dead cells in MC cultures was 10% higher than in HD cultures (Table 2B). This was found for each genotype. The survival of EKLF-KO cells was slightly better in HD than in MC cultures. Concordant with the absence of TER119 expression, cells that remained alive in the EKLF knockout cultures were larger than the live cells from wildtype littermates, and this could be observed in both HD (40% larger) and MC (35% larger) cultures (Table 2B).

We conclude that the differentiation potential of erythroid progenitors can be assessed conveniently and sensitively by the HD method. Application of the HD method is particularly powerful when combined with the MC cultures, which primarily determine the number of progenitors present in a particular tissue. Combination of these techniques provides a more comprehensive picture of the process of erythropoiesis and its failure, as exemplified by the analysis of EKLF mutant fetal liver cells. Therefore, the HD method is useful for analysing defects in terminal erythroid differentiation resulting from genetic perturbations.

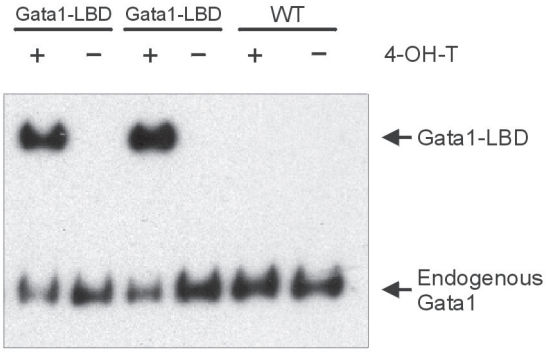

Figure 4. Tamoxifen treatment of Gata1-LBD expressing fetal liver cells during HD culture.
Gata1 was detected by Western blotting analysis in nuclear extracts of cells cultured in HD. WT, wildtype; 4-OH-T, 4-hydroxy-tamoxifen. The Gata1-LBD fusion protein is detected in the nuclear extracts of tamoxifen treated (+ 4-OH-T) transgenic cultures only.

Hanging drops as a tool for *in vitro* Tamoxifen-inducible expression

We previously generated transgenic mice bearing an inducible Gata1-Ligand Binding Domain (Gata1-LBD) fusion protein (Littlewood *et al*., 1995; Whyatt *et al*., 2000). This fusion protein allows the control of the nuclear localisation of Gata1 through treatment with 4-hydroxy-tamoxifen (4-OH-T) (Picard 1994; Metzger *et al*., 1995). Fetal liver cells isolated from the Gata1-LBD transgenic mice were cultured in HD in the absence or presence of 1μM 4-OH-T. The cells cultured with 4-OH-T showed a mild but significant reduction of $TER119^+$ $7AAD^-$ FSC^{low} cells (13%) (Whyatt *et al*., 2000).

After 2 days of HD culture, we collected cells from 30 drops and isolated nuclear proteins. The extracts were run on 10% SDS-PAGE gels, and after blotting, membranes were probed with an antibody against Gata1 (Figure 4). The results demonstrate that Gata1-LBD is clearly detected in the nuclear fraction after treatment with 4-OH-T, but not in the nuclear fractions of untreated samples. Therefore, the HD culture method allows utilisation of inducible systems, as exemplified by LBD fusion proteins, and analysis of protein expression by Western blotting. Furthermore, the system provides a convenient method to test the effects of pharmacological compounds on terminal erythroid differentiation.

Differentiation of adult primary cells derived from bone marrow

To test whether the HD system is also suitable for the analysis of erythroid progenitors derived from the bone marrow, we isolated total bone marrow cells from adult mice and subjected them to HD culture under standard conditions. FACS analysis shows the cell size of the erythroid live population ($TER119^+$ $7AAD^-$) during HD culture (Figure 5A). The cell composition through differentiation shows that the percentage of cell death increases slightly after 2 days of culture (Figure 5B). The percentage of live erythroid cells is reduced at day 2 compared to day 1, but the percentage of the smallest cells (terminally differentiated) almost doubles (Figure 5B-C). We conclude that the differentiation of cells from adult mouse bone marrow is very efficient in HD culture, and that terminal erythroid differentiation is already achieved after one day of culture.

Differentiation of human erythroid progenitors derived from cord blood

Human umbilical cord blood (UCB)-derived erythroid progenitors from two different batches were differentiated in HD culture. We cultured the cells in triplicate in HD standard medium and in synthetic media (Leberbauer *et al*., 2005) supplemented with either 2 U/mL or 10 U/mL of Epo. UCB-derived erythroid progenitors can be differentiated in suspension and after 6 days of culture they reach a minimum cell size of 7.5μM (Leberbauer *et al*., 2005). We collected cells after 3, 4 and 7 days of HD culture and analysed cell viability and size reduction by FACS after staining the cells with 7AAD (Figure 6A and 6B). We also measured cell diameter with the CASY counter (Figure 6C). There were no major differences in cell viability throughout the culture period and amongst the different media, with the exception of day 7, when cells cultured in the HD standard medium displayed slightly better survival rates (Figure 6A). By FACS, we analysed cell size reduction of live cells during differentiation by plotting them against the forward scatter. The size distribution of live cells differentiating in HD standard medium at day 0, 3, 4 and 7 is shown in Figure 6Bi. By day 4 of culture, efficient differentiation is observed. By day 7, a shoulder of smaller cells is visible and the average cell size is reduced (see also Figure 6C). Differentiation appears to proceed slightly faster in the HD standard medium than in the synthetic media at day 4 of culture (Figure 6Bii). However, by day 7 the differentiation

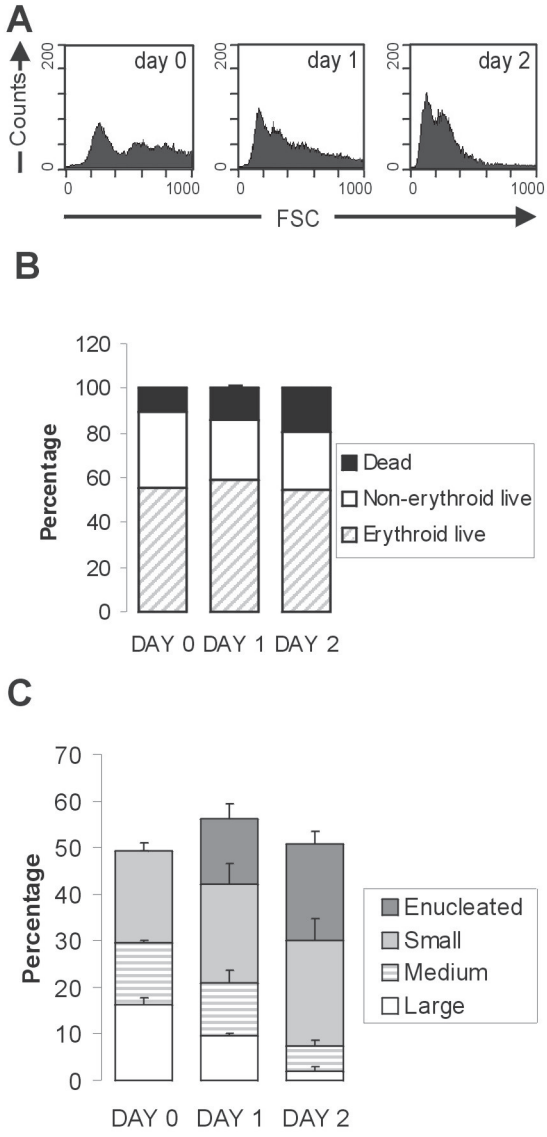

Figure 5. HD cultures of wildtype bone marrow cells from adult mice.
(A) Erythroid live (TER119$^+$ 7AAD$^-$) cells plotted against the forward scatter (FSC) at day of collection (day 0), day 1 and day 2 of HD culture.
(B) Cell composition. The dead (7AAD$^+$), erythroid live (TER119$^+$ 7AAD$^-$) and non-erythroid live (TER119$^-$ 7AAD$^-$) populations are depicted at day of collection, day 1 and day 2 of HD culture.
(C) Differentiation profile of the erythroid live (TER119$^+$ 7AAD$^-$) population at day of collection, day 1 and day 2 of HD culture. Cells were subdivided as enucleated, small, medium and large based on the forward scatter.

efficiency of cells is similar between the different media (Figure 6Biii). We measured cell diameters at different times of differentiation using a CASY cell counter (Figure 6C). The average diameter of differentiated cells at day 4 of culture was below 7.5μM, and a further reduction was observed by day 7. Collectively, we conclude that HD cultures can be used to study terminal differentiation of human erythroid progenitors.

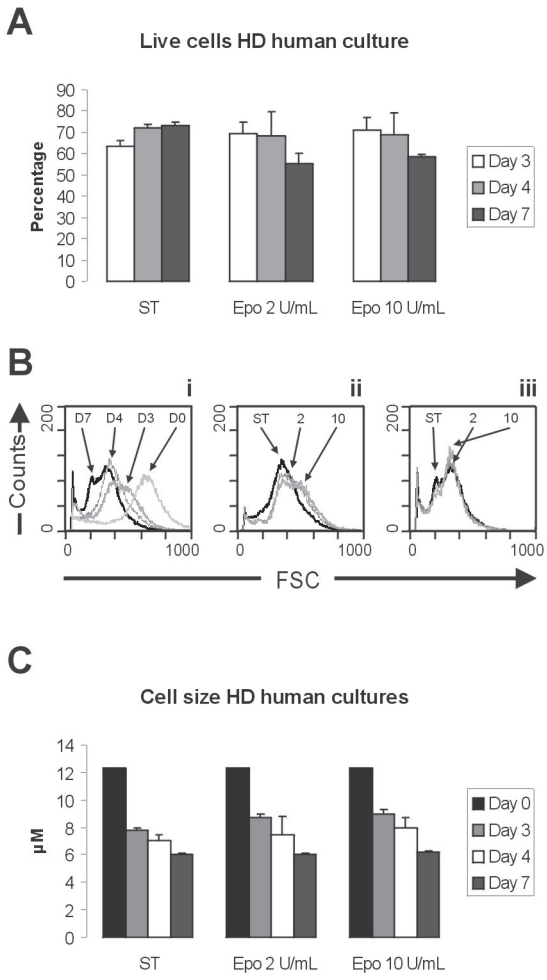

Figure 6: HD culture of human UCB-derived erythroid progenitors.
(A) Percentage of live cells during culture comparing standard (ST) and synthetic media with different concentrations of Epo, as indicated. Average and standard deviations are depicted.
(B) Graphs depicting differentiation of human erythroid progenitors. Live cells ($7AAD^-$) were plotted against the forward scatter (FSC). Standard medium cultures at day 0, 3, 4 and 7 (D0, D3, D4, D7) (i). Comparison of standard (ST) and synthetic media (2 U/mL Epo -2- and 10 U/mL Epo -10-) at day 4 (ii) and at day 7 (iii).
(C) Cell diameter during culture comparing standard and synthetic media with different concentrations of Epo, as indicated. Average and standard deviations are depicted.

DISCUSSION

We applied the previously described hanging drop (HD) method to differentiate primary erythroid cells (fetal or adult derived) *ex vivo*. In combination with FACS analysis, this method allows a consistent quantitative analysis of differentiation and cell composition of the cultured erythroid cells, which differentiate efficiently within two days of culture. The advantages of this culture method are that the numbers of cells, amounts of medium, expenses, and time to set up the assay have all been minimized. The HD assay is also very reproducible between independent experiments.

We compared cells cultured in HD with cells cultured in methylcellulose (MC) with the same concentrations of Epo, hemin and insulin, grown for the same time. We detected a 4- to 6-fold higher cell number in the HD cultures. The cell numbers are maintained in HD compared to the starting cell number, but they are reduced drastically in MC. Cells in MC culture grow isolated from each other and in the HD culture they are in forced proximity due to the action of gravity. Non-erythroid cells will eventually die in both cultures, because the growth conditions favour the survival of erythroid cells. The percentage of dead cells is consistently higher in MC cultures, suggesting that cell proximity enables mechanisms essential for cell survival in the erythroid compartment. Terminal differentiation is efficiently induced in the HD cultures. Terminal differentiation also occurs in the MC culture, but in our hands this is less reproducible and less efficient than in the HD system. Proliferation is required for differentiation (Allen and Testa 1991) and the forced cell proximity in the HD might favour proliferation towards differentiation of the cultured erythroid progenitors. Furthermore, auto-regulatory loops that stimulate differentiation are believed to exist *in vivo*. Such loops are part of homeostatic mechanisms that have been described as “community effects” (Gurdon 1988). It is likely that the high cell density and the 3D characteristic of HD cultures allow at least a partial reproduction of these community effects. In MC cultures, cells are grown in isolation and the initial events are therefore independent of neighbouring cells. In conclusion, HD cultures provide a convenient and efficient system for the study of terminal differentiation of erythroid cells. The combination with classical MC cultures is very powerful, because the MC cultures enable a precise determination of the number of erythroid precursors (BFU-E and CFU-E) present in a tissue or organ, while the HD method can be used to assess the differentiation potential of the erythroid cell population in a quantitative manner. Thus, the two methods address distinct properties of erythroid precursors. This is best exemplified by the analysis of EKLF-KO mice presented here. In classical BFU-E and CFU-E assays, EKLF knockout fetal livers contain similar numbers of erythroid progenitors as wildtype fetal livers (Nuez *et al*., 1995; Perkins *et al*., 1995). However, the EKLF knockout progenitors are defective in terminal differentiation, which is most clearly revealed by the HD assay.

In order to show the specificity of the HD culturing system, we cultured erythroid

cells overexpressing Gata1 in a pancellular (overexpressing males) and in a heterocellular (overexpressing heterozygous females) expression pattern. We also compared HD with MC cultures. Gata1 overexpressing erythroid cells survive beyond the proerythroblast stage before succumbing to defective differentiation (Whyatt *et al*., 2000). In both culture systems we could detect the defect associated with Gata1 overexpression.

We compared the HD culture with a described method to differentiate erythroid cells in suspension (Dolznig *et al*., 2001). Cell size reduction was accomplished efficiently in both culture systems, but we observed deregulated expression of surface markers in the suspension cultures. Cells differentiating in HD cultures showed the expected pattern of expression of the markers analysed. This coordinated differentiation might be favoured in the HD cultures through the forced close proximity of the cells, similar to the situation *in vivo*. The HD assay, however, might not completely mimic the cell-cell interactions that occur in the erythroblastic island *in vivo* (Bessis 1958; Breton-Gorius *et al*., 1991). The culture and reconstitution of erythroblastic islands *ex vivo* has been performed by other groups (Iavarone *et al*., 2004; Zackharov 2004). Thus, the combination of HD assays with these cultures might be very useful for functional studies of intercellular communication during erythroid differentiation.

To test other applications and possibilities of the HD culture system, we used erythroid cells expressing a Gata1-LBD fusion protein to show that the fusion protein can be activated by the addition of the pharmacological compound 4-OH-T to HD cultures. Activation of Gata1-LBD affects differentiation of the cells in the HD cultures in a manner similar to that observed with overexpression of Gata1 alone (Whyatt *et al*., 2000). Thus, the HD assay can be used to screen for the effects of pharmacological compounds on terminal erythroid differentiation and also in the molecular analysis of proteins, DNA and RNA.

We performed HD culture on human cells derived from UCB and confirmed that human erythroid progenitors derived from UCB can be differentiated in HD cultures using both standard and completely synthetic culture media. As expected, terminal differentiation of the human cells requires more time than observed with mouse cells. With the human cells, efficient differentiation is reached at day 4 of culture, as opposed to day 2 for the cultures of mouse cells. Interestingly, human progenitors appeared to differentiate faster in HD cultures than in suspension cultures (Leberbauer *et al*., 2005).

In summary, we have described a differentiation assay for primary erythroid cells that is complementary to the classical liquid and semi-solid based cultures. 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 genotypes, growth media, and biological and pharmaceutical compounds. Furthermore, erythroid cells terminally differentiate and enucleate quantitatively after two to four days of culture. The hanging drop culture method adds a useful tool to the arsenal available for the analysis of the biological characteristics

of erythroid cells.

Acknowledgements

We would like to thank Dr. M. de Bruijn for advice, assistance with mouse dissection and FACS analysis, C. Snoys for assistance with cell-sorting experiments, Dr. T. B. van Dijk for advice and Tom de Vries Lentsch for making the pictures of the HD cultures. This work was supported by NWO (Nederlandse Organisatie voor Wetenschappelijk Onderzoek) and KWF (Kanker Bestrijding) grants to S. P., F. G. and D. W.

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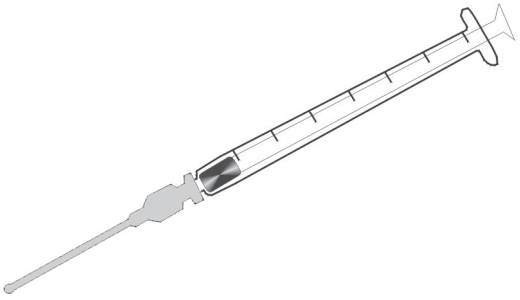
APPENDIX II

TAMOXIFEN INDUCED RECOMBINATION OF GATA1 IN ADULT MICE



Tamoxifen induced recombination of Gata1 in adult mice

Laura Gutiérrez, Frank Grosveld and Sjaak Philipsen.

ABSTRACT AND INTRODUCTION

We demonstrated that REDS signalling mechanism is homotypic by knocking out Gata1 thus ablating the wildtype erythroid population in Gata1 overexpressing females carrying a conditional *Gata1* locus on one X chromosome and an overexpressing *Gata1* allele on the other X chromosome. We reproduced the same crossing strategy and substituted the pEV-Cre mice with mice that ubiquitously express a tamoxifen inducible form of Cre protein with the aim of knocking out Gata1 in adult mice. We optimised the administration of tamoxifen in order to get efficient recombination in the erythroid compartment and we show that compound females suffer from severe anaemia as a consequence of the treatment.

MATERIAL AND METHODS

Mice

Mice overexpressing Gata1 were previously described (Whyatt *et al*., 2000). Gata1-lox mice were previously generated (Lindeboom *et al*., 2003; Gutierrez *et al*., 2004). Mice expressing ER-Cre were used (Vooijs *et al*., 2001). The breeding strategy is as in Chapter 3, but substituting the pEV-Cre mouse with the ER-Cre mouse.

Tamoxifen treatments

Mice were fed tamoxifen (Sigma) with a feeding needle. Tamoxifen was solved in sunflower oil (Sigma), sonicated, and stored in aliquots at –20°C during treatment.

Acute treatment:

Mice were fed during 5 days with 8mg/day and collected at day 8.

Chronic treatment:

Mice were fed during four days with 5mg/day and allowed to rest during the next three days. This sequence was repeated three times. At the end of the treatment, which lasts 21 days, efficient recombination of Gata1 was found.

Comments on the materials and methods:

The standard protocols described are based on injecting intraperitoneally 8mg/day for 5 days and collecting the animals on the 8th day. However, intraperitoneal injections of the tamoxifen solved in oil are very disturbing to the mice. We opted for feeding the tamoxifen to the mice instead of adding it to the drinking water in order to have a better control of the treatment. Administration of tamoxifen to the mice with a feeding needle induces more efficient recombination than intraperitoneally injected tamoxifen (Dr. A. Berns, personal communication). When we performed the acute protocol, we observed a fast recovery of the anaemia in the females were recombination was involved. This indicates that in the bone marrow, the target tissue, proliferation is occurring at a high rate, and if recombination is not efficient, wildtype progenitors will outgrow recombined progenitors. We thought of a chronic treatment as a solution. To avoid toxicity of the tamoxifen, the mice were allowed to rest for three days between series of four days of treatment. The duration of the treatment spans 18 to 21 days and full recombination of a gene in the bone marrow is assured.

Blood analysis

Blood of treated animals was collected by eye puncture at different time points during treatment and haemotological analysis was performed.

FACS analysis

Cells from bone marrow, blood, spleen and peritoneal wash of treated mice were analysed by FACS sorting and the following stains and antibodies were used: 7-actinomycin-C

(7AAD, BD Biosciences), CD71-FITC, TER119-PE, CD11c-APC, CD11b-PE, B220-FITC, MHCII-PE, CCR3-PE, F4/80-FITC (all BD Biosciences).

RESULTS

Acute treatment leads to a temporarily mild anaemia that is overcome shortly after the end of treatment.

We fed 8mg/day of tamoxifen during 5 days to the following mice: 3 ER-Cre males, 3 OX|Gata1-lox females, 2 heterozygous Gata1-lox females and 3 compound females. One heterozygous Gata1-lox|ER-Cre female died the last day of treatment but didn't present any symptoms of anaemia (data not shown). Blood was collected at day 7, day 20 and day 24 after the end of the treatment. At day 7 after treatment compound females showed a very mild anaemia, which was only detected in the haemoglobin and haematocrit levels but not in red cell blood numbers (Figure 1). Differences were not significant. At day 20 after treatment all mice had recovered and were normal at day 24 after treatment. Mice were not collected and recombination analysis was not done, as the phenotype was not as expected. This experiment led to the idea that efficient recombination in cells of the haematopoietic compartment would require a chronic treatment with tamoxifen.

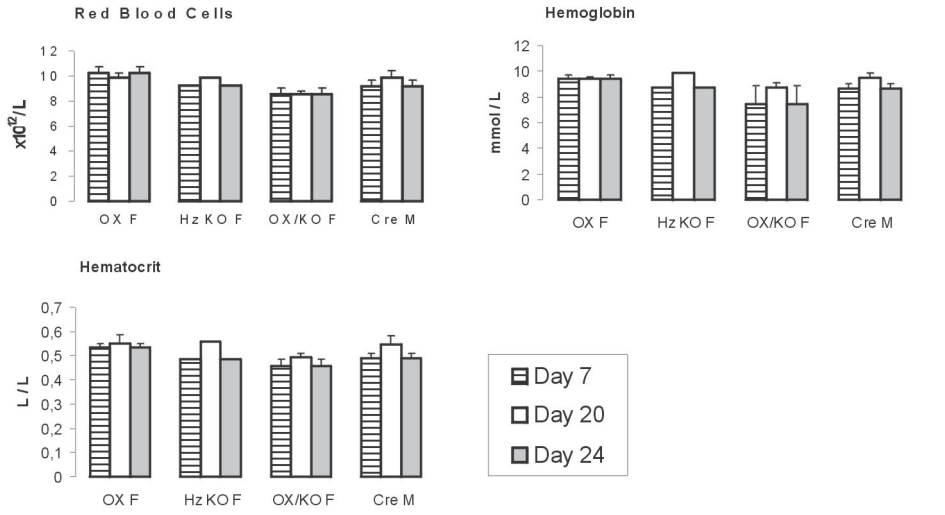


Figure 1. Acute tamoxifen treatment to induce recombination of Gata1.
Mice were treated with tamoxifen during 4 days. Blood was collected at day 7, 20 and 24 after treatment. The number of red blood cells, concentration of haemoglobin and haematocrit content are depicted per genotype. Treatment did not cause significant anaemia. Mice were almost normal at day 7 and fully recovered at day 20 after treatment.

Chronic intermittent tamoxifen treatment allows complete recombination of Gata1, which leads to severe anaemia in compound females.

We fed mice chronically with tamoxifen as explained in the Materials and Methods section. We analysed 2 ER-Cre males, 1 heterozygous Gata1-lox|ER-Cre female, 3 OX|Gata1-lox females and 3 compound females. At day 13 of treatment one ER-Cre male died due to stress after the injection. One compound female died with severe anaemia at day 16 and so did the heterozygous Gata1-lox|ER-Cre female at day 17. Blood was collected during treatment at days 10, 17 and 22. Mice were executed at day 22 for further analysis. The recombination event was measured by PCR and Southern Blot and was found to be 70-90% efficient in the expected genotypes. Data from haematological analysis of the blood is shown in Figure 2. As it can be seen, Gata1 OX females remain normal during treatment, so do the males, but the compound females are severely affected, showing that knocking out Gata1 affects the erythroid compartment.

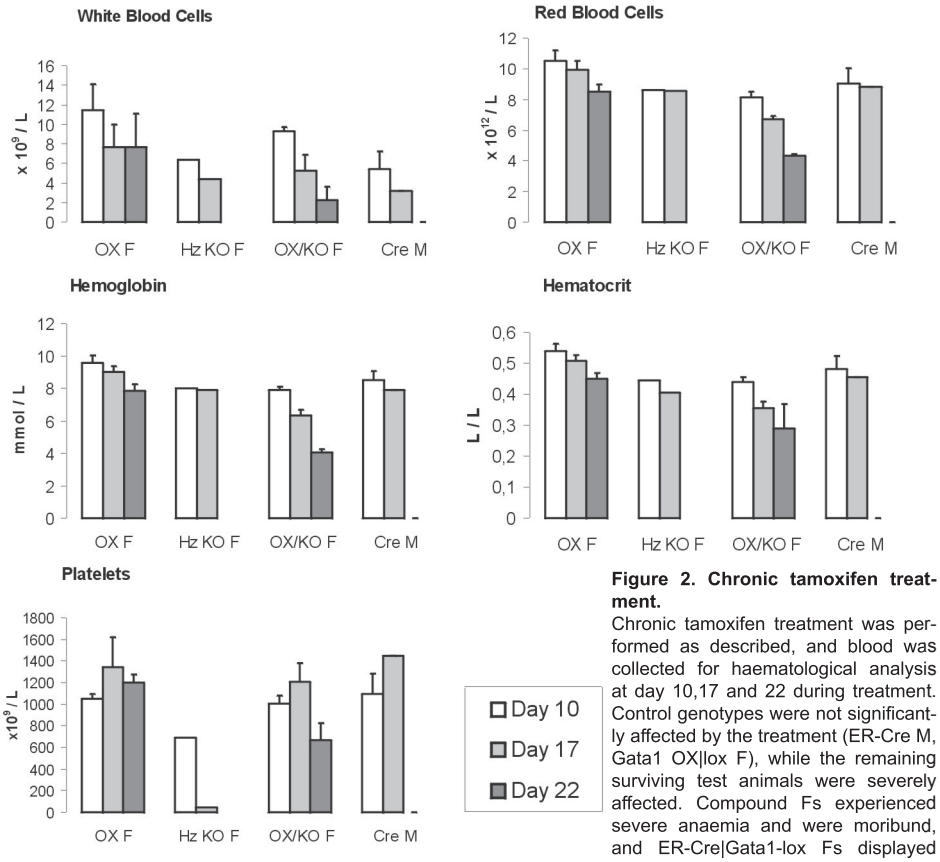


Figure 2. Chronic tamoxifen treatment.
Chronic tamoxifen treatment was performed as described, and blood was collected for haematological analysis at day 10,17 and 22 during treatment. Control genotypes were not significantly affected by the treatment (ER-Cre M, Gata1 OX|lox F), while the remaining surviving test animals were severely affected. Compound Fs experienced severe anaemia and were moribund, and ER-Cre|Gata1-lox Fs displayed very low platelet numbers.

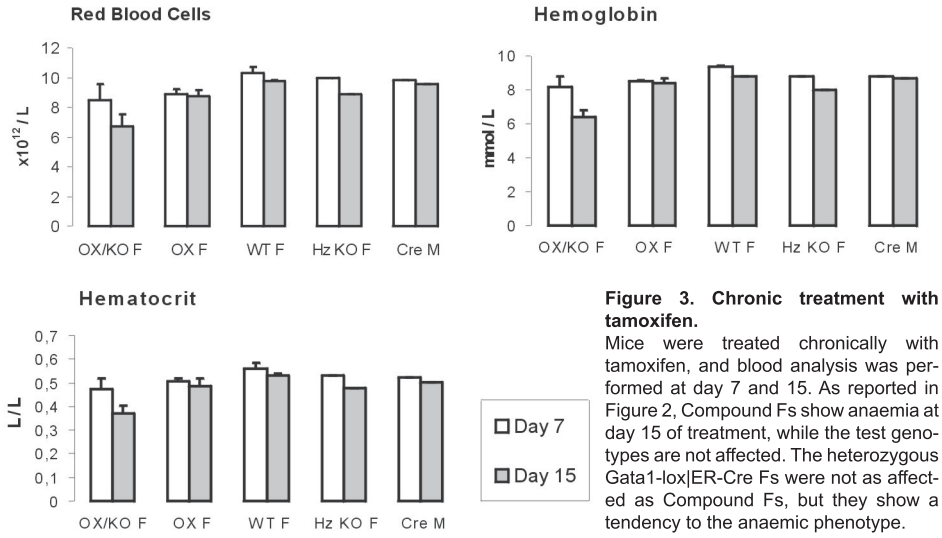


Figure 3. Chronic treatment with tamoxifen.
Mice were treated chronically with tamoxifen, and blood analysis was performed at day 7 and 15. As reported in Figure 2, Compound Fs show anaemia at day 15 of treatment, while the test genotypes are not affected. The heterozygous Gata1-lox|ER-Cre Fs were not as affected as Compound Fs, but they show a tendency to the anaemic phenotype.

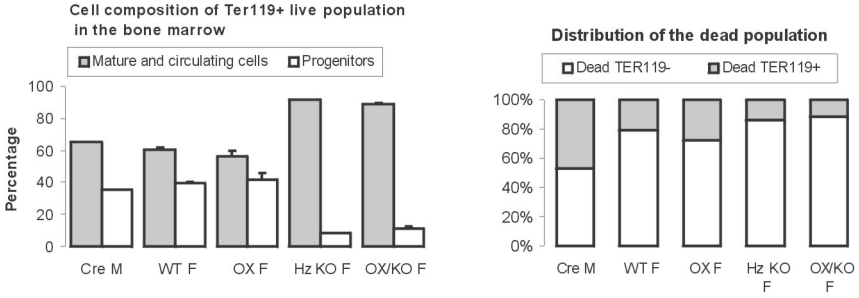
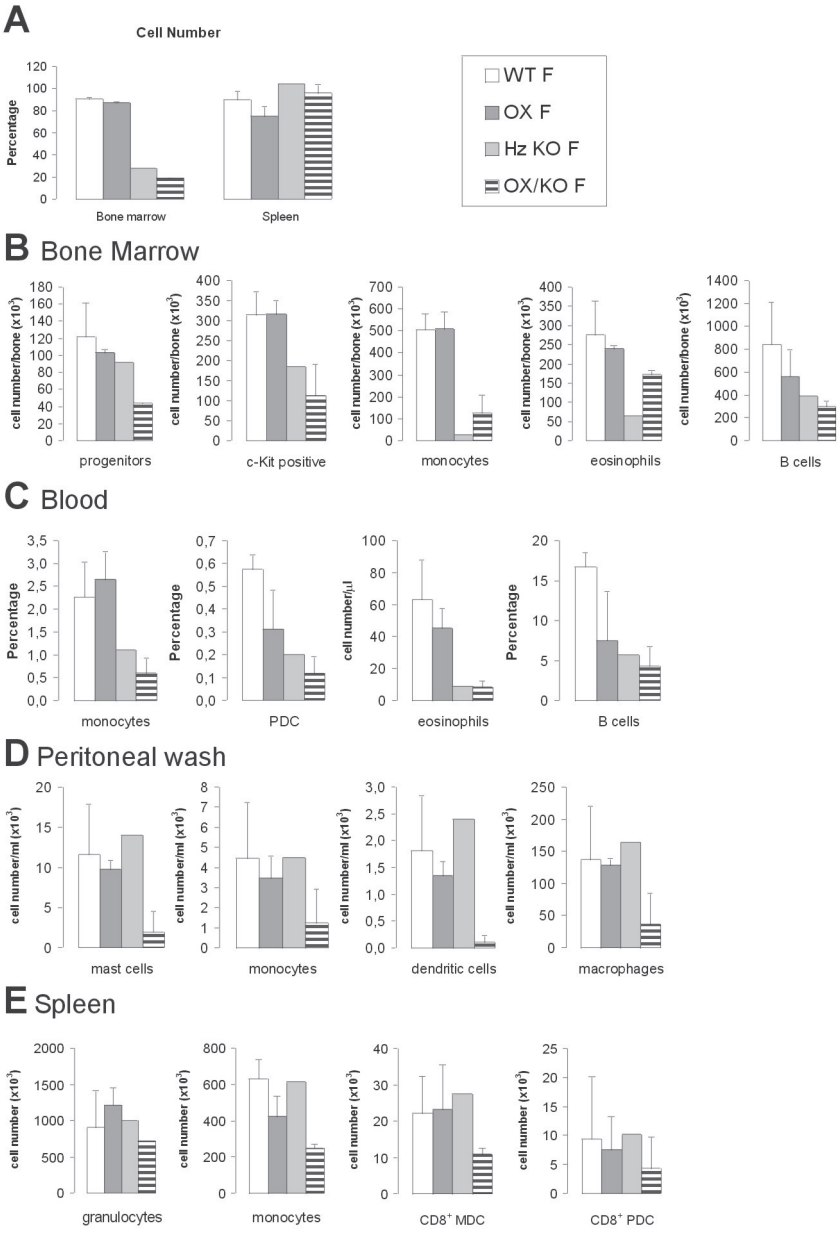


Figure 4. Analysis of the erythroid compartment of the bone marrows of tamoxifen treated mice.
The percentage of progenitor and circulating cells of the live fraction are depicted in the graph on the left. Note the lack of progenitors in the test genotypes. The percentage of TER119 positive cells of the dead fraction is depicted in the graph on the right. The majority of dead cells in the test genotypes are TER119 negative cells, indicating that the apoptotic events occurring in progenitors due to Gata1 loss occur prior to the TER119 expression stage.

Figure 5. Analysis of haematopoiesis in different organs (next page).
A.- The total cell number of the bone marrow and the spleen of treated mice are depicted. Note the reduction of cell number in the bone marrow of the test genotypes (Hz KO and OX/KO).
B.- The numbers of early progenitors, cKit positive cells, monocytes, eosinophils and B cells in bone marrow are depicted. All the cell types are reduced in the test genotypes, although the total cell numbers are already reduced in those bone marrows.
C.- In blood, the percentage of monocytes, PDCs, eosinophils and B cells are depicted.
D.- Peritoneal wash analysis. The numbers of mast cells, monocytes, dendritic cells and resident macrophages are reduced in test genotypes.
E.- In spleen, the numbers of granulocytes, monocytes, PDCs and MDCs are depicted. While the numbers of granulocytes seem to be not so affected, the numbers of monocytes and DCs in this tissue are reduced.


A
Cell Number
Percentage
120
100
80
60
40
20
0
Bone marrow
Spleen
WT F
OX F
Hz KO F
OX/KO F
B Bone Marrow
cell number/bone (x10³)
progenitors
c-Kit positive
monocytes
eosinophils
B cells
C Blood
Percentage
cell number/µl
monocytes
PDC
eosinophils
B cells
D Peritoneal wash
cell number/ml (x10³)
mast cells
monocytes
dendritic cells
macrophages
E Spleen
cell number (x10³)
granulocytes
monocytes
CD8⁺ MDC
CD8⁺ PDC

The same experiment was repeated with 1 ER-Cre male, 2 wildtype females, 1 heterozygous Gata1-lox|ER-Cre female, 4 Gata1 OX|Gata1-lox females and 2 compound females. Blood was collected at day 6 and day 14 during treatment and mice were collected at day 20. At the day of collection FACS analysis of the blood, spleen, bone marrow, and peritoneal wash was performed to analyse several haematopoietic lineages. The haematological analysis revealed anaemia of the compound females in the second week of treatment (Figure 3). On the day of collection, FACS analysis of the erythroid compartment showed a complete lack of progenitors in the bone marrow of compound females (Figure 4). Interestingly, in the animals where Gata1 recombination took place, the dead cell compartment in the bone marrow lacks TER119 positive cells, indicating that as previously described, Gata1 knockout erythroid cells apoptose in a stage prior to TER119 expression (Pevny *et al*., 1995). The FACS data analysis from the spleens of these animals showed enhanced extramedullar erythropoiesis, but there were almost no differences between animals (data not shown). Cell numbers of the bone marrow and spleen of treated mice revealed bone marrow cell loss in treated KO/WT and KO/OX (compound) females. FACS analysis of other haematopoietic lineages in blood, bone marrow, spleen and peritoneal wash revealed general depletion in the compound females (Figure 5). However, the depletion is more pronounced in eosinophils, DC, monocytes and early progenitors. B cells and resident macrophages were surprisingly affected in compound females, although not as significantly as the cell types mentioned above.

DISCUSSION

Tamoxifen usage to induce recombination of a gene in the haematopoietic compartment requires a chronic treatment to assure that the recombination event is efficient and occurs in a significant percentage of cells.

The effect of knocking Gata1 in the heterozygous Gata1 overexpressing females results in severe anaemia and death. These females lack erythroid progenitors and other cell lines that are Gata1 dependent, like eosinophils or plasmocytoid dendritic cells (PDC). Other cell lineages that are Gata1 independent, like macrophages or B cells, were also depleted in treated compound females, although not to the same extent as the cells mentioned above. We think that the depletion of Gata1 independent cell lines is due to general loss of progenitors and bone marrow failure, although we cannot dismiss the possibility that Gata1 ablation has a direct effect on these cell lines.

Acknowledgements

We are thankful to Dr. T. Nikolic for detailed FACS analysis in one of the treatments.

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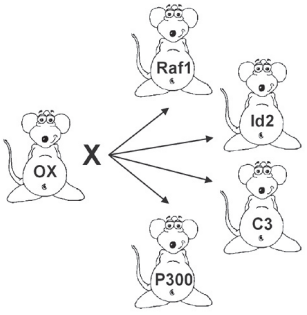
APPENDIX III

A GENETIC APPROACH TO STUDY GATA1 FUNCTION



A genetic approach to study Gata1 function

Laura Gutiérrez, Frank Grosveld and Sjaak Philipsen.

I have initiated the generation of several compound transgenic mice in the Gata1 overexpressing background in order to study the function of Gata1 and the pathways involved in the defect caused by Gata1 overexpression. With this aim, several transgenic mice that were previously generated were chosen for the crossing strategies.

Raf-1 null mice

Raf-1 inhibits caspases and this negative regulation is required for proper erythroid differentiation (Kolbus *et al*., 2002). Raf-1 *null* erythroid cells show accelerated differentiation and cannot be expanded in *ex vivo* cultures. Raf-1 knockout mice die of anaemia at 12.5 dpc (Mikula *et al*., 2001). We crossed the Gata1 overexpressing (OX) mice to the Raf-1 *null* background to determine whether the phenotype of the single mutants could be restored by mutual compensation. However, compound animals died between 12.5 and 13.5 (days *post coitum*) dpc, showing no complementation of the phenotypes (Table 1 and Figure 1). Analysis of the proliferation rate of cells from all genotypes collected at 12.5 dpc should still be performed in order to determine whether the accelerated differentiation found in Raf-1 *null* progenitors is changed in the Gata1 overexpressing background. Raf-1 interacts with other proteins, including pRb [Dasgupta, 2004 #388]. When this interaction is disrupted, cells become irregularly proliferative. Contrariwise, loss of Raf-1 in erythroid cells (and absence of the Raf-1-pRb interaction) does not cause a proliferative phenotype. Therefore, the defect generated in Raf-1 *null* erythroid cells does not seem to be induced by free pRb. Thus, although it is possible that high levels of Gata1 sequester free pRb (Whyatt *et al*., 1997), this would not overcome the defect generated by loss of Raf-1. The Raf-1 *null* state might simply be too deleterious as to detect a rescue in the Gata1 overexpressing background.

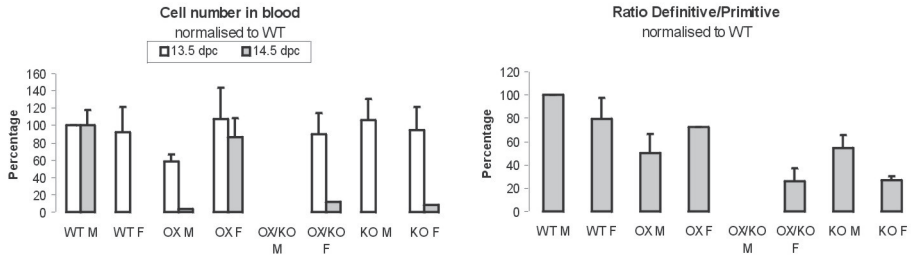


Figure 1. Raf-1 KO|Gata1 OX cross
The blood cell number (left) and the ratio of the number of definitive versus primitive cells (right), normalised to WT values, are depicted in respective graphs. Average and standard deviations are indicated.

Table 1. Raf-1 KO|Gata1 OX 13.5 dpc litters. Genotype and phenotype.

				Live	
Genotype	**Total**	**Expected**	**Dead**	**Normal**	**Anaemic**
WT M	8	9	1 (12.5%)	5 (62.5%)	2 (25%)
WT F	5	9	0	5 (100%)	0
OX M	14	9	1 (7%)	3 (21%)	10 (72%)
OX F	7	9	0	5 (71.5%)	2 (28.5%)
KO M	2	3	0	1 (50%)	1 (50%)
KO F	4	3	2 (50%)	1 (25%)	1 (25%)
OX/KO M	1	3	1 (100%)	0	0
OX/KO F	5	3	3 (60%)	0	2 (40%)
Total	**46**				

Pallor is scored as anaemia and lack of heartbeat is scored as death.

Id2 null mice

Id2 is an "inhibitor of differentiation" protein. It is known to be a pRb target during differentiation (Lasorella *et al*., 2000). Id2 activates the cMyc oncoprotein, thereby blocking differentiation. It has been described recently that pRb inhibits Id2 expression in the erythroblastic island macrophages thereby promoting erythroid differentiation (Iavarone *et al*., 2004). Loss of Id2 in the pRb *null* background rescues the erythroid phenotype of the pRb *null* mice (Lasorella *et al*., 2000). We crossed the Id2 knockout in the Gata1 OX background, based on the hypothesis that the defect generated by Gata1 overexpression is caused by sequestration of pRb by the excess Gata1 protein present in the erythroid cells. This sequestration would lead to a deregulated function of Id2, resulting in high levels of cMyc, and thus, a proliferative phenotype. Therefore, loss of Id2 in the Gata1 OX background might overcome the defect generated by sequestration of pRb, as Id2, a pRb target that may directly generate the defect, is no longer present. Preliminary results are presented in Table 2 and Figure 2. The fact that one Gata1-OX|Id2 *null* male was born (data not shown) makes this crossing strategy worth pursuing.

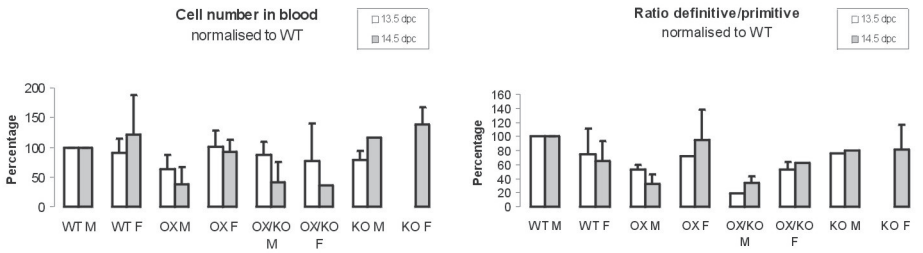


Figure 2. Id2 KO|Gata1 OX cross
The blood cell number (left) and the ratio of the number of definitive versus primitive cells (right) at 13.5 and 14.5 dpc, normalised to WT values, are depicted in respective graphs. Average and standard deviations are indicated.

Table 2a. Id2 KO|Gata1 OX 13.5 dpc litters. Genotype and phenotype.

				Live	
Genotype	Total	Expected	Dead	Normal	Anaemic
WT M	8	10	0	7 (87.5%)	1 (12.5%)
WT F	8	10	1 (12.5%)	6 (75%)	1 (12.5%)
OX M	14	10	0	4 (28.5%)	10 (71.5%)
OX F	6	10	0	5 (83.5%)	1 (16.5%)
KO M	5	3	1 (20%)	2 (40%)	2 (40%)
KO F	2	3	2 (100%)	0	0
OX/KO M	2	3	0	0	2 (100%)
OX/KO F	7	3	1 (14%)	3 (43%)	3 (43%)
Total	52				

Table 2b. Id2 KO|Gata1 OX 14.5 dpc litters. Genotype and phenotype.

				Live	
Genotype	Total	Expected	Dead	Normal	Anaemic
WT M	6	6	0	6 (100%)	0
WT F	4	6	0	4 (100%)	0
OX M	6	6	1 (16.5%)	0	5 (83.5%)
OX F	10	6	1 (10%)	4 (40%)	5 (50%)
KO M	1	2	0	1 (100%)	0
KO F	2	2	0	2 (100%)	0
OX/KO M	3	2	2 (67%)	1 (33%)	0
OX/KO F	1	2	0	0	1 (100%)
Total	33				

Pallor is scored as anaemia and lack of heartbeat is scored as death.

Caspase3 null mice

Caspase3 is a main effector caspase required during terminal erythropoiesis (Carlile *et al*., 2004). Whether it is involved in REDS signalling directly is unclear. With this cross we aimed to determine whether we could interfere with REDS signalling when knocking out caspase3 in the Gata1 OX females, causing anaemia during gestation and probably a low birth rate. Caspase3 mice are born and depending on the background, they die 3-5 weeks after birth or develop to adulthood. Deficiency of caspase3 generates defects in nervous system development due to absence of apoptosis in proliferating tissues (Pompeiano *et al*., 2000). However, caspase3 deficiency rescues the peripheral nervous system defect found in pRb null mice (Simpson *et al*., 2001). It is very difficult to assess a defect in Gata1 OX females due to intercepted REDS by loss of caspase3. This is mainly because both genotypes are born at apparently normal ratios, and a lot of animals need to be analysed

in order to detect a significant variation in the birth rate or in the anaemia status during gestation. In addition, compensation of caspase3 loss by other caspases makes it even more difficult. It has been reported that deficiency in caspase3 or caspase9 is compensated by other caspases (Zheng *et al*., 2000). The analysis presented here is preliminary, as it does not include sufficient numbers of mice (Table 3 and Figure 3). So far it appears that caspase3 loss has no deleterious effect to the Gata1 overexpression phenotype.

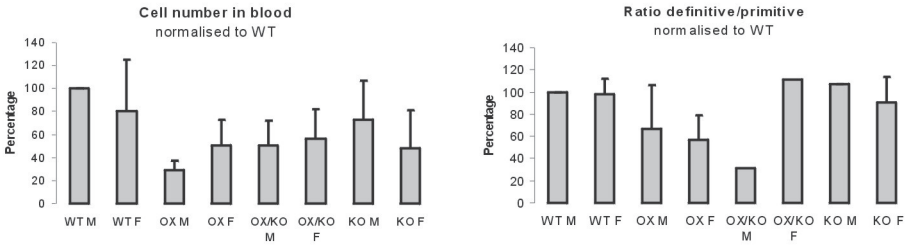


Figure 3. Caspase3 KO|Gata1 OX cross.
The blood cell number (left) and the ratio of the number of definitive versus primitive cells (right), normalised to WT values, are depicted in respective graphs. Average and standard deviations are indicated.

p300AS conditional mice

Finally, we are also crossing Gata1 OX mice with mice bearing a floxed mutated p300 allele that upon recombination produces a dominant negative form of p300 (Roth *et al*., 2003; Shikama *et al*., 2003). p300 is employed as a transcriptional co-regulator by Gata1 and, in addition, p300 stimulates Gata1 activity via acetylation (Boyes *et al*., 1998). However, the role of this interaction *in vivo* has not yet been analyzed. We wish to test whether impaired p300 function, and subsequently deregulated acetylation of Gata1 and reduction of Gata1 activity, will rescue the Gata1 overexpression phenotype. This work is ongoing.

Table 3a. Caspase3 KO|Gata1 OX 13.5 dpc litters. Genotype and phenotype.

				Live	
Genotype	**Total**	**Expected**	**Dead**	**Normal**	**Anaemic**
WT M	17	15	1 (6%)	15 (88%)	1 (6%)
WT F	19	15	1 (5%)	15 (80%)	3 (15%)
OX M	20	15	6 (30%)	2 (10%)	12 (60%)
OX F	8	15	0	6 (75%)	2 (25%)
KO M	6	5	1 (16.5%)	4 (67%)	1 (16.5%)
KO F	6	5	1 (16.5%)	5 (83.5%)	0
OX/KO M	3	5	0	1 (33%)	2 (67%)
OX/KO F	2	5	0	2 (100%)	0
Total	81				

Table 3b. Caspase3 KO|Gata1 OX 14.5 dpc litters. Genotype and phenotype.

				Live	
Genotype	Total	Expected	Dead	Normal	Anaemic
WT M	3	3	0	3 (100%)	0
WT F	2	3	0	2 (100%)	0
OX M	2	3	1 (50%)	0	1 (50%)
OX F	3	3	0	3 (100%)	0
KO M	2	1	0	2 (100%)	0
KO F	0	1	0	0	0
OX/KO M	2	1	0	1 (50%)	1 (50%)
OX/KO F	1	1	0	0	1 (100%)
Total	15				

Pallor is scored as anaemia and lack of heartbeat is scored as death.

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SUMMARY

SAMENVATTING

RESUMEN

SUMMARY

Gata1 is a zinc finger transcription factor essential for erythropoiesis. It is also expressed in other haematopoietic lineages and in Sertoli cells of the testis. Gata1 has been linked to the regulation of the cell cycle, differentiation and survival of the cells in which it is expressed. In the erythroid system, absence of Gata1 leads to apoptosis at the proerythroblast stage. Gata1 levels are downregulated at the last stages of normal erythroid differentiation. Gata1 overexpression causes a proliferative phenotype in cells beyond the proerythroblast stage that renders them unable to arrest the cell cycle, a requirement for terminal differentiation.

Gata1 overexpressing mice were generated in order to study the Gata1 gain-of-function phenotype *in vivo*. Males overexpressing Gata1 from a transgene located on the X chromosome die *in utero* due to lethal anaemia. However, heterozygous females are born normal and at expected rates. These females have wildtype- and Gata1-overexpressing erythroid populations due to the process of X-inactivation. It was observed that Gata1 overexpressing cells, which are intrinsically defective, were rescued in the heterozygous females. It was hypothesised that the rescue might be triggered by a signal originating from wildtype cells. The signal was named Red Cell Differentiation Signal or REDS.

One of the aims of my thesis project was to define the nature of REDS, as it could be a signalling mechanism involving cells of either the same or of a different type, *i.e.* it could be a homotypic or a heterotypic mechanism. By means of a "conditional" Gata1 allele, which is inactivated upon the action of Cre recombinase, I ablated the erythroid wildtype population in the Gata1-overexpressing heterozygous females. The compound females died of anaemia during gestation, showing that the ablated population of cells is the source of REDS, *i.e.* REDS is a homotypic signalling mechanism.

It is known that death receptors are expressed throughout erythroid differentiation and death receptor ligands are expressed at terminal stages. In the quest of finding the REDS counterparts, we hypothesised that REDS is mediated via activation of death receptor-like pathways. We have shown that activation of Fas (a death receptor) with the Jo2 antibody, or activation via oxidative stress of caspases, (a group of proteases that are effectors of the death receptor pathway), favours erythroid differentiation and results

in downregulation of Gata1 levels in *ex vivo* cultures. Furthermore, inhibition of caspases blocks *ex vivo* erythroid differentiation. Taken together, these data support the idea that death receptor-like proteins are involved in REDS signalling. However, we still have not identified the REDS receptor and this is the main aim of the ongoing work.

At the moment we are studying the candidature of EEG1 as REDS receptor. This protein was recently described. It is erythroid-specific and its levels are upregulated during terminal differentiation. It has a C1q domain that folds in a TNF (Tumour Necrosis Factor, a death receptor ligand)-like domain. It is cytoplasmic and transmembrane and can be shedded. Furthermore, it induces cell cycle arrest when expressed exogenously in Chinese Hamster Ovary (CHO) cells. We have detected upregulation of EEG1 in Gata1 overexpressing cells earlier than it occurs in wildtype cells. This upregulation, however, does not lead to cell cycle arrest. By chromatin immunoprecipitation analysis we have shown that Gata1 binds to the EEG1 promoter and that it might transactivate the expression of EEG1. We hypothesise that in a negative feedback loop, Gata1 might upregulate the levels of EEG1 that would end up with Gata1 degradation and cell cycle arrest, via activation of the EEG1 receptor itself, probably by excess of ligand shedded by mature erythroid cells.

We have detected Gata1 expression in murine and human dendritic cells, derived either from myeloid or lymphoid progenitors. Furthermore, by exploiting a tamoxifen-inducible loxP/Cre system, we have observed that Gata1 is required for the survival of *ex vivo* generated dendritic cells. This suggests that Gata1 plays an essential role in other haematopoietic lineages where its expression has not been described yet.

Gata1 is expressed in multipotent progenitors and the levels of Gata1 together with the interplay with other proteins might determine cell lineage commitment and the fate of committed haematopoietic progenitors, *i.e.* cell apoptosis versus survival, and proliferation versus terminal differentiation. Similar to REDS in the erythroid system, there might be other intra-lineage homotypic signalling mechanisms that regulate the homeostasis of haematopoietic cell production, and Gata1 or other lineage-specific transcription factors could be direct targets of their regulation.

SAMENVATTING

De zinkvinger transcriptiefactor Gata1 is essentieel voor erytropoiese, de vorming van rode bloedcellen. Gata1 komt ook tot expressie in andere celtypes van het hematopoietische (bloedvormende) systeem, en in de Sertoli cellen van de testis. Gata1 is in verband gebracht met het reguleren van de celcyclus, differentiatie en overleving van de cellen waarin het tot expressie komt. In erytroïde cellen leidt de afwezigheid van Gata1 tot geprogrammeerde celdood (apoptose) van pro-erytroblasten. Het niveau van Gata1 wordt verlaagd tijdens de laatste stadia van de normale erytroïde differentiatie. Overexpressie van Gata1 veroorzaakt een verhoogde celdeling na het pro-erytroblast stadium; de cellen zijn niet in staat om op te houden met delen en kunnen daardoor geen terminale differentiatie ondergaan.

Om de gevolgen van Gata1 overexpressie in een levend organisme te bestuderen, zijn er transgene muizen gemaakt waarin Gata1 specifiek in rode bloedcellen tijdens de laatste stadia van de differentiatie tot overexpressie komt. Mannetjes die Gata1 tot overexpressie brengen d.m.v. een transgen dat gelokaliseerd is op het X chromosoom sterven tijdens de ontwikkeling aan de gevolgen van bloedarmoede. Vrouwtjes met 1 normaal- en 1 X chromosoom met het Gata1 transgen worden gezond geboren, en in de normaal verwachte aantallen. Deze vrouwtjes hebben wildtype- en Gata1-overexpresserende erytroïde cel populaties dankzij het proces van X chromosoom inactivatie. De Gata1-overexpresserende cellen, die een intrinsiek differentiatie defect hebben, kunnen zich toch normaal ontwikkelen in deze heterozygote vrouwtjes. Op basis van deze observatie is de hypothese gebaseerd dat wildtype cellen een signaal produceren waardoor de Gata1-overexpresserende cellen normaal kunnen differentiëren. Dit signaal werd rode cel differentiatie signaal of REDS genoemd.

Een doel van mijn onderzoeksproject was om REDS verder te karakteriseren. Het zou een signaleringsmechanisme tussen cellen van hetzelfde type of tussen cellen van een verschillend type kunnen zijn: een homotypisch of een heterotypisch signaleringsmechanisme. Door gebruik te maken van een “conditioneel” Gata1 allel, dat geïnactiveerd wordt door het enzym Cre recombinase, heb ik de wildtype erytroïde cellen verwijderd uit Gata1-overexpresserende heterozygote vrouwtjes. Dit had tot gevolg dat deze vrouwtjes tijdens de ontwikkeling aan bloedarmoede stierven, waaruit we konden concluderen dat de wildtype erytroïde cellen de bron van REDS zijn en dus dat REDS een homotypisch signaleringsmechanisme is.

Het is bekend dat zgn. doodreceptors in erytroïde cellen tot expressie komen, maar dat de liganden van deze doodreceptors pas tijdens de laatste stappen van de differentiatie tot expressie komen. In de zoektocht naar REDS componenten hebben we de hypothese onderzocht dat door doodreceptoren geactiveerde signaaltransductieroutes betrokken zijn bij REDS. We hebben laten zien dat activering van Fas (een doodreceptor)

met het Jo2 antilichaam, en activering via oxidatieve stress van caspases (een groep van eiwit-afbrekende enzymen betrokken bij de uitvoering van apoptose), de differentiatie van erytroïde cellen stimuleert en de niveaus van Gata1 verlaagt in gekweekte cellen. Bovendien wordt de differentiatie geblokkeerd door de remming van caspases. Samengevat ondersteunen deze gegevens het idee dat doodreceptor-achtige eiwitten betrokken zijn bij REDS signalering. We hebben echter de REDS receptor nog niet geïdentificeerd en dat is een belangrijk doel voor de toekomst.

Op dit moment bestuderen wij het eiwit EEG1 als een kandidaat voor de REDS receptor. Dit eiwit is recent beschreven in de literatuur. Het is specifiek voor erytroïde cellen en wordt opgereguleerd tijdens de terminale differentiatie. Het heeft een C1q domein dat gevouwen wordt in een TNF (tumor necrose factor, een doodreceptor ligand)-achtig domein. Het wordt zowel in het cytoplasma als de celmembraan gevonden, en kan van de cel loskomen. Als het in Chinese hamster ovarium cellen tot expressie wordt gebracht, remt het de deling van deze cellen. We hebben gevonden dat EEG1 eerder opgereguleerd wordt in Gata1-overexpresserende erytroïde cellen dan in wildtype cellen. Dit resulteert echter niet in remming van de celdeling. Met chromatine immunoprecipitatie hebben we laten zien dat Gata1 aan de EEG1 promoter bindt; dit geeft aan dat het de expressie van EEG1 direct zou kunnen activeren. We veronderstellen dat er een negatieve terugkoppeling bestaat tussen Gata1 en EEG1: Gata1 stimuleert de transcriptie van EEG1 waarna activering van de EEG1 receptor leidt tot afbraak van Gata1 en stoppen van de celdeling. EEG1 wordt in dit model geactiveerd door ligand afkomstig van rijpe erytroïde cellen.

We hebben gevonden dat Gata1 tot expressie komt in dendritische cellen afkomstig van zowel myeloïde- als lymphoïde voorlopercellen in muis en mens. Door gebruik te maken van een tamoxifen induceerbaar Cre systeem in combinatie met het conditionele Gata1 allel hebben we kunnen laten zien dat Gata1 nodig is voor de overleving van in kweek gegenereerde dendritische cellen van de muis. Dit suggereert dat Gata1 een essentiële rol heeft in hematopoietische cellen waarin de expressie van Gata1 tot nog niet beschreven was.

Gata1 komt tot expressie in multi-potente voorlopercellen, en het niveau van Gata1 samen met interacties met andere eiwitten zou de keuze van het type cel waarin de voorlopercellen zich gaan ontwikkelen kunnen bepalen. Ook zou Gata1 het lot van hematopoietische voorlopercellen kunnen bepalen: apoptose versus overleving, celdeling versus terminale differentiatie. Vergelijkbaar met REDS in rode bloedcellen, kunnen er andere homotypische signaleringsmechanismen zijn die betrokken zijn bij de homeostase van hematopoietische celproductie. Gata1 en andere celtype-specifieke transcriptiefactoren kunnen directe doelwitten van deze signaleringsmechanismen zijn.

Vertaling: S. Philipsen

RESUMEN

Gata1, un factor de transcripción con dos dedos de zinc, es esencial durante la eritropoyesis. Su expresión no se limita al sistema eritroide y Gata1 se encuentra en otras líneas hematopoyéticas y también en las células de Sertoli de las gónadas masculinas. Las funciones de Gata1 se han relacionado con la regulación del ciclo celular, la diferenciación y la supervivencia de las células en las que se expresa. En el sistema eritroide, la ausencia de Gata1 resulta en la apoptosis de los precursores eritrocíticos en el estadío de proeritroblasto. Una diferenciación eritroide normal conlleva la reducción de los niveles de Gata1 en los últimos estadíos. La sobreexpression de Gata1 genera un defecto proliferativo en los precursores eritrocíticos, imposibilitándolos para detener el ciclo celular, un requisito que caracteriza los últimos estadíos de la diferenciación eritroide.

Con el objetivo de estudiar el fenotipo causado por la sobreexpresión de Gata1 in vivo, se generaron en el laboratorio ratones que sobreexpresan Gata1 desde un transgen ligado al cromosoma X, como el locus natural de Gata1. Los machos que sobreexpresan Gata1 mueren *in utero* debido a una anemia letal. Sin embargo, la hembras heterocigotas nacen con la frecuencia esperada y se desarrollan normalmente. Estas hembras contienen dos poblaciones de células eritrocíticas debido al proceso de inactivación del cromosoma X: una población normal, y otra población que sobreexpresa Gata1. En estas hembras se observó que las células que sobreexpresan Gata1, células que son intrínsecamente defectuosas, son rescatadas y se diferencian normalmente. Se hipotetizó que este rescate fenotípico debía de ser mediado por una señal originada en las células normales. Esta señal se denominó Señal de Diferenciación de las Células Rojas o REDS.

Uno de los objetivos del proyecto de mi tesis era definir la naturaleza de REDS, ya que podría ser que este mecanismo de señalización se originara en células normales del mismo tipo celular que las células diana (sistema homotípico) o en su defecto, que se originara en células normales de un tipo diferente al de las células diana (sistema heterotípico). Haciendo uso de un alelo condicional de Gata1, que es inactivado ante la acción de una Cre recombinasa, se eliminó la población eritroide normal en las hembras heterocigotas para la sobreexpresión de Gata1. Las hembras compuestas generadas murieron durante la gestación, mostrando que la población eliminada es la que suministraba REDS, es decir, REDS es un sistema de señalización homotípico.

Se sabe que los receptores de muerte se expresan durante todos los estadíos de la diferenciación eritroide y que sus ligandos se expresan en los últimos estadíos. Interesados en la identificación de las moléculas activas de REDS, pensamos que la señalización ocurre cuando los receptores de muerte, o moléculas similares, son activados. En la presente tesis se muestra que la activación de Fas (un receptor de muerte) por el anticuerpo Jo2, o bien la activación de caspasas (las moléculas efectoras de la ruta de los receptores de muerte) con la inducción de estrés oxidativo, favorece la diferenciación eritroide y resulta en la reducción de los niveles de Gata1 en cultivos ex vivo. A su vez,

la inhibición de las caspasas genera un bloqueo de la diferenciación eritroide ex vivo. De manera global, estos resultados apoyan la idea de que los receptores de muerte, o moléculas similares, están involucrados en la señalización de REDS. Desafortunadamente, todavía no hemos identificado el receptor de REDS, y éste es el principal objetivo del trabajo actual que desarrollo en el laboratorio.

En este momento estamos estudiando la candidatura de EEG1 como receptor de REDS. Esta proteína se ha descrito recientemente. Se expresa en el sistema eritroide y sus niveles aumentan durante los últimos estadíos de la diferenciación. Se caracteriza por un dominio C1q que se pliega dando lugar a un dominio TNF (Factor de Necrosis Tumoral, un receptor de muerte). Es una proteína citoplasmática y también de membrana, y puede ser segregada como receptor "señuelo". Curiosamente EEG1 induce la detención del ciclo celular cuando se expresa de manera exógena en células CHO (Células de Ovario de Hamster Chino). En células que sobreexpresan Gata1, los niveles de EEG1 son incrementados en un estadío más temprano que durante una diferenciación normal. Sin embargo, este incremento en los niveles de EEG1 no genera detención del ciclo celular como ocurre en células CHO. Mediante experimentos de immunoprecipitación de cromatina hemos detectado que Gata1 reconoce el promotor de EEG1 y pensamos que Gata1 pueda regular positivamente la expresión de EEG1. Nuestra hipótesis consiste en que Gata1 induce el incremento en los niveles de EEG1, que se acumula en la membrana de células más maduras y acaba siendo segregado como receptor "señuelo". El receptor "señuelo" segregado por células maduras actúa como ligando del receptor EEG1 que está en la membrana de células más inmaduras, y activa una ruta citoplasmática que induce la degradación de Gata1 (probablemente a través de la acción de caspasas) y la detención del ciclo celular. Resumiendo, Gata1 y EEG1 actúan en un bucle de retroalimentación negativo durante la diferenciación eritroide.

También se presenta en este libro que Gata1 se expresa en células dendríticas de origen murino y humano. En el ratón, tanto las células dendríticas de origen linfoide como las de origen mieloide expresan Gata1. Utilizando un sistema inducible para inactivar Gata1, hemos observado que Gata1 es necesaria para la supervivencia de las células dendríticas generadas en cultivos ex vivo. Esto sugiere que Gata1 juega un papel esencial en otras líneas hematopoyéticas donde su expresión no había sido descrita previamente.

Gata1 se expresa en progenitores multipotentes, y los niveles de Gata1, así como la interacción con otras proteínas, podría determinar la especialización de estos progenitores en las diferentes líneas hematopoyéticas y el destino celular de los progenitores de las diferentes líneas, por ejemplo, apoptosis celular o supervivencia, y proliferación o diferenciación. De manera similar a como actúa REDS en el sistema eritroide, podría haber otros mecanismos homotípicos dentro de cada línea hematopoyética que regulen la homeostasis de la producción celular, y Gata1, u otros factores de transcripción específicos de línea, podrían ser dianas de esta regulación.

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