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.

Key words: Erythropoiesis, Gata1, Homotypic signalling, REDS

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* knockout mice die of anaemia at around 11.5 dpc (Fujiwara et 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

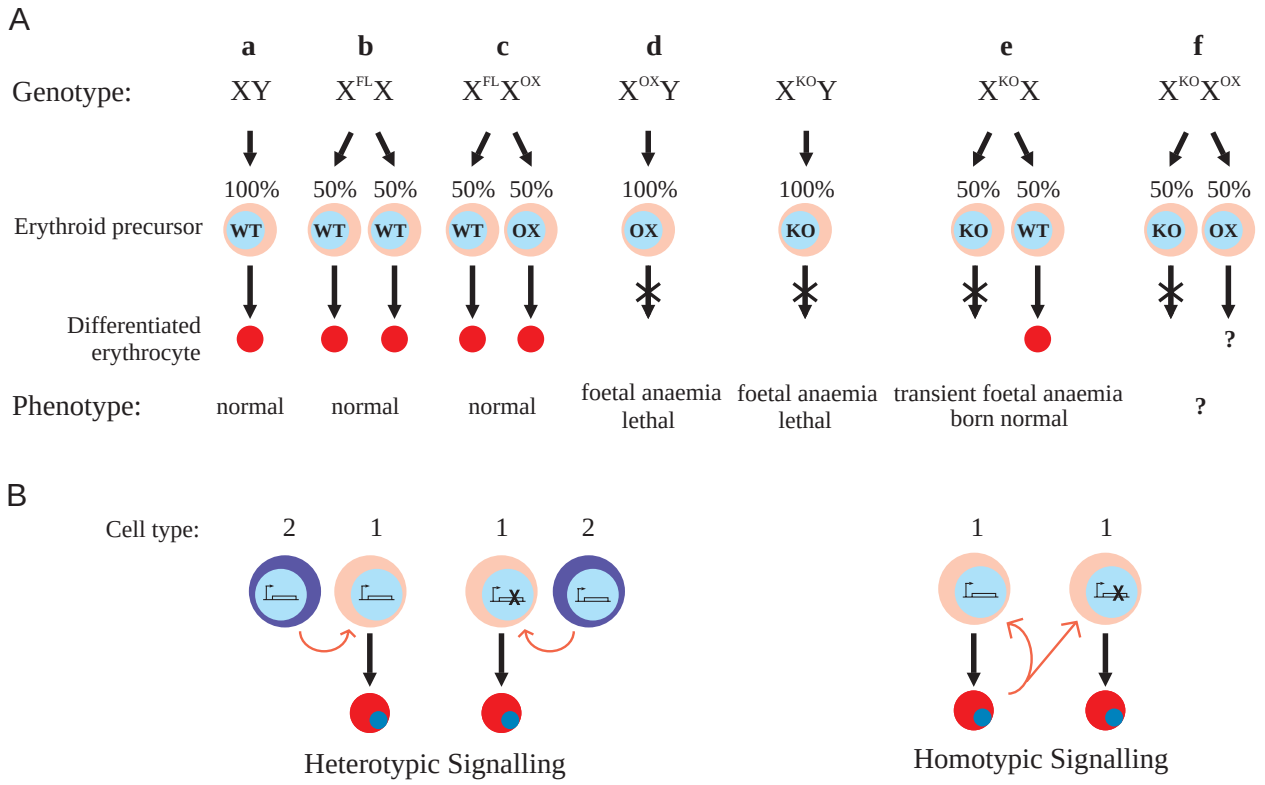

Fig. 1. (A) Summary of the different Gata1 mutants discussed and their outcome in terms of erythroid differentiation. FL, floxed; OX, overexpressing; KO, deleted Gata1 allele; WT, wild type. The progeny obtained in the crossing described in the present manuscript is indicated a-f, in accordance with 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.

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 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, 5×10^4 cells were resuspended in 20 μl hanging drop medium (DMEM supplemented with 20% FCS, 0.1% β-mercaptoethanol, 2×10^{-4} M hemin, 5 μg/ml penicillin/streptomycin, 2 U/ml erythropoietin, 5 μg/ml insulin) and cultured for 2 days (F.L., unpublished). Anti-mouse 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 5×10^4 events taken per sample at day of collection and after two days of hanging drop culture. Single-cell suspensions were incubated with R-PE-conjugated 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 2×Laemmli 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

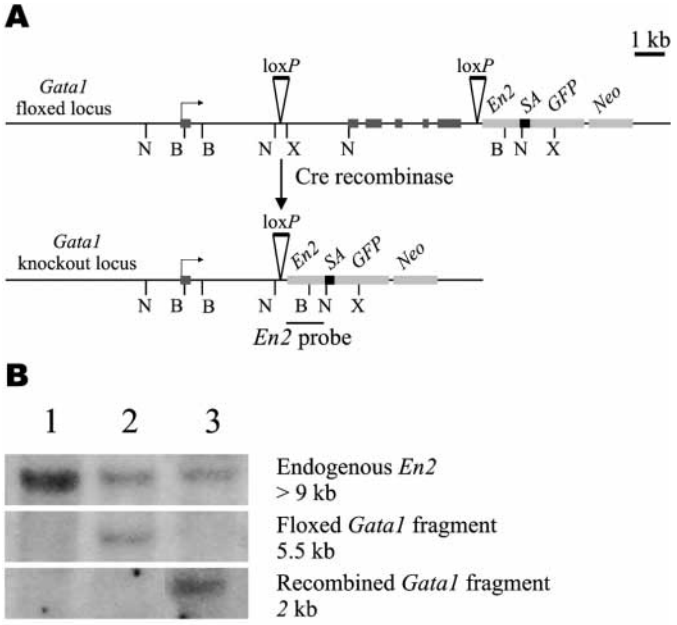

Fig. 2. Recombination of the *Gata1* floxed locus. (A) Maps of the floxed and the knockout *Gata1* locus after recombination. N, *Nco*I; B, *Bam*HI, X, *Xba*I; *En2*, murine engrailed 2 intronic sequence; *SA*, splice acceptor; *GFP*, green fluorescence protein. Lox*P* sites are indicated. (B) DNA samples of 13.5 dpc foetuses were digested with *Nco*I 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.

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-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 non-erythroid 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 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

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.

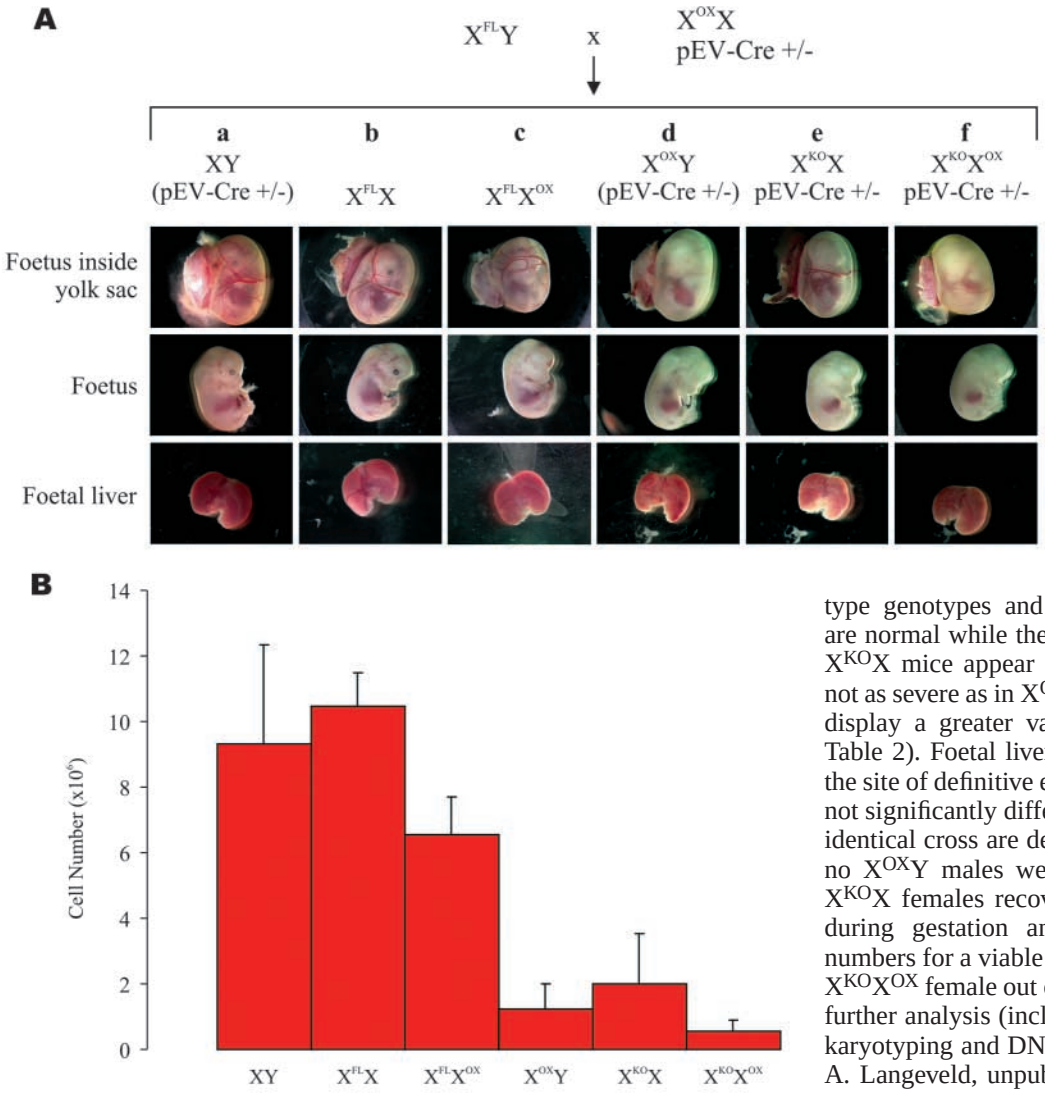

Fig. 3. (A) Crossing strategy and phenotype of the different Gata1 mutant foetuses obtained at 13.5 dpc. A photograph of the foetus and foetal liver is included for each genotype. Mice a and d are wild type or Gata1-overexpressing, respectively, regardless of expression of the pEV-Cre transgene (in brackets). 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.

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.

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

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*	Expected†	Expected‡
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		

*All animals born were normal.
†Assuming that $X^{OX}Y$ mice are not born.
‡Assuming that $X^{OX}Y$ and $X^{KO}X^{OX}$ mice are not born.

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 wild-type 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.

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

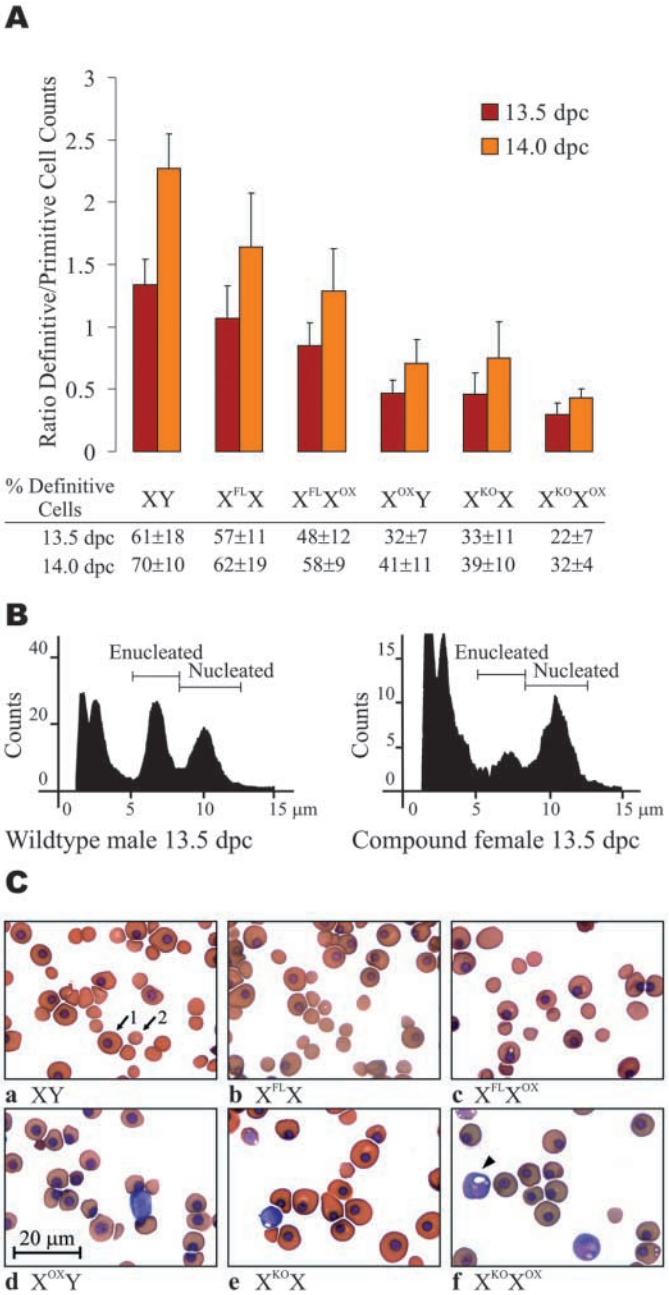

Fig. 4. (A) CASY analysis: the ratio of definitive versus primitive erythrocytes in foetal blood was compared at 13.5 and 14.0 dpc. The percentage of definitive cells in blood in each genotype at 13.5 dpc and 14.0 dpc is depicted below the graph. Average and s.d. are indicated. At least three foetuses 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 µm 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 100× magnification.

(Whyatt et al., 2000), $X^{OX}Y$ male foetuses have proportionally fewer definitive cells, with an additional population (~2% of the total) of immature precursors in the circulation. $X^{KO}X$

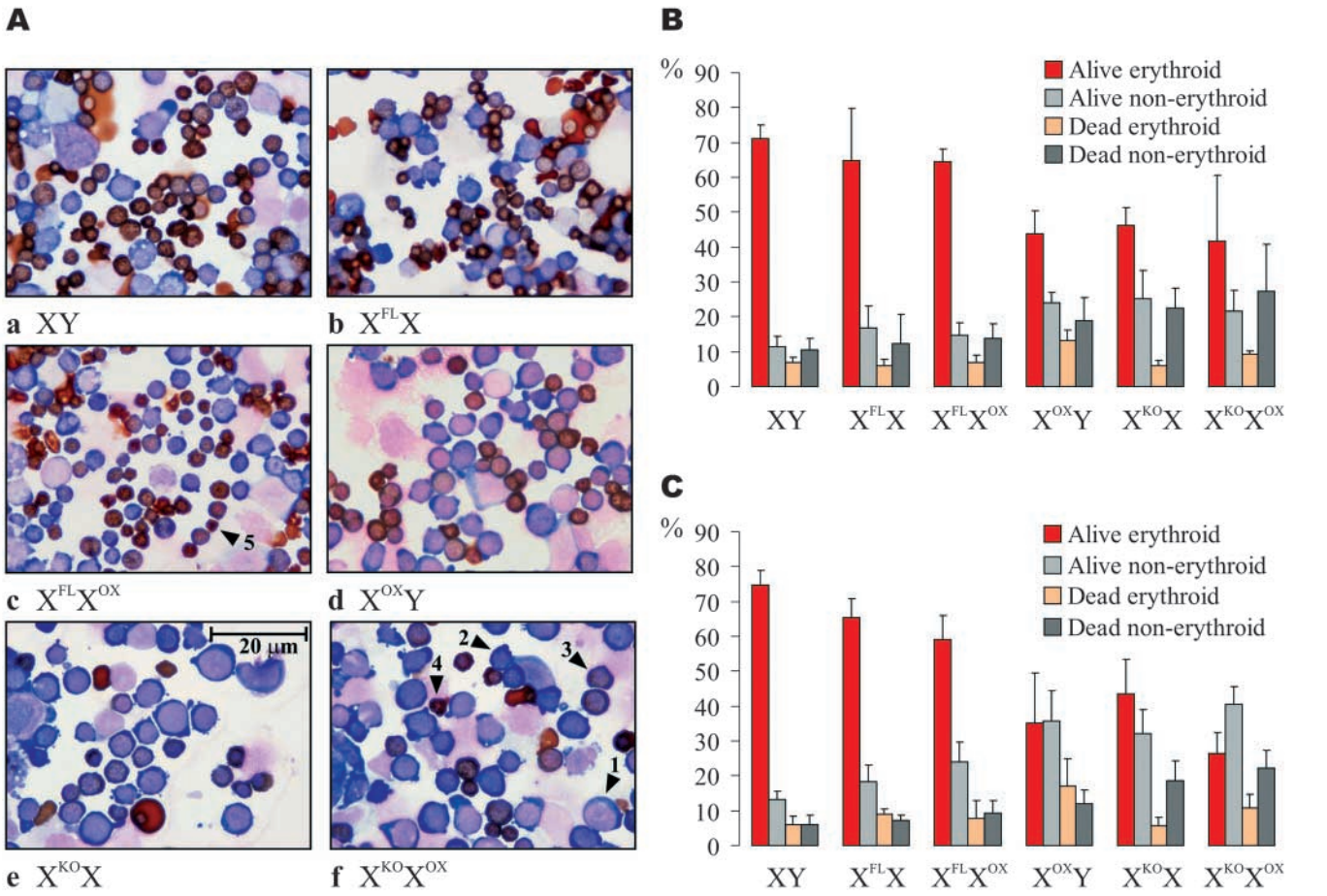

Fig. 5. (A) Foetal liver cytospins of each genotype at 14.0 dpc. Consecutive differentiation stages are indicated in photograph c and f: large proerythroblast (1), basophilic erythroblast (2), polychromatic erythroblast (3) (which exhibit both basophilia and benzidine positivity), orthochromatic erythroblast (4) (which are strongly benzidine positive) and enucleated definitive erythrocyte (5). Pictures were taken at 100× 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.

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 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. 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.

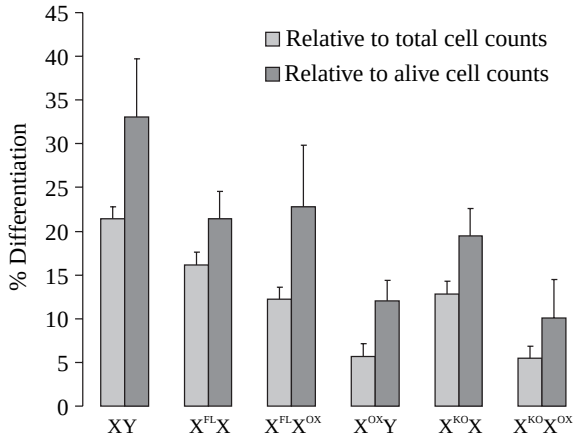

Fig. 6. FACS analysis of foetal liver cells at 14.0 dpc after 2 days of hanging drop culture to induce differentiation. Differentiation is estimated as the percentage of $TER119^{+}/7AAD^{-}/FSC^{low}$ of the 50,000 events measured in total (light-grey bars) and as the percentage of $TER119^{+}/FSC^{low}$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

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.

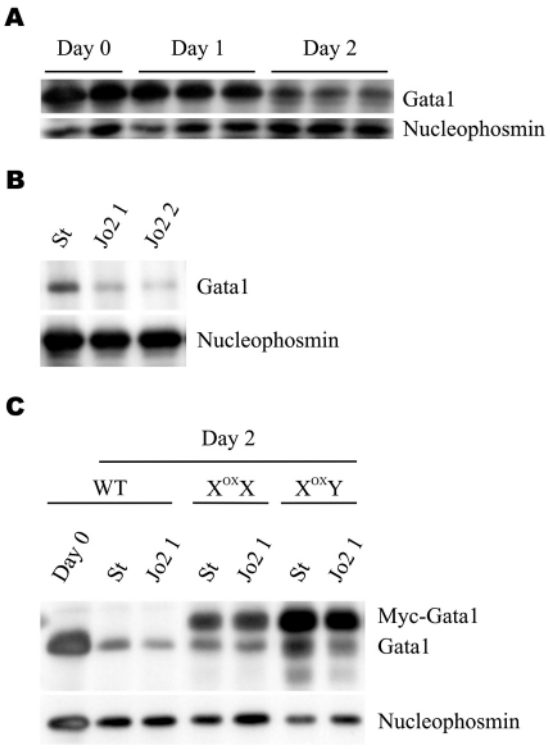

Fig. 7. Gata1 levels decrease during terminal erythroid differentiation. (A) Whole cell extracts from wild-type foetal liver cells, after day 0, 1 and 2 of hanging drop culture were analysed by western blotting using antibodies against Gata1 with nucleophosmin as loading control. Two samples at day 0 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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