MINIREVIEW

Transcription Factor GATA-1 and Erythroid Development (43519A)

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All mature hematopoietic cells, including erythrocytes, megakaryocytes, granulocytes, monocytes, and lymphocytes, are derived from pluripotent stem cells located in the bone marrow of the adult mammal. As stem cells proliferate, they become committed to the individual hematopoietic lineages. Lineage-specific transcription factors should play a key role in determining the developmental fate of precursor cells. The recent characterization of *cis*-acting DNA elements in lineage-specific genes has allowed the subsequent isolation of transcription factors that bind to these regulatory sites. To understand how red blood cell differentiation is programmed, erythroid transcription factors were identified on the premise that either these proteins or their regulators specify erythroid cell fate.

The zinc-finger protein GATA-1 is an example of a lineage-specific transcription factor (1). GATA-1 binds to GATA consensus elements in regulatory regions of the α- and β-globin gene complexes (2–4) and other red cell genes (5–7). GATA-1 is first expressed at low levels in multipotential hematopoietic progenitor cells and then up-regulated during erythroid maturation (8–10) and down-regulated during myeloid differentiation (9). The expression of GATA-1 is subsequently restricted to three different hematopoietic lineages: erythrocyte, megakaryocyte, and mast cell (11–14). GATA binding sites play an important *cis*-acting role in globin and non-globin genes in red cells; mutagenesis of GATA sites in these genes results in lower levels of transcription (2, 3, 6). Mutation of GATA motifs in the promoters of genes expressed in megakaryocytes and mast cells gives the same result, which indicates that GATA sites are important for transcriptional activity in these lineages too (14, 15). GATA-binding sites are also present in active regions of the globin locus control regions, DNase I hypersensitive segments required for activation of the entire chromatin domain and for high-level, position-independent expression of the α-globin (16–18) and β-globin genes (19–26). These results provide indirect evidence that GATA-1 is involved in the activation of lineage-specific genes during erythroid development. It seemed probable that GATA-1 was essential to red cell maturation as well.

GATA-1 is a member of a multigene family (27). Four distinct members of this family have been described in vertebrates thus far: GATA-1, GATA-2, GATA-3, and GATA-4 (27–29; T. Evans, personal communication; D. Wilson and S. Orkin, unpublished observations). These proteins are related by homologous zinc-finger domains with the configuration Cys-X_2-Cys-X_{17}-Cys-X_2-Cys. Members of the GATA-binding protein family differ in their tissue distribution. Whereas GATA-1 mRNA are only found in the three hematopoietic lineages mentioned previously, GATA-2 is expressed in a large number of different cell types, including endothelial cells (28, 29). GATA-3 is also found in a variety of cells, including T lymphocytes, and serves as a transcription factor for several genes expressed only in T cells, such as the subunits of the T cell antigen receptors (30–33). Cell types expressing GATA-4 are currently being investigated in the frog and the mouse.

GATA-2 and GATA-3 are expressed in addition to GATA-1 in erythroid cells at specific developmental stages (27). All three family members bind *in vitro* to

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P.S.E.B.M. 1993, Vol 202: 115–121

0037-9727/93/2022-0115$3.00/0

the consensus sequence (T/A)GATA(A/G) with high affinity and stimulate transcription from reporter constructs in heterologous cells (27, 34). Therefore, which family member functions in erythroid cell gene expression? Is GATA-1 essential to red cell development, or can other family members provide the functions initially ascribed to GATA-1? What is GATA-1's role in the development of megakaryocytes or mast cells? These questions must be answered by the systematic mutation of each member of the GATA-binding protein family to assess the developmental consequences of their lack of expression.

Targeted Mutation of the Murine GATA-1 Locus

To investigate the function of GATA-1 in hematopoiesis *in vivo*, the GATA-1 gene was disrupted by homologous recombination in male (XY) murine embryonic stem (ES) cells. Because the GATA-1 gene is X-linked, the mutant ES cells lack a normal allele and are completely GATA-1-deficient (GATA-1$^-$). Mutant ES cells were then assessed for their ability to contribute to peripheral blood in chimeric mice (35). These experiments allowed analyses to be performed without a germ line transmission of the targeted mutation. Cells derived from the donor ES cells in chimeras could be distinguished from those derived from the host blastocyst by a glucose phosphate isomerase polymorphism between the ES cells and the host embryos. The mutant ES cells contributed to all nonhematopoietic tissues and to a white blood cell fraction, but failed to produce circulating red blood cells (35). This demonstrates that GATA-1 is essential for the development of *definitive* erythrocytes that express α- and β-globin and circulate in the bloodstream. Other GATA-binding proteins cannot compensate for its absence.

Erythropoiesis in developing mammals is characterized by sequential changes in erythropoietic site and hemoglobin synthesis (see Ref. 36). Erythroid cell production first occurs in the visceral yolk sac of the embryo, shifts to the fetal liver, and then shifts again to the bone marrow around the time of birth. Accompanying these changes in erythropoietic site are modifications of human globin gene expression: ζ and ϵ in the yolk sac blood islands, α and γ in the fetal liver, and α and β in the bone marrow. This phenomenon is termed "hemoglobin switching" and is controlled by shifting patterns of transcription within the α- and β-globin gene complexes (see Ref. 1). Developmental switches in hemoglobin gene expression also occur in the mouse (37–39). Yolk sac erythrocytes contain $\alpha_2\beta H1_2$, $\zeta_2\epsilon_2$, and $\alpha_2\epsilon_2$ (37, 38, 40). Erythrocytes found in the fetal liver, spleen, and bone marrow contain adult hemoglobins ($\alpha_2\beta_2^{maj}$ and $\alpha_2\beta_2^{min}$) (40). There are two switches in globin gene transcription in fetal mouse ($\beta H1$ to ϵ and ϵ to β), similar to what takes place in the human embryo (41). Erythroid cell morphology also changes during development; yolk sac erythrocytes are nucleated, whereas erythrocytes in the fetal liver, spleen, and blood are nonnucleated.

We have established that GATA-1 is required for the production of definitive red cells in peripheral blood. Is GATA-1 also necessary for the development of *primitive* erythrocytes found in the embryonic yolk sac? GATA-1 is expressed during embryonic erythropoiesis at the time of blood island formation in the yolk sac (41). Functional binding sites in the promoters of the ζ- and ϵ-globin genes have been described (42–44). However, the level of GATA-1 mRNA is low compared with the levels detected later in the fetal liver and blood (41). On the other hand, GATA-2 is highly expressed in embryonic red blood cells of the chicken (27). To determine what role (if any) GATA-1 plays in yolk sac erythropoiesis, we employed an *in vitro* model of hematopoiesis in the developing embryo based on murine ES cells. If allowed to form three-dimensional structures known as embryoid bodies (EB), ES cells differentiate into many tissues, including hematopoietic cells (45–47). When ES cells are plated into methylcellulose media, the production of hematopoietic cells occurs at a high frequency (48). More than 80% of all EB contain red areas of visible erythrocytes, or "blood islands." These red cells appear normal in all ways that have been analyzed: they have the proper cytology by May-Grünwald-Giemsa staining, they express globin genes in the proper temporal order (see below), and retain their nucleus at earlier stages of differentiation like yolk sac erythrocytes (48–50). They can also be stained with benzidene, which indicates that they produce heme. Erythrocytes recovered at later times during *in vitro* differentiation have no nucleus, reminiscent of red cells detected at the adult stages of mouse erythroid development in the fetal liver, spleen, and bone marrow (48, 49).

The GATA-1$^-$ES cells were differentiated *in vitro* in methylcellulose and analyzed for the production of primitive erythrocytes that express embryonic hemoglobins (49). Under standard culture conditions (0.8% methylcellulose in Iscove's medium, 10% fetal calf serum, 4.5×10^{-4} *M* monothioglycerol, interleukin 1, interleukin 3, kit ligand, and erythropoietin), the production of hematopoietic cells is favored (48, 49). We have shown previously that GATA-1 is not expressed in undifferentiated CCE ES cells (35). However, GATA-1 mRNA levels increase during EB formation *in vitro* (50; M. C. Simon and S. Orkin, unpublished observations). When wild-type ES cells were differentiated, more than 80% of all EB observed had red-colored (hemoglobinized) cells by Days 8–10 in culture. Globinization began as a red glow in the center of each EB and proceeded until many EB ruptured, yielding a halo of red cells surrounding the original cell mass. Cytologic examination of May-Grünwald-Giemsa-stained smears

from these colonies revealed a large number of nucleated erythroid cells. In parallel experiments, GATA-1$^-$ EB never formed visible blood islands. The appearance of proerythroblastic cells in mutant cultures and the absence of any hemoglobinized cells suggest that GATA-1 is required for the further maturation of such cells in the embryonic yolk sac.

The visceral yolk sac in a developing mouse embryo expresses the embryonic globin βH1 as the predominant species first (Day 7.5–10.5), followed by ϵ-globin on Days 10.5–13.5 (45). ζ- and α-globin are also detected in the yolk sac by Day 7.5 of gestation (41). However, there is a gradual shift in embryonic erythroid cells from predominantly embryonic ζ-globin to adult α (37, 38, 41). RNase protection assays on RNA isolated from EB detect βH1 mRNA on Days 5–12 (48, 50), and ϵ mRNA on Days 9–12 (50). α-Globin and ζ-globin are detected from Day 6, but α stays on after ζ transcription ceases at Day 12 (50; M. C. Simon and S. Orkin, unpublished observations). If ES cells are regarded as similar to the inner cell mass of a 3.5-day blastocyst (45, 46), the onset of globin gene transcription in cultured EB parallels *in vivo* globin expression. These experiments mimic the developmental changes seen *in vivo* (41, 43, 47).

No globin gene expression was detected in EB derived from GATA-1-deficient ES cells (49). These results confirm that GATA-1 is necessary for the development of primitive erythrocytes that express embryonic globins. Furthermore, cells that transcribe adult globins are never generated by GATA-1$^-$ ES cells *in vitro*.

Rescue of the GATA-1 Mutation *In Vivo* and *In Vitro*

In an effort to "rescue" the mutant phenotype of the GATA-1$^-$ ES cells, a 12-kb genomic DNA fragment from the murine GATA-1 locus was introduced into these cells by electroporation (the first construct in Fig. 1). The 12-kb transgene includes 2.6-kb of 5′ flanking sequences and 1 kb of 3′ flanking sequences (49). Stable transformants harboring the GATA-1 transgene were differentiated *in vitro* and tested for their ability to produce erythroid cells. Roughly 50–60% made visible blood islands and transcribed ζ-globin mRNA (Fig. 1). Not all stable transformants containing transgenes were rescued due to variations in the level of expression of different transgenes. We assume that only those transformants expressing near normal levels of GATA-1 mRNA produced hemoglobinized red cells. In fact, one clone was examined for GATA-1 expression and was found to have wild-type levels of GATA-1 mRNA (49). Some clones were also tested for their ability to produce erythroid cells *in vivo*. ES cells were introduced into MF1 host blastocysts and resultant chimeric mice were analyzed for ES cell contribution to peripheral blood, as described previously (35). The rescued clones gave rise to circulating erythrocytes, as indicated by the appearance of the GPI-1C isozyme in blood samples from chimeric mice. Furthermore, the anemic phenotype observed in the mutant chimeras (35) is reversed by the presence of the transgene in GATA-1$^-$ ES cells (49).

Only half of the ES clones with intact GATA-1 transgenes exhibited erythroid cell production and ζ-globin transcription upon differentiation *in vitro*. This indicates that high levels of expression of GATA-1 are necessary for the proper development of this lineage. The clones that do not display erythropoiesis most likely are not synthesizing sufficient protein to overcome the mutant phenotype. Experiments that support this hypothesis include the analysis of the expression of a similar 12-kb construct with β-galactosidase (lacZ) sequences inserted as a reporter gene in ES and erythroleukemia cells (Fig. 1; M. C. Simon and S. Orkin, unpublished observations). The β-galactosidase experiments in murine erythroleukemia cells and human erythroleukemia (K562) cells demonstrate that only half of the stable transformants made with this construct have detectable levels of β-galactosidase enzymatic activity (Fig. 1). This is consistent with the ES cell results and is probably due to position effects on randomly integrated copies of exogenous DNA. Additional experiments in wild-type EB with the construct containing lacZ reporter sequences shown in Figure 1 confirm that approximately 50–60% of the clones express the transgene efficiently. Such effects have already been documented in a number of other experimental systems, including transgenic mice.

Based on these data, we conclude that the introduction of a 12-kb GATA-1 DNA fragment is sufficient to restore the ability of the mutant ES cells to differentiate into both primitive red blood cells (assayed *in vitro*) and definitive red blood cells (assayed *in vivo*). Using the mutant and rescued ES cells, we have established a model system that directly analyzes the role of the GATA-1 transcription factor in erythroid development. If GATA-1 is expressed, hemoglobinized red cells are produced. If GATA-1 is not expressed, hemoglobinized cells do not develop. We are now in a position to look at subtle modifications of GATA-1 coding sequences or of promoter elements that control GATA-1 expression. Functional analysis of the GATA-1 promoter in transient assays has shown that full promoter activity requires two CACCC boxes (at position −218 upstream of the last nucleotide of exon I) and a double GATA motif (at position −687; 51). The 5′-GATA motif is also contacted *in vivo* in mouse erythroleukemia cells (51). Therefore, it appears that GATA-1 is a positive regulator of its own promoter, at least in a committed erythroid cell line. What role does the GATA site in the GATA-1 promoter play in the early events in erythroid differentiation as analyzed in EB in

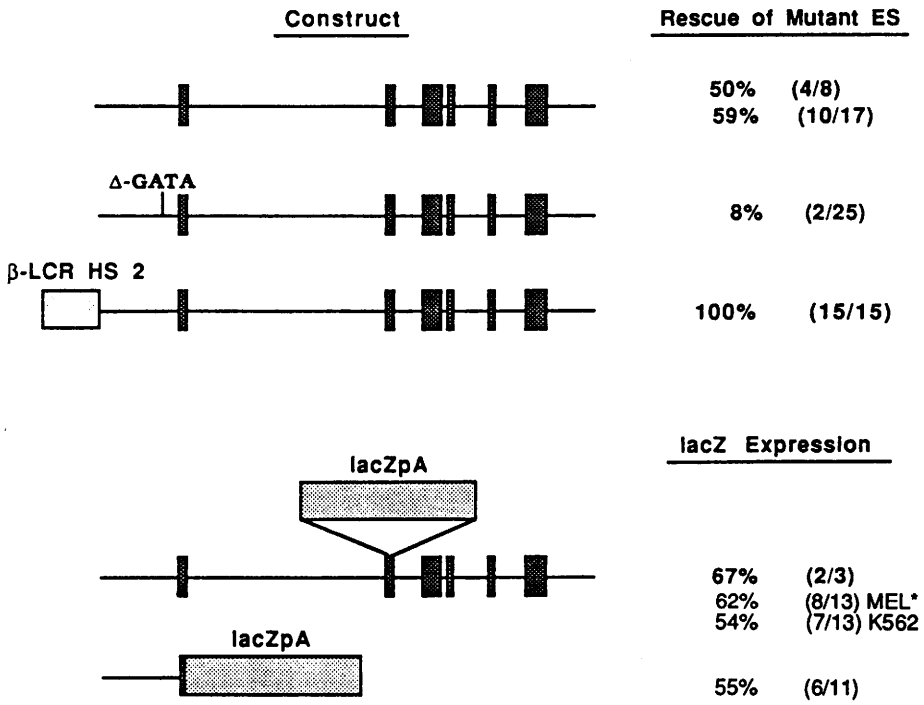

Figure 1. Constructs introduced into various ES cells or erythroleukemia cells and summary of the frequencies of rescue of erythroid differentiation of GATA-1$^-$ ES cells or lacZ expression in wild-type ES, murine erythroleukemia (MEL), or K562 cells. The number of clones analyzed are shown in parentheses. Details of the constructions are given in the Methods section of Ref. 49. LCR, locus control region.

methylcellulose? To address this question, we expanded the rescue experiments to include a larger number of clones containing an intact 12-kb transgene and a similar 12-kb construct with a short deletion at position −687 so that the double GATA sites were lost (Fig. 1). In all, 25 independent clones that had the original 12-kb fragment and 25 clones that contained the 12-kb fragment with the upstream GATA sites deleted were examined. Results from these experiments are presented in Figure 1. The number of clones containing the 12-kb construct that produced visible blood islands upon differentiation into EB was slightly better than one half (14 of 25). In contrast to these results, relatively few clones with the 12-kb fragment lacking the upstream GATA sites generated red cells within the EB. Only two out of 25 independent clones rescued the mutant phenotype. Taken together, these data suggest that GATA-1 influences its own expression in a positive manner. Another explanation would be that other GATA-binding proteins are required for efficient transcription at the GATA-1 locus. In fact, both GATA-2 and GATA-3 are expressed in ES cells at low levels (C. Simon and L. Zon, unpublished results). It remains a formal possibility that another family member(s) regulates GATA-1. Additional experiments involving gene targeting at the GATA-2 and GATA-3 loci are required to show definitively which GATA-binding protein acts on the GATA sites in the GATA promoter.

As stated above, only half of the stable transformants with intact 12-kb transgenes regained the ability to produce hemoglobinized erythrocytes. In an effort to increase the frequency of rescue in mutant ES cells, we introduced GATA-1 linked to a 1-kb segment from the locus control region including hypersensitive site 2 (5′HS2). When linked to the human β-globin gene, 5′HS2 confers high levels of expression and position independence to the exogenous β-globin gene in transgenic mice (52, 53). Fifteen independent clones were differentiated and scored for blood island formation. All 15 clones gave EB with hemoglobin-producing erythroid cells (Fig. 1). We conclude that 5′HS2 insulates the transgene from negative influences of flanking DNA, thereby boosting the level of exogenous GATA-1 expression. Including it in transgenes may reverse the mutant phenotype in 100% of all transformants. This finding may prove useful in future experiments on functional studies of the GATA-1 protein in this assay system.

GATA-1$^-$ EB are capable of producing early erythroid precursor cells called proerythroblasts; cells that resemble proerythroblasts in terms of morphology and staining characteristics have been detected upon cytologic examination of slides made from mutant embryoid bodies. No cells with the appearance of hemoglobinized erythrocytes have ever been detected on Giemsa-stained slides of cells associated with mutant EB. We suggest that the GATA-1 mutation exerts itself at this stage of red cell maturation: the protein is required for erythroid precursor cells to progress to the next stage of development (basophilic erythroblasts).

GATA-1 positively regulates the expression of the receptor for the erythroid growth factor erythropoietin (Epo) (54). It is possible that Epo is necessary for the viability and further differentiation of proerythroblasts. Without GATA-1, the Epo receptor is down-regulated and proerythroblastic cells undergo cell death.

Effect of the GATA-1 Mutation on Other Hematopoietic Lineages

When ES cells are differentiated into EB in methylcellulose, other hematopoietic cell lineages are obtained, including mast cells, macrophages, and neutrophils (48). The GATA-1-deficient ES cells have been tested for myeloid cell production and found to be capable of differentiating into both macrophages and neutrophils (Pevny *et al.*, manuscript in preparation). These results extend the previous findings in chimeric mice showing that mutant ES cells contributed to the white cell fraction in peripheral blood (35). We are currently investigating whether mutant ES cells make mast cells and megakaryocytes *in vitro*. This is of interest because both mast cells and megakaryocytes express GATA-1.

Hematopoietic colonies grown *in vitro* from clonogenic precursors in the yolk sacs of Day 10 chimeric embryos have been examined (L. Pevny, C. Simon, S. Orkin, and F. Costantini, manuscript in preparation). Individual colonies from methylcellulose cultures were picked and genotyped (by glucosephosphate isomerase analysis), and cell types present in each colony were determined by Wright-Giemsa staining of cytologic preparations. The *in vitro* differentiation of macrophages, neutrophils, megakaryocytes, and mast cells appeared to be unaffected by the mutation (Pevny *et al.*, manuscript in preparation). Mutant colonies also contained proerythroblasts, based on cellular morphology and staining (Pevny *et al.*, manuscript in preparation). Based on these results, GATA-1 is only required for erythroid cell development, and only beyond the proerythroblastic stage of development. The other lineages are not dependent on the GATA-1 transcription factor for survival or maturation, including mast cells and megakaryocytes. Other family members (GATA-2 or GATA-3) may be involved in the regulation of lineage-specific genes in these cells given the functional role of GATA binding sites in promoters of the lineage-specific genes.

Summary

In summary, we derived an experimental system that allows us to dissect the function of GATA-1 in red cell development at a genetic level. We have established the essential nature of GATA-1 during both primitive and definitive erythropoiesis. By ablating the expression of the endogenous GATA-1 gene, we are in a position to introduce a variety of constructs that harbor subtle modifications in flanking or protein-coding sequences. We can now study regulatory regions and functional domains of the protein in the context of a true erythroid environment, experiments that have not been possible heretofore. Although the assay involves the dramatic loss of red cell production, it should be possible to define important regulatory domains that can then be assayed using less stringent systems, such as cell-free extracts for *in vitro* transcription. The ideal situation would be analyses conducted in GATA-1$^-$ erythroid cells. However, these cells have been impossible to generate given the requirement of GATA-1 for Epo receptor expression and red cell viability (C. Simon and S. Orkin, unpublished observations). It may be possible to produce such cells by first expressing the Epo receptor under the influence of a constitutive promoter and then targeting the GATA-1 gene. If GATA-1$^-$ red cells were available, the analyses would involve the actual transcription of or chromatin structure surrounding the globin genes. Structure-function studies of the GATA-1 protein could be greatly simplified and a larger number of mutants studied. However, the ES cell system can be used as an alternative until targeted erythroleukemia cells become available. Other applications involve the introduction of other GATA-binding protein family members to determine whether they rescue the mutation. If they cannot, chimeric proteins can be tested to identify which amino acids distinguish the different family members.

We feel that these experiments are vital to understanding the function of GATA-1 during erythroid ontogeny. How does GATA-1 regulate red cell genes like globin or the Epo receptor? Once we identify the functional domains of the GATA-binding proteins, we hope to learn what proteins GATA-1 binds to in the basic transcription machinery or in chromatin. Is GATA-1 necessary for globin gene switching? GATA-1 may be modified differently during development so that the locus control region can interact with different globin promoters. We may find that one region of the protein is required for embryonic expression and another for adult globin gene expression.

The mutant ES cell system provides a novel approach to study directly the activity of a transcription factor in development *in vivo* and *in vitro*. It appears to recapitulate all that occurs in the developing mouse and we hope that this method can be successfully applied to other proteins important to red cell development.

The author wishes to thank her postdoctoral advisor, Dr. Stuart Orkin, for his support and guidance during the last 4 years and her and Dr. Orkin's collaborators on all experiments concerning mutagenesis of GATA-1, Dr. Frank Costantini and Larysa Pevny (Columbia University). Stuart Orkin, Merlin Crossley, and Gerd Blobel reviewed the manuscript prior to submission. M. C. S. is a fellow of the Medical Foundation, Inc.

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