Failure of megakaryopoiesis and arrested erythropoiesis in mice lacking the GATA-1 transcriptional cofactor FOG

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GATA transcription factors are required for the differentiation of diverse cell types in several species. Recent evidence suggests that their biologic activities may be modulated through interaction with multitype zinc finger proteins, such as Friend of GATA-1 (FOG) and U-shaped (Ush). In cell culture, FOG cooperates with the hematopoietic transcription factor GATA-1 to promote erythroid and megakaryocytic differentiation. We show here that mice lacking FOG die during mid-embryonic development with severe anemia. FOG−/− erythroid cells display a marked, but partial, blockage of maturation, reminiscent of GATA-1− erythroid precursors. In contrast to GATA-1 deficiency, however, megakaryocytes fail to develop in the absence of FOG. Although the FOG−/− erythroid phenotype supports the proposed role of FOG as a GATA-1 cofactor in vivo, the latter finding points to a pivotal, GATA-1-independent requirement for FOG in megakaryocyte development from the bipotential erythroid/megakaryocytic progenitor. We speculate that FOG and other FOG-like proteins serve as complex cofactors that act through both GATA-dependent and GATA-independent mechanisms.

[Key Words: Friend of GATA-1 (FOG); GATA-1; cofactor; gene targeting; hematopoiesis]

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GATA transcription factors have emerged as central regulators of diverse developmental processes in both vertebrate and invertebrate species. Members of the GATA family are distinguished by a DNA-binding domain comprised of one or two highly conserved, Cα-type zinc fingers that mediate sequence-specific binding to the consensus motif (A/T)GATA(A/G) (Evans and Felsenfeld 1989; Tsai et al. 1989). In vertebrates, six GATA factors (GATA-1 through GATA-6) have been identified, each serving distinct roles in development (Pevny et al. 1991; Laverriere et al. 1994; Tsai et al. 1994; Ting et al. 1996; Kuo et al. 1997; Molkentin et al. 1997). GATA factors have also been isolated from Drosophila (Raman et al. 1993), Caenorhabditis elegans (Spielth et al. 1991; Zhu et al. 1997), and fungi such as Aspergillus (Kudla et al. 1990).

GATA-1, the founding member of the GATA factor family, is perhaps the most extensively studied of the vertebrate GATA factors. A hematopoietic-restricted transcription factor, GATA-1 is expressed at high levels in erythroid, megakaryocytic, mast, and eosinophilic cells and at lower levels in multipotential progenitors (for review, see Orkin 1998). Consistent with the finding that virtually all characterized erythroid- and megakaryocytic-specific genes contain GATA motifs in critical cis-regulatory elements (Orkin 1992; Shivdasani 1997), gene targeting experiments in embryonic stem (ES) cells and mice have established an essential role for GATA-1 in both the erythroid and megakaryocytic lineages. Loss of GATA-1 in erythroid cells leads to a developmental arrest at the proerythroblast stage and apoptosis (Pevny et al. 1991, 1995; Weiss et al. 1994; Weiss and Orkin 1995), resulting in embryonic lethality by day 11.5 of gestation (Fujiwara et al. 1996). Megakaryocytes lacking GATA-1 also arrest in their maturation, but undergo unrestrained proliferation rather than apoptosis (Shivdasani et al. 1997). In addition to driving terminal maturation of erythroid and megakaryocytic cells, GATA-1 is also capable of influencing the cellular phenotype of recipient hematopoietic progenitors. Specifically, forced expression of GATA-1 induces differentiation of mouse myeloid 416B cells along the megakaryocytic lineage (Visvader et al. 1992) and myb–ets-transformed chicken myeloblasts along erythroid, thromboblastic (megakaryocytic), and eosinophilic lineages (Kullessa et al. 1995).

Fundamental to understanding how GATA-1 functions in differentiation and development is the elucidation of the mechanisms by which it acts in transcription. Although early studies implicated the amino-terminal activation domain of GATA-1 as critical for function based on its transactivation potential in fibroblastic cells (Martin and Orkin 1990), more recent studies have suggested that the biological activity of GATA-1 actually resides in its zinc finger DNA-binding domain, as ass...
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Targeted disruption of the murine FOG gene results in early embryonic lethality

To disrupt the FOG gene, we constructed two different targeting vectors, each of which replaced coding regions by selectable markers (Fig. 1A). Two independently targeted ES cell clones, one derived from each targeting vector (Fig. 1B), were injected into C57BL/6 blastocysts and gave germ-line transmission. Heterozygous mice (FOG+/−) appeared normal and were interbred to generate homozygous mutants (FOG−/−). Among 90 liveborn offspring of FOG−/− crosses, none was homozygous mutant (Table 1), indicating that FOG−/− embryos die in utero. To determine the developmental stage at which the FOG gene mutation is lethal, E10.5-E13.5 embryos were examined. Whereas at E10.5 ~26% of viable embryos were of the FOG−/− genotype (Fig. 1C), at E11.5 only ~14% of viable embryos were FOG−/−, and none survived to E12.5 (Table 1). Thus, homozygous FOG−/− embryos die between E10.5 and E12.5. No phenotypic differences were observed between the two independently targeted mouse lines. Absence of FOG expression in mutant embryos was confirmed by RT–PCR analysis for intact FOG transcripts (Fig. 1D) and by immunofluorescence analysis using antibodies directed against the amino terminus of the FOG protein (data not shown).

At E11.5, viable FOG−/−embryos were comparable in overall development to wild-type and heterozygous littermates, but were readily distinguished by their smaller size and marked pallor (Fig. 2). In contrast to the normal, easily visualized pattern of yolk sac vasculature in littermates (Fig. 2A), the yolk sac vessels of E11.5 FOG−/− embryos appeared very pale and thin (Fig. 2B). Upon closer inspection, the small capillaries of mutant yolk sacs appeared dilated to variable extents, presumably as a secondary consequence of anemia (see below). The fetal livers of mutant embryos were four to five times smaller than those of normal embryos and were also very pale (Fig. 2C,D), suggesting a significant reduction in effective fetal liver (definitive) erythropoiesis.

The phenotype of FOG−/− embryos was studied further by histological examination of E11.5 embryo sections. The fetal liver was of particular interest, as blood formation shifts from the yolk sac to the fetal liver at around E11.5–E12.5 of murine embryonic development. As shown in Figure 3A,B, the fetal livers of homozygous mutant embryos were smaller than those of wild-type embryos, yet contained dark-stained hematopoietic cells within the liver parenchyma, indicating that precursors for the definitive erythroid lineage were present at E11.5. Although smaller than wild-type, other organ systems in FOG−/− embryos also appeared to be normal. Cardiac development, for example, was not obviously impaired in mutant embryos (Fig. 3C,D). Of particular note, no differences were discernible between wild-type and mutant yolk sacs by histological analysis, and endodermal and endothelial cells lining the capillaries of mutant yolk sacs appeared histologically normal (Fig. 3E,F). In addition, the intraembryonic vasculature of mutants, including the aorta (Fig. 3G,H), intersomitic vessels (Fig. 3I,J), and branching head veins (data not shown), also appeared histologically normal.

Primitive hematopoiesis is defective in FOG-deficient embryos

The absence of gross morphological or histological ab-
normalities in FOG−/− embryos pointed to a specific failure of the primitive hematopoietic system in the absence of FOG. To address this, we first examined the peripheral blood of E10.5 and E11.5 embryos. At this stage of development, peripheral blood contains predominantly large primitive erythroid cells of yolk sac origin. These cells are nucleated and express primarily embryonic globin chains as well as small quantities of adult globins (Whitelaw et al. 1990). In contrast to the maturing primitive erythroblasts evident in normal blood (Fig. 4A, left), the primitive erythroid cells of mutant embryos appeared to be partially arrested, with many of the cells resembling proerythroblasts, the first morphologically identifiable erythroid precursors (Fig. 4A, right). In addition, a significant percentage of mutant red cells exhibited a prominent delay in nuclear versus cytoplasmic maturation (megaloblastosis) (Fig. 4A, right).

To determine the effect of this maturational defect on globin gene expression, we analyzed E11.5 peripheral blood RNA for embryonic and adult globin transcripts.
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Definitive erythropoiesis is severely impaired in the absence of FOG

As FOG−/− embryos die at or before the onset of the fetal liver stage of hematopoiesis, we examined a requirement for FOG in definitive erythropoiesis by in vitro hematopoietic colony-forming assays (Wong et al. 1986). Individual yolk sacs and fetal livers from mutant and control embryos were disaggregated into single cell suspensions and cultured in methylcellulose media supplemented with hematopoietic growth factors. Under assay conditions optimal for the differentiation of definitive hematopoietic progenitor cells, wild-type and heterozygous cells generated numerous, hemoglobinized colony-forming unit–erythroid (CFU-E) and burst-forming unit–erythroid (BFU-E) colonies when grown in erythropoietin (Epo) and erythropoietin plus kit ligand (Epo-KL), respectively. Staining of cytosin preparations revealed the presence of definitive erythroid cells at all stages of differentiation, including terminally differentiated, enucleated erythroblasts (Fig. 5A,C). In contrast, mutant progenitor cells yielded only pale CFU-E colonies that contained developmentally arrested proerythroblasts (Fig. 5B). Similarly, BFU-E colonies of FOG−/− origin were pale, stained weakly with benzidine, and contained morphologically aberrant erythroid precursors (Fig. 5D). In addition to large proerythroblasts, mutant colonies also contained cells with fragmented nuclei and clumped chromatin, as well as an abundance of other cell types, particularly mast and mast-like cells (Fig. 5D).

To evaluate the consequences of loss of FOG on definitive erythrocytes in vivo, FOG−/− ES cells were generated (see Materials and Methods) and injected into wild-type host blastocysts. The animals obtained were estimated to be ~50% chimeric based on agouti coat color. To assess contribution of FOG−/− ES cells to mature erythroid cells, we examined the hemoglobin in peripheral blood of adult chimeras. Host blastocysts of strain C57BL/6 are homozygous for the Hbbβ-globin haplotype, whereas CJ-7 ES cells of strain 129/5v are homozygous for the Hbbβ haplotype (Pevny et al. 1991). As shown in Figure 6, chimeras generated with FOG−/− ES cells showed ES cell contribution, as evidenced by the presence of Hbbβ (lane 3). In contrast, chimeras made with FOG−/− ES cells showed only host-derived Hbbα (lane 2), indicating no ES cell contribution. Thus, in accordance with the in vitro findings, FOG is required for production of mature red blood cells in vivo. We propose that the embryonic lethal phenotype of FOG−/− embryos is attributable to the combined failure of both primitive and definitive erythropoiesis.

Loss of FOG leads to the specific ablation of the megakaryocytic lineage at a very early stage

Previously, we have shown that FOG is highly expressed in the megakaryocytic lineage and, furthermore, cooperates with GATA-1 during megakaryocytic cell differentiation (Tsang et al. 1997). Because FOG−/− embryos die before the appearance of detectable numbers of megakaryocytes and platelets in the fetal liver and peripheral blood, respectively, we examined the role of FOG in the megakaryocytic lineage, as well as in other cell types, by cultivating yolk sac and fetal liver cells in the presence of hematopoietic growth factors known to stimulate formation of specific nonerythroid colonies. In the presence of thrombopoietin (Tpo), wild-type and heterozygous cells generated numerous megakaryocyte colonies (Fig. 7A), defined as clusters of >20–50 large cells. As shown in Figure 7B, these colonies contained largely mature megakaryocytes, as evidenced by their characteristic morphology and intense staining for acetylcholinesterase (AChE) activity, a specific marker of mouse megakaryocytes. Under identical assay conditions, no mature megakaryocyte colonies or cells were obtained from either the yolk sacs or fetal livers of viable FOG−/− embryos (Fig. 7A). Staining for AChE activity confirmed the complete absence of mature megakaryocytes, revealing only nonspecific staining of nuclei or, rarely, small, morphologically indistinct cells with weak AChE activity (Fig. 7B). Immunofluorescence staining with rabbit anti-mouse platelet antiserum (McDonald and Jackson 1990) yielded similar results as AChE staining (data not shown). In addition, the combination of Tpo with other growth factors, such as KL, interleukin (IL)-6, and IL-11, failed to enhance the proliferation or differentiation of mutant progenitors with megakaryocytic potential (data not shown).
To investigate further the nature of the megakaryocytic defect resulting from loss of FOG, we examined the expression of megakaryocyte-specific mRNAs by semi-quantitative RT–PCR. Consistent with the morphological and cytochemical data, transcripts for platelet factor 4 (PF4), an early marker of the megakaryocytic lineage (Poncz et al. 1987), were reduced >20-fold in mutant compared to control cultures (Fig. 7C). Similarly, transcripts for glycoprotein IIb (GPIIb), another early marker of the megakaryocytic lineage (Molla et al. 1992), were virtually undetectable in mutant cultures (Fig. 7C). The absence of FOG, therefore, results in an extremely early block in megakaryocyte development.

In contrast, the development of the myeloid lineage appeared normal. As shown in Figure 7A, approximately equal numbers of granulocyte-macrophage (GM) colonies were obtained when wild-type or mutant yolk sac cells were cultivated in the presence of granulocyte-colony-stimulating factor (G-CSF), GM-CSF, IL-3, and IL-11. Furthermore, macrophages and granulocytes recovered from wild-type and mutant GM colonies were morphologically indistinguishable (Fig. 8A–D). Mast cells derived from mutant progenitor cells appeared morphologically normal and stained with toluidine blue (Fig. 8E–H). However, mast and mast-like cells, the latter characterized by multilobed nuclei, mast cell-like granules, and AChE-negative staining, appeared to be abnormally abundant, particularly in cultures grown in Epo-KL or Tpo-KL, but not KL alone (data not shown). As FOG is not expressed in mast cell lines or in primary mast cells differentiated in vitro (Tsang et al. 1997), we speculate that these mast-like cells may represent aberrant bi- or tripotent precursors that cannot proceed normally along the megakaryocytic or erythroid differentiation pathways.

Figure 3. Histological analysis of wild-type and FOG−/− embryos. (A,B) Hematoxylin and eosin (H & E)-stained sagittal sections of wild-type and FOG−/− E11.5 embryos, revealing dark-stained hematopoietic elements within vessels and sinusoids of the fetal liver. Note that at this early stage of fetal liver hematopoiesis only nucleated definitive erythroblasts are observable in wild-type and mutant fetal livers. (FL) fetal liver; (H) heart. Original magnification, 200x (C,D) H&E-stained transverse sections through the fetal hearts of wild-type and FOG−/− E11.5 embryos. Although mutant hearts are smaller than those of wild-type embryos, cardiac development does not appear to be impaired. (a) atrium; (v) ventricle; (ec) endocardial cushion. Original magnification, 100x (E,F) H&E-stained sections of yolk sacs from wild-type and FOG−/− E10.5 embryos. No obvious defects in the vasculature of mutant yolk sacs are apparent by histological analysis. Note the presence of primitive blood cells in sections of mutant yolk sacs. Although not evident here because of H&E overstaining, these mutant cells are morphologically abnormal (see Fig. 4A). (En) extraembryonic endoderm; (EC) endothelial cell. Original magnifications, 400x and 1000x respectively. (G–J) H&E-stained sagittal sections showing the presence of endothelial cell-lined aortas (G,H) and intersomitic vessels (I,J) in both wild-type and mutant embryos. Note the abnormal morphology of mutant blood cells (asterisks). Arrows indicate endothelial cells. Original magnification, 1000x.
FOG−/− ES cells fail to differentiate into mature erythroid or megakaryocytic cells in vitro

To confirm the specific effects of the FOG null mutation on the erythroid and megakaryocytic lineages, we examined the hematopoietic development of wild-type and FOG−/− ES cells using a two-step in vitro differentiation assay (Keller et al. 1993). In this assay, the temporal appearance of hematopoietic precursors in developing embryoid bodies (EBs) parallels that found in the embryo. Embryoid bodies generated from two independent FOG−/− ES cell clones were disaggregated at select times and replated in the presence of various growth factors (see Materials and Methods). Both wild-type and FOG−/− EBs gave rise to primitive erythroid (EryP) precursors with replating in the presence of Epo. However, mutant EryP colonies were found at a reduced frequency and were typically pale or pink rather than red (data not shown). As shown in Figure 9B, cells within these colonies appeared to be partially arrested at a proerythroblast-like stage, closely resembling the primitive red blood cells of FOG−/− embryos (see Fig. 4A, right). Similarly, mutant EryD cells displayed a prominent defect in maturation, with many of the cells appearing proerythroblast-like or megaloblastic (Fig. 9D). Finally, in contrast to wild-type EBs, mutant EBs failed to generate any megakaryocytic colonies when replated in the presence of Tpo (Fig. 9E). In contrast, macrophage precursor-derived colonies developed from FOG−/− EBs (Fig. 9G), albeit at a slightly reduced frequency. Taken together, our in vivo and in vitro data demonstrate an intrinsic defect in FOG−/− hematopoietic progenitors that impairs selec-
C57BL/6; Hbbd (ES) is specific for ES cells of strain 129/Sv. The GATA-1− phenotype. In contrast to GATA-1 partial, arrest in erythroid maturation, closely resembling the GATA-1− phenotype. In contrast to GATA-1 partial, arrest in erythroid maturation, closely resembling the GATA-1− phenotype. In contrast to GATA-1 partial, arrest in erythroid maturation, closely resembling the GATA-1− phenotype. In contrast to GATA-1 partial, arrest in erythroid maturation, closely resembling the GATA-1− phenotype. In contrast to GATA-1 partial, arrest in erythroid maturation, closely resembling the GATA-1− phenotype. In contrast to GATA-1 partial, arrest in erythroid maturation, closely resembling the GATA-1− phenotype. In contrast to GATA-1 partial, arrest in erythroid maturation, closely resembling the GATA-1− phenotype. In contrast to GATA-1 partial, arrest in erythroid maturation, closely resembling the GATA-1− phenotype. In contrast to GATA-1 partial, arrest in erythroid maturation, closely resembling the GATA-1− phenotype. In contrast to GATA-1 partial, arrest in erythroid maturation, closely resembling the GATA-1− phenotype. In contrast to GATA-1 partial, arrest in erythroid maturation, closely resembling the GATA-1− phenotype. In contrast to GATA-1 partial, arrest in erythroid maturation, closely resembling the GATA-1− phenotype. In contrast to GATA-1 partial, arrest in erythroid maturation, closely resembling the GATA-1− phenotype. In contrast to GATA-1 partial, arrest in erythroid maturation, closely resembling the GATA-1− phenotype. In contrast to GATA-1 partial, arrest in erythroid maturation, closely resembling the GATA-1− phenotype. In contrast to GATA-1 partial, arrest in erythroid maturation, closely resembling the GATA-1− phenotype. In contrast to GATA-1 partial, arrest in erythroid maturation, closely resembling the GATA-1− phenotype. In contrast to GATA-1 partial, arrest in erythroid maturation, closely resembling the GATA-1− phenotype. In contrast to GATA-1 partial, arrest in erythroid maturation, closely resemble...
contrast to FOG and GATA-1, SCL/tal-1, and presumably Rbtn2/LMO2, function at the very earliest, definable stage of hematopoietic development. Taken together, these findings predict the existence of various, highly dynamic protein complexes involving factors such as GATA-1, FOG, Rbtn2/LMO2, and SCL/tal-1 that change in abundance and composition as hematopoietic progenitors arise, commit to specific lineages, and differentiate along single pathways (Orkin 1998).

FOG and other GATA transcription factors

Previously, we have shown that FOG is capable of interacting physically with other GATA factors in addition to GATA-1 (Tsang et al. 1997). This finding, together with the observation that FOG RNA is present in multipotential progenitor cell lines and at lower levels in certain T-lymphoid lines (Tsang et al. 1997) raised the intriguing possibility that FOG might act as a cofactor for the other hematopoietic GATA factors. Our present data, however, do not support an in vivo role for FOG in either GATA-2 or GATA-3 function. GATA-2, for example, has been shown by targeted gene disruption to be critical for maintaining the proliferative capacity of immature progenitor cells and for development of the mast cell lineage (Tsai et al. 1994; Tsai and Orkin 1997). In the absence of FOG, neither multipotential progenitors nor mast cells

Figure 7. Absolute requirement for FOG in early megakaryopoiesis in vitro. Yolk sac and fetal liver cells from wild-type and homozygous mutant (−/−) embryos were assayed in semisolid medium for megakaryocytic colonies. (A) Number of megakaryocytic (MK) and granulocyte-macrophage (GM) colonies observed after 6 days of cultivation. Numbers (N) at the top of each error bar indicate the number of embryos analyzed. Error bars represent SEM. (YS) yolk sac; (FL) fetal liver. (B) May–Grunwald-Giemsa and AChE staining of cells from wild-type and mutant (−/−) colonies. Wild-type megakaryocytes were readily identified by their large size, multilobed nuclei, granular cytoplasm, and AChE positivity, which appears as orange/brown cytoplasmic staining. Small, AChE-positive cells were very rarely observed in mutant cultures. Original magnification, 630×. (C) RT–PCR analysis of PF4 and GPIIb mRNAs from hematopoietic colonies cultured in the presence of Tpo. RT–PCR amplification of HPRT transcripts served as an internal control for quantitation. Samples were amplified for six cycles in the presence of HPRT primers, before addition of PF4 primers.
role for FOG in the development of the megakaryocytic lineage. In its absence, megakaryocytes fail to develop from either hematopoietic progenitors in the embryo or from FOG−/− ES cells differentiated in vitro. Although the presence of rare, AChE-positive cells and low levels of PF4 transcripts (Fig. 7B) suggests that megakaryocytic commitment takes place in the absence of FOG, at least to a small extent, these findings also demonstrate a nonredundant function for FOG in early megakaryocyte development. This profound consequence of FOG loss was unexpected given the proposed mechanism of action of FOG as a GATA-1 cofactor in both erythroid and megakaryocytic cells (Tsang et al. 1997). Although recent studies have demonstrated a critical role for GATA-1 in the megakaryocytic lineage, GATA-1 appears to be necessary at a somewhat later stage of development (Fig. 10). Specifically, GATA-1− megakaryocytes express PF4 and GPIIb, and exhibit deregulated proliferation and severely impaired cytoplasmic maturation (Shivdasani et al. 1997). The early effect of FOG deficiency on megakaryocyte development does not preclude a role for FOG as a GATA-1 cofactor during later stages. Rather, we speculate that FOG exhibits dual functions in megakaryocytes: an early, GATA-1-independent role, perhaps at the very earliest stage of development from the bipotential erythroid/megakaryocytic progenitor, and a later, GATA-1-dependent role during terminal megakaryocyte maturation.

To date, the specific ablation of the megakaryocytic lineage has not been a phenotypic consequence of any transcription factor gene knockout. Indeed, previous studies have identified only one other transcription factor besides GATA-1, the basic leucine zipper protein NF-E2 (Andrews et al. 1993), that is specifically required for the development of megakaryocytes. Like GATA-1, however, NF-E2 is only necessary for advanced stages of megakaryocyte maturation, as endomitosis and expression of lineage-specific markers such as AChE appear to be unaffected in the absence of NF-E2 (Shivdasani et al. 1995b). The only other genes required for the formation of megakaryocytes are those that are essential for the earliest hematopoietic progenitors, for example SCL/tal-1 (Porcher et al. 1996) and CBF (Okuda et al. 1996; Wang et al. 1996), and as such are required for the development of all hematopoietic lineages. Thus, among the known hematopoietic nuclear regulatory factors, FOG is unique in defining the earliest stage in megakaryocyte development. Although the identity of this stage has not been established, we speculate that FOG may be required for the proliferation and survival of an early megakaryocyte-specific progenitor or perhaps for the differentiation of a bi- or tripotent progenitor along the megakaryocytic lineage. The latter is indirectly supported by the abundance of peculiar cell types in mutant cultures characterized by multilobed nuclei, mast cell-like granules, and red cell morphology. In the absence of other known genes required specifically for early stages in megakaryocyte development, it is likely that FOG-regulated target genes will function as novel components of developmental pathways critical for the survival, prolif-

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**Figure 8.** Morphological assessment of myeloid and mast cells lacking FOG. Yolk sac and fetal liver cells from wild-type and mutant (−/−) embryos were assayed in semisolid medium for granulocyte-macrophage or mast colonies. Macrophages (A,B) and granulocytes (C,D) from wild-type and mutant myeloid colonies were morphologically indistinguishable. Cells with mast cell morphology (E,F) and toluidine blue-positive granules (G,H) were also identified in wild-type and mutant cultures. Original magnifications, 630× (macrophages), 1000× (granulocytes and mast cells).

A GATA-1 independent requirement for FOG in early megakaryopoiesis

Our findings establish a pivotal, GATA-1 independent
An essential role for FOG in hematopoiesis

Figure 9. In vitro differentiation of FOG−/− ES cells. (A–G) May–Grunwald–Giemsa staining of cells from primitive erythroid (EryP) colonies (A,B), definitive erythroid (EryD) colonies (C,D), megakaryocyte colonies (E), and macrophage colonies (F,G). Mutant EryP and EryD cells display a prominent defect in maturation, as evidenced by their large size and uncondensed nuclei. No megakaryocytic cells were obtained upon replating mutant embryoid bodies in the presence of thrombopoietin. Wild-type and mutant macrophages were morphologically indistinguishable. Original magnifications, 1000× (erythroid and megakaryocytic cells), 630× (macrophages).

Implications of FOG for other GATA cofactors

Recent evidence suggests that a novel family of complex zinc finger proteins, exemplified by FOG and Ush, function to modulate the transcriptional activities of GATA factors in mammalian and Drosophila development, respectively (Cubada et al. 1997; Haenlin et al. 1997; Tsang et al. 1997). Whereas FOG cooperates with GATA-1 in erythroid and megakaryocytic differentiation, Ush antagonizes the effects of the GATA factor Pannier during bristle patterning. Thus, the activities of GATA proteins may be modulated either positively or negatively through interaction with their respective cofactors. The aim of our present work was to address the validity of this paradigm. We have shown that FOG, like GATA-1, is indeed essential for terminal erythroid maturation, thereby supporting the proposed role of FOG as a GATA-1 cofactor in red blood cells. The unexpected finding of an absolute requirement for FOG during early, rather than late, megakaryocyte maturation, reveals an additional, GATA-1 independent role for FOG as an essential and specific regulator of early megakaryocyte development. Defining how FOG functions in early megakaryopoiesis will likely reveal novel GATA-independent mechanisms by which FOG and related finger proteins regulate transcription and development.

Materials and methods

All recombinant DNA work was done using standard techniques (Sambrook et al. 1989). Details of plasmid constructions are available upon request.

Targeted disruption of the murine FOG gene

Overlapping FOG genomic clones were isolated from a λFixII mouse strain 129 library (Stratagene). The exon/intron structure was determined by restriction enzyme mapping, DNA sequencing, and PCR. To generate the first targeting construct, a 2.9-kb XbaI–KpnI fragment containing FOG 5′ genomic DNA and a 2.8-kb HindIII fragment containing 3′ genomic DNA were cloned into pPNT (Tybulewicz et al. 1991). The second targeting vector was constructed by inserting a 10-kb 5′ BamHI–BstEII fragment and a 1.6-kb 3′ BglII–HindIII fragment into pTKLNC (a gift from R. Mortensen, Brigham and Women’s Hospital, Harvard Medical School, Boston, MA). The two targeting constructs were linearized with NotI and XhoI, respectively, and electroporated into C57BL/6 ES cells. Transfectants were selected in G418 (280 µg/ml) and ganciclovir (2 µM) and expanded for Southern blot analysis. The frequency of homologous recombination with each targeting vector was 1 in 132 and 1 in 124, respectively. Two independently generated, targeted ES cell clones with normal karyotypes were injected into C57BL/6 blastocysts. Male chimeras were mated to C57BL/6 females, and heterozygous offspring were intercrossed to generate homozygous mutants. Genotyping of pups and embryos was done by Southern blot analysis. No differences in phenotype were observed between mice derived from the two independent ES cell clones, nor between mice of different genetic backgrounds (mixed 129/Sv-C57BL/6 or inbred 129/Sv).

Generation of FOG−/− ES cells

Heterozygous FOG−/− ES cell clones were passaged on gelatin-treated plates as described (Weiss et al. 1994) and grown in high concentrations (1.2–3.0 mg/ml) of G418 (Mortensen et al. 1992). Homozygous mutant ES cell clones were identified by Southern blot analysis.

Figure 10. Developmental blocks imposed by loss of FOG or GATA-1. The consequences of FOG or GATA-1 deficiency for erythroid development are remarkably similar, strengthening the notion that the two proteins act in concert to drive erythroid-specific differentiation. In contrast to GATA-1 deficiency, loss of FOG leads to the specific ablation of the megakaryocytic lineage at a very early stage, pointing to a pivotal, GATA-1-independent role for FOG in early megakaryopoiesis.
Histology and cytology
E10.5 yolk sacs and E11.5 embryos were dissected from Bouin’s solution and embedded in paraffin. Sections (5 µm) were cut and stained with hematoxylin and eosin for histological examination. Cytocentrifuge preparations were stained with May–Grunwald–Giemsa or toluidine blue for morphological assessment, or for AChE activity according to standard protocol.

In vitro hematopoietic progenitor colony assay
Yolk sacs and fetal livers were dissected from E10.5 and E11.5 embryos under sterile conditions. Cells were disaggregated (Wong et al. 1986) and then plated in methylcellulose media containing 30% FCS and supplemented with one or more of the following recombinant growth factors: Epo (2 U/ml), rat KL (50 ng/ml), human Tpo (0.5 µg/ml), IL-3 (10 ng/ml), IL-6 (2 ng/ml), human IL-11 (5 ng/ml), human G-CSF (0.5 ng/ml), and GM-CSF (5 ng/ml).

In vitro hematopoietic differentiation
In vitro differentiation of ES cells was performed as described (Keller et al. 1993; Weiss et al. 1994). Primitive erythroid, EryD, and megakaryocyte (Mk) colonies were generated by cultivation in Epo, Epo-KL, and Tpo, respectively. Macrophage (MØ) colonies were obtained following growth in G-CSF, GM-CSF, IL-3, and IL-11.

Hemoglobin assay
Hemoglobin analysis was performed as described (Pevny et al. 1991).

Semiquantitative RT–PCR and RNase protection assay
Total RNA was isolated from cultured hematopoietic colonies or peripheral blood of viable E11.5 embryos by standard procedures (Chomczynski and Sacchi 1987). First strand cDNA synthesis and PCR (94°C for 1 min, 55°C for 1 min, 72°C for 1.5 min) in the presence of tracer [32P]dCTP were performed essentially as described (Weiss et al. 1994). Primer sets were PF4 and GPlbβ (Shivdasani et al. 1995b), GATA-1 and HPRT (Weiss et al. 1994), and FOG 5'-CACCCCTTGCAAGAACAGT-3' and 5'-GGTTTCTTCTTGCGCCG-3' (product 246 bp). RNase protection assays were performed according to standard protocol using previously reported RNA probes for βH1 globin (Perkins et al. 1995) and α-, β-, γ-, and ε-globins (Baron and Orkin 1986).

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