The EMBO Journal Vol.16 No.13 pp.3965–3973, 1997

A lineage-selective knockout establishes the critical role of transcription factor GATA-1 in megakaryocyte growth and platelet development

Ramesh A.Shivdasani[1–4], Yuko Fujiwara[2,4,5], Michael A.McDevitt[2–5] and Stuart H.Orkin[1,2,4–6]

Departments of Medicine, [1]Dana-Farber Cancer Institute, [2]Children's Hospital, [3]Brigham and Women's Hospital, [4]Harvard Medical School and [5]The Howard Hughes Medical Institute, Boston, MA 02115, USA

[6]Corresponding author
e-mail: orkin@rascal.med.harvard.edu

Y.Fujiwara and M.A.McDevitt contributed equally to this work

Transcription factor GATA-1 is essential for red blood cell maturation and, therefore, for survival of developing mouse embryos. GATA-1 is also expressed in megakaryocytes, mast cells, eosinophils, multipotential hematopoietic progenitors and Sertoli cells of the testis, where its functions have been elusive. Indeed, interpretation of gene function in conventional knockout mice is often limited by embryonic lethality or absence of mature cells of interest, creating the need for alternate methods to assess gene function in selected cell lineages. Emerging strategies for conditional gene inactivation through site-specific recombinases rely on the availability of mouse strains with high fidelity of transgene expression and efficient, tissue-restricted DNA excision. In an alternate approach, we modified sequences upstream of the GATA-1 locus in embryonic stem cells, including a DNase I-hypersensitive region. This resulted in generation of mice with selective loss of megakaryocyte GATA-1 expression, yet sufficient erythroid cell levels to avoid lethal anemia. The mutant mice have markedly reduced platelet numbers, associated with deregulated megakaryocyte proliferation and severely impaired cytoplasmic maturation. These findings reveal a critical role for GATA-1 in megakaryocyte growth regulation and platelet biogenesis, and illustrate how targeted mutation of *cis*-elements can generate lineage-specific knockout mice.

Keywords: conditional gene inactivation/differentiation/GATA transcription factors/megakaryocytes/platelets

Introduction

Many genes provide critical functions at different stages of development or in multiple tissues. When gene knockouts in mice are used to define *in vivo* function (Capecchi, 1989), embryonic lethality may obscure important roles later in development or in specific cell lineages. For example, the zinc finger transcription factor GATA-1 is essential for red blood cell formation (Pevny *et al.*, 1991) and, consequently, for survival of developing mouse embryos (Fujiwara *et al.*, 1996); in its absence, erythroid precursor cells display arrested maturation and undergo apoptosis (Weiss and Orkin, 1995). GATA-1 is also expressed in other hematopoietic lineages (megakaryocytes, mast cells, and eosinophils) and in Sertoli cells of the pubescent testis (Ito *et al.*, 1993; Yomogida *et al.*, 1994), where its functions *in vivo* are unknown.

Indirect evidence has suggested that GATA-1 might serve particularly important functions in megakaryocytes, the precursors of circulating blood platelets. Forced expression of GATA-1 re-programs chicken myeloblasts into erythroblasts, eosinophils or thromboblasts, the avian equivalent of megakaryocytes (Kulessa *et al.*, 1995), and overexpression of GATA-1 imparts a megakaryocytic phenotype to mouse myeloid 416B cells (Visvader *et al.*, 1992). Additionally, numerous *cis*-regulatory sequences of megakaryocyte-expressed genes depend on GATA consensus-binding motifs for full transcriptional activity *in vitro* (reviewed in Shivdasani, 1997). Nonetheless, study of mouse chimeras generated by introducing GATA-1$^-$ embryonic stem (ES) cells into wild-type blastocysts could not establish a role for GATA-1 in this cell lineage; although megakaryocytes and platelets of GATA-1$^-$ origin appear in such chimeras, neither the quality of megakaryocyte development nor the efficiency of platelet production could be evaluated accurately (Pevny *et al.*, 1995). Therefore, the unique role, if any, of GATA-1 in this lineage has remained uncertain.

To assess gene function in selected cell lineages, conditional gene inactivation may be achieved through the use of a site-specific recombinase, such as the Cre or FLP recombinases of bacteriophage P1 (Sauer and Henderson, 1990) and yeast (O'Gorman *et al.*, 1991), respectively. This and other strategies to create lineage-restricted gene knockouts (Gu *et al.*, 1994; Grupe *et al.*, 1995) rely heavily on the availability of transgenic mice expressing the recombinase or other genes in a restricted tissue distribution and on the efficiency of recombinase-mediated excision in specific cell lineages. When Cre recombinase is used to excise DNA sequences flanked by loxP recognition sequences, a high but variable frequency of *in vivo* excision has been observed (Gu *et al.*, 1994; Kuhn *et al.*, 1995; Feil *et al.*, 1996; Tsien *et al.*, 1996). While this is likely to prove satisfactory in many circumstances, the variable efficiency of excision in various tissues and the presence of residual cells harboring a functional gene may sometimes hinder the analysis.

We report that a targeted mutation of regulatory elements within the GATA-1 locus leads to a lineage-selective loss of expression and uncovers essential roles for the factor in megakaryocyte proliferation and maturation and in production of circulating blood platelets. This critical requirement for GATA-1 in megakaryocyte and platelet development could not be appreciated in chimeras or conventional knockout embryos. Our experience suggests that the targeting approach used here may prove advanta-

© Oxford University Press

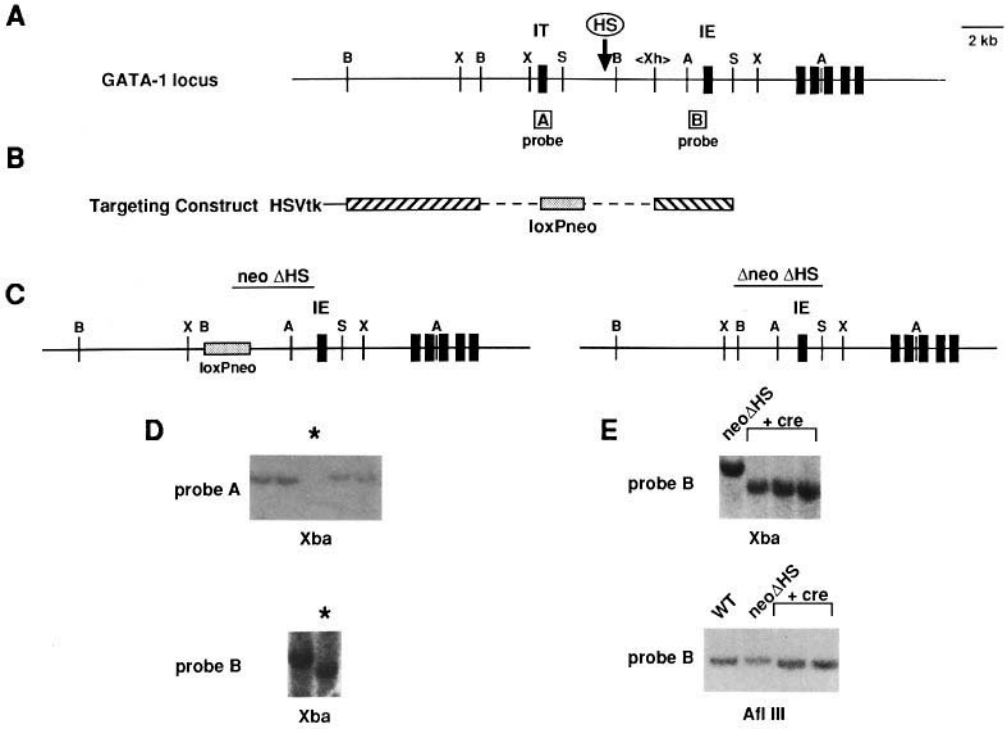

Fig. 1. Targeted modification of the GATA-1 locus. (**A**) Structural organization of the locus, indicating the relative positions of IT, IE, HS, selected restriction sites (A, *Afl*III; B, *Bam*HI; S, *Sma*I; X, *Xba*I; Xh, *Xho*I) and probes A and B for Southern analysis. (**B**) Regions of 5′- and 3′-homology in the targeting construct, indicated by hatched boxes. (**C**) In the modified GATA-1 loci, ~8 kb of upstream sequences are replaced by either the loxPneo cassette (neoΔHS) or a single loxP site (ΔneoΔHS). (**D** and **E**) Representative Southern blots confirming the desired modifications, as described in the text. The apparent, minor difference in migration of bands in (E) reflects slightly unequal loading of DNA in the gel.

geous in the study of other genes when embryonic lethality limits analysis of conventional knockout mice.

Results

Targeted mutation of the GATA-1 locus

The GATA-1 gene is expressed from two promoters, designated IT and IE (Figure 1A), located ~8 kb apart on the X chromosome (Ito *et al.*, 1993). IT is active predominantly in Sertoli cells, whereas IE is largely hematopoietic specific; a low level of cytokine-responsive IT promoter activity (2–5% of IE) can be detected in hematopoietic cells (Cairns *et al.*, 1994). To assess the impact of selective loss of GATA-1 expression on Sertoli cell function, we generated mice carrying germline replacement of the IT region with a PGK-Neo^R cassette (R.A.Shivdasani, M.Yamamoto, J.D.Engel, and S.H.Orkin, unpublished data) and noted that red blood cell production, fertility and Sertoli cell morphology were all intact in these animals. Residual, IE-derived GATA-1 expression in the testis, ⩽10% of the normal level, may account for the apparently normal phenotype.

Subsequently, we identified a region of DNase I hypersensitivity (HS) in the chromatin of an erythroleukemic cell line; this region lies between the two GATA-1 promoters (Figure 1A) and confers high level, hematopoietic-restricted expression of a reporter gene in transgenic mice (McDevitt *et al.*, 1997a). We therefore targeted ES cells to replace ~8 kb of upstream sequences, including the IT and HS regions, with a PGK-Neo^R cassette flanked by loxP sites (loxPneo, Figure 1B), and passed the mutation (designated neoΔHS) through the mouse germline (Figure 1C and D). We also removed the selectable marker from the targeted locus by expressing Cre recombinase in ES cells and produced a second mouse strain (ΔneoΔHS) in which the targeted upstream region is replaced by a single loxP site (Figure 1C and E). Representative Southern blots demonstrate deletion of IT sequences (Figure 1D), excision of PGK-Neo^R (Figure 1E, *Xba*I digest) and absence of rearrangements downstream of the targeted region (Figure 1E, *Afl*III digest).

Platelet numbers are markedly reduced in neoΔHS and ΔneoΔHS mice

In contrast to GATA-1$^-$ embryos, which invariably die by E10.5 (Fujiwara *et al.*, 1996), ~5% of hemizygous neoΔHS males survive fetal anemia into adult life. Their inefficient red blood cell production is accounted for by a modest (~5-fold) reduction in erythroid GATA-1 mRNA levels and delayed maturation of red cell precursors (McDevitt *et al.*, 1997b). Removal of the PGK-neo^R cassette restores viability substantially, and is accompanied by near-normal red cell maturation and GATA-1 expression. This latter finding indicates that the presence of the selectable marker in the neoΔHS locus interferes with transcription in erythroid cells, a phenomenon that has also been noted in several other instances (Fiering *et al.*, 1995; Olson *et al.*, 1996).

Remarkably, the number of blood platelets in hemizygous males of both mutant mouse strains is reduced to ~15% of normal (Figure 2A and B). Moreover, the few circulating platelets are considerably larger than normal

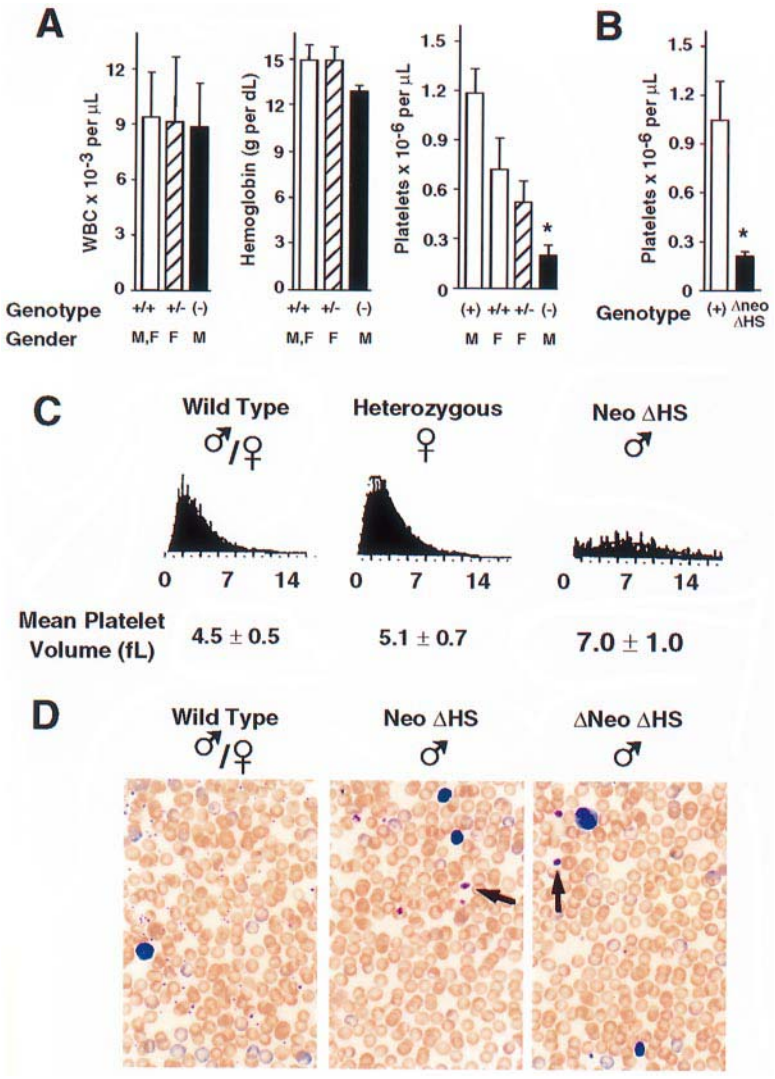

Fig. 2. Parameters of circulating blood cells in mutant and control animals. (**A**) Leukocyte and platelet numbers and hemoglobin concentration in adult wild-type males and females (+/+, $N = 12$); heterozygous females (+/–, $N = 15$) and neoΔHS hemizygous males (–, $N = 6$). (**B**) Platelet counts from 2-week-old wild-type (+, $N = 6$) and ΔneoΔHS hemizygous (–, $N = 4$) male mice. (**C**) Platelet volume histograms and mean platelet volumes (in femtoliters) of adult wild-type, heterozygous and hemizygous neoΔHS mice. (**D**) Peripheral blood smears confirm both the paucity of platelets in neoΔHS (center) and ΔneoΔHS males (right) relative to littermate controls (left), and their substantially larger size (arrows).

in size, as shown by measurement of volume distribution (Figure 2C) and examination of peripheral blood smears (Figure 2D). In contrast, red cell number and size are nearly normal in adult mice (Figure 2A and D), and leukocytes are not visibly affected. Liveborn mice of either mutant strain do not bleed spontaneously under standard housing conditions, indicating some preservation of platelet function.

The neoΔHS mutation leads to megakaryocyte-restricted loss of GATA-1 expression

GATA-1 mRNA is readily detected by RT–PCR in control but not in neoΔHS megakaryocytes cultured from adult bone marrow (Figure 3A) or fetal livers (data not shown); control reactions for hypoxanthine phosphoribosyl transferase (HPRT, Figure 3A) or actin (data not shown) transcripts verify equal amounts of input mRNA. Accordingly, neoΔHS megakaryocytes fail to stain with GATA-1-specific antiserum (Figure 3B), confirming the absence of GATA-1 expression in this lineage. In contrast, GATA-1 protein is readily detected in neoΔHS erythroid cells (Figure 3C), consistent with the very presence of red blood cells, which signifies substantial erythroid GATA-1 protein. These findings demonstrate that replacing ~8 kb of the upstream region with a PGK-neo^R cassette selectively abrogates GATA-1 expression in megakaryocytes while allowing for viability through sufficient expression in the erythroid lineage. Accordingly, the functions of GATA-1 in megakaryocytes can be assessed in animals spared from early embryonic lethality.

GATA-1 mRNA expression is partially restored in ΔneoΔHS megakaryocytes, albeit substantially reduced relative to the wild-type (Figure 3D), and little, if any, GATA-1 protein is detected by immunostaining (data not shown). This limited reactivation of GATA-1 expression is insufficient to restore normal platelet production

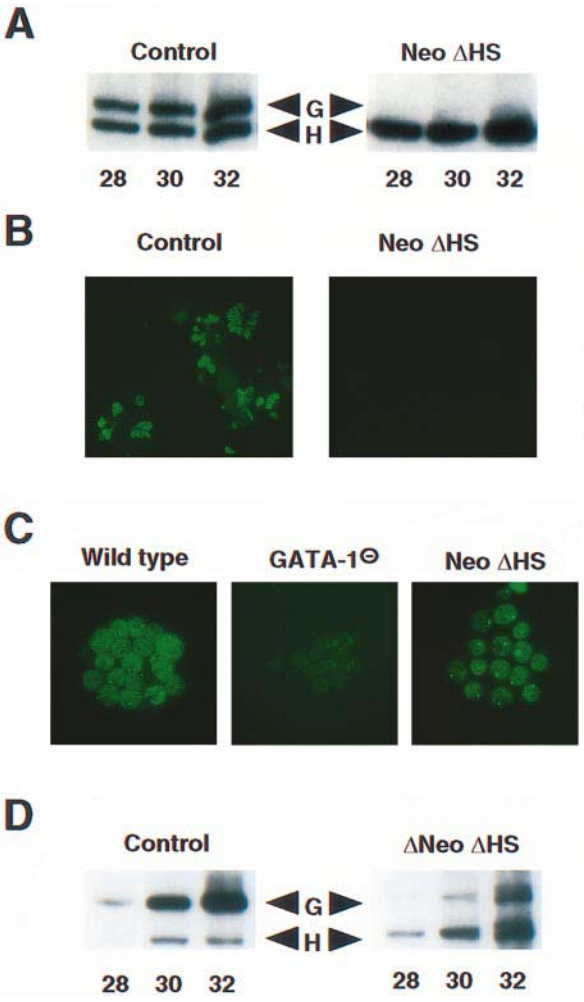

Fig. 3. Megakaryocyte-specific loss of GATA-1 expression upon targeted modification of upstream regulatory sequences. (**A**) RT–PCR analysis shows the absence of GATA-1 (G) mRNA in cultured megakaryocytes from neoΔHS mice, compared with equivalent amounts of HPRT (H) mRNA; numbers refer to PCR cycles. (**B** and **C**) Immunofluorescence with anti-GATA-1 antiserum confirms the absence of GATA-1 protein in neoΔHS megakaryocytes (B) but its presence in erythroid cells cultured *in vitro* from neoΔHS ES cells, in contrast to GATA-1-null ES cells (C). (**D**) Partially restored, low-level expression of GATA-1 (G) mRNA in megakaryocytes cultured from ΔneoΔHS mice, as judged by RT-PCR performed as in (A).

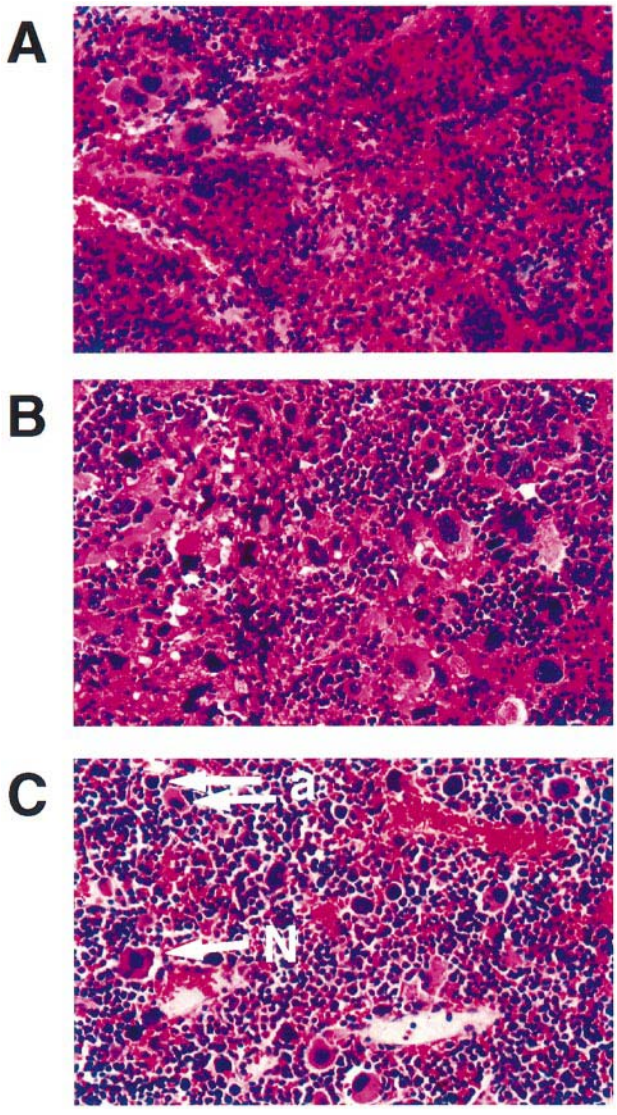

Fig. 4. Megakaryocytosis in hematopoietic tissues of hemizygous mutant males and heterozygous females. Histologic sections of the spleen from wild-type mice (**A**) reveal the normally sparse number, size and morphology of megakaryocytes. In contrast, a much larger number of small and poorly developed megakaryocytes is seen in specimens from hemizygous neoΔHS (**B**) or ΔneoΔHS (not shown) adult males. Females heterozygous for neoΔHS (**C**) or ΔneoΔHS (not shown) are mosaic for normal (N) and abnormal (a) megakaryocytes (original magnification ×200).

(Figure 2B), and all the megakaryocyte properties described below are indistinguishable between neoΔHS and ΔneoΔHS mice. We conclude, therefore, that the low level of GATA-1 mRNA in ΔneoΔHS megakaryocytes is below the threshold required for normal maturation and platelet formation.

Megakaryocytes are more abundant in neoΔHS and ΔneoΔHS mice

To determine the basis for thrombocytopenia in neoΔHS and ΔneoΔHS mice, we assessed the number of megakaryocytes. The spleen (Figure 4) and bone marrow (data not shown) of hemizygous males from both mutant strains contain up to 100- and up to 10-fold more megakaryocytes, respectively, than littermate controls. Moreover, these megakaryocytes are smaller than normal and reveal scant cytoplasm and condensed nuclei. Female mice heterozygous for the neoΔHS and ΔneoΔHS mutations have platelet numbers within the normal range (Figure 2A), yet the number of megakaryocytes in hematopoietic tissues is greatly increased (Figure 4C), as in mutant males. This suggests that the megakaryocytosis seen in these females and, by extension, in affected males is independent of low platelet numbers. Consistent with this view, inspection of megakaryocytes in female heterozygotes reveals a normal sub-population as well as a second, more abundant sub-population characterized by the abnormal morphology seen in affected males (Figure 4C). Such heterogeneity, best explained by mosaicism resulting from random inactivation of the X-linked GATA-1 locus, suggests that the pool of mutant megakaryocytes is intrinsically large. Indeed, mutant males and heterozygous females reveal an excess of abnormal megakaryocytes in the yolk sac and

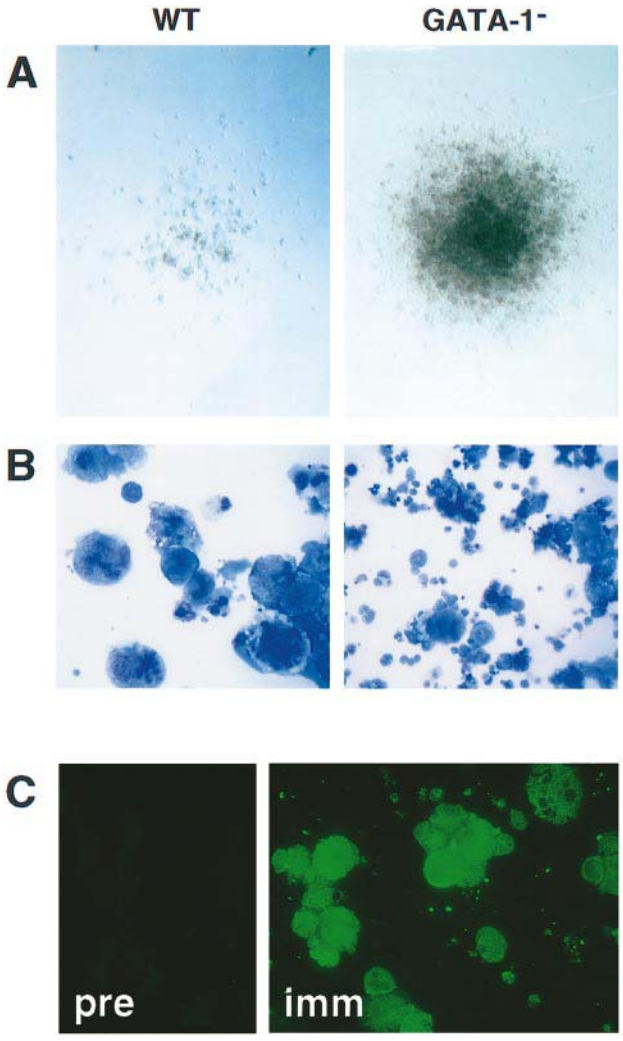

Fig. 5. Deregulated growth of neoΔHS and ΔneoΔHS megakaryocyte progenitors. Whereas roughly equal numbers of c.f.u.-Meg (**A**, WT) are present in cultures of wild-type and mutant hematopoietic tissues, the majority of colonies derived from neoΔHS and ΔneoΔHS fetal livers (A) and yolk sacs (not shown) are considerably larger. (**B**) May–Grunwald–Giemsa staining of cells from these colonies reveals maturing megakaryocytes, of smaller than normal size, as well as morphologically indistinct cells (original magnification ×400). (**C**) Immunofluorescence with anti-platelet antiserum (imm, right; pre = pre-immune serum control, left) confirms that the majority of cells derived from these colonies are of the megakaryocyte lineage.

early fetal liver (data not shown), prior to the onset of platelet production and the likely establishment of any feedback controls on platelet number.

Proliferation of GATA-1⁻ megakaryocytes is deregulated

To characterize further the apparent perturbation in megakaryocyte growth, we performed colony assays of hematopoietic cells harvested from yolk sacs and fetal livers. The frequency of megakaryocyte colony forming units (c.f.u.-Meg), defined as clusters of >20–50 mature cells (Figure 5A and B, WT), does not differ between mutants and littermate controls (3681 ± 405 c.f.u.-Meg per E12.5 fetal liver). However, the vast majority of colonies derived from neoΔHS or ΔneoΔHS fetal livers are significantly larger and exhibit a characteristic morphology (Figure 5A); such colonies appear rarely in control cultures (<1/100 c.f.u.-Meg). In addition to mature megakaryocytes, these colonies contain numerous small, morphologically indistinct cells that harbor few granules (Figure 5B) and display weak or no cholinesterase activity (data not shown). Immunostaining with anti-mouse platelet antiserum establishes that the majority of these cells are immature megakaryocytes (Figure 5C). Similar colonies are also observed when neoΔHS fetal liver cells are cultured in a combination of erythropoietin and stem cell factor (SCF, c-kit ligand), but they are not seen with either factor alone, or with granulocyte–macrophage colony-stimulating factor (GM-CSF) or interleukin (IL)-3 or -6 (data not shown). As expected, cells derived from heterozygous females yield similar colonies.

These results indicate that loss of GATA-1 results in greatly enhanced proliferation of megakaryocyte progenitors, a finding that is very likely to account for the abundance of megakaryocytes in heterozygous and hemizygous neoΔHS and ΔneoΔHS mice. Our observations also provide an explanation for the previously reported excess of GATA-1⁻ megakaryocytes in the fetal livers of mouse chimeras generated by introducing GATA-1⁻ ES cells into wild-type blastocysts (Pevny *et al.*, 1995).

Maturation of megakaryocytes is arrested in the absence of GATA-1

During their maturation, megakaryocytes initially undergo successive rounds of DNA replication without cytokinesis (endomitosis or endoreduplication), which result in a characteristically large, lobulated nucleus with polyploid DNA content. The hallmark of subsequent cytoplasmic maturation is the development of platelet fields or territories, collections of platelet-specific granules and other organelles within a system of demarcation membranes (Zucker-Franklin, 1989). On ultrastructural examination, GATA-1⁻ megakaryocytes contain large, highly segmented nuclei with condensed chromatin and a variably increased number of nucleoli (Figure 6A and B). Additionally, an inappropriately small cytoplasm harbors abundant endoplasmic reticulum and a wide, organelle-depleted peripheral zone (Figure 6C and D), features that indicate relative immaturity. Although frequently present, the demarcation membranes are either underdeveloped (Figure 6B and D) or highly disorganized (Figure 6E) and almost never observed to participate in differentiation of normal platelet fields; in many cells, demarcation membranes are absent altogether. Finally, platelet-specific granules are represented only sparsely or not at all within the cytoplasm of developing megakaryocytes (Figure 6B, D and E). Taken together, these findings indicate that megakaryocytes lacking GATA-1 are severely defective in their program of late cytoplasmic maturation and platelet biogenesis.

Although GATA-1⁻ embryos die at the yolk sac stage (Fujiwara *et al.*, 1996), precluding direct study of their megakaryocytes, female mice heterozygous for the X-linked mutation (GATA-1$^{+/-}$) are mosaic for normal and abnormal megakaryocytes, with the latter predominating, as noted in neoΔHS and ΔneoΔHS mice (data not shown). Moreover, the ultrastructure of the majority of megakaryocytes from such heterozygotes is indistinguishable from

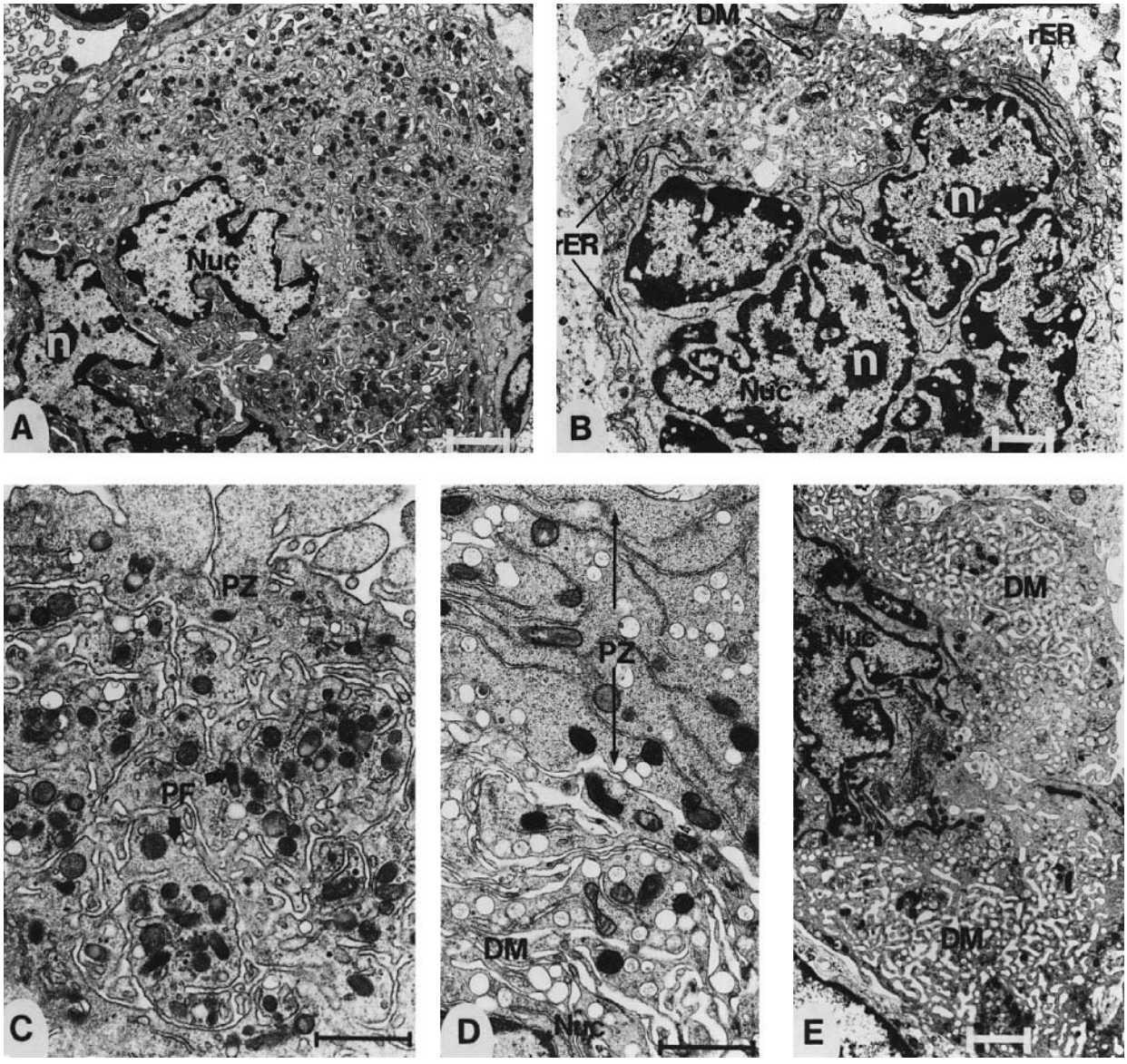

Fig. 6. Ultrastructure of neoΔHS and normal megakaryocytes. Low (**A**) and high (**C**) magnification views of mature wild-type megakaryocytes reveal features of normal cell differentiation, including abundance of platelet-specific granules, demarcation of platelet fields (PF) and a narrow peripheral zone (PZ). Nuc, nucleus; n, nucleolus. In contrast, low (**B**) and high (**D** and **E**) magnification views of neoΔHS megakaryocytes demonstrate multiple abnormalities, including large, segmented nuclei (Nuc), small cytoplasm with few granules, underdeveloped (B and D) or disorganized (E) demarcation membranes (DM), excess rough endoplasmic reticulum (rER) and a wide peripheral zone (PZ) bespeaking relative immaturity. n, nucleolus. Bars: (A), (B) and (E) 4 μm; (C) and (D) 2 μm.

that shown in Figure 6 (data not shown). We conclude, therefore, that the analysis of neoΔHS and ΔneoΔHS megakaryocytes provides an accurate view of the *in vivo* effects of GATA-1 loss on megakaryocyte maturation and platelet formation.

Discussion

The results described here are novel in two respects. First, we have established that the transcription factor GATA-1 serves a critical, and largely unanticipated, role in the growth control and maturation of megakaryocytes and production of blood platelets. Hence, this factor is vital for development of both erythroid and megakaryocytic lineages. Second, our experience illustrates how targeting of a *cis*-regulatory element may be used to generate lineage-selective loss of gene function and circumvent embryonic lethality of a conventional knockout mouse. This strategy should complement other conditional methods, such as site-specific recombination, for revealing temporal or lineage-restricted gene functions.

A requirement for GATA-1 in controlling megakaryocyte growth and differentiation

Although the major determinants of megakaryocyte lineage commitment and gene expression *in vivo* remain unknown, a role for GATA-1 in regulating megakaryocyte differentiation has been proposed. It is of particular note that forced expression of GATA-1 re-programs transformed avian hematopoietic progenitors and murine myeloid 416B cells toward a megakaryocytic pathway (Visvader *et al.*, 1992; Visvader and Adams, 1993; Kulessa *et al.*, 1995).

However, as suggested previously (Pevny *et al.*, 1995) and shown here, loss of GATA-1 activity is not associated with a failure of megakaryocyte lineage commitment. While it is possible that the related and co-expressed protein GATA-2 can substitute for GATA-1 in early aspects of megakaryocyte development, as seems to be the case in erythropoiesis (Weiss *et al.*, 1994), our studies establish an obligate requirement for GATA-1 in late megakaryocyte maturation. Further investigation may now permit identification of the relevant GATA-1-specific transcriptional targets.

GATA-1 is only the second transcription factor shown to be essential for megakaryocyte development (Shivdasani *et al.*, 1995b). The high expression and presumed function of the other factor, NF-E2, in erythroid cells raises interesting issues pertaining to a common differentiation program in the erythroid and megakaryocyte lineages. Furthermore, the related phenotypes of megakaryocytes lacking either GATA-1 or NF-E2 (marked reduction in cytoplasmic granules and impaired differentiation of platelets) raises the question of whether these factors function within the same or parallel pathways of thrombocytopoiesis and share target genes. Whereas transfection assays in cultured cells implicate GATA and Ets family proteins in regulating expression of several megakaryocyte-restricted genes, including glycoprotein (GP) IIb, components of the GP Ib complex and c-Mpl (Lemarchandel *et al.*, 1993; reviewed in Shivdasani, 1997), candidate target genes for the NF-E2 complex remain unknown. Our preliminary finding that GATA-1-deficient megakaryocytes may have decreased levels of platelet factor 4 and GP Ibα mRNAs (R.A.Shivdasani and S.H.Orkin, unpublished data) suggests that NF-E2 and GATA-1 regulate partially non-overlapping sets of genes involved in terminal megakaryocyte maturation. Clearly, the knockouts of these transcription factor genes provide an invaluable window into molecular aspects of platelet development.

Prior studies on chimeric mice generated by introduction of GATA-1^{-} ES cells into wild-type blastocysts failed to uncover enhanced proliferation of mutant megakaryocytes *in vitro* (Pevny *et al.*, 1995), very likely because they predated use of the c-Mpl ligand in megakaryocyte colony assays. Our observation that GATA-1-deficient megakaryocytes exhibit deregulated growth *in vitro* and *in vivo* points to a role for GATA-1 in orchestrating the transition from proliferation to maturation, analogous to its function in erythroid precursors (Briegel *et al.*, 1993; Weiss *et al.*, 1997). However, the megakaryocyte precursor targeted for deregulated proliferation in the absence of GATA-1 remains unknown; murine megakaryocyte progenitors, defined *in vitro* before the discovery of the c-Mpl ligand (Long *et al.*, 1985; Jackson *et al.*, 1994), exhibit different colony morphology, growth kinetics and growth factor requirements from those noted in neoΔHS and ΔneoΔHS fetal liver cultures. Moreover, most mature GATA-1-deficient megakaryocytes exhibit a dramatic discordance between nuclear and cytoplasmic maturation. The sum of these observations suggests that the role of GATA-1 in the balance between cell proliferation and nuclear versus cytoplasmic maturation will prove to be complex.

Lineage-selective knockout by cis-element gene targeting

Our analysis of the role of GATA-1 in megakaryocyte development was made possible through selective inactivation of its expression in a subset of the lineages in which it is normally expressed. This was achieved through targeted mutagenesis of a large upstream region, including a DNase I HS site identified in the chromatin of an erythroleukemic cell line (McDevitt *et al.*, 1977a). Our results suggest that the deleted region is essential for GATA-1 expression in megakaryocytes but dispensable for erythroid cell expression in the context of an intact locus. Although we did not fully anticipate the highly selective effect of this mutation on gene expression, our findings illustrate a strategy to complement the generation of lineage-specific knockouts through site-specific recombinases (Gu *et al.*, 1994; Kuhn *et al.*, 1995; Metzger *et al.*, 1995; Feil *et al.*, 1996; Tsien *et al.*, 1996). Indeed, each approach has specific advantages and drawbacks.

Cis-element targeting capitalizes on the often modular and redundant organization of regulatory elements governing expression of a gene in multiple tissue sites or at different stages of development. We achieved differential effects on gene expression through a replacement mutation that eliminates one of two distinct promoters as well as an upstream DNase I HS site and neighboring sequences. Although it will be of great advantage to the wider use of this approach if the critical *cis*-elements of a gene are known, this may not be a prerequisite. Rather, identifying regions of differential DNase I hypersensitivity of chromatin in individual tissues or stages of development may suffice for directing targeting experiments, as in this case, particularly if the consequences are examined with a selection cassette in place as well as after its removal. As lineage-specific DNase I HS sites are increasingly recognized to encompass regions critical for control of gene expression in various settings (Vidal *et al.*, 1990; Semenza *et al.*, 1991; Grewal *et al.*, 1992; Leroy-Viard *et al.*, 1994; May and Enver, 1995), we envisage the application of this strategy to study diverse gene functions *in vivo*.

Previous targeting of regulatory elements associated with DNase I HS sites or identified through functional mapping has focused on immunoglobulins and globins, gene loci with highly tissue-restricted expression (Fiering *et al.*, 1995; Ferradini *et al.*, 1996; Hug *et al.*, 1996). As such, these studies tested the relative contributions of *cis*-elements within a single cell type rather than in different lineages. With respect to effects on gene expression, however, they established an important principle: replacement of a putative regulatory element with a transcriptionally active selection cassette (e.g. PGK-neo^{R}) may interfere with transcription over large distances (Fiering *et al.*, 1995; Hug *et al.*, 1996). Rather than posing an inevitable limitation, this phenomenon may contribute toward selective effects on gene expression upon *cis*-element targeting of complex loci. Indeed, in neoΔHS mice, GATA-1 expression is selectively lost in megakaryocytes while partially preserved in the erythroid lineage, and loss of expression is more complete when the selectable marker is retained within the locus than when it is deleted.

While the success of *cis*-element targeting to achieve lineage-selective knockouts is subject to the organization of a given locus and the interplay among its regulatory elements in individual cell lineages, the approach can complement ongoing efforts toward conditional gene

targeting. Conditional gene knockouts with site-specific recombinases require transgenic mice that express the recombinase at high levels and in defined patterns (Gu *et al.*, 1994; Tsien *et al.*, 1996). Other important considerations relate to efficiency of DNA excision in different tissues and selective dominance of residual cells that express the targeted gene; the extent to which these may impose practical limitations on general use of this strategy is unknown. Ultimately, the application of multiple approaches will benefit dissection of gene function in selected tissues or lineages *in vivo*, as no single method is likely to fulfill all needs.

Materials and methods

Targeted modification of the GATA-1 locus

Fragments of the GATA-1 locus were cloned sequentially into a modified PUC polylinker. 5′- and 3′-homology regions were derived from a 5.9 kb *Bam*HI fragment and a 4 kb fragment extending from an artificial *Xho*I site to an intronic *Sma*I site, respectively. A PGK-neo^R cassette flanked by loxP sites (loxPneo, kindly provided by F.W.Alt) was introduced between these regions, and an HSVtk cassette was added at the boundary of the construct. DNA was linearized with *Asp*718 prior to electroporation into J1 ES cells (Li *et al.*, 1992); targeting (efficiency ~1%) was confirmed by Southern analysis using standard protocols (Ausubel *et al.*, 1987).

The loxPneo cassette was excised by introducing pBS185 Cre recombinase plasmid (Sauer and Henderson, 1990) ligated to a PGK-puromycin^R cassette. ES cells were selected in 1.5 μg/ml puromycin and assessed for deletion of the PGK-neo^R cassette by Southern analysis; deleted clones were subcloned for blastocyst injection. Unique sequence probes for Southern blotting (Figure 1) were generated by PCR. Generation of chimeric mice and subsequent breeding were as described (Shivdasani *et al.*, 1995a,b); mice were maintained on a mixed 129/Sv–C57Bl/6 background.

Histology, hematology and cytology

Freshly dissected tissues were fixed in buffered 10% formalin and embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin for light microscopy. Peripheral blood cell and platelet counts and parameters were measured on a Technicon H3 instrument. Blood was collected in EDTA-coated tubes (Becton-Dickinson) and either analyzed whole or following 1:3 dilution, as described previously (Shivdasani *et al.*, 1995b). Peripheral blood smears, prepared from fresh, undiluted blood collected in EDTA, and megakaryocyte colony cytospins, were stained with Wright–Giemsa and May–Grunwald–Giemsa stains, respectively.

In vitro hematopoietic colony assays

Single cell suspensions were prepared from aseptically harvested hematopoietic tissues by passage through a syringe and 25 gauge needle; yolk sacs were digested in 0.1% collagenase for 90 min at 37°C prior to mechanical disaggregation (Wong *et al.*, 1986). Between 5×10^4 and 5×10^5 cells were then plated in 0.9% methylcellulose in alpha medium (Stem Cell Technologies, Inc), as previously described (Shivdasani *et al.*, 1995a), supplemented with 30% fetal bovine serum and one or more of the following recombinant growth factors: 0.5 μg/ml human c-Mpl ligand, 50 ng/ml mouse SCF (both from Amgen), 2 U/ml murine erythropoietin, 10 ng/ml murine IL-3, 2 ng/ml murine IL-6, 0.5 ng/ml human granulocyte colony-stimulating factor (G-CSF) and 5 ng/ml mouse GM-CSF (all from R&D Systems, Inc.).

Ultrastructural analysis

Freshly excised hematopoietic organs were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer prior to routine processing for electron microscopy. Samples were post-fixed in 1% reduced osmium tetroxide, stained *en bloc* with uranyl acetate, dehydrated in graded ethanols and embedded in Epon LX112 (Ladd Res. Industries). Thin sections were stained with uranyl acetate and lead citrate, and examined with a Jeol 1200EX1 electron microscope at 120 kV.

Assessment of GATA-1 gene expression

Total RNA was extracted from cultured hematopoietic colonies using the single-step method (Chomczynski and Sacchi, 1987). Relative levels of mRNA species were assessed by semi-quantitative RT–PCR, as detailed previously (Shivdasani *et al.*, 1995b). The amount of cDNA template among samples under comparison was normalized using primers for HPRT or actin in the same reactions; these standard primers were added either at the outset of the reaction (Figure 3A) or after eight PCR cycles, with no difference in observed ratios. PCR primer references (with sizes of the amplified fragments) are GATA-1 (291 bp) (Shivdasani *et al.*, 1995a), actin (938 bp) and HPRT (249 bp) (Weiss *et al.*, 1994).

For immunofluorescence studies, erythroid colonies or megakaryocytes cultured *in vitro* were washed once in phosphate-buffered saline (PBS), cytospun onto coated glass slides and fixed in 4% paraformaldehyde for 5 min. Cells were treated with 0.1% Triton X-100 for 5 min and sequentially incubated with 5% goat serum in PBS at 4°C overnight; with either pre-immune serum, rat anti-GATA-1 antiserum L6 (gift of J.D.Engel) or rabbit anti-mouse platelet antiserum (McDonald and Jackson, 1990) for 1 h; and with either fluorescein isothiocyanate (FITC)-conjugated goat anti-rat (Pharmacia) or anti-rabbit (Tago, Inc) IgG for 30 min. Immunoreactivity was detected visually by fluorescence microscopy.

Acknowledgements

We thank Bethany Swencki, Karen Rockwell, Carol Browne, Haidi Yang and Sabra Goff for technical assistance; Marcia Lind for assistance with electron microscopy; Henry Warren and Ervin Meluleni for expert histology; J.D.Engel and T.P.McDonald for providing antisera; Amgen, Inc. for providing recombinant c-Mpl ligand; and Fern Tablin, Dorothea Zucker-Franklin and especially Paula Stenberg for critical review of electron micrographs. R.A.S. and S.H.O. are supported in part by National Institutes of Health grants. M.A.M. is the recipient of an HHMI Physician Scientist Fellowship and S.H.O. is an Investigator of the HHMI.

References

Ausubel,F.M., Brent,R., Kingston,R.E., Moore,D.D., Smith,J.A., Seidman,J.G. and Struhl,K. (1987) *Current Protocols in Molecular Biology*. Wiley, New York.

Briegel,K., Lim,K.-C., Plank,C., Beug,H., Engel,J. and Zenke,M. (1993) Ectopic expression of a conditional GATA-2/estrogen receptor chimera arrests erythroid differentiation in a hormone-dependent manner. *Genes Dev.*, **7**, 1097–1109.

Cairns,L.A. *et al*. (1994) Immortalization of multipotent growth-factor dependent hemopoietic progenitors from mice transgenic for GATA-1 driven SV40 tsA58 gene. *EMBO J.*, **13**, 4577–4586.

Capecchi,M.R. (1989) Altering the genome by homologous recombination. *Science*, **244**, 1288–1292.

Chomczynski,P. and Sacchi,N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal. Biochem.*, **162**, 156–159.

Feil,R., Brocard,J., Mascrez,B., LeMeur,M., Metzger,D. and Chambon,P. (1996) Ligand-activated site-specific recombination in mice. *Proc. Natl Acad. Sci. USA*, **93**, 10887–10890.

Ferradini,L., Gu,H., De Smet,A., Rajewsky,K., Reynaud,C.-A. and Weill,J.-C. (1996) Rearrangement-enhancing element upstream of the mouse immunoglobulin kappa chain J cluster. *Science*, **271**, 1416–1420.

Fiering,S. *et al*. (1995) Targeted deletion of 5′HS2 of the murine β-globin LCR reveals that it is not essential for proper regulation of the β-globin locus. *Genes Dev.*, **9**, 2203–2213.

Fujiwara,Y., Browne,C.P., Cunniff,K., Goff,S.C. and Orkin,S.H. (1996) Arrested development of embryonic red cell precursors in mouse embryos lacking transcription factor GATA-1. *Proc. Natl Acad. Sci. USA*, **93**, 12355–12358.

Grewal,T., Theisen,M., Borgmeyer,U., Grusenmeyer,T., Rupp,R.A., Stief,A., Qian,F., Hecht,A. and Sippel,A.E. (1992) The -6.1-kilobase chicken lysozyme enhancer is a multifactorial complex containing several cell-type-specific elements. *Mol. Cell Biol.*, **12**, 2339–2350.

Grupe,A., Hultgren,B., Ryan,A., Ma,Y.H., Bauer,M. and Stewart,T.A. (1995) Transgenic knockouts reveal a critical requirement for pancreatic β cell glucokinase in maintaining glucose homeostasis. *Cell*, **83**, 69–78.

Gu,H., Marth,J.D., Orban,P.C., Mossmann,H. and Rajewsky,K. (1994) Deletion of a DNA polymerase β gene segment in T cells using cell type-specific gene targeting. *Science*, **265**, 103–106.

Hug,B.A., Wesselschmidt,R.L., Fiering,S., Bender,M.A., Epner,E., Groudine,M. and Ley,T.J. (1996) Analysis of mice containing a targeted deletion of β-globin locus control region 5′ hypersensitive site 3. *Mol. Cell Biol.*,**16**, 2906–2912.

Ito,E., Toki,T., Ishihara,H., Ohtani,H., Gu,L., Yokoyama,M., Engel,J.D. and Yamamoto,M. (1993) Erythroid transcription factor GATA-1 is abundantly transcribed in mouse testis. *Nature*, **362**, 466–468.

Jackson,H., Williams,N., Bertoncello,I. and Green,R. (1994) Classes of primitive murine megakaryocytic progenitor cells. *Exp. Hematol*., **22**, 954–958.

Kuhn,R., Schwenk,F., Aguet,M. and Rajewsky,K. (1995) Inducible gene targeting in mice. *Science,* **269**, 1427–1429.

Kulessa,H., Frampton,J. and Graf,T. (1995) GATA-1 reprograms avian myelomonocytic cell lines into eosinophils, thromboblasts, and erythroblasts. *Genes Dev.*, **9**, 1250–1262.

Lemarchandel,V., Ghysdael,J., Mignotte,V., Rahuel,C. and Romeo,P.-H. (1993) GATA and Ets *cis*-acting sequences mediate megakaryocyte-specific expression. *Mol. Cell Biol.*, **13**, 668–676.

Leroy-Viard,K., Vinit,M.-A., Lecointe,N., Mathieu-Mahul,D. and Romeo,P.-H. (1994) Distinct DNase-I hypersensitive sites are associated with TAL-1 transcription in erythroid and T-cell lines. *Blood*, **84**, 3819–3827.

Li,E., Bestor,T.H. and Jaenisch,R. (1992) Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell*, **69**, 915–926.

Long,M.W., Gragowski,L.L., Heffner,C.H. and Boxer,L.A. (1985) Phorbol diesters stimulate the development of an early murine progenitor cell. The burst-forming unit-megakaryocyte. *J. Clin. Invest.*, **76**, 431–438.

May,G. and Enver,T. (1995) Targeting gene expression to haemopoietic stem cells: a chromatin-dependent upstream element mediates cell type-specific expression of the stem cell antigen CD34. *EMBO J.*, **14**, 564–574.

McDevitt,M.A., Fujiwara,Y., Shivdasani,R.A. and Orkin,S.H. (1997a) An upstream DNase I hypersensitive region of the hematopoietic-expressed transcription factor GATA-1 gene confers developmental specificity in transgenic mice. *Proc. Natl Acad. Sci. USA*, in press.

McDevitt,M.A., Shivdasani,R.A., Fujiwara,Y., Yang,H. and Orkin,S.H. (1997b) A 'knockdown' mutation created by cis-element gene targeting reveals the dependence of red blood cell maturation on the level of transcription factor GATA-1. *Proc. Natl Acad. Sci. USA*, in press.

McDonald,T.P. and Jackson,C.W. (1990) Thrombopoietin derived from human embryonic kidney cells stimulates an increase in DNA content of murine megakaryocytes *in vivo*. *Exp. Hematol.*, **18**, 758–763.

Metzger,D., Clifford,J., Chiba,H. and Chambon,P. (1995) Conditional site-specific recombination in mammalian cells using a ligand-dependent chimeric Cre recombinase. *Proc. Natl Acad. Sci. USA*, **92**, 6991–6995.

O'Gorman,S., Fox,D.T. and Wahl,G. (1991) Recombinase-mediated gene activation and site-specific integration in mammalian cells. *Science*, **251**, 1351–1355.

Olson,E.N., Arnold,H.-H., Rigby,P.W.J. and Wold,B.J. (1996) Know your neighbors: three phenotypes in null mutants of the myogenic bHLH gene MRF4. *Cell*, **85**, 1–4.

Pevny,L., Simon,M.C., Robertson,E., Klein,W.H., Tsai,S.-F., D'Agati,V., Orkin,S.H. and Costantini,F. (1991) Erythroid differentiation in chimeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. *Nature*, **349**, 257–260.

Pevny,L., Lin,C.-S., D'Agati,V., Simon,M.C., Orkin,S.H. and Costantini,F. (1995) Development of hematopoietic cells lacking transcription factor GATA-1. *Development*, **121**, 163–172.

Sauer,B. and Henderson,N. (1990) Targeted insertion of exogenous DNA into the eukaryotic genome by the Cre recombinase. *New Biologist*, **2**, 441–449.

Semenza,G.L., Koury,S.T., Nejfelt,M.K., Gearhart,J.D. and Antonarakis,S.E. (1991) Cell-type-specific and hypoxia-inducible expression of the human erythropoietin gene in transgenic mice. *Proc. Natl Acad. Sci. USA*, **88**, 8725–8729.

Shivdasani,R.A. (1997) Transcription factors in megakaryocyte differentiation and gene expression. In Kuter,D.J., Hunt,P., Sheridan,W. and Zucker-Franklin,D. (eds), *Thrombopoiesis and Thrombopoietins*. Humana Press, Totowa, NJ, pp. 189–202.

Shivdasani,R.A., Mayer,E.L. and Orkin,S.H. (1995a) Absence of blood formation in mice lacking the T-cell leukemia oncoprotein tal-1/SCL. *Nature*, **373**, 432–434.

Shivdasani,R.A., Rosenblatt,M.F., Zucker-Franklin,D., Jackson,C.W., Hunt,P., Saris,C. and Orkin,S.H. (1995b) Transcription factor NF-E2 is required for platelet formation independent of the actions of thrombopoietin/MGDF in megakaryocyte development. *Cell*, **81**, 695–704.

Tsien,J.Z., Chen,D.F., Gerber,D., Tom,C., Mercer,E.H., Anderson,D.J., Mayford,M., Kandel,E.R. and Tonegawa,S. (1996) Subregion- and cell type-restricted gene knockout in mouse brain. *Cell*, **87**, 1317–1326.

Vidal,M., Morris,R. and Grosveld,F. (1990) Tissue-specific control elements of the Thy-1 gene. *EMBO J.*, **9**, 833–840.

Visvader,J. and Adams,J.M. (1993) Megakaryocytic differentiation induced in 416B myeloid cells by GATA-2 and GATA-3 transgenes or 5-azacytidine is tightly coupled to GATA-1 expression. *Blood*, **82**, 1493–1501.

Visvader,J.E., Elefanty,A.G., Strasser,A. and Adams,J.M. (1992) GATA-1 but not SCL induces megakaryocytic differentiation in an early myeloid line. *EMBO J.*, **11**, 4557–4564.

Weiss,M.J. and Orkin,S.H. (1995) Transcription factor GATA-1 permits survival and maturation of erythroid precursors by preventing apoptosis. *Proc. Natl Acad. Sci. USA*, **92**, 9623–9627.

Weiss,M.J., Keller,G. and Orkin,S.H. (1994) Novel insights into erythroid differentiation revealed through differentiation of GATA-1^{-} embryonic stem cells. *Genes Dev.*, **8**, 1184–1197.

Weiss,M.J., Yu,C. and Orkin,S.H. (1997) Erythroid-specific properties of transcription factor GATA-1 revealed by phenotypic rescue of a gene-targeted cell line. *Mol. Cell Biol.*, **17**, 1642–1651.

Wong,P.M.C., Chung,S.W., Chui,D.H.K. and Eaves,C.J. (1986) Properties of the earliest clonogenic hematopoietic precursors to appear in the developing murine yolk sac. *Proc. Natl Acad. Sci. USA*, **83**, 3851–3854.

Yomogida,K., Ohtani,H., Harigae,H., Ito,E., Nishimune,Y., Engel,J.D. and Yamamoto,M. (1994) Developmental stage- and spermatogenic cycle-specific expression of transcription factor GATA-1 in mouse Sertoli cells. *Development*, **120**, 1759–1766.

Zucker-Franklin,D. (1989) Megakaryocytes and platelets. In Zucker-Franklin,D., Greaves,M.F., Grossi,C.E. and Marmont,A.M. (eds), *Atlas of Blood Cells: Function and Pathology.* Lea & Febiger, Philadelphia, PA, pp. 559–602.

Received on February 10, 1997; revised on March 19, 1997