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Embryonic stem cells and transgenic mice in the study of hematopoiesis

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ABSTRACT Blood formation (hematopoiesis) entails the generation of hematopoietic stem cells (HSCs) within the embryo and subsequent commitment of multipotential progenitors to differentiation along single lineages. These processes are controlled in large part by cell-restricted transcription factors which cooperate with more widely expressed factors to direct lineage-specific gene expression. Candidate hematopoietic transcriptional regulators have been identified by characterizing factors mediating cell-specific gene transcription and by defining genes involved in chromosomal rearrangements in leukemia. The application of transgenic and embryonic stem cell methods have provided insights into their *in vivo* functions and suggested mechanisms by which lineage selection may be achieved. One of the first, and best, characterized hematopoietic transcription factors is GATA-1. Herein studies of GATA-1 are reviewed to illustrate how manipulations of its locus in the mouse have contributed to current understanding in unique and unexpected ways.

KEY WORDS: *hematopoiesis, transcriptional factors, gene targeting, GATA-1, SCL/tal-1*

Introduction

Hematopoiesis is the process by which mature blood cells of distinct lineages are generated from multipotential progenitors or hematopoietic stem cells (HSCs) (Orkin, 1996). In the early mouse embryo the first blood cells, embryonic (or primitive) erythrocytes, are formed in the yolk sac blood islands starting at E7.5. Multiple lines of evidence suggest that the progenitors of these hematopoietic cells are descendants of the hemangioblast, a bipotential cell with the capacity to generate both hematopoietic and vascular cells. By E11.5 hematopoiesis then shifts to the fetal liver where adult (or definitive) erythroid precursors first develop, along with precursors of other blood lineages. HSCs, defined as cells that can reconstitute the hematopoietic system of an adult, irradiated animal, are first found within the embryo proper at ~E12 in the vicinity of the aorta-gonad-mesonephros (AGM) region (Medvinsky *et al.,* 1993; Medvinsky and Dzierzak, 1996). Whether HSCs alone arise within the yolk sac environment has been the subject of some controversy, although recent data suggest that they may, particularly if transplantation assays are performed in conditioned newborn mice (Yoder *et al.,* 1997). Following the fetal liver stage of hematopoiesis, blood formation shifts finally to the bone marrow where it is maintained throughout the lifetime of the individual. While the dynamics of hematopoiesis are well known, how the hematopoietic system is established during development and lineages are selected from HSCs or multipotential progenitors are less clear, and been subjects for intensive investigation in recent years.

The questions are numerous. What genes and growth factors are responsible for the induction of hematopoietic mesoderm within the early embryo? Do cytokines instruct lineage selection or merely provide a permissive environment for stochastic programs of differentiation? What mechanisms are employed to direct lineage-specific gene transcription and cellular maturation? How do "leukemia" oncoproteins intersect with and perturb the normal regulatory networks of hematopoietic cells?

The past several years have witnessed considerable progress in the identification and characterization of genes involved in programs of hematopoietic development. Much of what has been learned has benefited from use of the mouse as a genetic system in which to explore the relationship between specific gene products and *in vivo* function. Both transgenesis and gene targeting strategies have been used effectively to dissect the hematopoietic system in ways that could not have been imagined just a few years ago. In this brief review in honor of the seminal contributions of Ralph Brinster, I will touch on some of the recent findings from my laboratory, as examples of what has been accomplished and what needs to be addressed in the future.

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Search for "relevant" genes

It should be noted that "lower" eukaryotes (*Drosophila* and *C. elegans*, for example) do not have an hematopoietic system that is strictly analogous to that of vertebrates. Accordingly, it has not been possible to choose direct homologs of proteins in these species to analyze vertebrate hematopoiesis. Several approaches have been employed to identify candidate genes that might serve critical regulatory functions in hematopoiesis. These include "irrational" (i.e., random), "rational" (i.e., directed), and genetic strategies (see Table 1). The random approach relies on the *ad hoc* choice of potential candidates from randomly sequenced cDNAs, perhaps with interesting homologies to known proteins, or on sequences selected (perhaps by subtraction) for an intriguing pattern of expression. For the most part, this approach has contributed relatively little to progress in the area, although we may expect that cDNA "genomics" of recent years may soon begin to add to the field. The first regulatory factors critical for hematopoietic cells were identified through the directed search for DNA-binding proteins that mediate cell-specific gene transcription. Among these are the erythroid transcription factor GATA-1 (Evans and Felsenfeld, 1989; Tsai *et al.,* 1989) and B-lymphoid cell Oct-2 factor (Muller *et al.,* 1988; Staudt *et al.,* 1988). The study of such factors in the context of development is predicated on the presumption that they may regulate aspects of the early program, and not merely regulate the end-stage markers of a particular lineage. Indeed, this has often proved to be the case. Genetic strategies are just now beginning to provide new insights in hematopoiesis. Many leukemias are associated with chromosomal rearrangements that either deregulate expression of the affected locus or generate chimeric proteins (Rabbitts, 1994). *A priori*, there is no reason to presuppose that the genes involved in these chromosomal events serve important roles in normal hematopoiesis. Nonetheless, experience has shown that more often than not, they do. Finally, genetic studies in "lower" vertebrates provide additional promise. Surrogate genetics in *Xenopus*, an excellent system for the study of inductive events, allows dissection of signaling pathways involved in mesoderm specification. Moreover, recent mutant hunts in zebrafish, coupled with positional cloning, give hope for identification of novel genes important for blood cell development (Ransom *et al.,* 1996).

In this review, findings pertinent to genes discovered by the directed and genetic approaches will be discussed. The goal is to emphasize the versatility and power of the new techniques of mouse genetics for the elucidation of *in vivo* gene function in hematopoiesis.

TABLE 1

APPROACHES TO "RELEVANT" GENES

1. "Irrational" (i.e. random):
   - sequencing, homologies, subtraction

2. "Rational" (i.e. directed):
   - DNA-binding proteins to critical ces-elements

3. Genetic:
   - translocation-associated genes
   - model organisms, mutant hunts

GATA-1: a model hematopoietic transcription factor

In the pursuit of the molecular basis for erythroid-specific gene transcription we and others encountered the GATA-1, a DNA-binding protein that recognizes a consensus GATA-motif present in the cis-regulatory elements of virtually all erythroid-expressed genes (Evans and Felsenfeld, 1989; Tsai *et al.,* 1989). GATA-1 defined a new subfamily of zinc-finger transcription factors, which now includes members in all eukaryotes. Although GATA-1 was identified as an erythroid-specific transcription factor, soon after its isolation it became evident that it was also expressed in related hematopoietic lineages (megakaryocytes and mast cells), eosinophils, as well as multipotential progenitors (Martin *et al.,* 1990). Thus, if required for erythroid cell development, it could not be sufficient. Later studies showed it to be expressed also in the Sertoli cells of the testis (Ito *et al.,* 1993), the sole non-hematopoietic site demonstrated to date.

Besides typical biochemical experiments implicating GATA-1 in erythroid gene transcription, additional evidence pointed to a more direct, potential role in lineage commitment. Primary transformed chicken progenitors with a early myeloid phenotype are induced to differentiate along erythroid, megakaryocytic, or eosinophilic pathways by enforced GATA-1 expression, in part dependent on the concentration at which it is expressed (Kulessa *et al.,* 1995). Moreover, cells of a permanent murine myeloid cell line (416B) convert to megakaryocytes upon expression of GATA-1 (Visvader *et al.,* 1992). Thus, a positive role for GATA-1 in directing differentiation of three lineages may be inferred from these cell culture experiments.

GATA-1: essential for erythroid precursor cell maturation

To investigate whether GATA-1 is, as we anticipated, required for development of any of the lineages in which it is expressed, we disrupted the *GATA-1* locus in ES cells by gene targeting. Initially, this was performed in collaboration with the Costantini laboratory and the phenotypic consequences studied in the setting of mouse chimeras, since the gene was conveniently situated on the X-chromosome and the parental ES cells were of male origin (Pevny *et al.,* 1991). The effects of GATA-1 loss were also studied following *in vitro* hematopoietic differentiation of ES cells (Weiss *et al.,* 1994), taking advantage of the remarkable capacity of these cells under appropriate conditions to give rise to all myeloerythroid lineages in tissue culture from embryoid bodies (Keller *et al.,* 1993). Finally, we performed another targeting of the locus to generate a germline mutation so that the consequences of GATA-1 loss could be examined in the context of an intact, developing embryo (Fujiwara *et al.,* 1996). In aggregate, these experiments led to the following observations: first, GATA-1 is essential for mouse development, as GATA-1$^-$ embryos die by E10-11 of severe anemia. Indeed, mutant embryos are devoid of any visible red color. Second, despite profound anemia, cells with the appearance of proerythroblasts (the first identifiable erythroid precursor) are present at roughly normal numbers in the yolk sac blood islands. Thus, hematopoiesis including erythroid commitment appears to occur in the absence of GATA-1. Third, the arrested proerythroblasts

contain an elevated level of transcripts for the related transcription factor GATA-2, which we showed is essential in its own right for the proliferation or survival of hematopoietic progenitors (Tsai *et al.,* 1994). Hence, it is likely that a failure to repress GATA-2 in the absence of GATA-1 provides for partial erythroid differentiation due to overlapping functions of these related proteins. Fourth, the arrested proerythroblasts undergo apoptosis, indicating that GATA-1 acts to prevent death of erythroid precursors in which it is expressed (Weiss and Orkin, 1995). This provides a positive feedback mechanism to ensure the subsequent development of GATA-1$^+$ erythroid precursors. Curiously, although the initial motivation for studying GATA-1 in many laboratories was to explain how globin genes are activated in erythroid precursors, GATA-1$^-$ proerythroblasts express some globin transcripts, possibly under the aegis of GATA-2. Thus, activation of globin loci does not strictly require the action of GATA-1, as has been presumed by many in the field.

GATA-1: defining its requirement in megakaryocyte development by a lineage-restricted knockout

What about GATA-1's potential roles in other lineages in which it is expressed? As with many other essential genes, demonstrating function in other cells is complicated by the early death of conventional GATA-1$^-$ embryos. We have been particularly interesting in possible roles in megakaryocytes and in Sertoli cells. While the conditional cre-loxP targeting strategy pioneered by Rajewsky and colleagues (Gu *et al.,* 1994) might be applied to these issues, we came upon an alternative approach to reveal GATA-1's importance in megakaryopoiesis.

Our success in this venture came unexpectedly from other studies designed to identify *cis*-regulatory elements of the *GATA-1* gene. How the *GATA-1* gene itself is transcriptionally controlled is of particular interest in considering potential upstream factors in a regulatory hierarchy in hematopoietic cells. The *GATA-1* locus, though relatively compact, has two promoters, one of which is predominantly used in Sertoli cells and another, mostly in hematopoietic cells. To define regulatory elements a bacterial β-galactosidase *(lacZ)* gene was embedded in the GATA-1 gene at the position of the initiator ATG and various upstream sequences were included in constructs introduced into transgenic mice. Initial experiments in which the hematopoietic promoter was used proved disappointing. Inconsistent, low level expression of lacZ was observed but only in adult hematopoietic cells. In a wider search for potential regulatory elements we identified an upstream DNase I hypersensitivity site between the Sertoli and hematopoietic promoters (McDevitt *et al.,* 1997a). Inclusion of this region markedly enhanced both the frequency and strength of expression in transgenic mice, and also conferred appropriate developmental regulation. The expression of this GATA-1/lacZ transgene initiates at the onset of erythroid development in the yolk sac blood islands and continues in definitive erythroid and megakaryocytic cells.

To assess the *in vivo* function of this upstream region we were compelled to ask: what would happen to GATA-1 expression if it were removed? We modified the *GATA-1* locus by gene targeting once again, this time replacing the hypersensitive region plus the upstream Sertoli cell promoter with a neomycin-resistance cassette flanked by loxP sites (Shivadasani *et al.,* 1997). ES cells and mice were generated in which the neomycin-resistance cassette

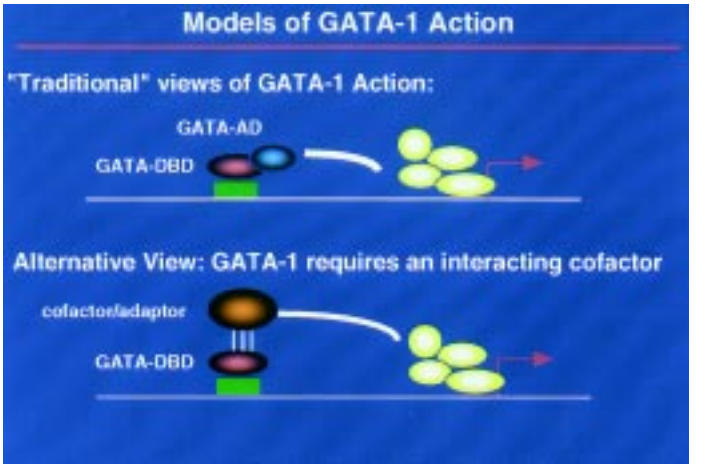

Fig. 1. Models of GATA-1 action. *At the top, the traditional view of GATA-1 as a transcriptional activator is depicted. The protein is modular with its DNA-binding domain (DBD) in contact with a GATA-motif and its activation domain (AD) providing a connection to the basal transcriptional machinery, shown to the right. At the bottom, the alternative model is presented in which the DBD is coupled to an adaptor or cofactor, which provides the link to the transcriptional machinery.*

was retained or removed by cre-mediated excision. The phenotypes of the embryos and mice obtained were informative in many respects. First, mice retaining the neomycin-resistance cassette were markedly anaemic at the fetal liver stage. Indeed, >90% of embryos died, although some managed to survive to term. These rare surviving pups were pale at birth, but later recovered and appeared well as adults. Remarkably, embryos and mice lacking the neomycin-resistance cassette exhibited no anemia. Thus, the presence of the selection cassette, rather than the removal of the hypersensitive region, was associated with a disturbance in red cell development. Other studies showed that GATA-1 RNA levels were reduced only ~4-fold in erythroid precursors in which the neomycin-resistance cassette was retained (McDevitt *et al.,* 1997b). Cellular maturation was markedly delayed. This example of transcriptional interference by an interposed transcribed element (Fiering *et al.,* 1995) establishes that erythroid maturation is highly sensitive to the concentration of GATA-1.

While the upstream hypersensitive region proved dispensable for erythroid GATA-1 expression, we were surprised to note that newborn and adult mice (either with or without the neomycin-resistance cassette) had only ~15% the normal number of circulating platelets (Shivadasani *et al.,* 1997). In addition, the few platelets produced were larger in size. These hematologic parameters predicted a defect in GATA-1 expression in megakaryocytes, which was confirmed by direct examination of GATA-1 RNA or protein. Mice in which the neomycin-resistance gene was present had no detectable GATA-1 expression. Those with the selection cassette excised expressed ~5-10% normal GATA-1 levels. Remarkably, our modification of the *GATA-1* locus generated a lineage-selective knockout. Expression was virtually eliminated in one lineage (megakaryocytes), yet preserved in another (erythroid). The targeting of *cis*-regulatory elements, particularly DNase I hypersensitive sites, offers another method for generating lineage-specific phenotypes, one that may complement conditional gene targeting methods (see below). The potential of modifying the

phenotype by either retention or excision of a selection cassette provides versatility to the approach. Finally, these experiments identified an essential regulatory element for megakaryocytic GATA-1 expression.

The direct study of GATA-1$^-$ megakaryocytes provided additional insight into the role of GATA-1 in this lineage, and allowed comparisons with its role in erythropoiesis to be made. Electron microscopy demonstrated that GATA-1$^-$ megakaryocytes are immature. Granules that normally fill the cytoplasm of megakaryocytes were sparse. Nuclear maturation appeared perturbed. In addition, *in vitro* colony assays showed that the number of megakaryocyte progenitors was normal in the absence of GATA-1, yet the number of megakaryocytes per colony was greatly elevated. Thus, GATA-1$^-$ megakaryocytes exhibit a disturbance of growth control, implicating GATA-1 in regulating the switch from proliferation to differentiation. In many respects, the effects of GATA-1 loss on megakaryopoiesis resembles that on erythropoiesis with the exception that GATA-1$^-$ erythroid precursors die rather than hyperproliferate. Nonetheless, GATA-1 appears to be required for both cellular maturation and proper growth control in both lineages.

GATA-1: is it a conventional transcriptional activator protein?

Studies of GATA-1 in transfected heterologous cells performed soon after the initial cloning of its cDNA indicated that GATA-1 can serve as a potent transcriptional activator in reporter assays (Martin and Orkin, 1990) (Fig. 1, top). A number of observations raised suspicions, however, that GATA-1 might not function in this manner in its normal cellular context. First, the primary structure of GATA-1s of various species is poorly conserved except in the region of the double zinc-finger domain. Second, megakaryocytic conversion of myeloid 416B cells was directed by GATA-1 variants lacking an "essential" N-terminal activation domain (Visvader *et al.,* 1995). The most compelling evidence in this regard emerged from studies of a novel cell line which was derived from GATA-1$^-$ ES cells (Weiss *et al.,* 1997). Reasoning that GATA-1$^-$ erythroid precursors undergo apoptosis, Mitch Weiss, then a postdoctoral fellow in the laboratory, introduced an expression construct containing human bcl-2 into GATA-1$^-$ ES in an attempt to forestall death on differentiation. ES cells were differentiated *in vitro* along an erythroid pathway in the presence of erythropoietin and kit-ligand (stem cell factor). Erythroid colonies were plucked and placed into liquid culture, whereupon they continued to grow. These cells, designated G1E (for GATA-1$^-$ erythroid), are dependent on both erythropoietin and kit-ligand and remain euploid. Remarkably, reintroduction of GATA-1 by retroviral infection triggers terminal erythroid maturation, thereby providing an authentic assay of GATA-1 function in an erythroid context. Although bcl-2 cDNA was introduced into the ES cells, human bcl-2 expression could not be demonstrated in the established G1E cells. Thus, we cannot fully explain how this extraordinary line came to be. While this is somewhat unsettling, hematopoietic cells can be routinely immortalized from differentiated ES cells by infection with myeloproliferative disease virus (MPLV) or hox-11-containing retrovirus. Hence, in the future we may witness greater use of targeted ES cells for the generation of genetically designed hematopoietic cell lines.

Nonetheless, the rescue of G1E cells has provided a useful assay for GATA-1 function. By testing a variety of GATA-1 mutants in this system, Weiss and associates made the following two observations: first, the "essential" N-terminal activation domain of GATA-1 appears entirely dispensable for GATA-1-dependent maturation from the proerythoblast stage onward. Second, the amino (N) zinc-finger of GATA-1 is strictly required for erythroid maturation, even though it is largely dispensable within the context of heterologous cell reporter assays. These finding led us to hypothesize that GATA-1 does not function as a typical transcriptional activator, but rather requires a cofactor to link DNA-bound GATA-1 to the transcriptional machinery (Fig. 1, bottom). We envisioned that this cofactor would interact physically with GATA-1, perhaps even within the N-finger domain.

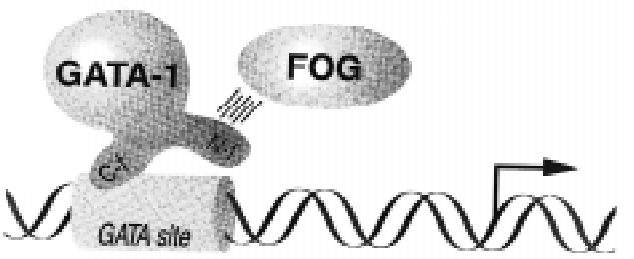

Fig. 2. FOG, a protein that interacts specifically with the N-finger of GATA-1, is shown as the principal cofactor for GATA-1 in erythroid cells.

GATA-1: discovery of the presumptive cofactor, FOG, and a new paradigm for GATA-factors in transcriptional control

To search for the predicted cofactor, Alice Tsang, then a Ph. D. student in the laboratory, performed a two-hybrid assay in yeast using as bait the N-finger of GATA-1 to screen a mouse erythroleukemia cDNA library (Tsang *et al.,* 1997). She obtained a single class of clones which encoded a novel complex, zinc-finger protein, designated FOG (for Friend of GATA-1). Circumstantial evidence supported FOG as the relevant cofactor. First, FOG is coexpressed with GATA-1 in yolk sac and fetal liver, as well as in erythroid and megakaryocytic lineages. Second, by one-hybrid assay it could be shown that FOG could form a ternary complex with DNA-bound GATA-1. Third, FOG and GATA-1 can be coimmunoprecipitated from native erythroid cells. Finally, FOG potentates GATA-1-dependent erythroid development of G1E cells and megakaryocytic development of myeloid 416B cells. Taken together, these findings suggest that FOG 'is able' to function as a cofactor for GATA-1, but do not indicate how many other (perhaps more critical) potential cofactors might exist or whether FOG and GATA-1 actually cooperate *in vivo* (Fig. 2).

To begin to address these issues, we disrupted the *FOG* gene (Tsang *et al.,* 1998). To our delight, FOG$^{-/-}$ embryos die of severe anemia at E11-12. FOG$^{-/-}$ erythroid cells closely resemble GATA-1$^-$ erythroid precursors in that they are largely arrested at the proerythroblast stage. The developmental block is slightly leaky but imposed at a stage similar to that in the absence of GATA-1. Also, FOG$^{-/-}$ precursors do not succumb to apoptosis as rapidly as those lacking GATA-1. On the whole, however, the results are consistent with the hypothesis that FOG is the principal cofactor for GATA-1 in erythroid cells, although models might be put forward to

explain these results without invoking direct physical interaction or cooperation.

Effects of FOG loss for megakaryopoiesis, however, were unexpected in that they were more extreme than those seen with absence of GATA-1 alone. Rather than giving rise to arrested precursor cells, FOG$^{-/-}$ progenitors are unable to produce megakaryocytes. This observation points to a GATA-1 independent role for FOG in megakaryocyte development. At the same time, though, an independent role in early megakaryocyte differentiation does not preclude a GATA-1 dependent action during mid-maturation, as might be anticipated on the basis of results with induction of megakaryocyte development in 416B cells. Thus, much remains to be learned about the intimate relationship between GATA-1 and FOG, and particularly how FOG may function independently.

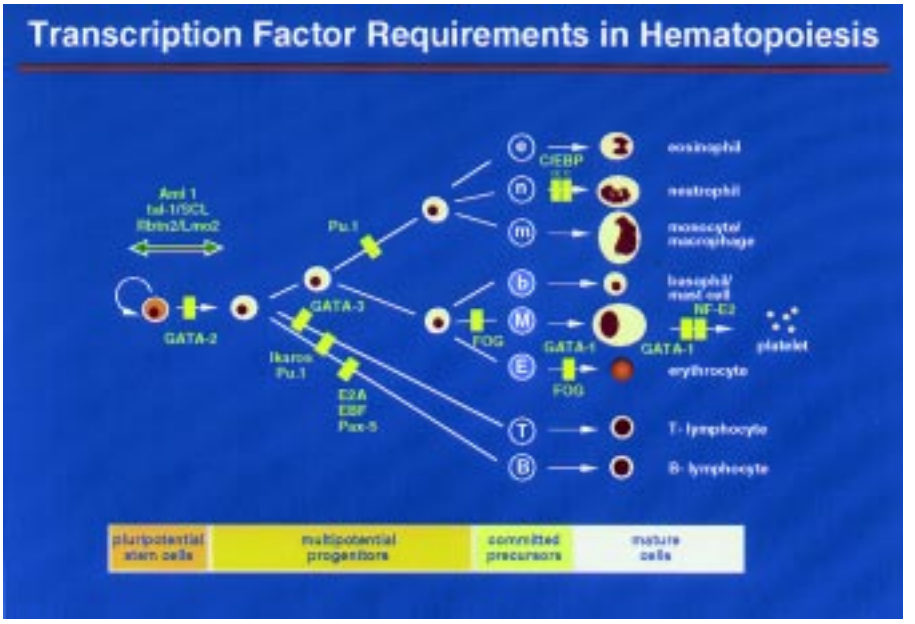

Fig. 3. Blocks to hematopoietic development in the absence of transcriptional proteins.

The association of GATA-1 and FOG provides a paradigm for a new protein partnership, as reinforced by the discovery of a similar interaction between the *Drosophila* GATA-factor pannier and a FOG-like complex zinc-finger protein, u-shaped (Cubadda *et al.,* 1997; Haenlin *et al.,* 1997). Remarkably, u-shaped interacts with the N-finger of pannier and GATA-1. Rather than acting to promote pannier function, u-shaped appears to antagonize its action. These findings raise the possibility that a family of FOG/u-shaped-like proteins regulate various GATA-factors either positively or negatively in different developmental settings. Given the involvement of GATA-factors in the development of many tissues, we anticipate complex interplay with the FOG/u-shaped family of regulators.

SCL/tal-1: a bHLH transcriptional regulator possibly tied into the GATA-factor regulatory network

Among the loci discovered through the study of chromosomal rearrangements in leukemia, the *SCL/tal-1* gene is of particular interest (Begley *et al.,* 1989). The *SCL/tal-1* locus is affected in approximately 20-25% of cases of T-ALL (acute lymphoblastic leukemia). Rearrangements may involve translocations or upstream interstitial deletions. Whichever is the case, the *SCL/tal-1* gene is deregulated such that it is expressed in the T-cell lineage, where it is typically silent. In the hematopoietic system SCL/tal-1 is expressed in a pattern remarkably similar to that of GATA-1 (Green *et al.,* 1992), although it is also expressed in vascular cells and the central nervous system. One of its two promoter contains a critical GATA-motif, lending support to the hypothesis that SCL/tal-1 may be regulated, in part, by GATA-factors, particularly in maturing erythroid precursors. Tissue culture evidence supports a positive role for SCL/tal-1 in erythroid maturation (Aplan *et al.,* 1992).

Knockout of the *SCL/tal-1* gene, however, provided a different perspective on its *in vivo* requirement. SCL/tal-1$^{-/-}$ embryos are entirely bloodless and die by E10 (Robb *et al.,* 1995; Shivdasani *et al.,* 1995). Subsequent studies of *in vitro* differentiated cells and chimeric mice revealed the inability of SCL/tal-1$^{-/-}$ to develop into any hematopoietic progenitors or lineages (Porcher *et al.,* 1996; Robb *et al.,* 1996). This findings has been widely inferred to suggest that SCL/tal-1 function is essential for the specification of mesoderm (or the hemangioblast) to an hematopoietic fate, although the alternative view that it is required for proliferation or maintenance of hematopoietic progenitors or HSCs is equally likely. The phenotype of the early SCL/tal-1$^{-/-}$ embryo is identical to that seen in the absence of the LIM protein, Rbtn2/Lmo2, another nuclear protein associated with leukemia (Warren *et al.,* 1994). Indeed, both proteins are often activated in T-ALL cells and induce lymphomas when expressed in early T-cells of transgenic mice (Kelliher *et al.,* 1996; Larson *et al.,* 1996). Remarkably, these proteins also physically associate (Wadman *et al.,* 1994). How SCL/tal-1 and Rbtn2/Lmo2 function in transcription is unclear, although recent evidence suggests that they participate as part of a larger complex that recognizes a composite GATA-E-box DNA element (Wadman *et al.,* 1997). *In vitro* a pentameric complex of SCL/tal-1, its heterodimeric partner E12/47, Rbtn2/Lmo2, GATA-1, and Lbd1 (a LIM-interacting protein) binds with high affinity to this unique element. These findings suggest further interplay between the GATA-factor regulatory network and the leukemic oncoproteins SCL/tal-1 and Rbtn2/Lmo2. The details remain to be dissected in future studies.

SCL/tal-1 in vascular development: use of a GATA-1-based transgene

As with GATA-1 knockout embryos, the early lethality of SCL/tal-1$^{-/-}$ embryos precludes ready examination of SCL/tal-1's roles in other developmental pathways. Given the extraordinary specificity of the GATA-1/lacZ transgene (described above) for directing hematopoietic-restricted transcription at the onset of blood cell formation in the yolk sac, Jane Visvader, a visiting fellow in our laboratory, considered the possibility that GATA-1-driven SCL cDNA might be used to rescue the hematopoietic defect of SCL/tal-1$^{-/-}$ embryos and reveal additional *in vivo* requirements for this

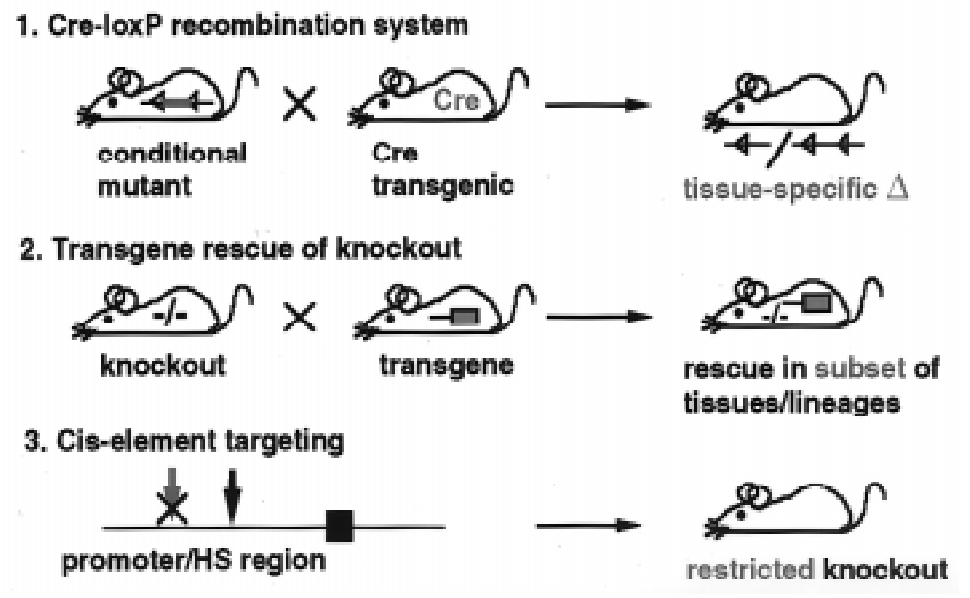

Fig. 4. Alternative approaches to the generation of lineage-selective knockouts in mice. (1) *Cre-loxP strategy in which a conditional mutant mouse is interbred with a cre-expressing transgenic mouse.* **(2)** *Rescue of a portion of the phenotype of a complex knockout mouse by a transgene directed to a specific tissue or cell-type. Rescue is achieved in a subset of lineages.* **(3)** *Use of cis-element gene targeting to generate restricted knockouts. For illustration, two DNase I hypersensitive sites (downward arrows), representative of cis-regulatory elements, are depicted upstream of a gene (solid box). One of the sites is obliterated by a targeting event, either with retention or removal of the selection cassette. Gene expression may be abolished in a restricted pattern, leading to a tissue-restricted knockout.*

factor (Visvader *et al.,* 1998). An underlying premise of such an experiment is that SCL/tal-1 might not be required in hematopoiesis until the time at which GATA-1 expression is first activated, or SCL/tal-1 and GATA-1 are expressed at a similarly early point in the program. Indeed, the GATA-1 driven SCL/tal-1 transgene rescues hematopoiesis of SCL/tal-1$^{-/-}$ embryos. Hematopoietic rescue revealed a second deficit of SCL/tal-1$^{-/-}$ embryos that had escaped prior examinations—yolk sac angiogenesis was abnormal. The inability of SCL/tal-1$^{-/-}$ vascular cells to contribute to the large vitelline vessels was confirmed by chimera studies using mutant ES cells tagged with an endothelial-expressed lacZ transgene. Beyond demonstrating a previously unsuspected role for SCL/tal -1 in vascular development, these studies illustrate the power of lineage-specific transgene rescue for the analysis of complex knockout phenotypes.

Some final thoughts

The studies reviewed above have benefited immeasurably from the new mouse genetics, in part a legacy of the pioneering studies of Brinster and his associates. No longer can *in vivo* functions of genes be presumed; they must be tested by generation of appropriate mutant animals. Through knockout, chimera, and *in vitro* ES cell experiments we have developed a rather precise view of where GATA-1 is required in development of both the erythroid and megakaryocytic lineages. Moreover, these experiments have stimulated complementary, molecular studies that have identified a transcriptional cofactor, FOG, and led to the demonstration of its *in vivo* requirements. This "to-and-fro" relationship between animal and molecular approaches enriches our understanding of the biological relevance and context of the molecules we study.

The study of other hematopoietic transcription factors, both in our laboratory and elsewhere, provides a more complete view of the components necessary for development of one, or multiple, lineages (see Fig. 3). Factors, such as SCL/tal-1, have been identified that are required in the earliest hematopoietic compartment, whereas others, exemplified by GATA-1 or the myeloid factor C/EBPα and ε, are essential to maturation of particular cell types. This "roadmap" of requirements is only an overview in that factors required early may also serve important functions later in hematopoietic development, and those seemingly required only late may have roles earlier that are partially obscured by proteins with overlapping functions. The potential compensation for GATA-1 deficiency by the action of the related factor GATA-2 may provide an example of this latter point.

What do these and other studies suggest about the way in which specific lineages are selected from multipotential progenitors? At the outset of these studies, many investigators supposed that HSCs or progenitors were naive and expressed none of the lineage-restricted regulatory factors (Orkin, 1995,1996). Subsequent expression of these factors, such as GATA-1, PU.1, GATA-3 among others, might then trigger differentiation along a specific path, perhaps analogous to the simplistic, early models of myoD action in myogenesis (Davis *et al.,* 1987). Precious little evidence now supports this simple "deterministic" model of hematopoietic lineage selection. Rather, we are now confronted with complex combinatorial scenarios in which HSCs or progenitors express a repertoire of "lineage-restricted" factors long before lineage commitment has occurred (Orkin. 1995,1996). Recent single cell RT-PCR studies support multilineage marker expression in hematopoietic progenitors preceding the choice of a particular pathway for subsequent maturation (Hu *et al.,* 1997). Lineage commitment, then, may involve the stabilization of sectors of regulatory networks, such as the enhanced expression or interaction of several components, along with the subsequent down-regulation or inhibition of other factors. Such models of selection are compatible with the combinatorial action of GATA-1 and FOG for erythroid and megakaryocyte differentiation and the proposed inhibitory role of the myeloid factor mafB on ets-1 transactivation in erythroid development (Sieweke *et al.,* 1996). Thus, lineage selection is likely to represent a fine balance of positive and negative influences, rather than cataclysmic changes in regulatory components. This may complicate life of the investigator in dissecting these pathways, but simplify the cell's ability to manage in a complex and changing environment.

Our studies also illustrate how various approaches may be used to circumvent the earliest developmental block in embryonic lethal knockout mice to examine other gene functions. While the cre-loxP conditional targeting system (Gu *et al.,* 1994) offers extraordinary promise in this regard, it suffers from practical limitations related to the availability of specific cre-expressing mouse strains and the completeness of cre-mediated excision in particular lineages. In some instances, these may represent significant hurdles to experimental success. Though the use of cis-element gene targeting to define a role for GATA-1 in megakaryocytes (Shivadasani *et al.,* 1997) had an aspect of uncertainty or serendipity, the principal of modifying regulatory elements to achieve lineage-selective effects remains valid. Indeed, the block to megakaryocyte-specific GATA-1 expression in mice retaining the neomycin-resistance cassette likely exceeds that which might have been obtained using conditional targeting and a megakaryocyte-specific cre-expressing transgenic mouse. Rescuing a portion of a complex phenotype by a transgene directed to specific tissues or stages of development constitutes yet another strategy for revealing additional *in vivo* consequences of a gene knockout. Thus, several options are now available for addressing the multiple functions of gene products in diverse developmental settings (see Fig. 4). The challenge for the future is how to apply these tools towards an improved understanding of mammalian development.

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