A Recurrent Network Involving the Transcription Factors PU.1 and Gfi1 Orchestrates Innate and Adaptive Immune Cell Fates

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SUMMARY

The transcription factor PU.1, encoded by the Sfpi1 gene, functions in a graded manner to regulate macrophage versus B cell generation; its higher concentration favors the macrophage fate. We demonstrated that Gfi1 reciprocally promoted B cell fate choice at the expense of myeloid progeny. Gfi1−/− multipotential progenitors (MPPs) were unable to constrain the expression of PU.1 because Gfi1 functioned to repress the Sfpi1 gene by displacing PU.1 from positive autoregulatory elements. Attenuating a transcriptional module composed of PU.1 and Egr suppressed the B lineage developmental defects of Gfi1−/− MPPs. Finally Ikaros, a transcription factor required for B cell development, promoted Gfi1 and antagonized PU.1 expression in MPPs. Our results reveal that a core transcriptional regulatory network used for directing cell fate choice in the innate immune system has been co-opted by Ikaros to orchestrate B lymphocyte generation. These findings have important implications for the evolution of the adaptive immune system.

INTRODUCTION

An emerging developmental framework for hematopoiesis posits that cells of the innate and adaptive immune system arise from a common lymphoid-primed multipotent progenitor (LMPP) lacking erythroid and megakaryocytic potential (Adolfsson et al., 2005; Laiosa et al., 2006a). Genetic and molecular analyses of various lineage-determining transcription factors have enabled the assembly of contingent gene regulatory networks that promote the generation of either myeloid or lymphoid progeny from multipotent hematopoietic progenitors (Laiosa et al., 2006a; Laslo et al., 2008; Rothenberg and Taghon, 2005; Singh et al., 2005). Although the transcription factors PU.1, Ikaros, Mef2c, E2A, and EBF1 have been implicated in regulating myeloid versus lymphoid cell fate choice (DeKoter and Singh, 2000; Dias et al., 2008; Pongubala et al., 2008; Reynaud et al., 2008; Stehling-Sun et al., 2009), the nature of the molecular circuits that underlie the onset of cell fate determination in MPPs remain to be elucidated. A deeper understanding of such transcriptional networks may provide insight into the molecular evolution of the adaptive immune cells from their primordial innate counterparts.

The existence of multipotent progenitors restricted to the generation of myeloid and lymphoid progeny was previously proposed on the basis of genetic analysis of the gene encoding PU.1 (Sfpi1) (Singh, 1996; Singh et al., 1999). PU.1 is a member of the Ets family of transcription factors and is expressed in multiple lineages of the hematopoietic system, including MPPs (Nutt et al., 2005). In the absence of PU.1, the development of myeloid and lymphoid lineages is severely impaired, whereas the generation of erythrocytes and megakaryocytes is largely unaffected (McKercher et al., 1996; Scott et al., 1994). The expression of many myeloid (DeKoter et al., 1998) and lymphoid-specific genes (DeKoter et al., 2002; Medina et al., 2004) is abolished in PU.1-deficient (Sfpi1−/−) hematopoietic progenitors. Graded amounts of PU.1 appear to regulate the development of myeloid and B lineage progeny, given that a low concentration of PU.1 induces the B cell fate, whereas a higher concentration promotes macrophage development at the expense of B cell generation (DeKoter and Singh, 2000). In addition, elevating expression of PU.1 has been shown to inhibit early T cell development (Anderson et al., 2002) and promote macrophage differentiation (Laiosa et al., 2006b). These results suggest that PU.1 expression needs to be constrained in MPPs in order to enable B lymphopoiesis in the bone marrow and T lymphopoiesis in the thymus. The molecular means by which this is achieved remains to be elucidated.

In myeloid progenitors, PU.1 has been shown to induce and resolve a mixed lineage pattern of gene expression resulting in the generation of macrophages and neutrophils (Laslo et al., 2006). In this cellular context, PU.1 is a component of a transcriptional regulatory circuit composed of the myeloid determinant C/EBPα and the counteracting repressors Egr1,2, Nab2, and Gfi1. High expression of PU.1 induces Egr2 and Nab2. Importantly, Egr2 functions in a feed-forward loop with PU.1 to activate macrophage-specific genes and with Nab2 to repress alternate lineage neutrophil genes, including Gfi1. Conversely, Gfi1 promotes neutrophil differentiation by antagonizing PU.1 and Egr activity, the former, presumably, via direct protein-protein interactions (Dahl et al., 2007) and the latter via transcriptional repression (Laslo et al., 2006). Because PU.1 expression
appears to be regulated by a positive autoregulatory loop (Okuno et al., 2005), these results raised the possibility that Gfi1 could attenuate expression of PU.1 by antagonizing PU.1 activity in MPPs, thereby lowering its expression to promote the generation of lymphocytes at the expense of myeloid progeny.

Like PU.1, Gfi1 is expressed in multiple hematopoietic lineages, including MPPs (Hock et al., 2004; Zeng et al., 2004). However, Gfi1 expression appears to be inversely correlated with that of PU.1 in hematopoietic cells. Gfi1−/− animals are neutropenic; the granulocytic intermediates that develop in the bone marrow misexpress PU.1-regulated genes such as Csf1r (Hock et al., 2003). Interestingly, the frequency of myeloid progeny is increased in the bone marrow of Gfi1−/− mice, whereas the number of B lineage cells in the bone marrow and T lineage cells in the thymus are significantly reduced (Hock et al., 2003; Yucel et al., 2003). Because high amounts of PU.1 function to induce myeloid development and Gfi1 activity is critical in early lymphocyte development, we considered whether PU.1 and Gfi1 might function in an antagonistic manner to regulate innate versus adaptive immune cell fate choice in MPPs as they do in orchestrating macrophage versus neutrophil development. Given that high amounts of PU.1 are inhibitory for early B and T cell development, we reasoned that the underlying basis might involve PU.1-mediated induction of the Egrs that could directly repress Gfi1 expression. Consistent with this possibility, the loss of Egr1 results in increased T lineage precursors in the thymus (Bettini et al., 2002). Given the aforementioned findings, we hypothesized that a network composed of PU.1, Egrs, and Gfi1 might function in a recurrent manner to regulate myeloid versus lymphoid cell fate choice in MPPs.

As with Gfi1, the loss of the zinc-finger transcription factor Ikaros has profound consequences on the development of both B and T lineage cells (Wang et al., 1996). Additionally, Ikaros has been implicated in the generation of LMPPs (Yoshida et al., 2006). Interestingly, Ikaros-deficient (Ikzf1−/−) pro-B cells retain myeloid developmental potential and misexpress multiple myeloid-specific genes, including Csf1r (Reynaud et al., 2008). These findings suggest similar roles for Ikaros and Gfi1 in promoting early B and T cell development and in repressing myeloid developmental potential. We therefore considered the possibility that Ikaros and Gfi1 may represent components of a regulatory network in which they collaborate to promote adaptive immune cell fates and repress innate immune cell fates in MPPs.

We designed a set of genetic and molecular experiments to test the existence of the proposed regulatory network controlling innate versus adaptive immune cell fates. We demonstrate that Gfi1 promoted B cell fate choice by antagonizing the expression of the gene encoding PU.1 and aspects of the myeloid gene-expression program in MPPs. Molecular analyses revealed that Gfi1 directly repressed the Sfpi1 gene by targeting the Sfpi1 promoter and a distal upstream regulatory element (URE). Consistent with our hypothesis, Egr transcription factors were shown to function in an opposing manner to Gfi1, given that they inhibited B cell development while enabling myelopoiesis. Finally, we showed that Ikaros positively regulated Gfi1 and antagonized the expression of PU.1 in MPPs. These data are consistent with a model whereby Ikaros and Gfi1 function within MPPs to inhibit myeloid lineage potential by attenuating PU.1 and Egr activity, thereby facilitating the specification of lymphoid cell fates.

RESULTS

Gfi1 Promotes B Cell Fate Choice

Because Gfi1−/− animals exhibit a decrease in the frequency of common lymphoid progenitors and an increase in granulocyte and macrophage progenitors (Zeng et al., 2004), we examined whether Gfi1 functions to promote B cell fate specification and repress myeloid development in the context of a MPP. We isolated MPPs from wild-type (WT) and Gfi1−/− mice (Figure S1A available online) and plated equivalent numbers on OP9 stroma under conditions that support the generation of both myeloid (Mac1+) and B lineage (CD19+) progeny. WT progenitors gave rise to both myeloid (21%) and B lymphoid cells (68%). In contrast, MPPs lacking Gfi1 were severely defective for B cell development in vitro (Figure 1A and Figure S1B). We note that the loss of Gfi1 in vivo impairs, but does not eliminate, B cell development (Hock et al., 2003). We also performed gain-of-function analyses to test whether an increased concentration of Gfi1 in MPPs enhanced the generation of B lineage progeny at the expense of myeloid precursors. Transduction of WT MPPs with a control vector (MIGR1) generated a higher proportion of CD19+ progeny (Figure 1B). With limiting-dilution assays, MPPs transduced with Gfi1 were seen to give rise to B lineage progeny at

Figure 1. Gfi1 Regulates B versus Myeloid Cell Fate Choice
(A) MPPs (Lin−Sca-1−c-Kit+) were isolated from the bone marrow of WT or Gfi1−/− animals. Cells were directly plated on OP9 stroma and analyzed by flow cytometry at day 12. Data are representative of at least three independent experiments.
(B) WT MPPs were transduced with a control vector (MIGR1) or one expressing Gfi1 (MIGR1-Gfi1). GFP+ transductants were plated on OP9 stroma and examined for the generation of myeloid and B lineage progeny after 7 days. Data are representative of at least three independent experiments.

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a frequency nearly 3.5 times higher than their control counterparts (Figure S1C). These results demonstrate that Gfi1 promotes B cell fate choice at the expense of myeloid options in the context of MPPs.

**Gfi1 Antagonizes PU.1 Expression in MPPs**

PU.1 expression is elevated in total bone marrow cells lacking Gfi1 (Hock et al., 2003). We specifically analyzed the expression of PU.1 and other hematopoietic determinants in WT and Gfi1−/− MPPs. Whereas the expression of Ikaros and Gata1 were unaffected in Gfi1−/− MPPs, PU.1 transcripts were elevated (Figures 2A and 2B). Moreover, the expression of the Csf1r gene, which is directly activated by PU.1 (Krysinska et al., 2007), was increased in Gfi1−/− MPPs (Figures 2A and 2B). We note that Flt3 transcripts are reduced in Gfi1−/− MPPs (Figure 2B), consistent with reduced Flt3 expression on the surface of Gfi1−/− MPPs (Hock et al., 2004; Zeng et al., 2004). We also assessed expression of c-fms and FcγRII and III on Gfi1−/− MPPs by using flow cytometry. Like c-fms, the FcγRII and FcγRIII receptors are expressed on myeloid precursors and their genes are directly activated by PU.1. The loss of Gfi1 resulted in increased expression of c-fms, FcγRII, and FcγRIII in MPPs (Figure 2C). We note that the expression of Ly6G and Mac1, two cell-surface markers that are expressed on more mature myeloid cells, was not affected (Figure S2A). Thus, loss of Gfi1 results in elevated expression of PU.1 and its myeloid target genes in MPPs.

We evaluated the developmental potential of c-fms expressing MPPs to test whether they were biased toward myeloid fates (Figure S2B). Gfi1−/− MPPs that were c-fms− had considerably lower B lineage developmental potential than WT MPPs (Figure S2B). Importantly, c-fms+ MPPs lacking Gfi1 were further impaired in their ability to give rise to B lineage progeny. Therefore, B lineage potential may be impaired in Gfi1−/− MPPs as a consequence of enhanced myeloid potential.

We predicted that augmented PU.1 expression in Gfi1−/− MPPs should result in enhanced macrophage potential. We analyzed Gfi1−/− MPPs for their ability to undergo multilineage erythro-myeloid differentiation. The absence of Gfi1 resulted in ~50% reduction in plating efficiency of MPPs relative to WT counterparts (Figure 2D). WT MPPs preferentially gave rise to granulocyte-macrophage colonies (~70%) and, to a lesser extent, macrophage (25%) and myelo-erythroid colonies (5%) (Figure 2E). In contrast, the preponderance of colonies generated from Gfi1−/− MPPs contained macrophages (85%). No discernable difference was noted between WT and Gfi1−/− MPPs in the generation of myelo-erythroid colonies, suggesting a role for Gfi1 in lineage restriction subsequent to the specification of megakaryocyte and erythrocyte fates. Taken together, these data suggest a critical role for Gfi1 in constraining the expression of PU.1 in the context of two distinct cell fate choices: (1) macrophage versus neutrophil and (2) myeloid versus B cell.

**PU.1 Heterozygosity Partially Rescues B Cell Development in Gfi1−/− Mice**

Given that the loss of Gfi1 results in enhanced expression of PU.1, we reasoned that this may partially account for the reduced B cell developmental potential of Gfi1−/− MPPs. To test this possibility, we determined whether reducing the dosage of PU.1 would suppress the B cell developmental defect caused by the loss of Gfi1. As previously described (Hock et al., 2003), loss of Gfi1 resulted in fewer committed B lineage cells (B220+CD19+) in the bone marrow (Figure 3A). Combining PU.1
heterozygosity with the Gfi1 mutation led to a 2-fold increase in the frequency as well as the absolute numbers of committed B lineage cells (Figures 3A and 3B). In addition, there was a 2-fold increase in the number of pro-B cells (B220+CD43+) in Gfi1+/− Sfpi1+ mice in comparison to Gfi1−/− controls (Figure 3B). These results demonstrate that B lineage defects in Gfi1−/− bone marrow can be partially suppressed by reducing Sfpi1 gene dosage.

To determine whether reduced Sfpi1 gene dosage increases the B lineage developmental potential of Gfi1−/− MPPs, we quantitatively analyzed their cell fate outputs in vitro. We note that loss of one allele of Sfpi1 was associated with a decrease in c-fms, FcγRII, and FcγRIII expression on the cell surface of Gfi1−/− Sfpi1−/− MPPs relative to their Gfi1−/− counterparts (Figure S3). WT MPPs gave rise to B lineage cells at a frequency of ~1/24, whereas loss of Gfi1 severely impaired the generation of such cells (1/1260) (Figure 3C). Importantly, we observed ~3.5-fold increase (1/360) in the frequency of B lineage cells generated from Gfi1−/− Sfpi1−/− MPPs compared with their Gfi1−/− counterparts. These data demonstrate that Gfi1-mediated antagonism of PU.1 expression in MPPs plays an important role in restraining myeloid differentiation and, in turn, promoting specification of the B cell fate.

**Attenuation of PU.1 in Gfi1−/− MPPs Promotes B Cell Development**

We utilized a shRNA approach to rigorously test whether Gfi1−/− MPPs are impaired in their B lineage potential because of increased expression of PU.1. Transduction of WT MPPs with a vector targeting PU.1 mRNA (MSCV-shPU.1) resulted in ~70% reduction in PU.1 transcripts and its target gene Csfr1 (Figure 4A). Reduced PU.1 expression impaired terminal myeloid differentiation, as evidenced by the accumulation of c-Kit+Mac1+ myeloid precursors (Figure 4B). This resembled the accumulation of similar myeloid precursors in the bone marrow of mice in which PU.1 expression is reduced by targeting an upstream distal enhancer (Rosenbauer et al., 2004). Importantly, attenuation of PU.1 expression in WT MPPs resulted in a higher frequency of committed B lineage progeny (27%) and diminished Mac1+ precursors (52%) compared to control transductants (Figure S4). Thus, lowering PU.1 expression in MPPs promoted the generation of B lineage cells at the expense of myeloid precursors.

We next examined whether attenuating PU.1 expression in Gfi1−/− MPPs restored their ability to give rise to B lineage progeny. Gfi1−/− MPPs were transduced with the control or MSCV-shPU.1 construct. Although Gfi1−/− MPPs primarily gave rise to Mac1+ precursors, attenuation of PU.1 strongly induced the generation of CD19+ B lineage cells (Figure 4C). These data establish that Gfi1-mediated antagonism of PU.1 expression in MPPs is used to regulate B versus myeloid cell fate choice.

**Inhibition of Egr Activity Promotes B Cell Fate Choice**

We have previously proposed that higher amounts of PU.1 antagonize Gfi1 expression via induction of the Egr’s which directly bind to the Gfi1 promoter and repress its activity (Laslo et al., 2006). Conversely, Gfi1 represses the transcription of Egr genes. Thus, perturbing Egr activity would be predicted to have the opposite consequence to that of manipulating Gfi1 on early B cell development. Furthermore, the Egrs should promote myeloid versus B cell fate choice in MPPs as is seen with a higher concentration of PU.1. To test this prediction, we transduced WT MPPs with a control vector (MIGR1) or one expressing
a dominant-negative version of Egr2 (MIGR1-ΔEgr). Whereas control transductants preferentially give rise to Mac1⁺ precursors, inhibition of Egr function in MPPs strongly promoted the development of B lineage progeny (Figure 5A). Limiting-dilution analyses revealed that blocking Egr activity in MPPs enhanced B cell development by more than an order of magnitude (Figure 5B). Thus, blocking Egr activity in MPPs has similar consequences on B cell development as enhancing Gfi1 expression (Figure 1B).

We next used genetic interaction analyses to test for the functional cross-antagonism between the Egrs and Gfi1 in B cell development. Interestingly, we observed that the removal of Egr1 partially suppressed the cell development defect observed in Gfi1⁻/⁻ mice (Figure 5C and Figure S5A). This paralleled the partial “rescue” of B cell development in Gfi1⁻/⁻ mice by reducing the dosage of PU.1 (Figures 3A and 3B). Egr1 and Egr2 function redundantly during macrophage differentiation (Laslo et al., 2006). To test whether Egr1 and Egr2 also function redundantly in antagonizing the generation of B cells, we generated mice lacking both Egr1 and Egr2 by using a conditional Egr2 allele (Taillebourg et al., 2002). This was necessary because Egr2⁻/⁻ mice die soon after birth (Swiatek and Gridley, 1993) and loss of Egr1 and Egr2 results in embryonic lethality. Using the Mx1-Cre transgene, we observed efficient excision of the Egr2 allele in total bone marrow cells 4 weeks after polyIC treatment (Figure S5B). Combined loss of Egr1 and Egr2 resulted in a ∼2-fold increase in the percentage of B lineage progeny in the bone marrow and a comparable reduction in myeloid precursors (Figures S5B and S5C). These results provide compelling genetic evidence in support of the hypothesis that Egrs antagonize B cell fate choice at the expense of myeloid options in MPPs.

**Gfi1 Targets the Sfpi1 Promoter and URE**

Given that Gfi1 functions as a transcriptional repressor, we examined whether Gfi1 targets the Sfpi1 gene, which encodes PU.1. Bioinformatic analysis revealed conserved putative Gfi1 binding sites in the PU.1 promoter and the URE (Figure S6A). Intriguingly, these presumptive Gfi1 binding sites were in close proximity to the PU.1 binding sites. PU.1 binding sites in the promoter and URE of the Sfpi1 gene have been implicated in a positive autoregulatory feedback loop (Okuno et al., 2005). We considered the possibility that Gfi1 could repress the Sfpi1 gene by displacing PU.1 from its own promoter and URE.

We performed electrophoretic mobility-shift assays (EMSAs) to determine whether PU.1 and Gfi1 recognize the predicted binding-site motifs in the promoter and URE. PU.1 and Gfi1 bound to their respective motifs in the URE with higher affinity than to the corresponding motifs in the promoter (Figure S6B). Given that the Gfi1 and PU.1 sites overlap within the URE, we tested whether the proteins compete for DNA binding. Competition reactions revealed PU.1 and Gfi1 displace each other when binding to the composite element in the URE (Figure 6A).

To determine whether Gfi1 and PU.1 bind to the URE in a reciprocal manner in vivo, we performed chromatin immunoprecipitation (ChIP) assays with a cell line that expresses an inducible PU.1 protein (PUER) (Walsh et al., 2002). In the absence of tamoxifen (OHT), these cells express Gfi1 (Laslo et al., 2006), which could be seen to crosslink to the URE (Figure 6B) as well as the PU.1 promoter (Figure S6C, left panel). A basal amount of crosslinking of PU.1 was also detectable at these regions (Figure 6B and Figure S6C, left panel). Upon stimulation with OHT, PU.1 binding at the URE and promoter increased, whereas Gfi1 crosslinking was diminished (Figure 6B and Figure S6C, left panel). Increased PU.1 binding accompanied by the loss of Gfi1 was also observed at the Csf1r promoter (Figure S6C, right panel), consistent with recent findings implicating Gfi1 in the repression of the Csf1r gene (Zarebski et al., 2008). Although induction of PU.1 activity resulted in downregulation of Gfi1, we did not observe reduced Gfi1 crosslinking to an autoregulatory site in the Gfi1 promoter (data not shown, Yucel et al., 2004). Thus, loss of Gfi1 binding at the URE is likely a consequence of displacement by PU.1. Collectively, these data demonstrate that Gfi1 targets multiple regulatory regions within the PU.1 locus and suggest that it represses PU.1 expression by interrupting an autoregulatory loop.

**Apposed Gfi1 Motifs in PU.1 Target Sequences**

To explore whether the molecular antagonism of PU.1 action by Gfi1 via apposed binding sites may occur at other loci,
we performed ChIP-on-chip assays by using a promoter array to identify PU.1 target genes in OHT-induced PUER cells. Bioinformatic analysis (see Experimental Procedures) revealed that 3170 PU.1 target sequences contained presumptive Gfi1 binding sites. Of these, 19% contained a putative PU.1 site 10 bp or less. Notably, PU.1 binding sites for the two transcription factors separated by 10 bp or less.

Interestingly, PU.1 and Gfi1 motifs were seen to overlap in nearly half of this subset of ChIP sequences. As a control, we generated a set of randomized DNA sequences in silico with the same base composition and length as the PU.1 target set. Comparison between PU.1-bound and randomized DNA sequences revealed a statistically significant difference given that only 9% (SD = 0.44) of randomized sequences contained presumptive binding sites for the transcription factors.

Strikingly, the most notable arrangement of presumptive PU.1 and Gfi1 binding sites consisted of ones in which the core motif of PU.1 (GGAA) and Gfi1 (AATC) overlapped by a single nucleotide (Figure S6D). The fact that the same configuration was retained as a predominant feature in the randomized sequences, albeit at a substantially lower frequency, led us to consider the possibility that the large frequency of overlapping sites might be explained by compatibilities within the extended binding motif of PU.1 and Gfi1.

In fact, inspection of the Gfi1 binding site matrix revealed preferences for specific nucleotides flanking the core base pairs of the Gfi1 site that potentially encode PU.1 sites (Figure 6D). Detailed analysis of the Gfi1 binding matrix revealed a number of preferred PU.1 site configurations in relation to the Gfi1 core, as evidenced in both the PU.1 ChIP-on-chip and randomized DNA analysis (Figure 6D). These analyses suggest that a large number of PU.1 target genes may be repressed via competitive binding or proximal action of Gfi1.

Ikaros Regulates Gfi1 and Constrains PU.1 Expression

Given that Ikaros is required for B cell development and repression of myeloid potential, we examined whether its loss, like that of Gfi1, also results in the enhanced expression of PU.1 in MPPs. Utilizing a Gfi1-GFP reporter allele (Yucel et al., 2004), we observed that the loss of Ikaros was associated with the generation of a subset of MPPs expressing lower amounts of Gfi1 (Figure 7A and Figure S7A). In addition, the loss of Ikaros resulted in a substantial increase in the expression of both c-fms and FcγRII and III (Figure 7B). The similarities in the myeloid signature observed in Gfi1−/− and ikzf1−/− MPPs suggested a potential relationship between Ikaros and Gfi1 activity in restraining myelopoiesis. Therefore, we sought to determine whether Ikaros activity was required for the expression of Gfi1 in MPPs. Utilizing a Gfi1-GFP reporter allele (Yucel et al., 2004), we observed that the loss of Ikaros was associated with the generation of a subset of MPPs expressing lower amounts of GFP (Figure 7C). We note that a larger subset of ikzf1−/− MPPs expressed higher amounts of GFP relative to their control counterparts. These cells also expressed higher amounts of c-fms (data not shown) and may represent MPPs that are primed to the myeloid lineage (Ng et al., 2009). Bioinformatic analysis revealed several putative Ikaros binding sites in the Gfi1 locus. Interestingly, two such sites were positioned downstream of the Gfi1 gene in a ~170 bp region that is duplicated (Figure S7B). EMSA and ChIP assays demonstrated that Ikaros bound these sites in vitro and in vivo (Figures S7C and S7D). These data suggest that Ikaros directly promotes Gfi1 expression in a subset of MPPs.

DISCUSSION

Using Sfp1−/− hematopoietic progenitors, we have previously reported that a graded concentration of PU.1 regulates B lymphocyte versus macrophage cell fate choice (DeKoter and Singh, 2000). Consistent with these findings, it has been reported that antagonizing the expression of PU.1 in differentiating mouse embryonic stem cells with siRNA enhances their B lineage...
developmental potential (Zou et al., 2005). However, demonstrating a role for PU.1 dosage in innate versus adaptive cell fate “choice” in vivo has proven to be elusive. Using a sensitized genetic background, a null mutation in the Gfi1 locus, we show that reducing PU.1 concentration via a gene-dosage strategy can negatively regulate the expression of Gfi1 via the Egrs (Laslo et al., 2006). Gfi1 expression is likely to be increased in MPPs of PU.1-enhancer-deficient animals. In this context, increased Gfi1 expression would function in concert with C/EBPα to promote neutrophil development. Nonetheless, our results demonstrate that PU.1 and Gfi1 have opposing roles in promoting myeloid versus B cell development and establish that these counteracting regulators function in a recurring manner to regulate cell fate choice in the innate as well as the adaptive immune systems.

How does Gfi1 restrain the expression of PU.1 in MPPs to specify the B cell fate? Previously, Gfi1 has been shown to interact with the PU.1 protein, and this protein-protein interaction has been suggested to inhibit PU.1 mediated transactivation (Dahl et al., 2007). Although we cannot rule out the involvement of this protein interaction in antagonism of Spi1 gene expression by Gfi1, we show that Gfi1 directly competes for PU.1 binding in vitro and in vivo to autoregulatory sites in the PU.1 locus. We therefore propose that Gfi1 represses Spi1 gene activation by binding to DNA sites in the locus and disrupting a PU.1-dependent autoregulatory loop. Importantly, increased expression of PU.1 can induce Egr expression, and the latter represses Gfi1. Finally, Gfi1 has been shown to bind to its own promoter and function in a negative autoregulatory loop (Yucel et al., 2004). Thus, the two autoregulatory feedback loops and the cross-antagonism between PU.1 and Gfi1 may generate alternate gene-expression states within this network that are driven by either higher PU.1 and Egr activity or higher Gfi1 activity.

Gfi1−/− MPPs are poised to differentiate along the myeloid lineage at the expense of the B lineage as a consequence of misexpression of PU.1 and other myeloid genes. In addition to the PU.1 locus, Gfi1 targets many myeloid genes, including Csf1r (Zarebski et al., 2008). Accordingly, the absence of Gfi1 in MPPs may augment the expression of PU.1 target genes, such as Csf1r, via derepression. Our analysis of PU.1 target sequences has revealed an intriguing feature: a large number of PU.1 core motifs are embedded within the extended binding matrix for Gfi1. Therefore, robust activation of such PU.1 target genes would be dependent on displacement of Gfi1 repressor complexes with PU.1 activator complexes at these composite elements, and vice versa. We note that the predicted composite sites are found in a varied set of genes, including growth factors and their receptors, lineage-determining transcription factors, and histone-modifying enzymes. We envision that the antagonistic regulation of a large battery of genes by PU.1 and Gfi1 would have evolved more readily by selecting for favorable composite binding sites for the two factors rather than independent selective events that generate two separated sites. This may represent a general strategy for rapidly evolving counteracting regulatory modules by utilizing transcription factors whose individual binding motifs are compatible with the generation of overlapping composite elements.
Figure 7. Ikaros Promotes Gfi1 Expression and Represses PU.1
(A) RT-PCR analysis of the indicated transcripts in WT and ikzf1−/− MPPs.
(B) Flow cytometric analysis of c-fms and FcγRII&III expression on WT (gray shaded area) and ikzf1−/− (black line) MPPs. Data are representative of three independent experiments.
(C) Expression of the Gfi1-GFP targeted allele in MPPs from Gfi1+/− (gray shaded area) or ikzf1−/−/C0 (black line) mice by flow cytometry. Data are representative of two independent experiments.
(D) A proposed gene regulatory network (circuit diagram) that dictates myeloid versus B cell fate choice in the context of a MPP. Arrows represent gene activation and barred lines represent gene repression. Hatched lines represent regulatory connections whose molecular basis remains to be elucidated. The shaded region encompasses a core gene regulatory network that is used in a recurring manner for orchestrating innate as well as adaptive immune cell fates.

On the basis of the aforementioned data, we propose a transcriptional regulatory network (circuit diagram) that appears to function in a recurring manner to govern cell fate choice in the immune system. In MPPs, PU.1 is proposed to function in a graded manner to regulate B lymphoid versus macrophage cell fates. A higher concentration of PU.1 represents a primary input to the macrophage developmental program and can induce the Egrs (module a). These regulators activate both myeloid gene expression and the Id genes that inhibit E2A activity (module b) and consequently the priming of B-lymphoid developmental potential (Dias et al., 2008). A lower concentration of PU.1 along with Ikaros and E2A function as primary inputs to activate the B lymphoid program. These factors induce the B cell fate determinant EBF1 (module c) (Laslo et al., 2006). In our model, the reduced concentration of PU.1 that promotes B lymphoid development is achieved by Ikaros, in part, through the induction of Gfi1. Ikaros and Gfi1 constrain the expression of PU.1 while promoting the expression of B lymphoid genes. We note that Ikaros has been implicated in repression of the myeloid determinant C/EBPα (Reynaud et al., 2008; Ng et al., 2009). This repressive activity of Ikaros is likely to be important for B cell development, given that C/EBPα can reprogram committed B cells into macrophages (Laiosa et al., 2006a).

An additional consequence of Ikaros and Gfi1-mediated repression of the myeloid program in MPPs could be to promote T cell developmental potential. Along these lines, Gfi1 activity appears to be critical for the generation of the earliest T lineage progenitors in the thymus (Yucel et al., 2003). It is noteworthy that the loss of Ikaros and Gfi1 is associated with the increased expression of Id genes (Yucel et al., 2003; Ng et al., 2009). These genes encode proteins that inhibit E2A family transcription factors that are required for early B and T cell development (Quong et al., 2002). Conversely, we have demonstrated that high concentrations of PU.1 induce Id2 during macrophage differentiation (Laslo et al., 2006). Similarly, increasing PU.1 expression in fetal thymic progenitors induces Id2 and aspects of the myeloid program (Franco et al., 2006). These findings suggest a general mechanism by which Gfi1 and PU.1 could function in a counteracting and recurring manner to promote B and T lymphoid versus myeloid cell fates via the antagonistic regulation of Id genes. Consistent with our model, Egr1 has been identified as a positive regulator Id3 gene expression (Bain et al., 2001; Quong et al., 2002). Loss of Egr1 results in an increase in the absolute numbers of the earliest T lineage progenitors (Bettini et al., 2002). We note that multiple defects observed in Gfi1−/− mice during T cell development, including reduced cellularity and impaired progression through the double-negative and double-positive stages of development, are partially rescued upon the loss of Egr1 in vivo (C.J.S. and H.S., unpublished data). These findings are also in keeping with our proposal that the Egrs and Gfi1 comprise a counteracting regulatory module that directs cell fate options or developmental transitions in multiple cellular contexts (Laslo et al., 2006). On the basis of the above results, the Ids would represent an additional node within this module whose expression would be counteracted by Gfi1 to promote lymphopoiesis or induced by PU.1 and the Egrs to inhibit B and T cell potential.

Our proposed core transcriptional network for lymphoid versus myeloid cell fate determination is derived from one that regulates macrophage versus neutrophil cell fate choice (Laslo et al., 2006). In the former, Ikaros has replaced C/EBPα as a pivotal primary determinant, but the remaining circuit is conserved both with respect to the nature of the regulatory molecules and to their connectivity. The modified network architecture has two important evolutionary implications for the emergence of the adaptive immune system from an innate primordial precursor. First, it suggests that the conserved core network...
composed of the counteracting PU.1, Egrs, and Gfi1 modules predates the origin of lymphocytes and was co-opted by Ikaros. Second, it suggests that Ikaros has played a key role in the evolutionary emergence of adaptive immune cells (i.e., lymphocytes). Ikaros manifests two major regulatory functions that are consistent with this proposition: (1) It is able to restrain myeloid developmental potential by antagonizing expression of PU.1 and other myeloid genes, and (2) it directly activates Rag gene expression and promotes the recombination of antigen receptor gene segments, a hallmark of adaptive immune cells (Reynaud et al., 2008). Molecular phylogenetic analysis of our network components and their connectivity will be necessary to test this evolutionary proposition.

**EXPERIMENTAL PROCEDURES**

**Mice**

Sfi1−/− (Scott et al., 1994), Egr1−/− (Sviatsek and Gridley, 1993), Egr2−/− (Taillebourg et al., 2002), Ikarf−/− (Wang et al., 1996), and Gfi1−/− (Yucel et al., 2004) mice have been previously described. The Gfi1 mutant allele represents a GFP knockin, and the mutant mice are phenotypically indistinguishable from mice have been previously described. The viable and dead cells. MPPs were isolated and cultured as previously outlined in Table S5. DAPI was used for discriminating between thrin-Cy7 (PE-Cy7), and allophycocyanin (APC) were used against cell-surface fluorescein isothiocyanate (FITC), phycoerythrin (PE), PerCP-Cy5.5, phycoerythin-Cy7 (PE-Cy7), and allophycocyanin (APC) were used against cell-surface molecules outlined in Table S5. DAPI was used for discriminating between viable and dead cells. MPPs were isolated and cultured as previously described (Karsunky et al., 2002). Therefore, Gfi1−/−/GFP mice are referred to as Gfi1−/− in these studies. Wild-type C57BL/6 mice were purchased from the Jackson Laboratory. Mice were maintained in pathogen-free conditions in accordance with guidelines approved by the Institutional Animal Care and Use Committees of the University of Chicago.

**Flow Cytometry and Cell Culture**

Bone marrow single-cell suspensions were cultured in PBS containing 5 mM EDTA and 0.5% BSA were analyzed by flow cytometry with an LSRII (Becton Dickinson) and FlowJo software. The following antibodies conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), PerCP-Cy5.5, phycoerythin-Cy7 (PE-Cy7), and allophycocyanin (APC) were used against cell-surface molecules outlined in Table S5. DAPI was used for discriminating between viable and dead cells. MPPs were isolated and cultured as previously described (Medina et al., 2004) and analyzed for the presence of Mac1+ and CD19+ cells between days 7–12 by flow cytometry.

**Retroviral Transduction**

WT MPPs were isolated as described above and transduced by coculture with GFP (MIGR1), Gfi1 (MIGR1-Gfi1), or ΔEgr2 (MIGR1-ΔEgr) as previously described (DeKoter and Singh, 2000). After 2 days, GFP+ transductants were sorted and cultured on OP9 stroma in previously described B lineage conditions (Medina et al., 2004) and analyzed for the presence of Mac1+ and CD19+ cells by flow cytometry 7 days after sorting.

**Flow Cytometry and Cell Culture**

WT MPPs were isolated as described above and transduced by coculture with GFP (MIGR1), Gfi1 (MIGR1-Gfi1), or ΔEgr2 (MIGR1-ΔEgr) as previously described (DeKoter and Singh, 2000). After 2 days, GFP+ transductants were sorted and cultured on OP9 stroma in previously described B lineage conditions (Medina et al., 2004) and analyzed for the presence of Mac1+ and CD19+ cells by flow cytometry 7 days after sorting.

**Flow Cytometry and Cell Culture**

**Expression Analysis**

RNA was isolated with TRIzol reagent (Invitrogen) and reverse-transcribed with M-Mulv reverse transcriptase and oligo-dT primers as described in Appendix A. Primers were designed to amplify fragments of the transcript of interest and to amplify a constitutively expressed internal control (β-actin). PCR products were visualized on a gel and the fragments were excised, purified, and sequenced to confirm their identity. Gene expression was normalized to the expression of β-actin. Primers used for quantitative PCR analyses are listed in Table S1 and S2.

**Colony-Forming Assays**

Clonogenic assays were performed by sorting ~300 MPPs from WT or Gfi1−/− mice in 3 ml of Methocult M3434 (Stem Cell Technologies) supplemented with 584 nM of FGF ligand (R&D Systems). Approximately 100 MPPs were plated in duplicate in 35 mm petri dishes. After 8 days in culture, individual colonies were counted, subjected to cytoxin, and analyzed for the presence of erythroid and/or myeloid colonies by Wright staining.

**REFERENCES**

Regulation of B versus Myeloid Cell Fates


