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Multilineage Transcriptional Priming and Determination of Alternate Hematopoietic Cell Fates

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SUMMARY

Hematopoietic stem cells and their progenitors exhibit multilineage patterns of gene expression. Molecular mechanisms underlying the generation and refinement of these patterns during cell fate determination remain unexplored because of the absence of suitable experimental systems. Using *PU.1*$^{-/-}$ progenitors, we demonstrate that at subthreshold levels, this Ets transcription factor regulates a mixed pattern (macrophage/neutrophil) of gene expression within individual myeloid progenitors. Increased PU.1 levels refine the pattern and promote macrophage differentiation by modulating a novel regulatory circuit comprised of counter antagonistic repressors, Egr-1,2/Nab-2 and Gfi-1. Egr-1 and Egr-2 function redundantly to activate macrophage genes and to repress the neutrophil program. These results are used to assemble and mathematically model a gene regulatory network that exhibits both graded and bistable behaviors and accounts for the onset and resolution of mixed lineage patterns during cell fate determination.

INTRODUCTION

Hematopoietic multipotential progenitors exhibit low levels of multilineage gene expression patterns (Hu et al., 1997; Miyamoto et al., 2002). This phenomenon has been termed transcriptional priming and it appears to reflect, at a molecular level, the developmental potency of a multilineage progenitor. These observations suggest that hematopoietic cell fate specification depends on induction as well as repression of subsets of lineage-specific genes. Regulatory mechanisms that initiate and resolve mixed lineage gene expression patterns during cell fate specification remain unexplored because of a lack of suitable experimentally manipulable cellular systems.

Antagonistic interplay between primary lineage determining factors has been proposed as a molecular mechanism for initiating and resolving mixed lineage states (Orkin, 2000). This widely accepted model is based on studies which have demonstrated that transcription factors such as GATA-1 and PU.1 physically interact and can functionally antagonize one another in transactivation and differentiation assays (Rekhtman et al., 1999; Zhang et al., 1999). Although this is an attractive regulatory model, its key features remain to be rigorously tested in a suitable experimental system that dynamically primes and resolves alternate lineage expression patterns during cell fate determination. Furthermore, the model does not account for specification of cell fates that involve cooperative rather than antagonistic interplay between primary lineage-determining transcription factors; although GATA-2 and PU.1 have also been shown to mutually antagonize one another, the development of mast cell precursors requires their cooperative interplay (Walsh et al., 2002).

Macrophage and neutrophil cell fate specification require the transcription factors PU.1 and C/EBPα, respectively (Scott et al., 1994; Zhang et al., 1997). The relative levels of PU.1 and C/EBPα in granulocytic-macrophage progenitors have been suggested to regulate macrophage versus neutrophil cell fate choice (Dahl et al., 2003), although the underlying molecular mechanism remains obscure. Paradoxically, both transcription factors are highly expressed in macrophages and neutrophils and synergistically regulate transcription of genes that are active in one or the other cell type (Smith et al., 1996; Zhang et al., 1996). This regulatory feature is not taken into consideration by the aforementioned regulatory model.

We establish a cellular system to analyze mixed lineage transcriptional priming and its resolution in the context of macrophage versus neutrophil cell fate determination. The analysis uncovers a novel regulatory circuit comprised of counter-acting (mutual) repressors. Mathematical modeling of the assembled gene regulatory network shows that such a network architecture can exhibit both graded and bistable (switch-like) behaviors. The model accounts for both the onset and resolution of mixed lineage patterns during cell fate determination. In particular, the model shows how upregulation of a common pair of primary lineage determinants can contribute to specification and commitment from a cell state in which the potential for two distinct fates coexists.

RESULTS

Subthreshold Levels of PU.1 Activate a Mixed Lineage Pattern of Gene Expression

PU.1$^{-/-}$ myeloid progenitors can be expanded in culture using the cytokine IL-3 and established as cell lines (Walsh et al., 2002). These cells can be induced to differentiate into macrophages or neutrophils by re-expression of PU.1. Cell fate specification in this system is dictated by the relative levels of PU.1 and C/EBPα at the onset of differentiation (Dahl et al., 2003; Walsh et al., 2002). The *PU.1* gene is expressed at low levels in common myeloid progenitors (CMP) and its expression is induced in vivo and in vitro as these cells differentiate into macrophages and neutrophils (Nutt et al., 2005; see Figure S1 in the Supplemental Data). To examine the consequences of differing levels of PU.1 on the differentiation fate of *PU.1*$^{-/-}$ myeloid progenitors, we made use of mutant clones expressing varied physiological levels of a conditionally active PU.1 protein fused to the ligand binding domain of the estrogen receptor (PUER). The PUER^{hi} cells express approximately 5-fold higher levels of the PUER transcript (Figure S1) and the fusion protein (Figure 1A) compared with PUER^{lo} counterparts. The levels of PU.1 RNA in PUER^{lo} and PUER^{hi} cells are physiologically appropriate as they are comparable to those observed in CMPs and macrophages, respectively (Figure S1).

In the absence of 4-hydroxy-tamoxifen (OHT), both PUER^{lo} and PUER^{hi} cells retain myeloid progenitor morphology (Figure 1A) and are c-kit^{hi}Gr-1^{lo}F4/80^{-} (Figure S2). Upon OHT addition, the PUER^{hi} cells undergo rapid growth arrest and differentiate within 4 to 7 days into adherent c-kit^{-}CD11b^{hi}F4/80^{+} macrophages (Figure 1A, Figure S2). In contrast, the PUER^{lo} cells do not undergo discernable morphological differentiation with OHT addition (Figure 1A, Figure S2). PUER^{lo} and PUER^{hi} cells were analyzed for the expression of macrophage- or neutrophil-specific genes (Figure 1B). Strikingly, the induction of PU.1 activity in PUER^{lo} cells appears to activate a mixed (macrophage/neutrophil) pattern of gene expression within 24 hr. This pattern is sustained over the course of 4 to 7 days and is not accompanied by overt differentiation (Figures 1A and 1B). Induction of PU.1 activity in PUER^{hi} cells also seemingly activates a mixed lineage pattern of gene expression within 24 hr (Figure 1B). However, this pattern, with the exception of G-CSFR, is resolved into a macrophage-specific subset. As noted above, the PUER^{hi} cells undergo overt differentiation into macrophages within 4 to 7 days.

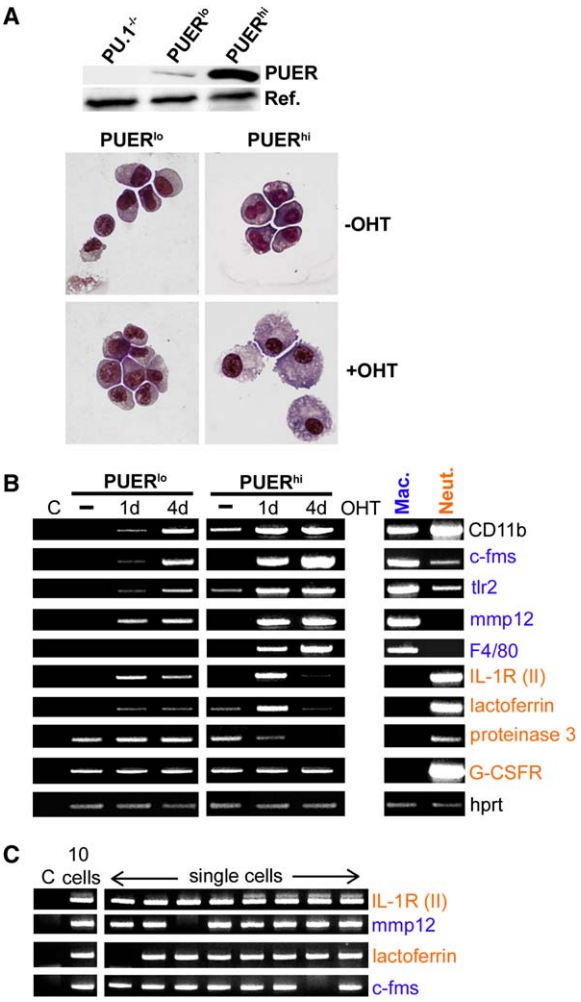

Figure 1. PU.1 Transiently Activates a Mixed Lineage Pattern of Gene Expression in Myeloid Progenitors

(A) Immunoblot analysis of IL-3 cultured *PU.1*$^{-/-}$ cell clones expressing different levels of a PU.1 fusion protein containing the ligand binding domain of the estrogen receptor (upper panel). Protein levels were normalized using a cross-reactive band. PUER activity was induced by the addition of 100 nM OHT. Cellular morphology was examined by Wright staining after 7 days (lower panel).

(B) Myeloid gene expression patterns in PUER^{lo} and PUER^{hi} cells induced with OHT for 1 and 4 days. Transcripts for the indicated myeloid genes were analyzed by RT-PCR after normalization with hprt. Total RNA from purified macrophages (Mac.) and neutrophils (Neut.) were used as controls.

(C) Single-cell multiplex RT-PCR analysis of OHT treated (1 day) PUER^{hi} cells.

To test if PU.1 activates a mixed lineage pattern of gene expression within individual cells, we treated PUER^{hi} cells for 24 hr with OHT and performed single-cell multiplex

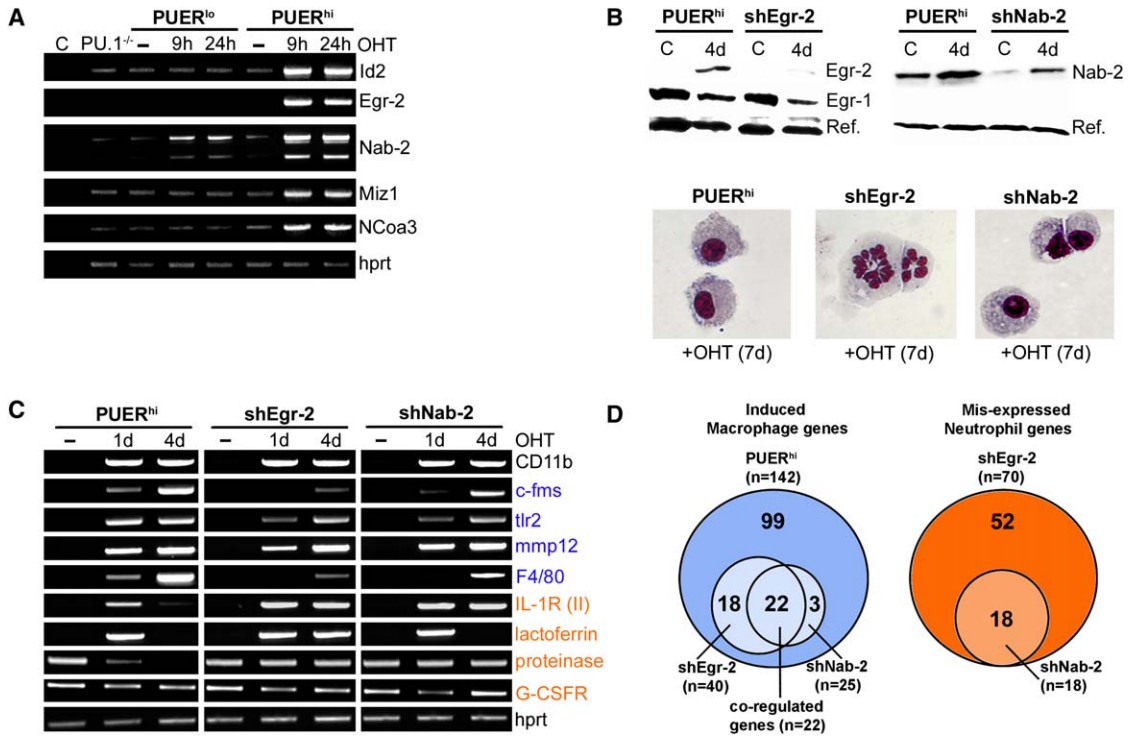

Figure 2. High Levels of PU.1 Induce Egr-2 and Nab-2, which Repress Neutrophil Genes

(A) Expression of PU.1-regulated transcription factors in PUER^{lo} and PUER^{hi} cells. PU.1 activity was induced with OHT for the indicated times.

(B) Immunoblot analysis of PUER^{hi} clones stably expressing either a shEgr-2 RNA or shNab-2 RNA (upper panel). The ability of shEgr-2 and shNab-2 cells to differentiate into macrophages was analyzed by Wright staining (lower panel).

(C) Expression of various macrophage or neutrophil transcripts was analyzed by RT-PCR.

(D) Genome-wide expression analysis using Affymetrix gene chips. Venn diagrams of macrophage (left panel) and neutrophil (right panel) genes expressed in PUER^{hi}, shEgr-2, or shNab-2 cells after induction of PU.1 activity (OHT, 4 days). Of the 142 macrophage genes induced by PU.1, the expression of 40 is impaired by knockdown of Egr-2, and the expression of 25 is impaired by reduction of Nab-2 levels. Seventy neutrophil-specific genes are mis-expressed in shEgr-2 cells. Eighteen of these genes are also mis-expressed in shNab-2 cells.

RT-PCR (Figure 1C). Of 21 single cells that were analyzed, all coexpressed at least one macrophage- and neutrophil-specific gene. These results reveal that the PUER cells represent an experimentally manipulable model system for analyzing regulatory mechanisms that underlie transcriptional priming and resolution during cell fate determination. Furthermore, the experiments demonstrate that subthreshold levels of PU.1 activate a mixed lineage pattern of gene expression within individual myeloid progenitors. An increase in PU.1 activity beyond the threshold induces resolution of the mixed lineage pattern and is accompanied by overt differentiation into macrophages.

PU.1 Induces Egr-2 and Nab-2, which Repress Neutrophil Genes during Macrophage Differentiation

The repression of neutrophil genes during macrophage differentiation could be directly mediated by higher levels of PU.1 or indirectly mediated via the induction of a negative regulator(s). We explored these possibilities by performing genome-wide expression analysis. Among the large set of genes whose expression was induced by PU.1, ten encoded regulatory factors (data not shown). The expression of five of these regulatory genes (*Id2*, *Egr-2*, *Nab-2*, *Miz1*, and *NCoA3*) was strongly induced in PUER^{hi}, but not PUER^{lo}, cells (Figure 2A).

Egr-2 (*Krox-20*) and Nab-2 were of interest since the related zinc finger family transcription factor Egr-1 (*Krox-24*) has been implicated in regulating macrophage differentiation (Nguyen et al., 1993). Furthermore, Egr-1 and Egr-2 interact with the corepressor Nab-2, which can inhibit the ability of Egr proteins to activate transcription (Svaren et al., 1996). These results suggested that PU.1-induced Egr/Nab complexes could function in the repression of neutrophil-specific genes. To test this, we employed a shRNA-based approach. PUER^{hi} cells expressing shRNA directed against Egr-2 (shEgr-2) showed a reduction (approximately 5-fold) in the accumulation of Egr-2 protein upon activation of PUER (Figure 2B). We note that PUER^{hi} cells express Egr-1 and that its expression is downregulated by inducing high PU.1 activity. Importantly, the Egr-2 shRNA did not perturb the levels of Egr-1 protein.

Induction of PU.1 activity in the shEgr-2 cells resulted in a striking morphological alteration (Figure 2B). The cells increased in size with an extensive cytoplasm resembling

that of a macrophage but contained segmented nuclei with numerous lobes, a feature characteristic of neutrophils. Additionally, these cells did not adhere and failed to express the F4/80 antigen (Figure S2). The shEgr-2 cells not only induced but also sustained a mixed pattern of gene expression, with high levels of certain macrophage (*mmp12*) and neutrophil (*IL-1R (II), lactoferrin, proteinase 3*) RNAs (Figure 2C). These results demonstrate that PU.1 initially activates a mixed lineage pattern of gene expression in myeloid progenitors, then utilizes Egr-2 to resolve this pattern into a macrophage-specific output.

To determine if Nab-2 functions in concert with Egr-2, we stably expressed a shNab-2 construct. This construct resulted in a 3-fold reduction of the basal and PU.1-induced levels of the Nab-2 protein (Figure 2B). Attenuation of Nab-2 levels did not appreciably affect the PU.1-dependent morphological differentiation (Figure 2B). However, the knockdown of Nab-2 protein resulted in both a marked reduction of F4/80^{+} cells (Figure S2) and, importantly, a failure to downregulate the neutrophil genes *IL-1R (II)* and *proteinase 3* (Figure 2C).

To examine the subsets of macrophage and neutrophil genes that are coregulated by Egr-2 and Nab-2, we performed genome-wide expression analysis with the shEgr-2 and shNab-2 cells. The induction of PU.1 in the PUER^{hi} cells resulted in the expression of 142 macrophage-specific genes (Figure 2D). Expression of 22 of these genes was found to be dependent on both Egr-2 and Nab-2 (see Table S1 in the Supplemental Data). Conversely, attenuation of Egr-2 resulted in the mis-expression of 70 neutrophil genes, 18 of which were also improperly expressed as a consequence of reduction of Nab-2 (Figure 2D, Table S2). The finding that Egr-2 mediates repression of a larger set of neutrophil genes than Nab-2 may be due to the fact that Egr-2 is capable of interacting with a related corepressor, Nab-1 (Russo et al., 1995). Nevertheless, our results demonstrate that Egr-2 and Nab-2 function in concert to repress a subset of 18 neutrophil-specific genes.

We note that both Egr-2 and Nab-2 reinforce macrophage gene activity while antagonizing neutrophil-specific gene expression. Egr-2 can function as a positive regulator of transcription and therefore likely upregulates the expression of a large subset of macrophage genes (Figure 2D). The molecular function of Nab-2 in promoting macrophage differentiation is analyzed below.

Egr-2/Nab-2 and Gfi-1 Represent Counter Antagonistic Repressors

The phenotypes of the shEgr-2 and shNab-2 cells paralleled those of myeloid cells lacking the zinc finger transcription factor, Gfi-1. In *Gfi-1*$^{-/-}$ mice, neutrophil development is impaired and the mutant granulocytic cells mis-express the macrophage genes, *c-fms* and *Mac-3* (Hock et al., 2003). Collectively, these analyses led us to consider that Egr-1,2/Nab-2 and Gfi-1 represent lineage-specific counter-acting repressors. Consistent with this possibility, *Egr-1,2/Nab-2* and *Gfi-1* exhibited a reciprocal pattern of expression in macrophages and neutrophils (Figure S3). We next analyzed the dynamics of *Egr-2/Nab-2* and *Gfi-1* expression using PUER^{hi} cells, under conditions that favor the generation of macrophages (IL-3) or neutrophils (G-CSF) (Dahl et al., 2003). Macrophage differentiation was accompanied by the induction of *Egr-2* and *Nab-2* and the concomitant downregulation of *Gfi-1* (Figure 3A). Conversely, differentiation of PUER^{hi} cells into neutrophils resulted in sustained expression of *Gfi-1* and a failure to significantly upregulate *Egr-2* or *Nab-2* in spite of induction of high PU.1 activity.

Impairing Egr-2 or Nab-2 induction resulted in sustained expression of *Gfi-1*, demonstrating that Egr-2 and Nab-2 negatively regulate *Gfi-1* expression (Figure 3B). We suggest that the macrophage differentiation defect observed in the shNab-2 cells (i.e., the failure to induce 22 macrophage genes, Figure 2D) is due to sustained expression of Gfi-1, which likely represses these genes. Interestingly, loss of Egr-2 did not affect the induction of *Nab-2* and vice versa, suggesting that both Egr-2 and Nab-2 are independently regulated by PU.1 during macrophage differentiation (Figure 3B).

Based on the above analysis, we predicted that Egr-1,2/Nab-2 should bind the Gfi-1 gene to directly mediate its repression. By DNA sequence analysis, we identified several putative Egr binding sites in the promoter region of the Gfi-1 gene. One of these sites, positioned 176 bp upstream of the transcription start site, is bound with high affinity by Egr-2 (Figure S4) as well as Egr-1 (data not shown). It has not been possible to detect the assembly of Egr-1,2/Nab cocomplexes on DNA by gel electrophoresis (Svaren et al., 1996). To determine if Egr-2 and Nab-2 associate with the *Gfi-1* promoter, we carried out chromatin cross-linking and immunoprecipitation assays. Induction of Egr-2 and Nab-2 by PU.1 resulted in their cross-linking to the *Gfi-1* promoter region containing the Egr binding site (Figure 3B). Importantly, the *Gfi-1* promoter was repressed via the Egr site by coexpression of Egr-2 and Nab-2 (Figure 3C). Thus, Egr-2 and Nab-2 directly repress the *Gfi-1* gene.

To test if Gfi-1 can counter antagonize the expression of Egr-1,2/Nab-2, we utilized PUER^{hi} cells expressing elevated levels of Gfi-1 (Figure 3B, right panel). Sustained expression of Gfi-1 inhibits PU.1-induced macrophage differentiation (R. Dahl, personal communication and data not shown). Importantly, overexpression of Gfi-1 in these cells resulted in the attenuation of both *Egr-1* and *Egr-2* expression, but not *Nab-2*. To determine if Gfi-1 repression of *Egr-1* and *Egr-2* can occur in the absence of PU.1 and other myeloid regulatory factors, we expressed Gfi-1 in NIH3T3 fibroblasts. Egr-1,2/Nab-2 are induced in fibroblasts upon serum starvation and restimulation (Svaren et al., 1996). Gfi-1 inhibited the growth factor-induced expression of both *Egr-1* and *Egr-2* (Figure 3B, right panel). As was the case with PUER^{hi} cells, *Nab-2* expression was not affected by Gfi-1. The *Egr-2* promoter region (−650 to +150) contains multiple putative Gfi-1 binding sites (data not shown). Importantly, Gfi-1 could be shown

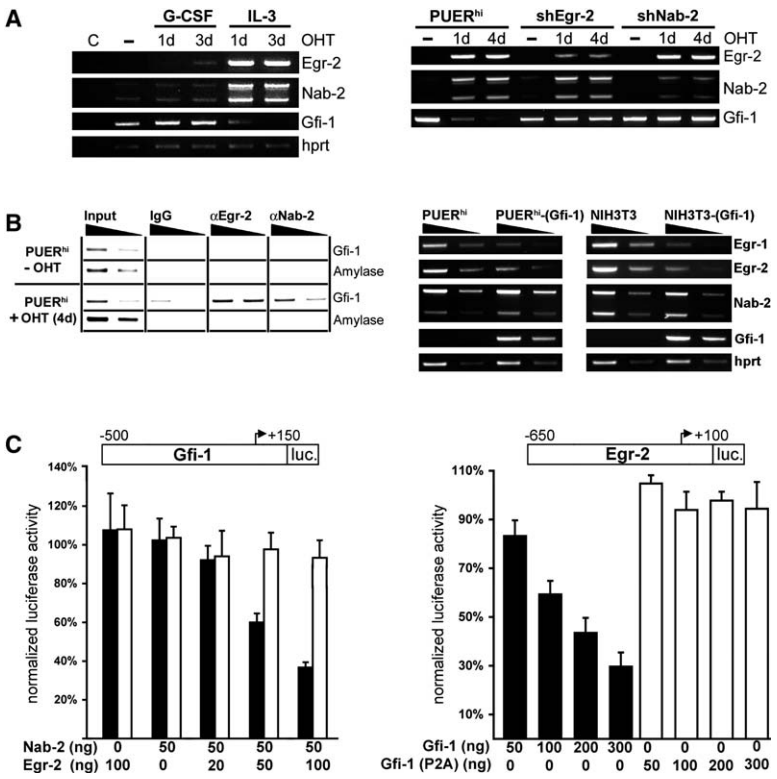

Figure 3. Egr-2/Nab-2 and Gfi-1 Represent Counter Antagonistic Repressors

(A) Expression of Egr-2, Nab-2, and Gfi-1 in PUER^{hi} cells after differentiation under conditions that favor generation of macrophages (IL-3) or neutrophils (G-CSF) (left panel). Expression of Egr-2, Nab-2, and Gfi-1 in PUER^{hi} cells upon attenuation of Egr-2 or Nab-2 (right panel).

(B) ChIP analysis of Egr-2 and Nab-2 binding to *Gfi-1* promoter in OHT-treated PUER^{hi} cells (left panel). Amylase gene primers were used as a control. RT-PCR analysis (1:5 cDNA dilution) of Egr-1, Egr-2, Nab-2, and Gfi-1 transcripts in the indicated cells (right panel). PUER^{hi}-Gfi-1 and NIH3T3-Gfi-1 cells were generated by retroviral transfection using a Gfi-1 vector. PUER cells were induced with OHT for 48 hr before RNA isolation. NIH3T3 cells were serum starved overnight and then stimulated with 20% FCS for 1.5 hr before RNA isolation.

(C) NIH3T3 cells were transiently transfected with luciferase reporter plasmids containing the Gfi-1 promoter (left panel, shaded bars) or the Egr-2 promoter (right panel). Open bars in left panel represent Gfi-1 promoter with mutation in the Egr binding site. Promoter activity was measured in response to levels of indicated expression plasmids. Gfi-1 (P2A) is a repression-defective mutant protein. Data is from three independent experiments. Error bars represent mean ± SEM.

to repress the Egr-2 promoter (Figure 3C, right panel). Repression by Gfi-1 required a functional SNAG domain as it was not observed with the P2A mutant (Grimes et al., 1996). Although the Gfi-1 binding site(s) required for Egr-2 repression remain(s) to be delineated, our results establish that Gfi-1 can counter-repress the *Egr-1* and *Egr-2* genes in two different cellular contexts.

Functional Redundancy of *Egr-1* and *Egr-2* in Macrophage Differentiation

Egr-2$^{-/-}$ mice exhibit perinatal lethality (Swiatek and Gridley, 1993), but the development of macrophages in *Egr-2*$^{-/-}$ embryos has not been examined. We analyzed the generation of myeloid cells in the fetal liver of E14.5 embryos. Loss of *Egr-2* did not impair the production of CD11b^{+}Gr-1^{+} or CD11b^{+}Gr-1^{-} myeloid cells (data not shown). Furthermore, *Egr-2*$^{-/-}$ Lin^{-} hematopoietic progenitors could be induced to differentiate into macrophages by culturing in M-CSF (Figure 4A).

Since macrophages express Egr-1, which is also not essential for macrophage differentiation (Lee et al., 1996), we reasoned that the two regulators may functionally substitute for one another. *Egr-2*$^{-/-}$ macrophages express wild-type levels of Egr-1, but not detectable levels of Egr-3 and Egr-4 (Figure 4A). Furthermore, in the *PU.1*$^{-/-}$ progenitors, induction of PUER results in the expression of *Egr-2* and downregulation of *Egr-1* (Figure 4A), thereby likely accounting for our ability to reveal a nonredundant function for *Egr-2* in macrophage differentiation in these cells. We directly tested the redundant functions of Egr-1,2 by using the *PU.1*$^{-/-}$ cell system. Initially, we complemented shEgr-2 cells by expressing human Egr-2 (Figure 4B). This resulted in the restoration of PU.1-dependent macrophage differentiation comparable to that of control cells based on morphology, F4/80 expression, and the downregulation of neutrophil genes. Importantly, increased expression of murine Egr-1 in shEgr-2 cells also complemented the mutant phenotype (Figure 3B). We note that neither Egr-1 nor Egr-2 promoted macrophage differentiation in the absence of PU.1 induction (data not shown). Thus, *Egr-1* and *Egr-2* function redundantly in promoting macrophage differentiation and repressing neutrophil genes.

To test the redundant role of Egr-1,2 in primary hematopoietic progenitors, we employed a dominant-negative Egr protein. This protein (δEgr-2) represents the zinc finger DNA binding domain (amino acids 330–470) of murine Egr-2 and lacks both trans-activation and Nab interaction domains. We generated PUER^{hi} cells stably expressing the δEgr-2 construct. Induction of PU.1 activity in these cells resulted in a significant loss of F4/80^{+} cells, and importantly, mis-expression of *Gfi-1* (Figure 4C). These phenotypes paralleled those of the shEgr-2 cells, thereby validating the utility of the δEgr-2 protein. Fetal liver Lin^{-} hematopoietic progenitors from *Egr-1*$^{-/-}$ mice can differentiate into macrophages as a result of *Egr-2* expression. Expression of δEgr-2 in these progenitors impaired their

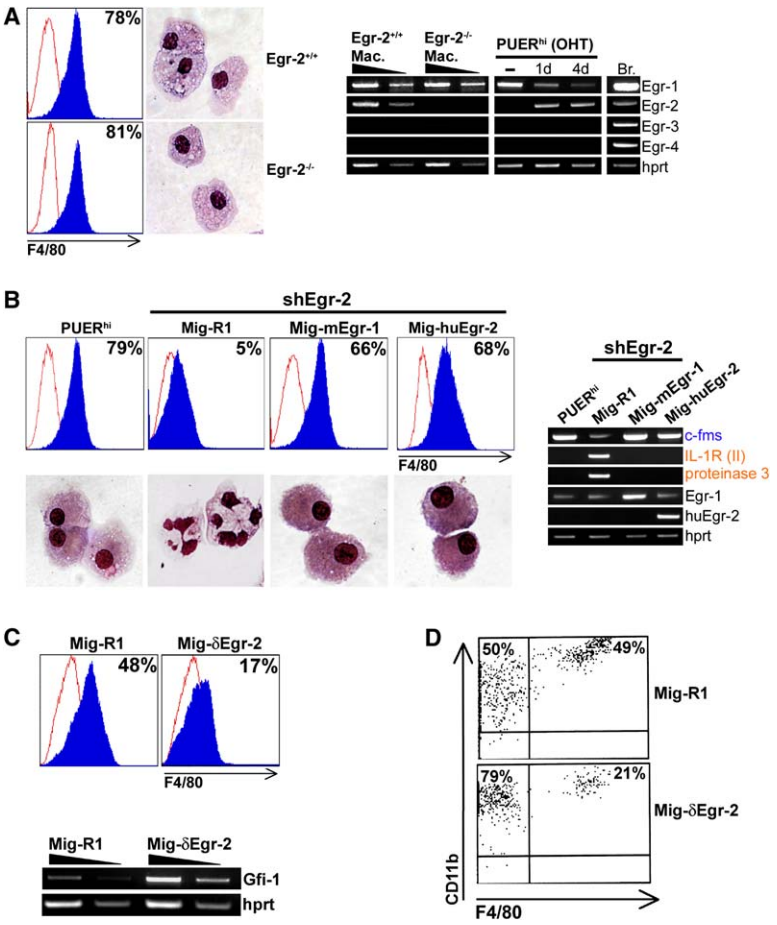

Figure 4. Functional Redundancy of Egr-1 and Egr-2 in Macrophage Differentiation

(A) Fetal liver (FL) hematopoietic Lin$^-$ progenitors (E14.5) were isolated from *Egr-2*$^{+/+}$ and *Egr-2*$^{-/-}$ embryos and cultured for 4 days in the presence of M-CSF. Macrophage differentiation was analyzed by F4/80 expression and Wright staining (left panel). RT-PCR analysis of the Egr family members in macrophages derived from M-CSF-cultured Lin$^-$ progenitors (1:5 cDNA dilution) and PUER^{hi} cells induced with OHT for 1 and 4 days (right panel). Total RNA from mouse embryonic brain was used as control.

(B) shEgr-2 cells were complemented with murine Egr-1 or human Egr-2 vectors. Mig-R1 is the control vector. Macrophage differentiation was examined after 7 days by F4/80 expression and Wright staining (left panel). RT-PCR analysis of myeloid transcripts in the indicated cells after PU.1-dependent differentiation (right panel).

(C) PUER^{hi} cells were stably transduced with dominant Egr-2 mutant (Mig-δEgr-2) or control Mig-R1 vectors. Macrophage differentiation was examined after 24 hr by F4/80 expression (upper panel). RT-PCR analysis (1:5 cDNA dilution) of Gfi-1 transcripts in the indicated cells after PU.1-dependent differentiation (lower panel).

(D) FL Lin$^-$ progenitors (E14.5) were isolated from *Egr-1*$^{-/-}$ mice and infected with Mig-δEgr-2 or Mig-R1 vectors. Cells were cultured for 3 days in presence of M-CSF. Myeloid and macrophage differentiation were analyzed by CD11b and F4/80 expression, respectively.

M-CSF-dependent differentiation into CD11b$^+$F4/80$^+$ macrophages (Figure 4D). These results strongly suggest that Egr-1 and Egr-2 function redundantly to regulate the differentiation of primary hematopoietic progenitors into macrophages.

Egr-1$^{-/-}$*Egr-2*$^{+/-}$ Hematopoietic Progenitors Are Defective in Undergoing M-CSF-Dependent Macrophage Differentiation

Loss of *Egr-1* and *Egr-2* results in early embryonic lethality (Grose et al., 2002). Therefore, to genetically test the redundant functions of *Egr-1* and *Egr-2* in regulating macrophage development, we made use of *Egr-1*$^{-/-}$*Egr-2*$^{+/-}$ compound mutant mice. These mutant mice appeared healthy yet displayed severe growth retardation when compared to their wild-type, *Egr-1*$^{-/-}$, or *Egr-2*$^{+/-}$ littermates (Figure 5A, data not shown). This size difference correlated with a decrease (control 21.41 ± 2.82 g; *Egr-1*$^{-/-}$ *Egr-2*$^{+/-}$ 15.08 ± 2.14 g; n = 5) in total body weight. There were no notable abnormalities in the hematopoietic system in 5-week-old compound mutant mice in relation to their *Egr-1*$^{-/-}$ counterparts as determined by peripheral blood and bone marrow differentials (data not shown).

We analyzed hematopoietic progenitors from these mice for their ability to undergo M-CSF-dependent differentiation. The compound mutant progenitors gave rise to fewer CFU-M colonies in methylcellulose assays, and the majority of these colonies were smaller in size than those of their *Egr-1*$^{-/-}$ control counterparts (Figure 5B). In contrast, macrophage differentiation in response to GM-CSF was unimpaired. When plated in liquid culture in M-CSF, the *Egr-1*$^{-/-}$*Egr-2*$^{+/-}$ progenitors gave rise to substantially fewer (6-fold) F4/80$^+$ macrophages (Figure 5C). Importantly, the CD11b$^+$Gr-1-F4/80$^-$ macrophage precursors generated from the compound mutant progenitors expressed higher levels of Gfi-1 (3-fold) and lower levels of PU.1 (3-fold) transcripts (Figure 5D). Thus, *Egr-1*$^{-/-}$*Egr-2*$^{+/-}$ hematopoietic progenitors are defective in undergoing M-CSF-dependent macrophage differentiation and fail to properly repress the *Gfi-1* gene. In vivo, homeostatic mechanisms, e.g. GM-CSF signaling, may enable restoration of the macrophage compartment. Nevertheless, these results provide genetic support for the redundant roles of Egr-1 and Egr-2 in promoting macrophage differentiation and repressing the *Gfi-1* gene.

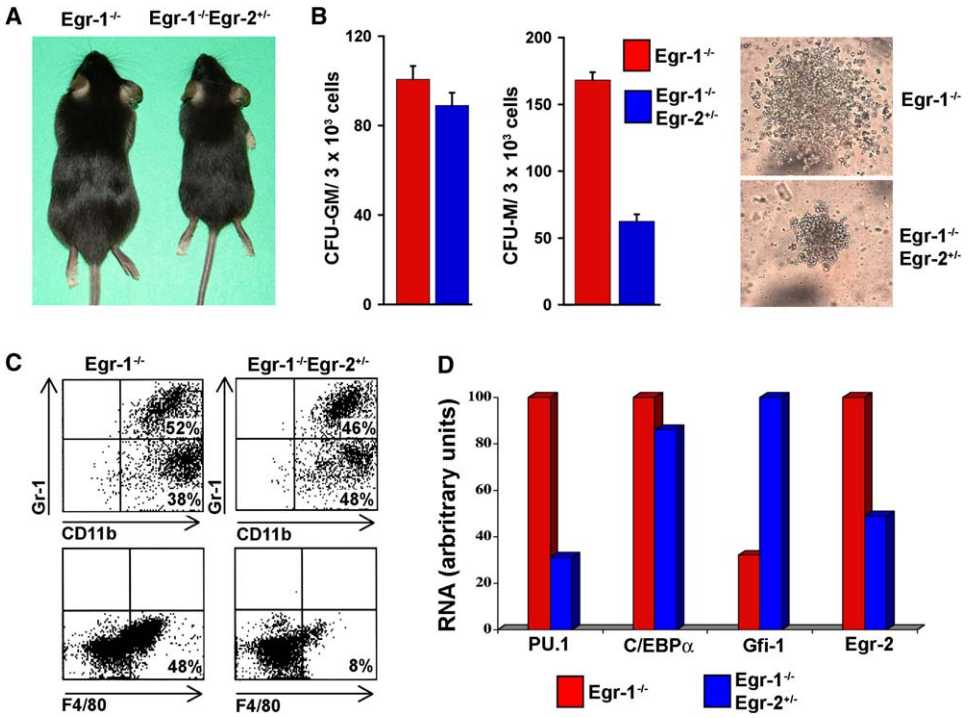

Figure 5. *Egr-1*$^{-/-}$*Egr-2*$^{+/-}$ Hematopoietic Progenitors Are Defective in Undergoing M-CSF-Dependent Macrophage Development

(A) 5-week-old *Egr-1*$^{-/-}$ and *Egr-1*$^{-/-}$*Egr-2*$^{+/-}$ littermates.

(B) Bone marrow (BM) hematopoietic Lin^{-} progenitors were isolated from *Egr-1*$^{-/-}$ and *Egr-1*$^{-/-}$*Egr-2*$^{+/-}$ mice and cultured in methylcellulose in GM-CSF or M-CSF. Colony numbers were scored after 9 days. Phase contrast images of representative CFU-M colonies (right panel). Data is from two independent experiments. Error bars represent mean ± SEM.

(C) BM Lin^{-} progenitors were isolated from *Egr-1*$^{-/-}$ and *Egr-1*$^{-/-}$*Egr-2*$^{+/-}$ mice and cultured for 3 days in M-CSF. Myeloid and macrophage differentiation were analyzed by Gr-1/CD11b and F4/80 expression, respectively.

(D) Macrophage precursors (CD11b^{+}Gr-1^{-}F4/80^{-}) were FACS purified from 3 day M-CSF-cultured progenitors (Figure 5C). PU.1, C/EBPα, Gfi-1, and Egr-2 transcripts were quantitated by Q-PCR.

Cross-Antagonism Yields Both Graded and Bistable Behaviors

To analyze how the cross-antagonism between Egr-1,2/Nab-2 and Gfi-1 initiates and resolves a mixed lineage pattern of gene expression, we assembled a minimal regulatory network for the macrophage-neutrophil developmental program based on our results and earlier findings (Dahl et al., 2003; Hock et al., 2003; Smith et al., 1996; Zhang et al., 1996, 1997). As shown in Figure 6A, there are two primary cell fate determinants, PU.1 and C/EBPα. PU.1 upregulates the secondary cell fate determinants Egr-2 and Nab-2 (Figure 2A). We note that Egr-1 is also induced during macrophage differentiation (Figure S3A). Egr-1 and Egr-2 are treated as a single species (denoted Egr) in the model due to the redundancy demonstrated above (Figures 4 and 5). We propose that C/EBPα regulates the secondary determinant, Gfi-1, based on the observation that pretreatment of PUER^{hi} cells with G-CSF results in increased expression of C/EBPα (Dahl et al., 2003) and Gfi-1 (Figure 3A). The primary and secondary determinants together regulate the expression of many downstream macrophage and neutrophil genes. In particular, both PU.1 and C/EBPα regulate each of these genes synergistically in the model (Supplemental Data). Negative feedback from Gfi-1 to PU.1 was included to account for the increased expression of PU.1 in *Gfi-1*$^{-/-}$ bone marrow (Hock et al., 2003) and the downregulation of PU.1 accompanied by increased expression of Gfi-1 in *Egr-1*$^{-/-}$*Egr-2*$^{+/-}$ macrophage precursors (Figure 5D). The genome-wide analysis (Figure 2D) suggests some macrophage and neutrophil genes are regulated autonomously of this circuit (Figure 6A), but there are insufficient data to include them in the model at present.

This network can be represented mathematically by a set of differential equations that are integrated forward in time (t) numerically (see Experimental Procedures and the Supplemental Data). Combinations of expression levels that do not change in time are called "fixed points." Stable fixed points are robust to perturbations. We show below that they correspond to the mixed lineage, macrophage, or neutrophil developmental states. Unstable fixed points are not readily observable in experiments but are important because they define the boundaries (in expression levels) between developmental states.

Consistent with the behavior observed for PUER^{lo} cells (Figure 1), a mixed lineage pattern of gene expression is

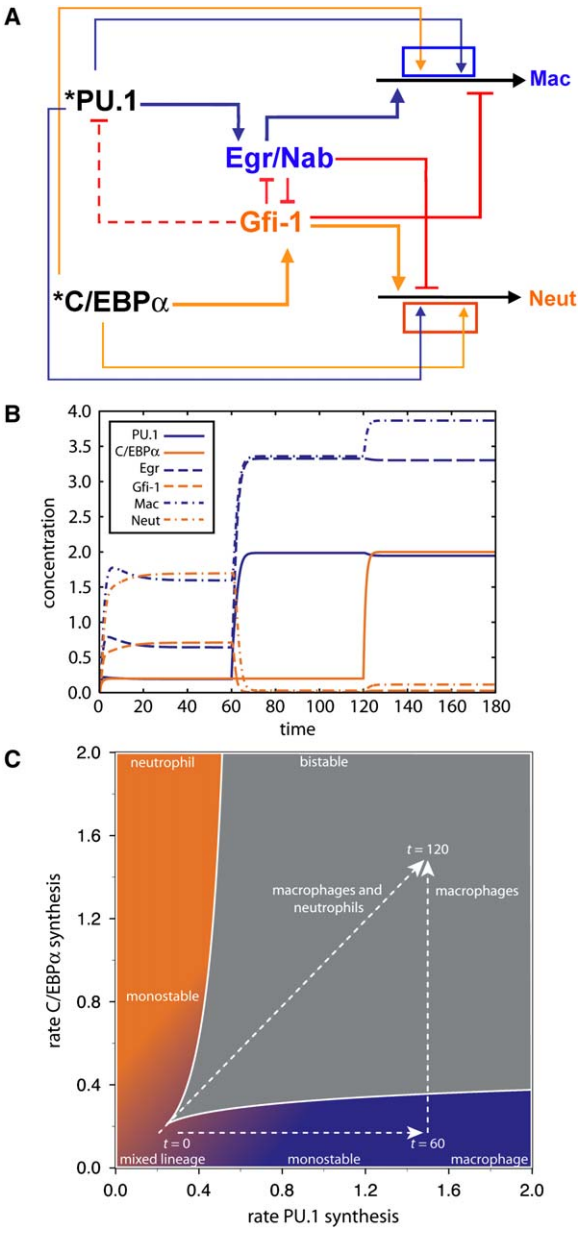

Figure 6. Mathematical Model of a Gene Regulatory Network Underlying Macrophage and Neutrophil Cell Fates

(A) The network depicts primary (PU.1 and C/EBPα) and secondary (Egr/Nab and Gfi-1) cell fate determinants and macrophage (Mac) or neutrophil (Neut) target genes. Arrows represent positive regulation and barred lines represent repression. In the model, PU.1 and C/EBPα are constitutively synthesized (*). Boxes represent synergistic activation of transcription. For simplicity, Egr-1 and Egr-2 are treated as a composite species labeled Egr.

(B) Simulations of enforced expression experiments. PU.1 is induced to high expression levels at $t = 60$, and C/EBPα is induced at $t = 120$.

(C) Diagram showing the boundary between the monostable and bistable regimes as a function of the rates of synthesis of PU.1 and C/EBPα. Perpendicular arrows correspond to the inductive events shown in (B). Diagonal arrow represents a coordinate increase in both PU.1 and C/EBPα synthesis, resulting in stochastic cell fate determination upon entering the bistable region.

observed when the rates of synthesis of both primary determinants are low ($0 \leq t < 60$ in Figure 6B). In this case, the cross-antagonism between Egr and Gfi-1 is weak. Because each of these factors cannot fully repress the other, imbalances in their expression levels cannot be maintained, and there is only a single possible long-time outcome (the system is "monostable").

Inducing PU.1 prior to C/EBPα, as in PUER^{hi} cells ($60 \leq t < 120$ in Figure 6B), shifts the pattern of gene expression to that of a macrophage. At $t = 120$, the rate of C/EBPα synthesis is also increased, but, due to the imbalance of the secondary determinants already established, the cell remains a macrophage. If instead C/EBPα is induced prior to PU.1, a pattern of gene expression that corresponds to a neutrophil results (Figure S5), which is in agreement with earlier work (Dahl et al., 2003). Because the cross-antagonism between Egr and Gfi-1 is strong when PU.1 and C/EBPα expression levels are high, one secondary determinant can fully repress the other. As a result, imbalances between Egr and Gfi-1 are self-reinforcing in this case, and mixed patterns of gene expression are unstable. The two possible long-time patterns of gene expression that are robust to perturbations correspond to the differentiated states (the system is "bistable").

The different behaviors illustrated in Figure 6B are placed in a broader context in Figure 6C, which shows the number of stable states of the network as a function of the rates of synthesis of the primary determinants. As indicated by the perpendicular arrows, induction of PU.1 at $t = 60$ promotes a macrophage-like fate, but cells remain in the monostable region of parameter space. Subsequently increasing the other factor (C/EBPα) moves cells into the bistable region, and the resulting switch-like behavior ensures that they remain committed to their differentiated state even when the primary factor that directs the alternate fate is induced. This behavior mirrors the experiments described above and corresponds to an instructive pathway for specification.

Along the diagonal arrow in Figure 6C, the levels of both the primary and secondary determinants are comparable when the mixed lineage transitions from stable to unstable at the boundary between the colored and gray regions (see Supplemental Data for a quantitative discussion based on an analytically tractable model). Small cell-to-cell variations in the relative amounts of the secondary determinants, Egr and Gfi-1, can then cause the population to partition into macrophages and neutrophils. Thus, the model encompasses both instructive as well as stochastic determination of cell fates.

DISCUSSION

Our analysis reveals that Egr-1 and Egr-2 function redundantly to regulate the macrophage cell fate. They do so not only by positively regulating the expression of macrophage specific genes, but also by repressing the alternate lineage neutrophil genes. Importantly, Egr-1,2 counteract an alternate regulator, Gfi-1, which is required for

neutrophil cell fate determination and repression of macrophage genes. This counter-acting circuit explains the generation of alternate cell fates (macrophage versus neutrophil) from a shared pair of lineage determinants (PU.1 and C/EBPα).

We note that, during hindbrain development, Egr-2 is required for generation of r3 and r5 rhombomeres (Swiatek and Gridley, 1993). As with macrophage development, dual regulatory functions for Egr-2 as a positive regulator of the r3/r5 gene expression program (Giudicelli et al., 2001) and as a repressor of the alternate r2/r4 program (Voiculescu et al., 2001) have been suggested. Previous reports have demonstrated roles for Gfi-1 in the context of cell fate determination of secretory intestinal cells (Shroyer et al., 2005) and inner hair cells (Wallis et al., 2003). These analyses lead us to propose that Egr-1,2 and Gfi-1, or the related family member Gfi-1b (Duan and Horwitz, 2003), may represent a conserved counter-regulatory switch that functions to resolve mixed lineage gene expression patterns and thereby regulate cell fate determination in a variety of developmental contexts. It should be noted that Egr-1,2 and Gfi-1 were discovered in the context of analyses focusing on control of cell growth and proliferation (Gilks et al., 1993; Sukhatme et al., 1988). Therefore, they may also function as a counter-regulatory switch controlling exit from quiescence (G_0) into a proliferative state. Consistent with this proposal, Gfi-1 has been implicated in regulating hematopoietic stem cell self-renewal by restricting proliferation (Hock et al., 2004).

Transcriptional priming of distinct cell fates within a multipotential progenitor likely reflects developmentally specific alterations of chromatin structure at lineage-specific loci. Such loci are poised for physiologically relevant levels of lineage-specific gene expression upon differentiation. We propose that primary cell fate determinants such as PU.1 and C/EBPα induce mixed lineage transcriptional priming by functioning as "pioneer transcription factors" to induce initial alterations of chromatin structure at lineage-specific target genes (Cirillo et al., 2002). Subsequently, secondary cell fate determinants (such as Egr-1,2) function in concert with their corresponding primary regulators (such as PU.1) to promote the physiological levels of transcription of the relevant subset of genes. We note that transcription of the macrophage *c-fms* gene is dependent on both PU.1 and Egr-1,2. Whereas PU.1 binds to the *c-fms* promoter, Egr-2 appears to regulate *c-fms* transcription by interacting with a downstream enhancer element (H. Krysinska and C. Bonifer, personal communication). Thus, concerted activation of transcription between primary and secondary determinants would enable physiological levels of c-fms expression during macrophage differentiation. How might Egr-1,2 repress the transcription of Gfi-1 and neutrophil genes? We propose that repression is dependent on corepressors such as Nab-2. Consistent with this idea, δEgr-2, which lacks the Nab-2 interaction domain, fails to repress *Gfi-1* (Figure 4C). Furthermore, Nab-2 is required along with Egr-2 to repress the *Gfi-1* promoter (Figure 3C). We posit that Nab-2 is stably associated with Egr-1,2 on repressed, but not activated, target genes and that such molecular discrimination is dictated by the context of the *cis*-regulatory elements within which Egr binding sites are embedded.

We note that our regulatory framework of primary and secondary cell fate determinants applies to several cell fate choices in the hematopoietic system. In particular, during B cell development, the transcription factors E2A and EBF appear to function as primary cell fate determinants (Busslinger, 2004; Singh et al., 2005). The transcription factor Pax-5 represents a secondary cell fate determinant analogous to Egr-1,2 and Gfi-1. Its expression is dependent on EBF and it functions to positively regulate B lineage genes and to repress myeloid genes. In the context of B lymphoid versus myeloid cell fate choice, Pax-5 activity appears to be counteracted by C/EBPα; the latter can reprogram B cells into macrophages, in part by antagonizing Pax-5 (Xie et al., 2004). In this regard, it is intriguing that C/EBPα reprograms B cells into macrophages rather than neutrophils. B cells express Egr-1,2, Nab-2, and low levels of PU.1 (data not shown). Addition of lymphoid determinants to our mathematical model predicts that C/EBPα is unable to induce Gfi-1, as the bistable switch discussed in the present study is thrown to the macrophage-promoting side by the presence of PU.1, Egr-1,2 and Nab-2 (A.W., H.S. and A.R.D., unpublished data). Finally, in the case of erythrocytic versus megakaryocytic cell fate choice, GATA-1 represents a shared primary determinant (Cantor and Orkin, 2002; Orkin et al., 1998), whereas EKLF and Fli-1 may function as a counter-acting circuit to stabilize one or the other cell fate (Starck et al., 2003).

Our model reveals general features of transcriptional networks that may regulate cell fate decisions in diverse cellular patterning events, including those associated with embryonic development (Stathopoulos and Levine, 2005). Low levels of a combination of lineage-determining transcription factors could be generally used to activate a mixed lineage pattern of gene expression. Such transcriptional priming would represent an obligatory transitional state, manifested by multipotential progenitors. Cross-antagonism among secondary cell fate determinants is likely to be a key feature of gene regulatory networks; it can be used to refine mixed lineage transcription patterns manifested by multipotential progenitors, thereby ensuring proper cell fate specification and stabilization. An outstanding question is how cells like natural killer T cells modulate the elements of their gene regulatory network to support stable mixed patterns of gene expression at physiologic levels (Godfrey et al., 2000).

It is well known that cross-antagonism can give rise to switch-like behavior (Angeli et al., 2004; Gardner et al., 2000; Ozbudak et al., 2004). That such a network architecture supports graded responses to stimuli as well is less appreciated (Ozbudak et al., 2004). Although both types of behaviors have been elicited synthetically by manipulation of biological circuits (Acar et al., 2005; Biggar and

Crabtree, 2001; Ozbudak et al., 2004), to the best of our knowledge, our study is the first to identify a regulatory network in which naturally arising changes in the number of stable states regulate patterns of gene expression.

The mathematical model resolves a seeming paradox concerning myeloid cell development. Counterintuitively, expression levels of PU.1 and C/EBPα increase when cells differentiate, and there are binding sites for both these factors on the downstream macrophage and neutrophil genes. Expressing both primary determinants at high levels disfavors mixed patterns of gene expression because the secondary cross-antagonism is sufficiently strong to make imbalances in Egr-1,2/Nab-2 and Gfi-1 self-reinforcing. The resulting bistability then serves to lock the cell into one of two well-defined differentiated states, both of which are robust to further changes in the primary determinants. The inducible switch-like nature of the cross-antagonism identified here is thus likely to play an important role in lineage commitment even in instructive situations, such as that considered in Figure 6B.

We note that the bistable state could also be exploited during the course of cell fate specification. We see in Figure 6C that coordinately increasing the synthesis of both primary cell fate determinants causes cells to transition into the bistable region with comparable levels of the secondary determinants. Amplification of imbalances in Egr-1,2/Nab-2 and Gfi-1 through the self-reinforcing mechanism described above could then allow cell-to-cell variation to determine cell fates. This stochastic pathway obviates the need for generating separate instructive cues in the same compartment. In the hematopoietic system there is evidence for this model, particularly with regard to the development of erythroid and myeloid cell types (Enver et al., 1998; Metcalf, 1998).

EXPERIMENTAL PROCEDURES

Isolation and Culture of Cells

Isolation of primary cells is detailed in the Supplemental Data. For macrophage development, hematopoietic progenitors were cultured in IMDM with 10 ng/ml M-CSF for 4 days. For clonogenic assays, hematopoietic progenitors were cultured in Methocult 3424 supplemented with 1 ng/ml GM-CSF or 10 ng/ml M-CSF (DeKoter et al., 1998). For experiments involving serum starvation, NIH3T3 cells were plated in media containing 0.15% FCS overnight and subsequently restimulated with 20% FCS for 1.5 hr.

Establishment and Analysis of IL-3-Dependent *PU.1*$^{-/-}$ Cell Lines

Generation of PUER^{hi} cells has been described previously (Walsh et al., 2002). The PUER^{lo} cells were generated by infection of a *PU.1*$^{-/-}$ IL-3-dependent line with a bicistronic retrovirus encoding PUER and GFP. After infection, cells were FACS sorted for low levels of GFP expression and subsequently cloned by limiting dilution. *PU.1*$^{-/-}$ cell lines were cultured in IL-3 (5 ng/ml) except where noted for G-CSF (10 ng/ml). Differentiation of PUER cells was induced by culture in the presence of OHT (100 nM) and analyzed by cytochemical staining and flow cytometry (Dahl et al., 2003; Walsh et al., 2002). Total RNA was isolated using RNAzol (Tel-Test, Inc.) and expression of various genes was analyzed by RT-PCR or Q-PCR after normalization with hprt. Sequences of PCR primers are available upon request. Immunoblots were performed as previously described (DeKoter et al., 1998) and details are available in the Supplemental Data.

Mice

Timed matings between *Egr* mutant mice (Lee et al., 1996; Swiatek and Gridley, 1993) were performed (*Egr-2*$^{+/-}$ × *Egr-2*$^{+/-}$ or *Egr-1*$^{-/-}$ × *Egr-1*$^{+/-}$) and embryos obtained at day 14.5 gestation. *Egr-1* or *Egr-2* mutant embryos were identified by genotyping. Primer sequences are available upon request. *Egr-1*$^{-/-}$*Egr-2*$^{+/-}$ compound mice were generated by *Egr-1*$^{-/-}$*Egr-2*$^{+/-}$ or *Egr-1*$^{+/-}$*Egr-2*$^{+/-}$ (male) × *Egr-1*$^{+/-}$*Egr-2*$^{+/-}$ (female) matings.

Single-Cell RT-PCR

Multiplex single-cell RT-PCR was performed as previously described (Hu et al., 1997). Details are available in the Supplemental Data.

Microarray Analysis

Biotin labeled cRNA was generated and hybridized to the Mouse Genome 430 2.0 Array according to manufacturer's instructions (Affymetrix). Details are available in Supplemental Data.

Construction and Use of Small Hairpin Interfering RNA Plasmids

Plasmids encoding shRNA oligonucleotides were constructed as described by Hannon et al. (http://katahdin.cshl.org/; Gregory Hannon Lab under the Labs option on the navigation bar). Details are available in Supplemental Data.

Complementation of shEgr-2 Cells

Mig-mEgr-1 and Mig-huEgr-2 were generated by cloning murine Egr-1 and human Egr-2 cDNA fragments into the Mig-R1 vector (DeKoter et al., 2002). Complementation of shEgr-2 cells was performed by electroporation using linear Mig-R1, Mig-mEgr-1, or Mig-huEgr-2 DNA. After 48 hr, GFP-expressing cells were isolated by FACS sorting. Cells were expanded for 7 to 10 days and subsequently differentiated in the presence of OHT (100 nM).

Chromatin Immunoprecipitation Assays

Chromatin cross-linking and immunoprecipitation assays were performed as previously described (DeKoter et al., 2002). Details are available in Supplemental Data.

Retroviral Transduction

Retroviral transfection of PUER^{hi} or NIH3T3 cells was performed by spin infection (Dahl et al., 2003) using Mig-Gfi-1 or Mig-δEgr-2 retroviral supernatants. After 2 days, GFP^{+} cells were sorted and propagated.

Transient Transfections and Reporter Assays

The proximal promoters of murine *Egr-2* (−650 to +100) and *Gfi-1* (−500 to +150) genes were cloned into the pGL3-basic vector (Promega). An Egr binding site was identified within the mouse *Gfi-1* promoter region (CAGTGGGCG; position −176) using TRANSFAC (http://www.gene-regulation.com/). Mutation of the Egr site (CAGTcGGCG) within the *Gfi-1* promoter was performed by site-directed mutagenesis. Reporter assays were performed using NIH3T3 cells as previously described (Russo et al., 1995). Details are available in the Supplemental Data.

Mathematical Modeling of Gene Regulatory Network

The model for gene expression and its analysis are similar in form to earlier such treatments (Acar et al., 2005; Elowitz and Leibler, 2000; Gardner et al., 2000; Ozbudak et al., 2004) and are described in the Supplemental Data.

Supplemental Data

The Supplemental Data for this article can be found online at http://www.cell.com/cgi/content/full/126/4/755/DC1/.

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