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Regulation of B Lymphocyte and Macrophage Development by Graded Expression of PU.1

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The ets family transcription factor PU.1 is required for the development of multiple lineages of the immune system. Using retroviral transduction of *PU.1* complementary DNA into mutant hematopoietic progenitors, we demonstrate that differing concentrations of the protein regulate the development of B lymphocytes as compared with macrophages. A low concentration of PU.1 protein induces the B cell fate, whereas a high concentration promotes macrophage differentiation and blocks B cell development. Conversely, a transcriptionally weakened mutant protein preferentially induces B cell generation. Our results suggest that graded expression of a transcription factor can be used to specify distinct cell fates in the hematopoietic system.

The ets family transcription factor PU.1 represents a unique transcriptional regulator within the hematopoietic system (*1*). It is required for the proper generation of both myeloid lineages (macrophages and neutrophils) and lymphoid lineages (B and T lymphocytes) (*2*). The blocks to B cell and macrophage development caused by the loss of *PU.1* function are more severe than defects in neutrophil (*3, 4*) and T cell (*5*) development. *PU.1* is essential for regulating the proliferation and differentiation of macrophage and B lineage progenitors (*3, 6*). PU.1 regulates the expansion of such progenitors by controlling the expression of the *c-fms* and *IL-7Rα* genes, which encode receptors for macrophage colony-stimulating factor (M-CSF) and interleukin 7 (IL-7), respectively (*3, 7*). Macrophage- and B lineage–specific gene expression programs are also severely affected by the *PU.1* mutation (*2, 3, 8*).

To analyze the function of PU.1 in B cell and macrophage development, we established a system for efficient retroviral transduction of murine fetal liver hematopoietic progenitors. On embryonic day 14.5, $PU.1^{+/-}$ or $PU.1^{-/-}$ progenitors were enriched using a lineage-depletion protocol (*9*), then infected by coculture with retroviral packaging cells, which stably produce high titers of murine stem cell virus (MSCV) vectors (*10*). $PU.1^{+/-}$ lineage-depleted (Lin^-) progenitors infected with a control virus (MSCV-EGFP) proliferated and differentiated on S17 stromal cells (*10, 11*) into pro-B cells ($CD19^+$, $B220^+$, $CD43^+$, and c-kit^+) and macrophages (Mac-1^+) in 10 to 14 days. The majority of cells were pro-B (86 ± 4%, $n = 7$); a minor fraction were macrophages (7 ± 3%, $n = 7$). $PU.1^{-/-}$ Lin^- progenitors could not be productively infected with the control virus, because they failed to proliferate and died during the initial coculture. In contrast, $PU.1^{-/-}$ progenitors infected with the PU.1 virus (MSCV-EGFP-PU.1) and cultured on S17 survived and proliferated in response to IL-7. We found it intriguing that after 10 to 14 days these cultures contained many more macrophages (56 ± 4%, $n = 6$) than pro-B cells (19 ± 5%, $n = 6$). Identical results were obtained using M-CSF–deficient OP9 stromal cells (*12*), suggesting that the increased proportion of macrophages observed with PU.1-transduced mutant progenitors was not simply due to increased M-CSF–dependent proliferation. As did their heterozygous counterparts, PU.1-rescued pro-B cells expressed CD19, B220, CD43, and c-kit; the B-lineage–specific genes *mb-1*, *B29*, *λ5*, and *VpreB*; and they underwent V(D)J recombination at the *IgH* locus. Further analysis of the flow cytometry data from these experiments revealed that most macrophages expressed high levels of green fluorescent protein (GFP), whereas the pro-B cells were low or lacking in GFP (Fig. 1). If one assumes, in our vector system, that expression of PU.1 from the viral promoter is correlated with expression of GFP from an internal promoter (*10*), these results suggest that differing concentrations of PU.1 protein are required to promote development of B cells or macrophages.

To analyze PU.1 expression, rescued cells were expanded under conditions that select for pro-B cells or macrophages (*13*). After expansion, rescued pro-B cells no longer expressed GFP as seen by flow cytometry (Fig. 2A) or Western blotting. However, these cells expressed detectable PU.1 transcripts and protein but at substantially lower levels than their macrophage counterparts (one-fifth to one-seventh as much) (Fig. 2B) (*14*). We suggest that the two distinct levels of *PU.1* gene expression observed in the rescued pro-B cells versus macrophages are due to selection imposed on the differential activity of the retroviral long terminal repeat (LTR), which is caused by integration in distinct chromatin environments (*15*). It is noteworthy that wild-type macrophages express higher levels of PU.1 protein than their pro-B counterparts (Fig. 2C) (*16*). These results agree with earlier reports that *PU.1* RNA is expressed at higher levels in myeloid than in B lymphoid cell lines (*17*). Because the levels of PU.1 in wild-type and rescued macrophages are equivalent (Fig. 2, B and C), it is unlikely that the higher levels of PU.1 in rescued macrophages compared with pro-B cells are a consequence of retroviral expres-

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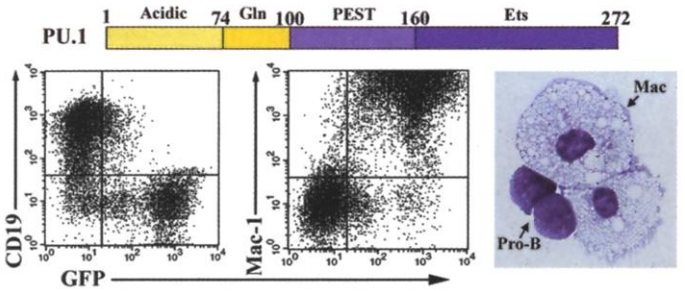

Fig. 1. Analysis of pro-B cells and macrophages induced by transduction of *PU.1*$^{-/-}$ hematopoietic progenitors with MSCV-EGFP-PU.1 retrovirus. Schematic depicts various domains of the PU.1 transcription factor. Embryonic day 14.5 *PU.1*$^{-/-}$ Lin$^-$ fetal progenitors were infected with the PU.1 retrovirus and differentiated on S17 stromal cells with IL-7. Cells from these cultures were analyzed by flow cytometry for expression of GFP and CD19 (left panel) or GFP and Mac-1 (center panel). Morphological analysis of pro-B cells and macrophages was performed by Wright's staining (right panel, original magnification 1000×).

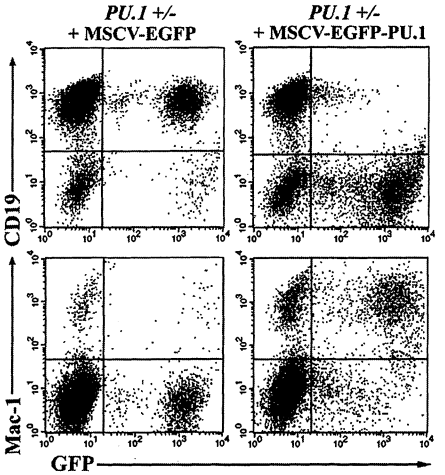

Fig. 3. Effect of PU.1 overexpression on differentiation of *PU.1*$^{+/-}$ progenitors. Embryonic day 14.5 *PU.1*$^{+/-}$ Lin$^-$ progenitors were infected with MSCV-EGFP (left panels) or MSCV-EGFP-PU.1 (right panels) retrovirus and differentiated on S17 stromal cells with IL-7. Cells were analyzed by flow cytometry for expression of GFP and CD19 (upper panels) or GFP and Mac-1 (lower panels).

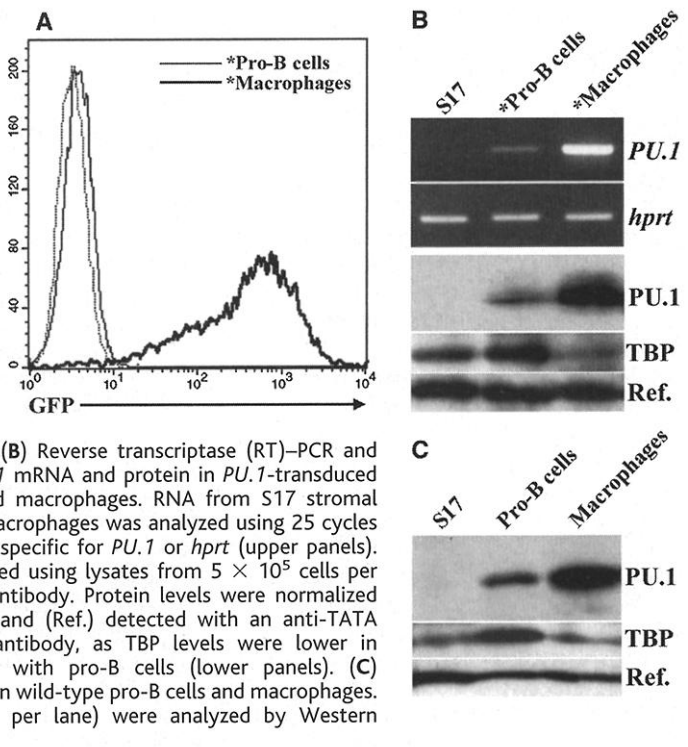

Fig. 2. PU.1 mRNA and protein levels in rescued or normal pro-B cells and macrophages. (**A**) GFP expression in rescued *PU.1*$^{-/-}$ pro-B cells and macrophages. IL-7–dependent Pro-B cells (*Pro-B cells) and M-CSF–dependent macrophages (*Macrophages) generated by *PU.1* transduction of *PU.1*$^{-/-}$ progenitors were analyzed by flow cytometry for GFP expression. Non-transduced, wild-type pro-B cells were used as a control (dashed line). (**B**) Reverse transcriptase (RT)–PCR and Western analysis of *PU.1* mRNA and protein in *PU.1*-transduced *PU.1*$^{-/-}$ pro-B cells and macrophages. RNA from S17 stromal cells, *Pro-B cells, or *Macrophages was analyzed using 25 cycles of RT-PCR with primers specific for *PU.1* or *hprt* (upper panels). PU.1 protein was analyzed using lysates from 5×10^5 cells per lane and an anti-PU.1 antibody. Protein levels were normalized using a cross-reactive band (Ref.) detected with an anti-TATA binding protein (TBP) antibody, as TBP levels were lower in macrophages compared with pro-B cells (lower panels). (**C**) Analysis of PU.1 protein in wild-type pro-B cells and macrophages. Lysates (5×10^5 cells per lane) were analyzed by Western blotting as above.

sion and in vitro differentiation. Thus, a higher concentration of PU.1 protein is required to promote macrophage rather than B lymphocyte development.

If maintaining a low concentration of PU.1 is a requirement for B cell specification, then overexpression of PU.1 in normal progenitors should block B cell development and promote macrophage differentiation. To test this hypothesis, *PU.1*$^{+/-}$ Lin$^-$ fetal progenitors were infected with either the control or PU.1 retrovirus and differentiated on S17 cells with IL-7. With the control virus, 85 ± 3% ($n = 3$) of the GFP$^+$ cells were pro-B cells, whereas 12 ± 3% ($n = 3$) were macrophages (Fig. 3), demonstrating that high- level GFP expression from the retroviral vector is sustainable in pro-B cells. In contrast, infection of *PU.1*$^{+/-}$ progenitors with the PU.1 virus resulted in only 3 ± 2% ($n = 3$) of GFP$^+$ cells being pro-B. The majority of the GFP$^+$ cells (88 ± 4% , $n = 3$) were macrophages. Notably, the GFP$^+$ pro-B cells expressed GFP at low levels (Fig. 3, upper right panel). Because GFP expression is correlated with that of PU.1, these results support the hypothesis that overexpression of PU.1 in normal progenitors blocks B cell development and promotes macrophage differentiation.

PU.1 contains multiple activation domains embedded in acidic (amino acids 7 to 74) and glutamine-rich (75 to 100) segments (Fig. 1) (*18*). To test whether these activation domains are differentially required in B cell and macrophage development, we constructed retroviral vectors expressing proteins designated ΔN100, ΔN74, and ΔN75–100, which lack amino acids 1 to 100, 1 to 74, and 75 to 100, respectively (*19*). *PU.1*$^{-/-}$ Lin$^-$ progenitors were infected with the various mutant PU.1 retroviruses and cultured on S17 cells with IL-7. Only the ΔN75–100 PU.1 protein restored the ability of these progenitors to proliferate on the stroma with IL-7. In a striking way, this mutant protein, unlike its wild-type counterpart (compare Fig. 4 with Fig. 1), preferentially induced the generation of pro-B cells (72 ± 5%, $n = 3$) rather than macrophages (16 ± 3, $n = 3$). A comparison of Fig. 4 with Fig. 1 also reveals that many of the pro-B cells rescued with the ΔN75–100 PU.1 mutant express higher levels of GFP, suggesting that higher concentrations of this transcriptionally weak activator can promote B cell development. To quantitatively assess the ability of the ΔN75–100 PU.1 mutant to induce the generation of macrophage progenitors, we infected *PU.1*$^{-/-}$ progenitors in the presence of multilineage cytokines and plated them in methylcellulose cultures containing M-CSF (*20*). Under these conditions, ΔN75–100, but not ΔN74 or ΔN100, PU.1 proteins induced the development of macrophage progenitors. However, wild-type PU.1 induced approximately 70 times as many macrophage colonies per 10^5 transduced progenitors (535 ± 18, $n = 2$) as ΔN75–100 (8 ± 3, $n = 2$). It is noteworthy that the colonies induced with ΔN75–100 were similar in size to those obtained with the wild-type protein and contained morphologically normal macrophages. Thus the mutant protein is not defective in supporting the proliferation of macrophage progenitors. Collectively, these results demonstrate a quantitative effect of deletion of the glutamine-rich transactivation domain on generation of macrophage progenitors. These experiments suggest that lower levels of PU.1 activity are required

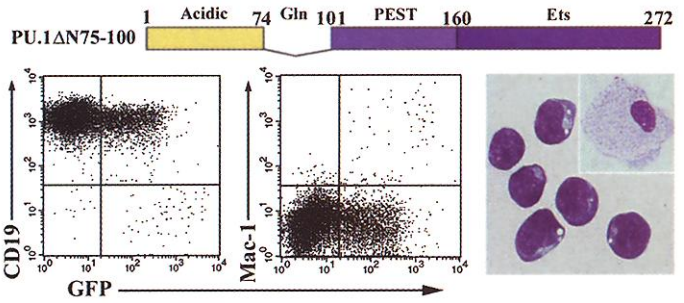

Fig. 4. Analysis of pro-B cells and macrophages induced by transduction of *PU.1*$^{-/-}$ hematopoietic progenitors with MSCV-EGFP-PU.1 ΔN75–100 retrovirus. Schematic depicts the PU.1 ΔN75–100 cDNA. Embryonic day 14.5 *PU.1*$^{-/-}$ Lin$^-$ fetal progenitors were infected with the PU.1 ΔN75–100 retrovirus and differentiated on S17 stromal cells with IL-7. Cells from these cultures were analyzed by flow cytometry for expression of GFP and CD19 (left panel) or GFP and Mac-1 (center panel). Morphological analysis of pro-B cells and macrophages was performed by Wright's staining (right panel, original magnification 1000×).

for B cell than for macrophage development.

Our findings have two possible explanations. In one scenario, differing concentrations of PU.1 protein function to specify distinct cell fates (B cell or macrophage), perhaps from a common lymphoid-myeloid progenitor (*6, 21*). Alternatively, B cell and macrophage development may be initiated with equivalent concentrations of PU.1, but higher concentrations are needed to complete macrophage differentiation. We favor the former possibility, because the *PU.1* gene is expressed at low levels in multipotential progenitors and is induced specifically at the onset of myeloid differentiation (*22*). Although the level of PU.1 expression in lymphoid-restricted progenitors remains to be determined, the lower concentrations in pro-B cells appear unchanged during differentiation into B lymphocytes (*23*). The high concentration or a high activity state of the PU.1 protein appears to be required for inducing macrophage differentiation rather than simply promoting the proliferation of macrophage progenitors. Furthermore, a PU.1-estrogen receptor fusion protein preferentially induces macrophage rather than neutrophil differentiation of *PU.1*$^{-/-}$ myeloid progenitors at high concentrations of tamoxifen, in the absence of proliferation (*24*). Thus, we suggest that in vivo, high concentrations of PU.1 function to specify the macrophage cell fate. Although specification of alternative cell fates by graded concentrations of a transcription factor is a widely considered mechanism in developmental biology, demonstration of its utilization in vertebrate systems is lacking. The best defined examples of this regulatory mechanism involve specification of anterior-posterior cell fates in the developing *Drosophila* embryo by *bicoid* (*25*) and *hunchback* (*26*) transcription factors. Although an earlier study has shown a correlation between lineage choice and transcription factor dosage in transformed hematopoietic progenitors (*27*), our work strongly suggests a physiologically regulated requirement for different concentrations of a transcription factor in the specification of distinct cell fates in the hematopoietic system.

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9. The polymerase chain reaction (PCR) amplified genotyping of fetuses resulting from timed matings of *PU.1* $^{+/-}$ mice has been described (*3*). Embryonic day 14.5 (d14.5) *PU.1*$^{+/+}$, *PU.1*$^{+/-}$, or *PU.1*$^{-/-}$ fetal liver cells were lineage-depleted using biotinylated antibodies against CD4, CD5, CD8a, CD3ε, Gr-1, Mac-1 (CD11b), Ter119, and B220, streptavidin-conjugated magnetic beads, and MACS depletion columns. Lineage-depleted (Lin$^-$) cells contained less than 1% Lin$^+$ cells and were greater than 80% c-kit$^+$.
10. The vectors MSCV-EGFP and MSCV-EGFP-PU.1 have been described (*3*). In this vector system, expression of the gene for GFP is driven by an internal phosphoglycerate kinase (pgk-1) promoter, whereas PU.1 expression is dependent on the viral LTR. To establish retroviral packaging cell lines, retrovirus produced by transient transfection of ΦNX cells [T. M. Kinsella and G. P. Nolan, *Hum. Gene Ther.* **7**, 1405 (1996)] was used to infect GP+E-86 cells [D. Markowitz, S. Goff, A. Bank, *J. Virol.* **62**, 1120 (1988)] after overnight treatment with tunicamycin. After 2 days, the brightest GFP-expressing cells were sorted using a Becton-Dickinson FACScalibur system and expanded under gpt selection. For infection of Lin$^-$ cells, GP+E-86 lines were irradiated using 2500 rad from a cesium source and plated at 10^5 cells per well in a 24-well plate. After an overnight incubation, 10^5 Lin$^-$ fetal liver progenitors were introduced in each well and were infected by coculture for 2 days in Iscove's modified Dulbecco's medium containing 10% fetal bovine serum, 5×10^{-5} M β-mercaptoethanol, 1 U/ml penicillin-streptomycin (complete IMDM), 4 μg/ml polybrene, and 5% conditioned medium from an IL-7–producing cell line [T. H. Winkler, F. Melchers, A. G. Rolink, *Blood* **85**, 2045 (1995)]. Infected cells were removed by gentle pipetting and transferred onto a monolayer of irradiated S17 (*11*) or OP9 (*12*) stromal cells. Cultures were fed with fresh IL-7–containing medium every 4 days and analyzed after 10 to 14 days.
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13. *PU.1*$^{-/-}$ fetal liver progenitors were infected and cultured as described (*10*). Pro-B cells were selected by passaging for two rounds on S17 with IL-7. Macrophages were generated by transfer of infected progenitors to S17 stroma in the presence of 10 ng/ml M-CSF.
14. RNA prepared from *PU.1*-transduced *PU.1*$^{-/-}$ pro-B cells or macrophages was reverse-transcribed using a first-strand cDNA synthesis kit. Serial dilutions of cDNA were analyzed with either 20 or 25 cycles of PCR using primers specific for *PU.1* (forward 5′-CGGATGTGCTTCCCTTATCAAAC-3′, reverse 5′-TGACTTTCTTCACCTCGCCTGTC-3′ or *hypoxanthine phosphoribosyl transferase* (*hprt*) (forward 5′-GCTGGTGAAAAGGACCTCTC-3′, reverse 5′-CACAGGACTAGAACACCTGC-3′). PCR products were visualized by ethidium bromide staining or by Southern blotting using ^{32}P-labeled *PU.1*- or *hprt*-specific probes. Western blotting was performed using an affinity-purified polyclonal anti-PU.1 antibody (*6*). A polyclonal anti-TATA binding protein (TBP) antibody (Santa Cruz) was used as a control. Western blots were quantified by serial dilution of protein samples.
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16. Wild-type macrophages were generated by culture of unfractionated day 14.5 *PU.1*$^{+/+}$ fetal liver cells in complete IMDM medium containing 10 ng/ml M-CSF for 6 days. Wild-type pro-B cells were generated by culture of unfractionated day 14.5 *PU.1*$^{+/+}$ fetal liver cells on irradiated S17 stromal layers in complete IMDM containing 5% IL-7–conditioned medium.
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19. Mutant *PU.1* cDNAs were constructed using PCR as described [R. C. Fisher *et al.*, *Mol. Cell. Biol.* **18**, 4347 (1998)] except for the 5′ external primer (5′-CGGCGGCCGCTTACAGGCGTGCAAAATGGAAGGGT-3′) and 3′ external primer (5′-CCGGGCCCTCAGTGGGGCGGGAGGCGCCG-3′) and verified by DNA sequencing. All *PU.1* mutant forms contained a 5′ HA-tag sequence. All constructs were blunt-end–ligated into the Hpa I site of MSCV-EGFP. Packaging cell lines were constructed as described (*10*) and expressed similar concentrations of the various PU.1 derivatives.
20. *PU.1*$^{-/-}$ fetal liver progenitors (10^5) were infected for 2 days by coculture with virus-producing GP+E-86 cells in 1 ml of complete IMDM containing, 4 μg/ml polybrene, 20 ng/ml IL-3, 100 ng/ml stem cell factor (SCF), and 10 ng/ml IL-6. Ten percent of infected cells were analyzed by flow cytometry for GFP expression. After washing, infected cells were plated at 5×10^4 cells per plate in 2 ml of methylcellulose medium containing 10 ng/ml M-CSF. Colonies were scored using an inverted microscope after 6 days. Colonies were picked, cytospun, and stained with Wright's stain to verify macrophage morphology.
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