ELSEVIER

EXPERIMENTAL HEMATOLOGY

Experimental Hematology 33 (2005) 395–402

Visualizing PU.1 activity during hematopoiesis

Jonathan Back[a], David Allman[b], Susan Chan[a], and Philippe Kastner[a]

[a]*Institut de Génétique et de Biologie Moléculaire et Cellulaire, IGBMC CNRS/INSERM/ULP, Illkirch, CU Strasbourg, France;* [b]*Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, Pa., USA*

(Received 9 December 2004; revised 21 December 2004; accepted 23 December 2004)

***Objective*. PU.1 is a critical transcription factor for hematopoietic development that is required for the early differentiation of myeloid, erythroid, and B lineage cells. To gain a better insight into PU.1 function, we performed a comprehensive analysis of PU.1 gene activity in the hematopoietic system, using a green fluorescent protein reporter mouse line.**

***Methods*. We used flow cytometry to analyze green fluorescent protein (GFP) expression, along with various cell surface markers, in heterozygote mice that harbor a GFP reporter knocked into exon1 of the PU.1 gene. Phenotypic and functional properties of GFP^+ and GFP^- precursors were studied.**

***Results*. We show that PU.1 is dynamically and heterogeneously expressed in many hematopoietic lineages, from the stem cell stage to terminally differentiated cells, suggesting that PU.1 is not only important in early differentiation events but also may play a role in mature hematopoietic cell function. Further, examination of GFP^+ vs GFP^- populations shows that differentiation, but not commitment, to the myeloid lineage requires PU.1. In contrast, B cell commitment is associated with low levels of PU.1 expression.**

***Conclusion*. Our study provides a detailed visualization of PU.1 gene activity in hematopoietic cells, and shows that highly dynamic regulation of PU.1 accompanies cell fate decisions during hematopoiesis.** © **2005 International Society for Experimental Hematology. Published by Elsevier Inc.**

Hematopoiesis is coordinated by sets of transcription factors that function in concert to influence self-renewal, cell fate choice, and differentiation. PU.1, an ets family member expressed exclusively in hematopoietic cells, plays an important role in these processes. PU.1 has been characterized as a master regulator of myeloid and B-cell development, as graded levels of this factor in multipotent progenitors alters the cell fate choice between the B cell and myeloid lineage [1]. Graded levels of PU.1 in myeloid cells also block differentiation and cause myeloid leukemias [2], or regulate macrophage vs neutrophil cell fate [3]. Furthermore, PU.1 is required for mast and dendritic cell differentiation, and plays a role in early T lymphopoiesis [4–8]. More recently, we have shown that very low levels of PU.1 in erythroid progenitors control the self-renewal capacity of these cells [9]. Clearly, slight changes in PU.1 activity can have a significant impact on hematopoietic differentiation and/or function.

Despite its importance, little is known about the potential role of PU.1 in other hematopoietic cell types, such as pluripotent progenitors and mature cell populations. Previous attempts to evaluate PU.1 function in these cells have been hampered by the lack of suitable physiological tools, and instead have relied on traditional mRNA detection methods to investigate PU.1 expression in bulk populations [10–12]. Caveats to these studies included lack of information at the single cell level, relative levels of expression, and the inability to follow live the PU.1-expressing cells in question. Although progress has been made to examine PU.1 mRNA expression in single cells [13], many limitations remain.

We recently described the generation of a green fluorescent protein (GFP) reporter line of PU.1-deficient mice [9]. The $PU.1^G$ mutation was engineered by targeting EGFP cDNA into exon 1 of the PU.1 locus by homologous recombination. The resulting heterozygote $PU.1^{+/G}$ mice expressed PU.1 from one allele and GFP from the other. In this study, we present an in-depth analysis of GFP expression, and its

Offprint requests to: Susan Chan, Ph.D., and Philippe Kastner, Ph.D., Institut de Génétique et de Biologie Moléculaire et Cellulaire, IGBMC, BP 10142, 67404 Illkirch, CU Strasbourg Cedex, France.; E-mail: scpk@igbmc.u-strasbg.fr

0301-472X/05 $–see front matter.
doi: 10.1016/j.exphem.2004.12.010

relative levels, from hematopoietic stem cells (HSCs) to mature cell types. Our results show that PU.1 is dynamically expressed, and reveal an unexpected heterogeneity within previously defined populations of multipotent progenitors.

Materials and methods

Mice

The PU.1^{G} mouse line has previously been described [9]. The 6- to 10-week-old mice used in this study were maintained under specific pathogen-free (SPF) conditions, and have been backcrossed more than 7 generations onto the C57Bl/6 genetic background.

Antibodies and flow cytometry

For staining of HSCs, common myeloid progenitors (CMPs), granulocyte-myeloid progenitors (GMPs), and megakaryocyte-erythroid progenitors (MEPs), the following antibodies were used: biotin-anti-CD34, phycoerythrin (PE)-anti-IL-7Rα A7R34), PE-anti-lineage (Lin; B220, CD3, NK1.1, Gr-1, CD5, TER119, CD19), PE-Cy5.5-anti-Sca-1, PE-Cy7-anti-CD16, APC-Cy7-anti-c-kit (2B8), APC-anti-AA4.1. For staining of CLPs and B lineage cells, we used biotin-anti-IL7R, PE-anti-Flt3, PE-Cy5.5-anti-Sca-1, PE-Cy7-anti-Lin, APC-Cy7-anti-c-kit, APC-anti-AA4.1, PE-anti-CD43, PE-Cy7-anti-B220, APC-Cy7-anti-IgM or PE-anti-IgM, PerCP-Cy5.5-anti-CD19, biotin-anti-CD11b. The above antibodies were purified and conjugated in our laboratories according to standard protocols. For staining of DC subsets, we used PE-anti-CD11c, APC-anti-120G8, biotin-anti-CD8α, APC-anti-CD11b. For staining of natural killer (NK) and natural killer T (NKT) cells, we used PE-anti-NK1.1, purified anti-CD3. For staining of ETPs, we used purified anti-Lin antibodies (B220, CD3, CD8, NK1.1, TCRγδ, CD11b, Gr-1, TER119), PE-anti-CD25, biotin-anti-c-kit. Secondary antibodies included: streptavidin (SA)-CascadeBlue, SA-PerCP, SA-Cy5, and Cy5-anti-rat IgG (the latter two from Jackson ImmunoResearch Labs, West Grove, PA, USA). For sorting experiments, Lin^{+} cells were depleted using the following antibodies: B220, CD3, Gr-1, F4/80, TER119, and NK1.1. Unless specified, all antibodies were from BD Biosciences (BD Pharmingen, San Diego, CA, USA). Samples were analyzed or sorted using a FacsCalibur (BD Biosciences, San Jose, CA, USA), an Epics Elite (Coulter Electronics, Hialeah, FL, USA), a FACSVantage SE option DiVa (BD Biosciences), or a MoFlo cell sorter (Cytomation, Fort Collins, CO, USA). Sort purity was greater than 90% for culture assays and greater than 95% for RNA experiments. Results were analyzed using the FlowJo software (Tree Star Inc., Ashland, OR, USA).

Methylcellulose colony assays

Lin^{-} populations were obtained from wild-type (WT) and PU.1$^{+/G}$ bone marrow (BM) cells, or PU.1$^{+/G}$ and PU.1$^{G/G}$ fetal liver (FL) cells. Colonies were obtained as follows: cells were plated in triplicate in 3.5-cm plates with 1.1 mL of the semi-solid methylcellulose medium M3434 (StemCell Technologies, Vancouver, BC, Canada) containing erythropoietin (EPO), stem cell factor (SCF), interleukin (IL)-3, and IL-6, according to the manufacturer's recommendations, and cultured at 37°C. After 7 days, the cultures were stained with a benzidine solution (2 mg/mL benzidine, 0.5% glacial acetic acid, 0.5% H_2O_2). Large colonies containing only darkly stained cells were counted as BFU-E (erythroid burst-forming unit); colonies containing a mix of darkly stained and unstained cells were counted as mixed erythro-myeloid colonies. Pure unstained colonies were counted as myeloid-only colonies (CFU-GM, CFU-M, CFU-G). In some cases, colonies were picked and their cells stained with May-Grünwald-Giemsa to confirm their identity.

May-Grünwald-Giemsa staining

Cells were cytospun (~10^5 cells per slide) at 700 rpm for 5 minutes onto glass slides, and stained with May-Grünwald followed by Giemsa, according to standard protocols.

Western blotting

Detection of PU.1 protein by Western blot was performed as described previously [9].

Reverse transcriptase-polymerase chain reaction (RT-PCR)

RNA was isolated from 1 to 3 × 10^5 cells using the RNeasy kit (Qiagen SA, Valencia, CA, USA), and resuspended in 12 μL H_2O. Three μL RNA were used in the reverse transcriptase (RT) reactions (20 μL). One to 2 μL of cDNA was used for the polymerase chain reaction (PCR). PCRs were performed as follows: 94°C for 5 minutes, followed by n cycles of 94°C 1 minute, 60°C 1 minute, and 72°C 1 minute (n = 26–35 depending on the gene). Primers used are shown in Table 1. The following primers were used:

Table 1. Forward and reverse primers

Gene	Forward primers (5′ to 3′)	Reverse primers (5′ to 3′)
C/EBPα	AAG GCC AAG AAG TCG GTG GA	CAG TCC ACG GCT CAG CTG TT
C/EBPε	ACC AGT CGA GGC AGC TAC AA	CCC GAC ACC CTT GAT GAG
SCL	ACC TCA CGG CAA GCT AAG TAA	ACG CCG TTG AGC AGG ACT A
EKLF	ACC ACC CTG GGA CAG TTT CT	GAA GGG TCC TCC GAT TTC AG
GATA-1	GGG AGC TGA CTT TCC CAG T	GTC TCC TCT GCC ACA AGG TC
GATA-2	CAA GGA TGG CGT CAA GTA CC	ACA GTA ATG GCG GCA CAA G
GATA-3	GTC GGC CAG GCA AGA TGA	CAG GGC TCT GCC TCT CTA AC
GM-CSFR	GAG GTC ACA AGG TCA AGG TG	GAT TGA CAG TGG CAG GCT TC
IL7R	GGA GGA TCA CTC CTT CTG GT	CCC ATC CTC CTT GAT TCT TG
β Actin	GTG ACG AGG CCC AGA GCA AGA G	AGG GGC CGG ACT CAT CGT ACT
c-fms	CTGAGTCAGAAGCCCTTCGACAAAG	CTTTGCCCAGACCAA AGGCTGTAGC
EBF	AGC CCG TGG AGA TTG AGA G	CGG ATG GCA TGA GGA GTT AT
GFP	GTG GAT CGA TCT GAG AAC TT	GCG GAT CTT GAA GTT CAC
PU.1	GGA TCT GAC CAA CCT GGA GC	AGC ACC TCG CCG CTG AA