The transcription factor PU.1 does not regulate lineage commitment but has lineage-specific effects

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Abstract: PU.1 is a transcription factor shown to regulate the expression of many important genes in myeloid and B cells. At birth, mice homozygous for the disruption of the PU.1 gene have erythrocytes, megakaryocytes, and T cells, but no mature myeloid or B cells. Cells with an inability to develop to maturity were found in this mouse for B cells, neutrophils, eosinophils, mast cells, and monocytes. Rescue of early monocytic cells by transfection with the PU.1 gene results in renewed development to macrophages. These results demonstrate that PU.1 is an important regulator in the development of cells in the hematopoietic system. *J. Leukoc. Biol.* 66: 727–732; 1999.

Key Words: *hematopoiesis · macrophage · myeloid · oncogene · spi-1*

INTRODUCTION

PU.1 is a transcription factor found exclusively in cells of the hematopoietic system [1]. PU.1 is also known as the oncogene spi-1 because it was independently discovered as the gene induced by insertion of the Friend murine leukemia virus [2]. The purine-rich DNA binding sequence, 5'-GAGGAA-3' (the PU box), was characterized by methylation interference and DNase I protection. The protein contains three domains. The amino terminus contains both a highly acidic and a glutamine-rich transactivation domain [3]. The carboxy-terminal region encompasses the DNA-binding domain, including 99 amino acids that have approximately 40% sequence identity to the DNA-binding domains of ets-1, ets-2, elk-1, and erg-1 [1].

The transactivation and DNA-binding domains are linked by a PEST (P-proline, E-glutamic acid, S-serine, T-threonine) domain. Regions rich in these amino acids have been linked to control of protein degradation [4]. It is suggested that this may be one of its functions in PU.1 by the increased resistance to protease degradation when Ser148 is phosphorylated by casein kinase II [5]. The phosphorylation of Ser148 in this sequence has also been shown to be important in protein-protein interactions. NF-EM5 [6, 7], which was subsequently cloned, identified as a member of the interferon regulatory factor (IRF) family, and called PIP [8], was shown to associate with PU.1 after phosphorylation at Ser148. The two associated proteins bind to the immunoglobulin κ 3' and λ2-4 enhancers and have been shown to increase expression of a reporter construct sixfold above PU.1 alone.

PU.1 has also been implicated in the regulation of the expression of many other myeloid and B cell genes. Examples include CD18 [9], CD11b [10], macrophage colony-stimulating factor (M-CSF) receptor [11], granulocyte-macrophage CSF (GM-CSF) receptor α [12], G-CSF receptor [13], the mannose receptor [14], macrosialin [15], the scavenger receptor [16], the immunoglobulin J chain [17], IL-4 in mast cells [18], and may even be autoregulatory [19]. Many of these genes play important roles in the growth, development, and function of these lineages.

To investigate the nature of the interaction between the transcription factor PU.1 and its DNA binding sequence, the DNA binding domain of PU.1 (the ets domain) in complex with DNA was crystallized [20] and the crystal structure determined [21]. It was found that PU.1 binds DNA with a unique loop-helix-loop interaction that results in a uniform 8-degree curvature of the DNA. Analysis of data from other ets transcription factors suggests that this may be a paradigm for ets protein interactions with DNA [22].

TARGETING THE PU.1 GENE

To investigate the role that PU.1 plays in the hematopoietic system, we targeted the gene by homologous recombination to produce a knockout mouse [23]. PU.1-null mice were born at the expected Mendelian frequency, appeared normal at a gross level, however, they lacked all peripheral blood leukocytes, were severely osteopetrotic [24], and died of acute septicemia within 48 h. Antibiotic therapy prevented bacterial infection and kept the pups alive for over 2 weeks; rearing under axenic conditions allowed them to live for just over 3 weeks. However, all pups eventually died of unknown causes [23, S.R. McKercher, unpublished results].

THE PU.1-NULL MOUSE HEMATOPOIETIC SYSTEM

Table 1 summarizes our current understanding of the various hematopoietic lineages in PU.1-null mice. Erythroid cells express PU.1 at a very early stage of their development [25], but PU.1 protein disappears before maturation [26]. PU.1-null mice

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TABLE 1. Cells in Various Hematopoietic Lineages in PU.1-null Mice

	Fetal Liver	Bone Marrow		Spleen		Thymus	
Age	E16	P1	P12	P1	P12	P1	P12
Cell Lineage							
Erythrocyte	+	+	+	+	+	nd	
Megakaryocyte	+	+	+	+	+	nd	
Macrophage	−	−	−	−	−	−	−
Osteoclast	nd	−	−	nd		nd	
Neutrophil	−	−	(+)	−	(+)	nd	
Eosinophil	−	−	−	−	−	nd	
B Lymphocyte	−	−	−	−	−	nd	
T Lymphocyte	−	nd		−	+	−	+

are born with apparently normal erythrocytes, suggesting that PU.1 does not play a dominant role in development of this lineage. The peripheral blood reticulocyte count of PU.1-null pups is considerably elevated compared to normals, and their extruded nuclei and other debris clutter the blood [S. McKercher et al., unpublished results]. Most of these phenomena suggest secondary effects on the erythroid lineage in PU.1-null mice, although direct effects due to lack of the gene at early stages of development have not been ruled out.

We find megakaryocytes in liver and the small amount of bone marrow present in PU.1-null neonates. Platelets are seen in blood smears stained with Wright-Giemsa and the blood of PU.1-null mice clots [23]. Because it is known that PU.1 is expressed in megakaryocytes [27], it would appear that its role in this lineage is reduced.

Of the granulocytic lineages, neutrophils have been most thoroughly characterized. Neutrophils are not seen in fetal or early neonate PU.1-null mice and are designated as missing in Table 1. However, the parenthetical plus signs indicate that cells with neutrophil-like morphology, including a segmented nucleus, can be found by postpartum day 5 in the peripheral blood and hematopoietic organs. The number of these cells is much reduced compared to normal neutrophils [23]. These cells can also be cultured from neonate liver and spleen [28]. However, these cells are not functional neutrophils by most criteria [29]. Though possessing the surface markers Gr-1 and CD18, they express little or no CD11b. These cells have azurophilic granules and are positive for chloroacetate esterase, but fail to migrate in response to chemotactic stimuli and will not activate as judged by a lack of production of superoxide in response to various agents including PMA. These PU.1-null neutrophil-like cells also have reduced phagocytic capacity and lack secondary granule components, including gelatinase, lactoferrin, and lysozyme.

The presence of mature eosinophils has been investigated and they are absent in the PU.1-null mouse. The presence of immature cells of this lineage in PU.1-null mice is suggested by reverse transcriptase-polymerase chain reaction (RT-PCR) data showing expression of eosinophil peroxidase and granule major basic protein in 16.5-day embryos and 9-day pups. IL-5 receptor α subunit mRNA is not found in fetal liver but is seen at levels reduced from those of normal mice in 9-day-old PU.1-null spleen [30].

Peritoneal lavages of PU.1-null mice have shown cells that appear morphologically like mast cells. Mast-like cells have also been found in colony-forming unit (CFU) assays [28]. These cells from PU.1-null mice have not been further characterized, but demonstrate that commitment occurs.

Cells positive for B220 and BP-1 have been identified in PU.1-null mouse fetal liver and neonate spleens and bone marrow by flow cytometry [23]. These cells have not undergone rearrangement of their immunoglobulin heavy chain gene, but RT-PCR analysis shows that they express Rag-1 and Rag-2. λ5 was not expressed, whereas some mu° was expressed in fetal liver, but not in 10-day neonate bone marrow. These findings show that coordinate expression of B lineage traits is disrupted but there is commitment to the B cell lineage.

PU.1-null mice have a rudimentary thymus with no T cells at birth. Antibiotic-treated pups demonstrate thymic development by postpartum day 8, including a normal distribution of cells that are CD4- and CD8-single and -double positive and express TCR α/β/CD3. The number of T cells is much reduced compared to normals but some enter the peripheral circulation and are found in the spleen [23]. PU.1 has not been found to be expressed in any T cell line tested [1] and the presence of T cells in the null mouse shows that PU.1 is not a transcription factor used by this lineage. It is likely that this delayed thymic development is due to secondary factors related to the absence of macrophages and/or dendritic cells.

THE MONOCYTIC LINEAGE

Table 1 shows that macrophages, osteoclasts [24], and microglia as determined by F4/80 staining [S. McKercher, unpublished results] have not been found in any tissue. The demonstration of cells committed to the other lineages suggested that there may be cells of the monocytic lineage blocked at an early stage. These cells would not be readily detected if they did not mature to the stage at which F4/80 and other mature monocyte/macrophage markers are expressed. Detection of early monocytic cells is complicated by the poor characterization of these cells, in contrast to those of the lymphoid lineages. However, we reasoned that if cells committed to the granulocytic lineages, then the multipotent precursor GM cell may be present. If this cell is present, then perhaps cells also commit to the monocytic lineage.

We investigated the possibility that cells early in the monocyte development pathway were present in PU.1-null mice. The earliest identified cell of this lineage is the monoblast, which gives rise to the promonocyte and finally to the monocyte. Because unique stage-specific markers are not available for monocytes, we decided on a combination of available antibodies for initial identification of monoblasts/promonocytes [31]. Moma-2 is specific for monocyte/macrophage lineage cells, recognizing mature macrophage subsets and bone marrow precursors [32]. Although the antibodies are not individually monocyte-specific, the cell phenotypes ER-MP12^{hi}/ER-MP20^{-}, ER-MP12^{+}/ER-MP20^{+}, and ER-MP12^{-}/ER-MP20^{+} define early, intermediate, and late stages, respectively, of monocyte development [33, 34]. We used a combination of these three antibodies to identify a population of cells present in normal and PU.1-null mice.

Figure 1 shows flow cytometry of cells from normal and PU.1-null mouse neonate livers that were triple stained. A population of cells exists from both mice that is double positive for Moma-2 and ER-MP12. This phenotype corresponds to an early to intermediate stage of monocytic development. This was confirmed by the third antibody staining of the normal cells, which were predominantly positive for F4/80, CD11b, and Gr-1. On the other hand, the corresponding population of PU.1-null cells is absolutely negative for F4/80 and Gr-1, and contains only a few CD11b$^{low/intermediate}$ cells.

The Moma-2/ER-MP12 population of cells was isolated by antibody-conjugated magnetic bead sorting (Miltenyi Biotec) and centrifuged onto glass slides. Normal and PU.1-null cells were found to be positive for nonspecific esterase, using α-naphthyl acetate as substrate (**Fig. 2**), and the activity was inhibited by the addition of sodium fluoride. This was strong evidence for identification of these cells as early monocytic lineage cells, but we wanted to confirm this.

We had previously shown that a variety of colonies containing morphologically characterized neutrophils, mast cells, and megakaryocytes, but no macrophages, could be grown under standard CFU conditions in medium containing stem cell factor (SCF), interleukin (IL)-3, and IL-6. No colonies were produced from PU-null fetal or neonate hematopoietic cells with either GM-CSF or M-CSF as the only added growth factor [28, G. Henkel, unpublished results]. We established growth conditions that supported primarily macrophage development using SCF, IL-3, GM-CSF, and M-CSF in the CFU assay system. Under these conditions normal neonate liver cells produced predominantly macrophage colonies and some mixed granulocyte/macrophage and neutrophil colonies. The mixed colonies also contained predominantly macrophages [31].

PU.1-null neonate liver cells produced fewer and smaller colonies that were consistent with a monocyte morphology. We designated them as myeloid colonies. In fact, a few of the colonies contained only cells displaying neutrophil morphology. However, most of these colonies contained only blast-like mononuclear cells with a large nucleus/cytoplasm ratio or were predominantly of this cell type with a few neutrophil-like cells. Some of the mononuclear cells contained vacuoles and were similar in appearance to immature cells present in the normal macrophage colonies. These cells are also Moma-2 and NSE positive, showing that they are early monocytic cells (**Fig. 3**).

PRIMARY CELL CULTURE

Primary liver cultures from normal fetal or neonate mice will produce macrophages in the presence of GM-CSF or M-CSF. However, primary liver cultures of cells from PU.1-null mice did not yield macrophages [23]. After the discovery of early monocytic cells in the PU.1-null mice, we investigated primary cultures again. We were unable to grow early myeloid cells from neonate liver or spleen in either M-CSF or GM-CSF, nor from fetal liver in M-CSF. However, early myeloid cells grew from

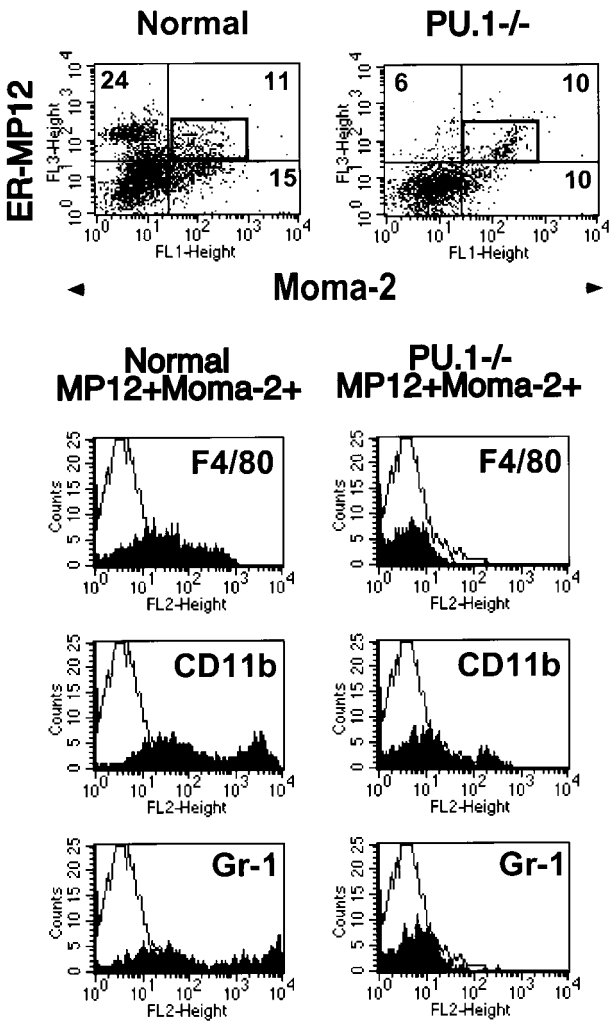

Fig. 1. Identification of monocytic precursor cells in PU.1-null neonate livers. Cells from livers of PU.1-null or normal neonates were triple-stained with Moma-2^{FITC} and unconjugated ER-MP12 and either F4/80^{PE}, CD11b^{PE}, or Gr-1^{PE}. ER-MP12 was then stained with a biotinylated isotype-specific (IgG2a) secondary antibody followed by Streptavidin Cy-Chrome. Dot plots of Moma-2/ER-MP12 staining is divided into quadrants based on isotype-matched control background staining and the numbers indicate the percentage of cells in each quadrant. Histogram plots show the indicated third antibody staining profile of the double-staining population defined by the rectangular box gate in the dot plots.

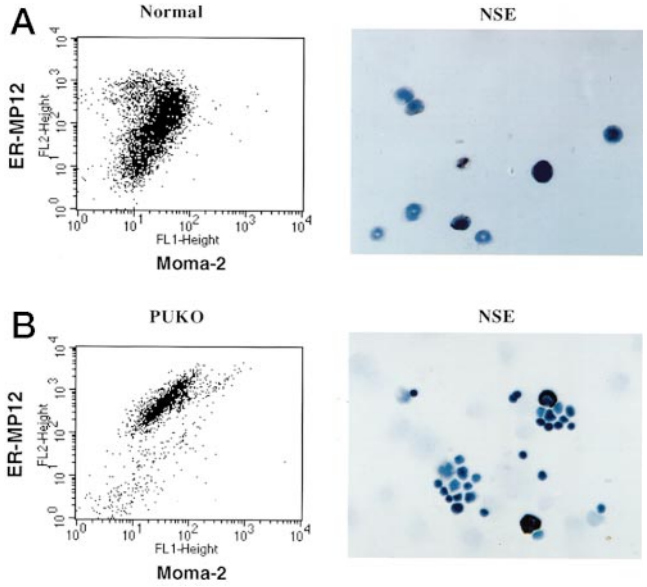

Fig. 2. Cells isolated from neonate livers are positive for monocyte-specific nonspecific esterase (NSE) enzyme activity. Normal cells (A) and PU.1-null cells (B) were isolated from neonate liver by positive selection over a magnetic column (Miltenyi Biotec). Cells were first stained with Moma-2^{FITC} antibody followed by an iron bead-conjugated anti-FITC antibody and passage over the magnetic column. These cells were stained with unconjugated ER-MP12, followed by PE-conjugated anti-IgG2a antibody, and assayed by FACS. These cells were also assayed for NSE activity through the use of α-naphthyl acetate as substrate that yields a black precipitate.

fetal liver cultured with GM-CSF as the only added growth factor [31].

A clone (GMpu4) derived from these cells can be grown in suspension away from stromal cells and is GM-CSF-dependent. These cells have a morphology similar to the early monocytic

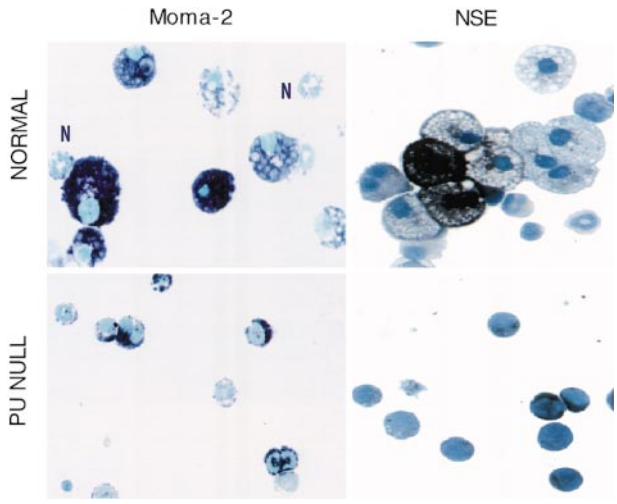

Fig. 3. Characterization of cells from colony-forming assays. Normal and PU.1-null neonate liver cells were grown in methylcellulose for 7 days with 50 ng/mL stem cell factor, 100 U/mL IL-3, 2.5 ng/mL GM-CSF, and 5,000 U/mL M-CSF. Pooled normal or PU.1-null colonies were tested for Moma-2 expression and NSE activity with α-naphthyl acetate as substrate. Biotinylated secondary antibody and avidin-peroxidase produced purple Moma-2-positive cells (left panels); N, neutrophils that are negative. Black staining of cells (right panels) indicates NSE positivity.

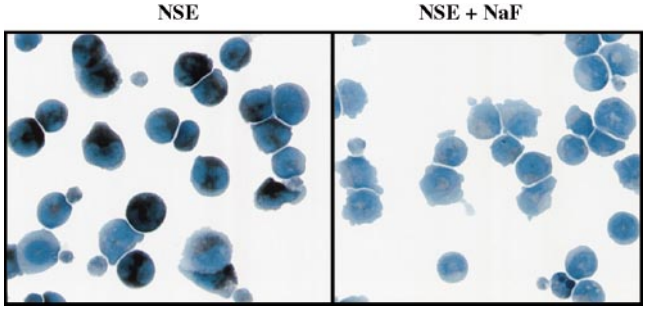

Fig. 4. GMpu4 cells are NSE-positive. Cytospin preparations of GMpu4-cultured cells were made and assayed for NSE activity using α-naphthyl acetate as substrate. Black staining indicates a positive reaction. Assays run in parallel with sodium fluoride added to the reaction inhibited the NSE enzyme activity.

cells, are Moma-2 positive and NSE positive (**Fig. 4**). The GMpu4 cells have now been kept in culture for many generations, cycle continuously in the presence of GM-CSF, but become dormant and eventually die in its absence. These cells cannot be sustained with M-CSF and are further proof that committed monocytic lineage cells are present in the PU.1-null mouse.

To further characterize the GMpu4 cells we have rescued them by transfection with a retroviral construct containing the PU.1 gene. These transfected cells express PU.1 but show only a very minimal degree of morphological change and become CD11b positive [G. Henkel, unpublished results]. They are still dependent on GM-CSF. However, we have isolated clones that respond to the addition of M-CSF by attaching to the culture dish and assuming the morphology of a macrophage (**Fig. 5**). These cells are now positive for F4/80, scavenger receptor, and MHC class II and remain Moma-2-positive.

CONCLUSIONS

We have shown that deletion of the PU.1 gene in mice results in the elimination of mature myeloid and B cells. We have also shown, however, that commitment to each of these lineages occurs in the absence of PU.1 expression. Another PU.1 knockout mouse has been reported [35] that displays differences in phenotype from ours, including embryonic death. Published results reported for this mouse support our hypothesis that PU.1 does not regulate lineage commitment. These results demonstrate expression of early myeloid genes in embryos and ES cells [36], *in vitro* proliferation of granulocytic precursors in response to growth factors [37], and the expression of some eosinophil mRNA species in fetal liver [30]. The differences between the two PU.1-null mice remain to be explained, but it has now been demonstrated that hematopoietic cells from both mice can be rescued by reintroduction of the PU.1 gene and matured to macrophages [36, 37, 38, G. Henkel, unpublished results] (Fig. 5).

Our results show cells at a unique developmental stage for each hematopoietic lineage. These results are not consistent with a hypothesis for PU.1 as a regulator of lineage commitment. We feel that the evidence is now very clear that PU.1 does

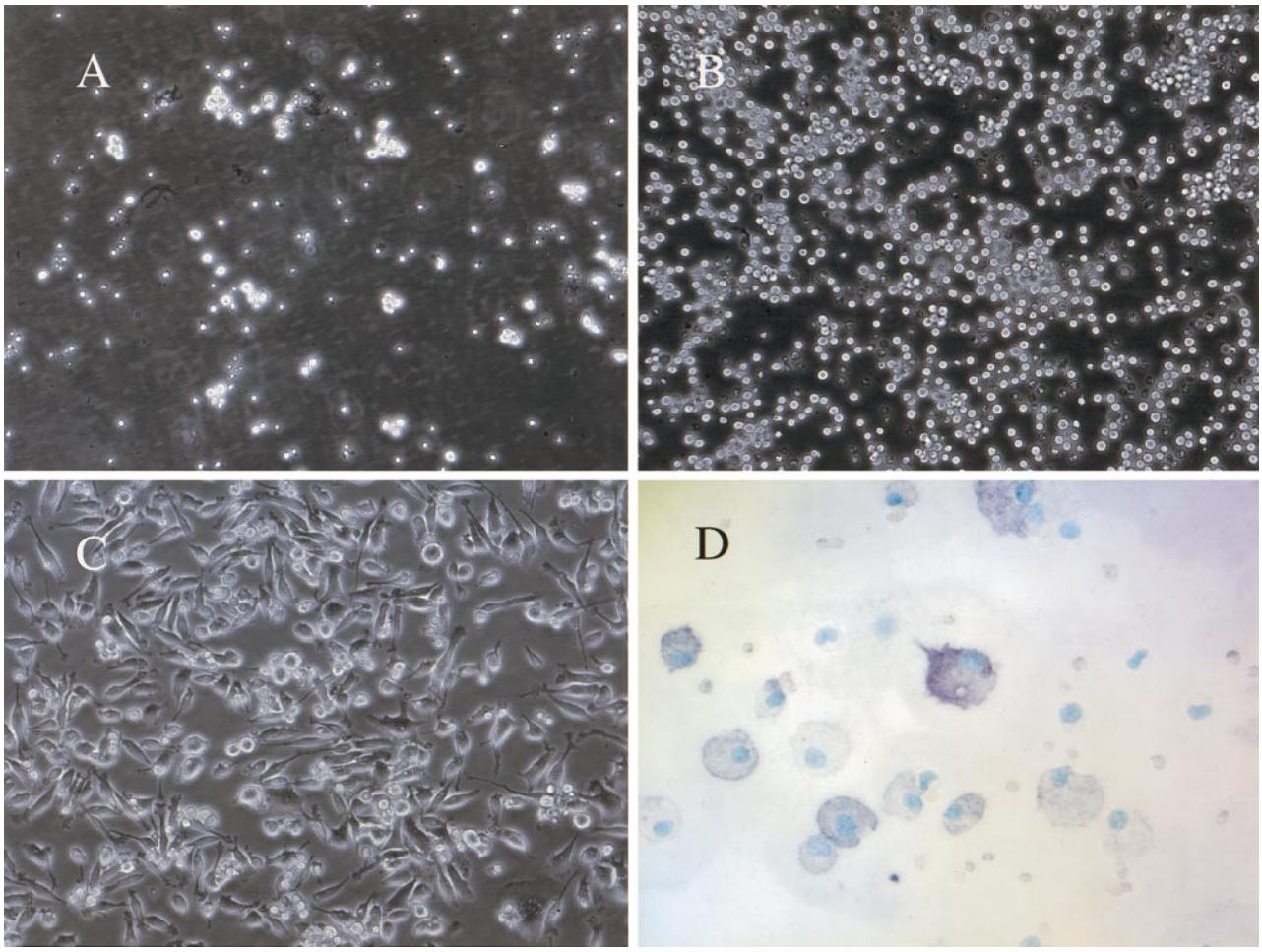

Fig. 5. Rescued GMpu4 cells can become macrophages. (A) GMpu4 cells cultured in the presence of GM-CSF and M-CSF. (B) GMpu4 cells rescued by transfection with a PU.1 gene expression vector (GMpu4R cells) and cultured in the presence of GM-CSF. (C) GMpuR cells cultured in the presence of M-CSF. (D) Cytospin preparation of GMpu4R cells cultured in the presence of M-CSF and stained with F4/80 antibody.

not control commitment to any of the hematopoietic lineages but acts in each, except T cells, in a lineage-specific manner.

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