

# Down-regulation of PU.1 by Methylation of Distal Regulatory Elements and the Promoter Is Required for Myeloma Cell Growth

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## Abstract

**The transcription factor PU.1 is essential for myeloid and B-cell development. Down-regulation of PU.1 by disruption of its 14-kb 5' upstream regulatory element induced acute myeloid leukemia, T-cell lymphoma, and chronic lymphocytic leukemia-like disease in murine models. In the present study, we found that PU.1 was down-regulated in the majority of human myeloma cell lines and a subset of freshly isolated myeloma cells, in contrast to relatively high expression of PU.1 in normal plasma cells. Patients in this low PU.1 expression subset may have a poor prognosis. In human myeloma cell lines, the 17-kb 5' upstream enhancer and the promoter region of the *PU.1* gene were highly methylated, and this is consistent with disappearance of DNase I-hypersensitive sites in these regions. To elucidate the significance of down-regulation of PU.1, we generated stable myeloma cell lines with an inducible PU.1 expression system. Exogenous expression of PU.1 in PU.1 null myeloma cell lines, U266 and KMS12PE, induced complete growth arrest and cell death. Up-regulation of PU.1 by 5-aza-2'-deoxycytidine also induced growth arrest of KMS12PE and KHM11 myeloma cells. These data suggest that down-regulation of PU.1 is an essential step for the survival of a subset of myeloma cells and that up-regulation of PU.1 by demethylation agents or other types of agents may represent a new therapeutic strategy for treatment of multiple myeloma patients. [Cancer Res 2007;67(11):5328–36]**

## Introduction

PU.1 is a critical transcription factor for the development of myeloid and lymphoid lineages. Its expression is tightly regulated through hematopoiesis and disruption of this tight regulation can lead to leukemias in the myeloid and lymphoid lineages in experimental models. *PU.1* was originally identified as an oncogene activated by spleen focus-forming virus (SFFV) integration into a region 14 kb upstream of the *PU.1* transcription start site, resulting in erythroleukemia in mice (1). Proper gene expression *in vivo* generally requires combinatorial interactions of *cis*-elements, which are often located many kilobases upstream or downstream of the promoter region (2–8). We identified a 14-kb 5' upstream regulatory region in the murine *PU.1* gene, which is also a SFFV

integration site of *PU.1*, and found that PU.1 itself can bind to this element (3, 6). Knocking out this 14-kb 5' upstream regulatory region induced down-regulation of PU.1 to 20% of the wild-type (WT) level, and all the knockout mice developed acute myeloid leukemia, T-cell lymphomas, and/or chronic lymphocytic leukemia-like disease (9, 10). These findings suggest that PU.1 may show tumor suppressor activity against leukemogenesis. In addition, *PU.1* is frequently silenced by deletion and point mutations of the PU.1 coding region in a radiation-induced murine leukemia model (11). In human leukemia, *PU.1* was found to be mutated in one allele in 7% of subjects in a Japanese cohort (12). The role of PU.1 in myelopoiesis has been well elucidated and PU.1 is an essential transcription factor for myeloid cells. In contrast, in lymphopoiesis, PU.1 is necessary for commitment of hematopoietic stem cells to the common lymphoid progenitor stage but is not required for maturation of B lymphocytes after the common lymphoid progenitor stage (13–15). It has been reported that PU.1 is expressed in normal plasma cells (16), but its role in plasma cell development has not been elucidated. PU.1 is down-regulated in myeloma cell lines (17), but the functional significance of this down-regulation is not clear. Here, we report that PU.1 is down-regulated in freshly isolated myeloma cells from a subset of patients and the majority of myeloma cell lines and that conditional expression of PU.1 induces growth arrest and cell death of PU.1 null myeloma cell lines. These data suggest that PU.1 may have tumor suppressor activity for plasma cells and that down-regulation of PU.1 is related to the growth advantage and poor prognosis of myeloma patients.

## Materials and Methods

**Cell culture.** Human myeloma cell lines (KMML1, KHM4, KHM11, U266, KMS12BM, KMS12PE, and RPMI8226), human myeloid U937 and HL-60 cells, and B lymphocyte KMS1B, KMS2B, and Raji cells and T lymphocyte Jurkat and CEM cells were grown in RPMI 1640 containing 10% fetal bovine serum at 37°C.

**Patients.** Myeloma patient samples and non-myeloma plasma cell samples were obtained from the Hematology Division of Kumamoto University of Medicine. Written informed consent was obtained according to the Declaration of Helsinki in keeping with institutional policies.

**Purification of myeloma cells and normal plasma cells.** Myeloma cells from patient samples and normal plasma cells obtained from bone marrow samples of malignant lymphoma patients without invasion of lymphoma cells were purified as CD138-positive cells using anti-CD138 antibody-conjugated magnetic beads (Miltenyi Biotec). To obtain highly purified myeloma cell samples, samples from patients were stained with phycoerythrin-conjugated anti-CD138 antibody and FITC-conjugated anti-CD11b, anti-CD33, and anti-CD19 antibodies or anti-CD5 antibody, and the myeloma cells and normal plasma cells were sorted as CD138 single-positive cells using a FACS Vantage (Becton Dickinson). Sorting was carried out thrice and the purities of the cells were >96%.

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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**Semiquantitative reverse transcription-PCR and real-time PCR.**

Quantitative Taqman PCR was done with commercially available assay-on-demand probe primer sets for PU.1 and  $\beta$ -actin (Applied Biosystems) and Taqman Universal PCR Master Mix reagent using an ABI Prism 7700 Sequence Detection System. The expression level of  $\beta$ -actin was used to standardize the relative expression level of PU.1. The expression level of PU.1 in PU.1-positive myeloma cell line KMS12BM was set as 100.

Semiquantitative PCR analysis was done with the following primers: 5'-GCCGAGAACAACCTTCACGG-3' and 5'-CAGATGCTGCTTCATGTCG-3' (PU.1) and 5'-GGAGCCAAAAGGTCATCATCT-3' and 5'-TCAAAGGTG-GAGGAGTGGGTGT-3' [glyceraldehyde-3-phosphate dehydrogenase (GAPDH)].

**Bisulfite sequencing.** We treated genomic DNA with sodium bisulfite as described previously (18) and then subjected the treated DNA to 35 cycles of PCR. We found CpG islands around the 17-kb 5' upstream region and intron 1 of the PU.1 gene using software created by the University of California to search for CpG islands.<sup>4</sup> A 326-bp DNA fragment of the PU.1 17-kb 5' upstream region containing the CpG islands was amplified with the primers 5'-GTTGGATATTTTTGAGGTTTGG-3' and 5'-TAAACCTAAAACCTAC-TAAACCA-3', whereas a 448-bp PU.1 promoter-intron 1 region containing the CpG islands was amplified with the primers 5'-GAGATTTTTGTATG-TAGYGTAAAGA-3' and 5'-TAACTCCCACTAATAACAAACCA-3'. PCR products were directly sequenced and also subcloned into the pGEM-T Easy vector. Four clones for each myeloma cell line were sequenced for evaluation of their methylation status.

**DNase I-hypersensitive site assay.** DNase I-hypersensitive sites (DHS) were assayed as described previously (19). Probe 1 for the 17-kb 5' upstream region was amplified by PCR with the primers 5'-GAGGGATCTTCCTGC-CATGT-3' and 5'-GTGGTCACCCAGAAAAGGA-3', whereas probe 2 for the promoter region was amplified by PCR with the primers 5'-CCACTCTC-TGTCCAGTTC-3' and 5'-TTAGGAAACCTGCCAACCA-3'.

**Constructs.** pCAG20-1, pUHD-3 puromycin, and pUHD-10-3 IRES-GFP plasmids were kind gifts from Dr. Takumi Era (Laboratory for Stem Cell Biology, RIKEN Center for Developmental Biology, Kobe City, Hyogo, Japan; ref. 20). Human PU.1 cDNA was subcloned into the blunt-ended EcoRI site of pUHD10-3 IRES-GFP, resulting in pUHD10-3 PU.1-IRES-GFP.

**Stable transformants with PU.1 expression.** To obtain tetracycline-off ("tet-off") PU.1-inducible myeloma cell lines,  $1 \times 10^7$  U266 or KMS12PE cells were cotransfected with 10  $\mu$ g each of Sca-1-digested pCAG20-1 and a pUHD-3 puromycin plasmid by electroporation. After puromycin-resistant stable cell lines, designated U266-puro and KMS12PE-puro, were obtained, U266-puro cells were transfected with 10  $\mu$ g Sca-1-digested pUHD10-3 PU.1-IRES-GFP and 1  $\mu$ g HindIII-digested pPGKneo, whereas KMS12PE-puro cells were transfected with 10  $\mu$ g Sca-1-digested pUHD10-3 PU.1-IRES-GFP and 1  $\mu$ g Sca-1-digested pZeoSV2\*. After isolation of G418- or zeocin-resistant clones, tetracycline was removed and the cells were analyzed for their green fluorescent protein (GFP) expression by fluorescence-activated cell sorting (FACS) analysis.

**Electron microscopy.** Cell pellets of U266<sup>tetPU.1</sup> cells were fixed with 1.0% glutaraldehyde for 60 min and postfixed with 1.0% osmium tetroxide for 30 min at 4°C. After dehydration in a graded ethanol series, the cells were embedded in epoxy resin. Ultrathin sections stained with uranyl acetate and lead citrate were observed using an H-7500 electron microscope (Hitachi).

**Western blot analysis.** Cell lysates were separated by SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were incubated with anti-PU.1, anti-p27, anti-L-MYC, anti-Bcl-2, anti-Bcl-xL, anti-A1/Bfl-1, anti-caspase-3 (Santa Cruz Biotechnology), anti-cyclin D1, anti-cyclin E (BD PharMingen), or anti- $\beta$ -actin (Sigma Chemical Co.) primary antibodies for 1 h. Finally the membranes were incubated with peroxidase-labeled secondary antibodies for 1 h and developed using an enhanced chemiluminescence system (Amersham Life Science, Inc.).

**Cell cycle analysis.** Cells were stained with 50  $\mu$ g/mL propidium iodide and treated with 10  $\mu$ g/mL RNase A for 30 min at room temperature. Cell cycle analyses were done using a flow cytometer (EPICS V, Coulter).

**Detection of apoptosis.** Apoptosis was quantified using an Annexin V-Phycoerythrin Apoptosis Detection kit (Medical and Biological Laboratories).

**5-Aza-2'-deoxycytidine treatment.** KMS12PE, KHM11, and RPMI8226 cells were treated with 250 or 500 nmol/L 5-aza-2'-deoxycytidine. Total RNA was extracted from the 5-aza-2'-deoxycytidine-treated cells and subjected to 30 cycles of RT-PCR for PU.1 and 25 cycles for GAPDH.

**Results**

**PU.1 is down-regulated in myeloma patients and myeloma cell lines.** Using real-time PCR, we found that PU.1 was completely down-regulated in five of seven myeloma lines (U266, KMS12PE, KHM4, KHM11, and KMM1), whereas the other two cell lines (KMS12BM and RPMI8226) contained similar levels of PU.1 mRNA to those observed in B-cell lines (Fig. 1A). Next, we evaluated whether PU.1 expression was also down-regulated in freshly isolated primary myeloma cells. To purify myeloma cells, we used an anti-CD138 antibody and magnetic bead isolation technique (Miltenyi Biotec). Compared with the relatively high levels of PU.1 expression in normal plasma cells, PU.1 was down-regulated to various levels in several myeloma patients (Fig. 1B). Although the myeloma cells purified with the anti-CD138 antibody and magnetic beads had purities of 90% to 95%, they nevertheless included contaminating granulocytes, monocytes/macrophages, and B cells, all of which express PU.1 at relatively high levels. Therefore, the patients with low PU.1 expression in Fig. 1C may have almost complete loss of PU.1 expression. To improve the purity of the primary myeloma cells, we did FACS of CD138-positive myeloma cells from patients and subjected these more highly purified populations to RT-PCR for PU.1. We also purified normal plasma cells, sorted as CD138-positive cells, from bone marrow samples from malignant lymphoma patients lacking invasion of lymphoma cells. The purities of the sorted myeloma cells and normal plasma cells were >95% (Fig. 1C). We did not detect any PU.1 expression after 35 cycles of PCR in purified myeloma cells from one myeloma patient, suggesting that at least some of the patients with low PU.1 expression do not have detectable PU.1 expression in their myeloma cells, similar to the majority of myeloma cell lines (Fig. 1C). In contrast, we detected relatively high expression levels of PU.1 in FACS-purified normal plasma cell samples. These data indicate that PU.1 is not down-regulated in normal plasma cells but is completely down-regulated in several myeloma patients.

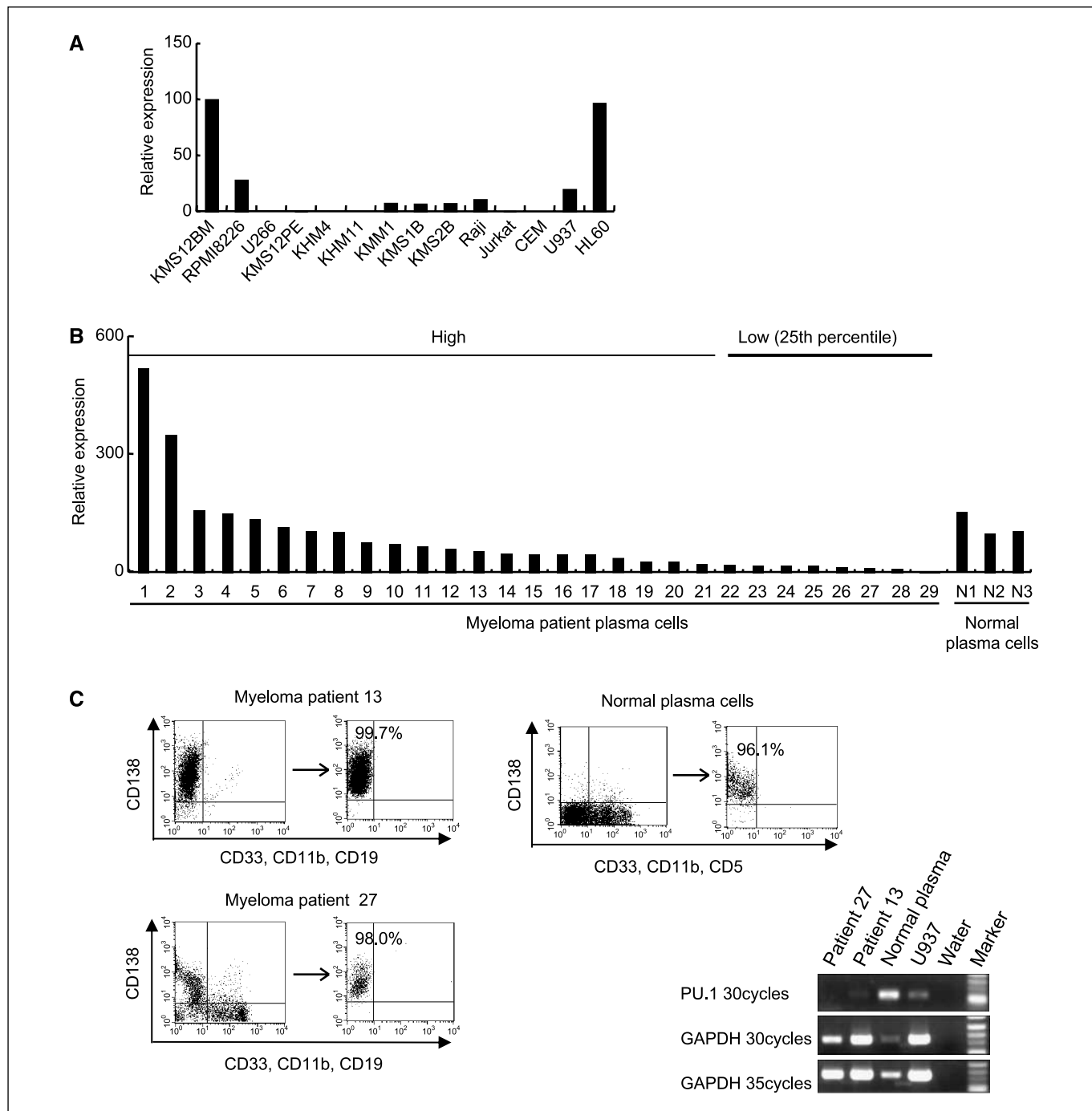
Two patients exhibited very high relative levels of PU.1 RNA in their myeloma cells (patients 1 and 2). Because the expression level of PU.1 was standardized to that of  $\beta$ -actin, it is possible that these high PU.1 expression levels might be artificially elevated as a result of low levels of  $\beta$ -actin. Indeed, we did not observe such high expression levels of PU.1 RNA in these patient samples using semiquantitative PCR (data not shown).

**Low PU.1 expression may identify a subset of myeloma patients a poor prognosis.** Based on their PU.1 expression levels, we provisionally divided the myeloma patients into two groups (i.e., PU.1 high and PU.1 low-to-negative) using a cutoff index of 25 (approximately corresponding to the lower 25th percentile of the PU.1 expression level distribution among all patients; PU.1 high was patient 21 and up and low-to-negative was patients 22-29) and evaluated the clinical features of the two subsets. There were no differences for the immunoglobulin subtypes, ages, or gender that were associated with PU.1 expression levels. In contrast, median survival was 5.6 months for PU.1 low-to-negative patients ( $n = 8$ ) and 27.0 months for the PU.1 high patients ( $n = 21$ ;  $P < 0.05$ ).

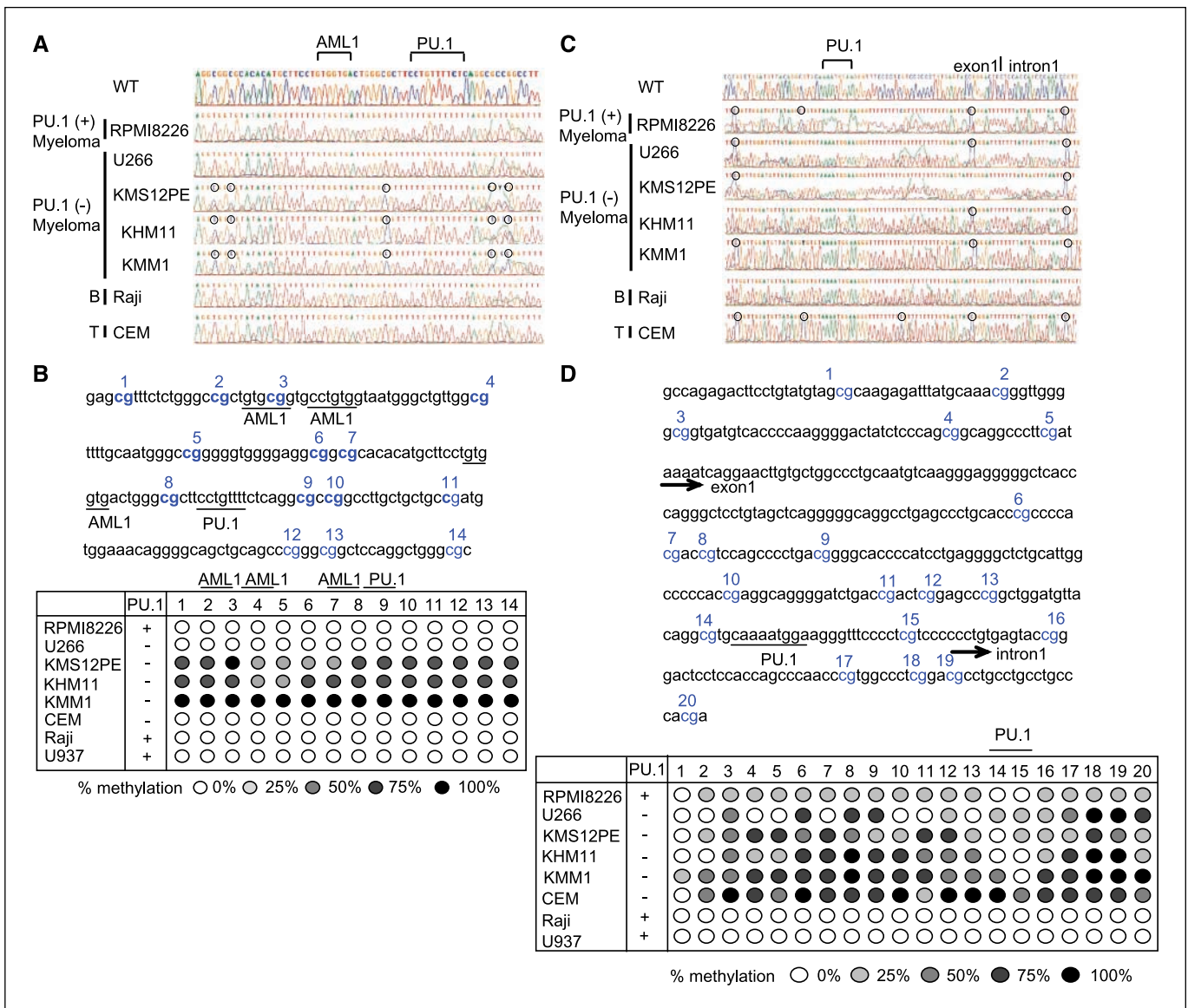
<sup>4</sup> <http://genome.ucsc.edu/cgi-bin/hgBlat>

However, the number of patients analyzed was small ( $n = 29$ ); therefore, additional follow-up studies will be required to conclude that loss of PU.1 expression is a prognostic factor in myeloma. These data suggest that down-regulated PU.1 expression might be related to an aggressive phenotype of myeloma or refractoriness to chemotherapeutic agents, including thalidomide and stem cell transplantation.

**Methylation of 17-kb 5' upstream cis-elements and the region from the PU.1 promoter to intron 1 of the PU.1 gene in myeloma cell lines.** PU.1 was reported to be down-regulated through methylation of intron 1 in T-cell lines (21). Therefore, we investigated whether the methylation status of the PU.1 gene could induce down-regulation of PU.1 gene expression. As mentioned earlier, we reported previously that a 14-kb 5' upstream regulatory



**Figure 1.** PU.1 is down-regulated in myeloma cell lines and freshly isolated primary myeloma cells. **A**, real-time PCR analysis of PU.1 expression in myeloma cell lines, B and T lymphocyte cell lines, and myeloid cell lines. **B**, real-time PCR analysis of PU.1 expression in freshly isolated primary myeloma cells. Taqman PCR was done using cDNAs from each cell line and patient sample. The PU.1 expression levels were normalized to the  $\beta$ -actin expression level. **C**, PU.1 expression in sorted myeloma cells or normal plasma cells was analyzed by 30 cycles of RT-PCR. CD138 single-positive cells were sorted thrice as purified myeloma cells or normal plasma cells. Compared with the relatively high expression of PU.1 in purified normal plasma cells, PU.1 is completely down-regulated in purified myeloma cells from one patient and relatively low in those from the other patient (*top left, top right, and bottom left*).



**Figure 2.** Methylation status of the PU.1 17 kb 5' upstream and promoter regions in human myeloma cell lines. **A**, direct sequencing of the PU.1 17-kb 5' upstream region using bisulfite-treated genomic DNA derived from myeloma cell lines. A PU.1-binding site and one of three AML1-binding sites are indicated. Methylated cytosines (○). **B**, methylation analysis of subcloned PCR products of the 17-kb 5' upstream region derived from bisulfite-treated genomic DNA. The CG sequences were numbered and their methylation statuses were determined. A total of four clones were sequenced and the results in each circle indicate the percentage methylation status. **C**, direct sequencing of the PU.1 promoter region using bisulfite-treated genomic DNA in myeloma cell lines. The PU.1-binding site at the translation start site is indicated (41). Methylated guanines (○). **D**, methylation analysis using subcloned PCR products of the promoter region derived from bisulfite-treated genomic DNA. The CG sequences were numbered and their methylation statuses were determined. A total of four clones of each cell line were sequenced and the results in each circle indicate the percentage methylation status.

*cis*-element, working in concert with the promoter, is critical for proper expression of the murine *PU.1* gene (6). Hence, we analyzed the 17-kb 5' upstream conserved region of the human *PU.1* gene, which is homologous to the 14-kb 5' upstream regulatory elements of the mouse *PU.1* gene. We identified a PU.1-binding site in this region that was critical for *PU.1* gene expression (6). We searched for CpG islands around the 17-kb 5' upstream conserved region and the promoter region and found CpG islands in the 17-kb 5' upstream conserved region itself and intron 1. We did bisulfite treatment of the genomic DNA and subsequently sequenced these regions. In three of four PU.1-negative myeloma cell lines (KMM1, KHM11, and KMS12PE), the 17-kb upstream region was highly methylated (Fig. 2A and B), whereas RPMI8226, a PU.1-positive

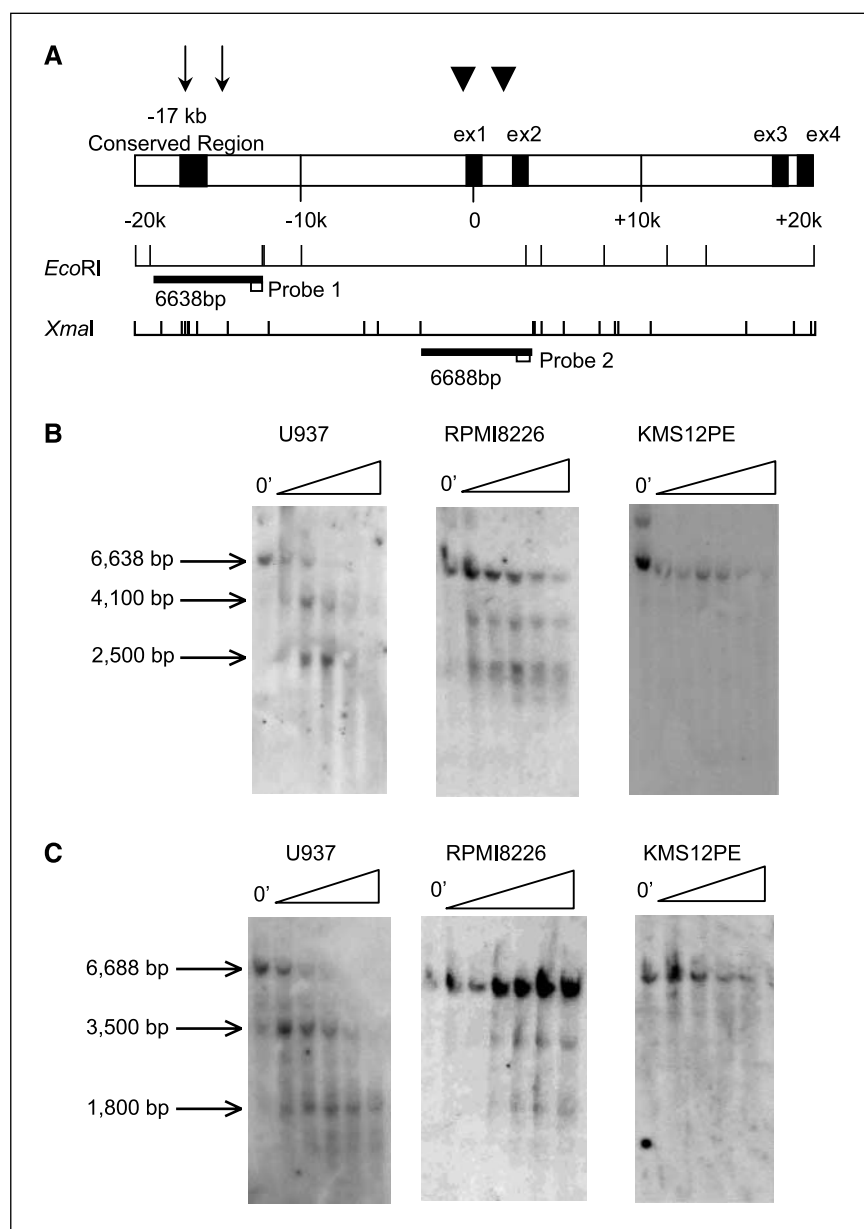
myeloma cell line, did not show methylation in this region. In contrast, the region from promoter to intron 1 was methylated in all myeloma cell lines examined (Fig. 2C and D). Therefore, the level of PU.1 expression may be highly dependent on the methylation status of the 17-kb 5' upstream conserved region in myeloma cells.

We further evaluated the chromatin structure in myeloma cell lines using a DHS assay. We first evaluated the -17-kb 5' upstream regulatory element using a probe detecting a 6.6-kb *EcoRI* fragment (Fig. 3A). In both the PU.1-positive myeloma cell line, RPMI8226, and the PU.1-positive myelomonocytic cell line, U937, we detected two additional bands (~4.1 and 2.5 kb in size) following DNase I treatment. These results show the presence of one DHS in the -17-kb

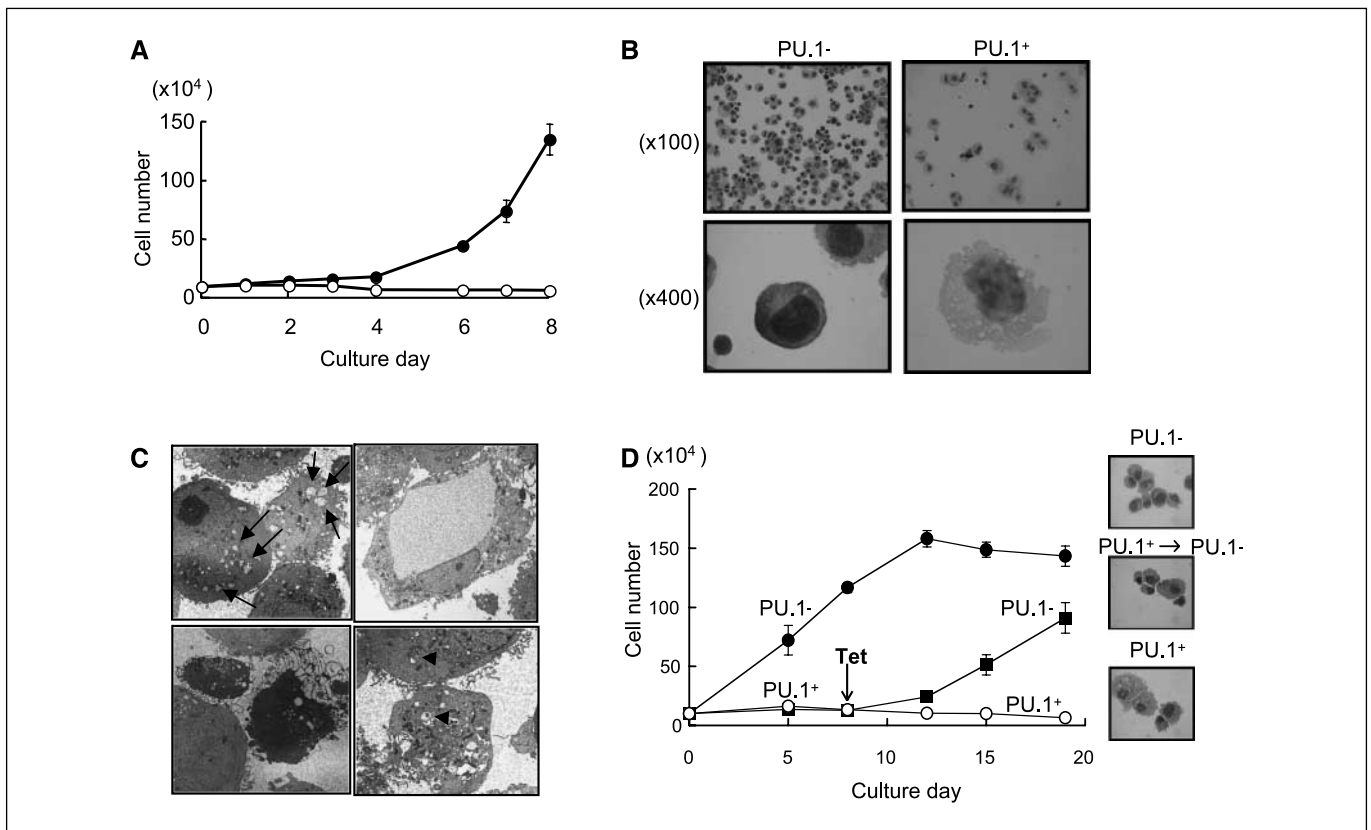
5' upstream regulatory region and the located ~15.5 kb upstream of the transcriptional start site (Fig. 3A and B). We also evaluated the region from the *PU.1* promoter to intron 1 with a probe detecting a 6.7-kb *Xma*I fragment (Fig. 3A). Both RPMI8226 and U937 cells showed an additional two bands (~3.5 and 1.8 kb in size), indicating the presence of DHS in the *PU.1* promoter and intron 1 (Fig. 3A and C). These data suggest that PU.1-positive RPMI8226 and U937 cells have an open chromatin structure in these regions, which is correlated with transcriptional activation. In contrast, there were no DHS in these regions in a PU.1-negative myeloma cell line, KMS12PE (Fig. 3B and C). Therefore, the methylation status in these regions highly correlated with the tightly closed chromatin context observed in PU.1-negative KMS12PE cells.

**Conditional expression of PU.1 in a PU.1-negative myeloma cell line induces cell growth arrest and cell death.** We next examined whether this down-regulation of PU.1 was related to

oncogenesis or growth advantage of myeloma cells. To achieve this, we generated a stable cell line, designated U266<sup>tetPU.1</sup>, with a "tet-off" inducible PU.1 expression system from the PU.1-negative cell line U266 (20). When PU.1 was expressed after removal of tetracycline, U266<sup>tetPU.1</sup> cells underwent complete cell growth arrest (Fig. 4A). Morphologically, some of these cells also underwent cell death, whereas others showed an enlarged cytosol and vacuoles around the nucleus (Fig. 4B). Electron microscopy observation revealed liquefaction and degeneration of mitochondria with vacuolization (Fig. 4C, top left, arrows). Some of these cells had large vacuoles, which possibly developed due to the liquefaction and degeneration of mitochondria (Fig. 4C, top right). There were also apoptotic cells with large complex vacuoles, which appeared to resemble the dilated endoplasmic reticulum that was abundant in myeloma cells (Fig. 4C, bottom left). Degeneration of mitochondria may induce leakage of cytochrome *c* into the cytosol, provoking activation of the apoptotic cascade. Some cells also contained autophagosomes (Fig. 4C, bottom



**Figure 3.** Loss of DHS in the 17-kb 5' upstream conserved region and the promoter region in PU.1-negative myeloma cell lines. **A**, diagram of the *PU.1* gene. The 17-kb 5' upstream conserved region and exons 1 to 4 (*ex1*–*ex4*) are shown as black bars. The restriction maps for *Xma*I and *Eco*RI are shown below. Probe 1 detected 6,638 bp length of *Eco*RI-digested DNA fragment containing –17-kb 5' upstream region, and probe 2 detected 6,688 bp length of *Xma*I-digested DNA fragment containing promoter, exon 1 and intron 1. DHS in the 17-kb 5' upstream conserved region (arrows) and the promoter (arrowheads) are based on the results shown in (B and C; arrows). **B** and **C**, DHS in the 17-kb 5' upstream region (B) and the *PU.1* promoter (C). The triangle above the lanes indicates increasing time of digestion of genomic DNA with DNase I. There are two DHS around both the *PU.1* 17-kb 5' upstream conserved region and the promoter region in the human myelomonocytic cell line U937 and PU.1-positive myeloma cell line RPMI8226 but not in the PU.1-negative myeloma cell line KMS12PE.



**Figure 4.** Conditional expression of PU.1 induces growth arrest and apoptotic cell death of U266 myeloma cells. *A*, conditional expression of PU.1 (○; without tetracycline) induces growth arrest of U266<sup>tetPU.1</sup> cells compared with their exponential growth without PU.1 (●; with tetracycline). Points, cell number (from three independent experiments); bars, SD. *B*, PU.1 induces morphologic changes and apoptosis in U266<sup>tetPU.1</sup> cells (*top right*) compared with control cells (*top left*). Higher magnification views reveal that PU.1 induced enlargement of the cytosol and nucleus and the formation of variable-sized vacuoles in myeloma cells (*bottom right*) compared with control cells (*bottom left*). *C*, ultrastructural morphology of U266<sup>tetPU.1</sup> cells conditionally expressing PU.1. PU.1 induces liquefaction and degeneration of mitochondria (*top left*; arrows) and the formation of large vacuoles (*top right*). Apoptotic cells also contain complex vacuoles (*bottom left*). Autophagy is also induced by PU.1, as indicated by an autophagosome in the cytosol (*bottom right*; arrowheads). *D*, growth arrest of U266<sup>tetPU.1</sup> cells is reversed by suppression of PU.1 expression. After a 7-d culture without tetracycline, U266<sup>tetPU.1</sup> cells begin to grow again following PU.1 suppression after the addition of tetracycline (■) compared with control cultures, in which PU.1 is expressed in the absence of tetracycline (○). Right, morphologies of U266<sup>tetPU.1</sup> cells. Morphologic changes are reversible after suppression of PU.1.

*right*), suggesting autophagic cell death (22–25). Taken together, these data indicate that the inducible expression of PU.1 mediates growth arrest and apoptosis through degradation of mitochondria and/or the autophagic cell death pathway. In contrast, when PU.1 expression was completely suppressed again by tetracycline addition at 7 days after its original removal, the cells began growing with a normal morphology, indicating that the cell growth arrest and morphologic changes of U266<sup>tetPU.1</sup> cells induced by PU.1 were reversible phenomena (Fig. 4D). We isolated three independent U266<sup>tetPU.1</sup> cell lines, and all three clones showed the same phenotype after PU.1 expression.

We also generated another stable cell line, designated KMS12PE<sup>tetPU.1</sup>, with the same “tet-off” inducible PU.1 expression system from the PU.1-negative cell line KMS12PE. Expression of PU.1 also induced complete growth arrest of all four independent KMS12PE<sup>tetPU.1</sup> clones (Supplementary Fig. S1A). Morphologically, these cells also contained vacuoles in their cytosol, similar to the case for U266<sup>tetPU.1</sup> cells (Supplementary Fig. S1B). Therefore, we conclude that PU.1 down-regulation is essential for the growth and survival of two independent human myeloma cell lines.

Next, we evaluated which stage in the cell cycle of U266<sup>tetPU.1</sup> cells is affected by the induction of PU.1 expression. M phase cells were decreased from 19.2% to 8.4% after the induction of PU.1

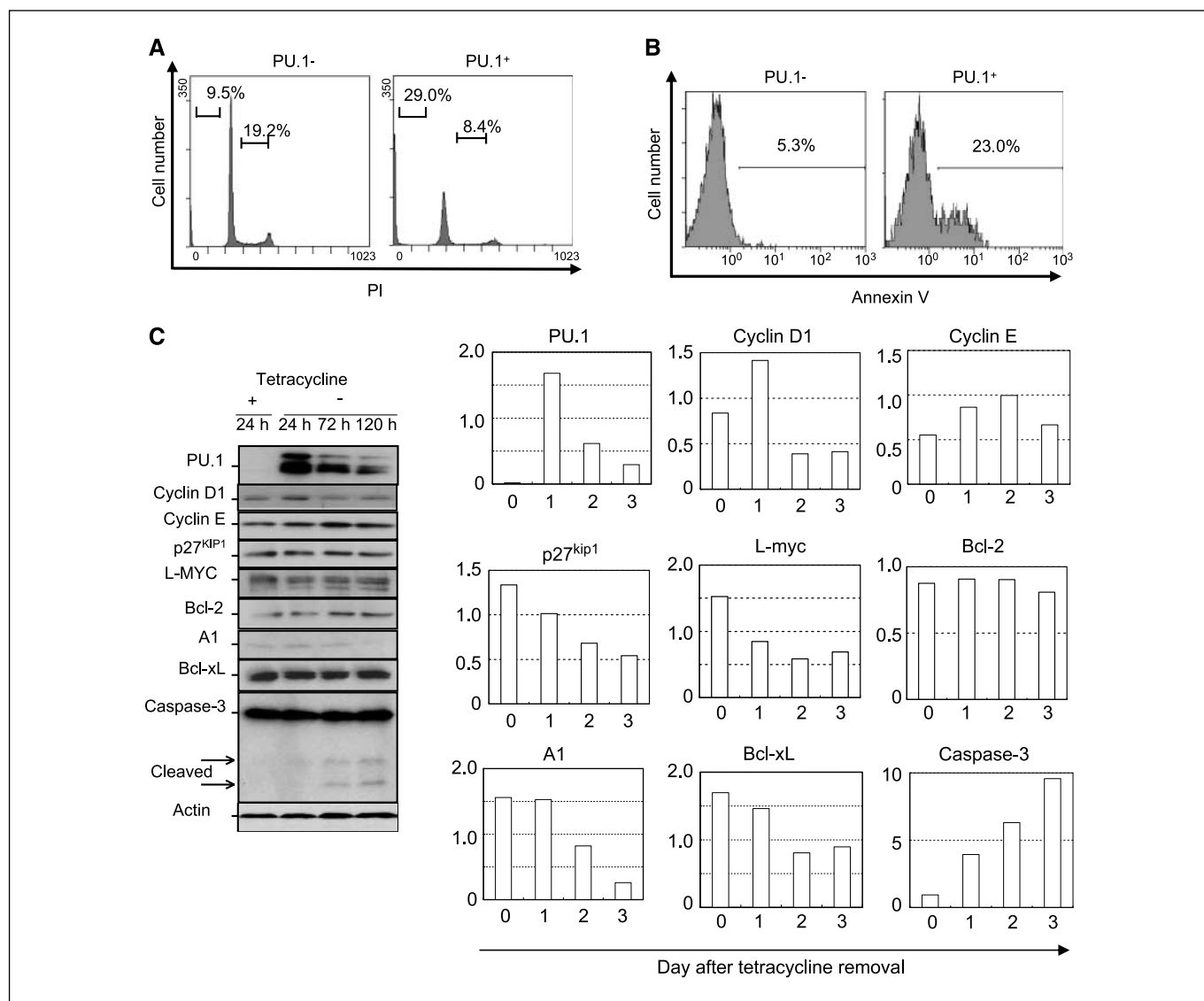
expression, indicating that the cells underwent G<sub>1</sub> arrest (Fig. 5A). There was also an increase in the number of apoptotic cells in the sub-G<sub>1</sub> phase from 9.5% to 29.0%. The induction of apoptosis of U266<sup>tetPU.1</sup> cells by PU.1 was confirmed by the increased number of Annexin V–positive cells from 5.3% to 23.0% (Fig. 5B). KMS12PE<sup>tetPU.1</sup> cells also underwent G<sub>1</sub> arrest and apoptosis after PU.1 induction (data not shown). We further evaluated the levels of cell cycle- and apoptosis-related proteins before and after PU.1 induction in U266<sup>tetPU.1</sup> cells. Among cell cycle-related genes, cyclin D1 and L-myc, which is activated by translocations to IgH loci in U266 cells (26), were down-regulated to ~50% of the original levels after PU.1 induction (Fig. 5C, *right*). In contrast, among apoptosis-related genes, A1 and Bcl-xL were down-regulated to <50% of the original levels, and cleavage of caspase-3 was increased after PU.1 induction (Fig. 5C). The decreased protein expression of cell cycle-related and antiapoptotic genes are relevant to the arrest in cell growth and apoptosis of U266<sup>tetPU.1</sup> cells following induction of PU.1 expression, albeit these expression changes could represent indirect effects of PU.1 expression in U266<sup>tetPU.1</sup> cells.

**Up-regulation of PU.1 in myeloma cell lines by 5-aza-2'-deoxycytidine also induces growth arrest and apoptosis of myeloma cells.** Because PU.1 was down-regulated by methylation in myeloma cell lines and up-regulation of PU.1 induced growth arrest

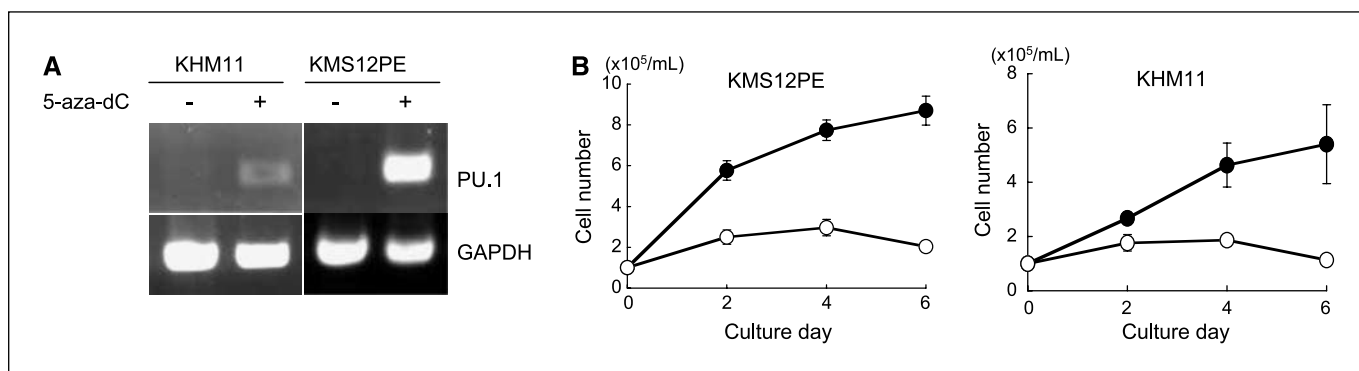
and apoptosis of myeloma cells, we next investigated whether a demethylation agent, 5-aza-2'-deoxycytidine, could induce the same effects in myeloma cells through PU.1 induction. Treatment of KMS12PE and KHM11 myeloma cells, which were PU.1 negative and had a heavy methylation status of the 17-kb 5' upstream regulatory element and the region from the *PU.1* promoter to intron 1, with 5-aza-2'-deoxycytidine induced PU.1 expression and growth suppression (Fig. 6A and B). These data indicate that induction of PU.1 by 5-aza-2'-deoxycytidine may also induce cell growth arrest and apoptosis of myeloma cells, although it remains possible that some other genes induced by 5-aza-2'-deoxycytidine may mainly induce these effects. Nevertheless, these data suggest that more powerful inducers of PU.1, including demethylation agents, should be effective tools for treating myeloma patients.

## Discussion

Our present results show the importance of loss of PU.1 expression for the maintenance of myeloma cell growth and survival. Specifically, we found that (a) in a subset of myeloma patients, PU.1 is down-regulated as in the majority of myeloma cell lines, and PU.1 low-to-negative patients may have a poorer prognosis than PU.1 high patients; (b) PU.1 is down-regulated through methylation of the 17-kb 5' enhancer region and the promoter; and (c) exogenous introduction of PU.1 expression or induction of PU.1 by 5-aza-2'-deoxycytidine treatment in the PU.1-negative cell lines U266, KMS12PE, and KHM11 induces growth arrest and apoptotic cell death. Therefore, we hypothesize that there is a subset of myeloma patients with low to negative levels of PU.1 expression. Furthermore, we propose that down-regulation of



**Figure 5.** Expression of PU.1 evokes growth arrest in the G<sub>1</sub>-G<sub>0</sub> phase and apoptosis of U266<sup>tetPU.1</sup> cells. A, conditional expression of PU.1 induces G<sub>1</sub> arrest in U266<sup>tetPU.1</sup> cells. After conditional expression of PU.1, cells in M phase are decreased and sub-G<sub>1</sub> cells, which represent apoptotic cells, are increased (right) compared with control cultures (left). B, conditional expression of PU.1 induces apoptosis of U266<sup>tetPU.1</sup> cells. After conditional expression of PU.1, Annexin V-positive apoptotic cells increase from 5.3% [tetracycline positive (+)] to 23.0% [tetracycline negative (-)] at 2 d after induction of PU.1 expression. C, down-regulation of cell cycle-related and antiapoptotic proteins and the cleavage of caspase-3 in U266<sup>tetPU.1</sup> cells after PU.1 induction. Western blot analyses of cell cycle-related and apoptosis-related genes in U266<sup>tetPU.1</sup> cells before and after PU.1 induction. Right, based on densitometry of the (left). The relative ratio of protein to  $\beta$ -actin was calculated by densitometry for each day except caspase-3. For caspase-3, the percentage of cleaved caspase-3 to total caspase-3 is shown.



**Figure 6.** Cell growth arrest of KMS12PE and KHM11 myeloma cells is induced by 5-aza-2'-deoxycytidine (5-aza-dC) treatment through up-regulation of PU.1. **A**, PU.1 expression is restored by 5-aza-2'-deoxycytidine treatment. RT-PCR was done for PU.1 and 25 cycles for GAPDH using almost equal amounts of cDNA. **B**, restoration of PU.1 expression by 5-aza-2'-deoxycytidine induces growth arrest of KMS12PE and KHM11 myeloma cells. Induction of PU.1 (○; with 500 nmol/L 5-aza-2'-deoxycytidine) induces growth arrest of KMS12PE and KHM11 cells compared with their exponential growth without 5-aza-2'-deoxycytidine (●). Points, cell number (from three independent experiments); bars, SD.

PU.1 through methylation represents an important genetic event for oncogenesis and/or growth advantage of myeloma cells. To understand the meaning of the down-regulation of PU.1 expression in myeloma cells, we need to know the function of PU.1 in normal plasma cells. We reported previously that PU.1 has a tumor suppressor activity for leukemogenesis because knockout of the 14-kb 5' regulatory elements of the mouse *PU.1* gene leads to down-regulation of PU.1 to 20% of the WT level and subsequent leukemia in mice (9). Recently, it is reported that conditional knockout of PU.1 in early B-cell precursors does not affect their further maturation (13). Nevertheless, the function of PU.1 during the late stage of B-cell lymphopoiesis, including the plasma cell stage, is unclear. Our present data suggest that PU.1 may be necessary for the growth arrest and apoptotic pathways that prevent uncontrolled growth of plasma cells and that disruption of these pathways may lead to multiple myeloma. We are currently investigating the mechanisms that evoke the growth arrest and cell death of myeloma cell lines after PU.1 induction. We are also trying to generate mice with conditional knockout of PU.1 only in plasma cells.

Proper gene expression *in vivo* generally requires not only the promoter region but also *cis*-elements in introns and/or distal 5' regions and/or 3' regions. Nevertheless, it is usually the case that methylation studies are only done for CpG islands in promoter regions to explain the silencing of tumor suppressor genes or genes related to growth arrest or apoptosis. In myeloma cells, the promoter regions of tumor suppressor genes, including *p15*, *p16*, *SHP-1*, and *SOCS1*, are highly methylated (27–33). In the present study, we detected methylation of CpG islands within the 17-kb 5' upstream highly conserved region in addition to the promoter. This is the first report to show that methylation of such distal *cis*-elements critical for gene expression may contribute to tumor formation in humans.

Recently, a study involving fluorescence *in situ* hybridization identified translocations of the IgH locus, including t(4;14), t(6;14), t(11;14), t(14;16), and t(14;20), in monoclonal gammopathy of undetermined significance (MGUS), myeloma patients, and mye-

loma cell lines that directly dysregulate FGFR3 and MMSET, cyclin D3 and IRF4, cyclin D1, c-MAF, and MAFB, respectively (34–39). The prevalences of the IgH translocations increase according to the myeloma disease stages (50% in MGUS, 55–73% in multiple myeloma, and 90% in myeloma cell lines), and some of them are closely related to a poor prognosis (40). We did not recognize PU.1 down-regulation in MGUS patients (data not shown), whereas PU.1 was down-regulated in myeloma cell lines, specifically KHM11 with t(4;14), KMM1 with t(6;14), and U266 and KMS12PE with t(11;14). These data suggest that PU.1 down-regulation may not be an early genetic event for oncogenesis of myeloma, but rather a genetic alteration dependent on disease progression and a poor prognosis, although it remains possible that down-regulation of PU.1 may be an initial event leading to oncogenesis in some cases of myeloma.

Our present results have shown that expression of PU.1 in PU.1-negative myeloma cells can induce growth arrest and cell death of these cells, suggesting that reintroduction of PU.1 may represent a novel molecularly targeted therapeutic strategy for myeloma patients. We have further shown that demethylation agents, such as 5-aza-2'-deoxycytidine, can restore PU.1 expression and suppress the growth of some myeloma cells, although 5-aza-2'-deoxycytidine should activate many other genes that may also induce cell growth arrest and apoptosis. Therefore, more effective derivatives or other type of agents that specifically increase PU.1 expression levels may represent new therapeutic agents for aggressive myeloma.

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## Down-regulation of PU.1 by Methylation of Distal Regulatory Elements and the Promoter Is Required for Myeloma Cell Growth

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