Cancer Science
The official journal of the Japanese Cancer Association

Hmga1 is differentially expressed and mediates silencing of the *CD4/CD8* loci in T cell lineages and leukemic cells

Yang Xi,[1] Sugiko Watanabe,[1] Yuko Hino,[1] Chiyomi Sakamoto,[1] Yuko Nakatsu,[1] Seiji Okada[2] and Mitsuyoshi Nakao[1,3]

[1]Department of Medical Cell Biology, Institute of Molecular Embryology and Genetics, and Global Center of Excellence ''Cell Fate Regulation Research and Education Unit''; and [2]Okada Project Laboratory, Center for AIDS Research, Kumamoto University, Kumamoto, Japan

(Received October 5, 2011/Revised November 11, 2011/Accepted November 19, 2011/Accepted manuscript online November 22, 2011/Article first published online December 28, 2011)

High-mobility group A1 (Hmga1) protein is an architectural chromatin factor, and aberrant Hmga1 expression in mice causes hematopoietic malignancies with defects in cellular differentiation. However, the functional involvement of Hmga1 in hematopoietic development and leukemic cells remains to be elucidated. Using *Hmga1-green fluorescent protein* (*GFP*) knock-in mice that endogenously express an Hmga1-GFP fusion protein, we examined Hmga1 expression in undifferentiated and differentiated populations of hematopoietic cells. During early T cell development in the thymus, Hmga1 is highly expressed in CD4/CD8-double negative (DN) cells and is transiently downregulated in CD4/CD8-double positive (DP) cells. Consistently, Hmga1 directly binds to *cis*-regulatory elements in the *CD4/CD8* loci and the heterochromatin foci in DN-stage cells, but not in DP cells. Interestingly, CD4/CD8 expression in DN-stage leukemic cells is induced by inhibition of Hmga1 binding to nuclear DNA or RNA interference-mediated Hmga1 knockdown. In addition, Hmga1-depleted leukemic T cells markedly diminish proliferation, with transcriptional activation of cyclin-dependent kinase inhibitor genes as a direct target of Hmga1. The data in the present study reveal a role of Hmga1 in transcriptional silencing in T cell lineages and leukemic cells. (*Cancer Sci* 2012; 103: 439–447)

Hematopoietic development has been investigated extensively through identification of stage-specific cell surface markers, as well as multiple transcription factors, which play an important role in establishing lineage diversity.[1–3] Recent studies have emphasized that the cell-fate decision is ultimately determined by the chromatin and nuclear machineries that affect protein-DNA structures and histone modifications.[1,2,4,5] Therefore, it is crucial to investigate the involvement of DNA/chromatin-binding factors to understand key events during hematopoietic differentiation and malignant transformation.

High-mobility group A1 protein (Hmga1) is a non-histone architectural chromatin protein and is characterized by the presence of three AT-hook DNA-binding motifs that preferentially bind AT-rich DNA.[6–10] Hmga1 binding to DNA acts as the architectural structure, which alters the chromatin conformation of target DNA and facilitates the assembly of a high-order multiprotein transcription complex called enhanceosome on transcriptionally active promoter/enhancer regions in specific genes.[11–15] However, Hmga1 is present in the nucleus and predominantly localized to condensed chromatin, suggesting that the major sites of Hmga1 accumulation are not transcriptionally active in somatic cells.[16,17] Thus, Hmga1 possesses distinct and independent biological functions within cells.

The *Hmga1* gene is highly expressed in early embryos, whereas its expression is commonly downregulated in differentiated cells, suggesting its biological role during mammalian development.[18] *Hmga1* haploinsufficiencies in mice cause myeloid leukemia and B cell lymphoma in the peripheral spleen.[19] Conversely, mice with *Hmga1* overexpression develop T and natural killer cell lymphomas.[20,21] In addition, altered hematopoietic differentiation that leads to reduced T cell precursors and preferential B cell differentiation is observed in *Hmga1* null mouse embryonic stem cells.[22] Thus, Hmga1 possesses a crucial role in hematopoietic development and malignant transformation. However, the molecular mechanism of Hmga1 that regulates hematopoietic cells remains to be elucidated.

Hmga1 overexpression is a common feature in the transformation and progression of human cancers, including leukemia and lymphoma.[20,23–26] In our previous study, we showed that Hmga1 overexpression can abolish the retinoblastoma protein (RB) function through direct binding in glioblastoma cells.[27] Furthermore, an anti-apoptotic function has been suggested through a direct Hmga1-p53 interaction with both proteins overexpressed *in vitro*.[28] Because the loss of RB protein and/or the p53 mutations are frequently found in leukemia and lymphoma cells,[29] Hmga1 overexpression may have an uncovered role in hematopoietic tumors.[20,24]

Using *Hmga1-green fluorescent protein* (*GFP*) knock-in mice that express an Hmga1-GFP fusion protein,[25] here we found that Hmga1 has a distinct expression level and mediates silencing of the *CD4/CD8* loci during early T cell development in the thymus. We also demonstrate that Hmga1-depleted T leukemic cells decrease proliferation, together with transcriptional activation of the cyclin-dependent kinase (Cdk) inhibitor genes *p21* and *p27*. Collectively, our results suggest that Hmga1 is actively involved in the transcriptional silencing of these genes in T cell lineages and leukemic cells.

Materials and Methods

Animals. *Hmga1-EGFP* knock-in mice were prepared as previously described.[25] All procedures and protocols were approved by the Committee on Animal Research at Kumamoto University, Japan.

Cell culture and treatments. Human T cell acute lymphoblastic leukemia (T-ALL) Jurkat cells and mouse thymoma EL4 cells were cultured in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% (v/v) heat-inactivated FBS and 50 μM β-mercaptoethanol. To study inhibition of Hmga1 binding to DNA, EL4 cells (2×10^5 cells/mL) were treated with Hoechst 33342 (0.1, 0.2 and 0.5 μg/mL) (Dojindo, Kumamoto, Japan) or Hoechst 33258 (1.0, 2.5 and 5.0 μg/mL) (Dojindo) for 24 h.

[3]To whom correspondence should be addressed.
E-mail: mnakao@gpo.kumamoto-u.ac.jp

doi: 10.1111/j.1349-7006.2011.02159.x

Antibodies and cell sorting. The following anti-mouse antibodies were used for flow cytometry analyses: APC-conjugated CD4 (GK1.5), B220 (RA3-6B2), TER-119 (TER-119), Gr-1 (RB6-8C5), CD3ε (145-2C11), PE-conjugated CD4 (GK1.5), CD3 (17A2), CD8α (53-6.7), CD19 (6D5), CD71 (RI7217), Mac-1 (M1/70) and PE/Cy7-conjugated CD45 (30-F11). All antibodies were purchased from Biolegend (San Diego, CA, USA). Flow cytometry data were generated using FACSCanto or FACSAria II (BD Bioscience, Franklin Lakes, NJ, USA) and analyzed using FlowJo software (Tree Star, Ashland, OR, USA). FACS was performed using FACSAria II. CD4/CD8-double negative (DN) ($CD3^-$ $CD4^-$ $CD8^-$) cells and CD4/CD8-double positive (DP) ($CD4^+$ $CD8^+$) cells were collected with a purity of more than 95%.

***In vitro* differentiation of CD4/CD8-double negative cells.** Sorted DN cells were cultured on more than 90% confluent OP9 or OP9-DL1 cells in RPMI-1640 medium supplemented with 10% FBS, 1 ng/mL IL-7 (402-ML) and 5 ng/mL FLT3-L (308-FK). IL-7 and FLT3-L were purchased from R&D Systems (Minneapolis, MN, USA). Cells were harvested and analyzed by flow cytometry after 7 days.

Quantitative reverse transcription-polymerase chain reaction. Two micrograms of the total RNA were reverse transcribed using a High Capacity cDNA Reverse Transcription kit (Applied

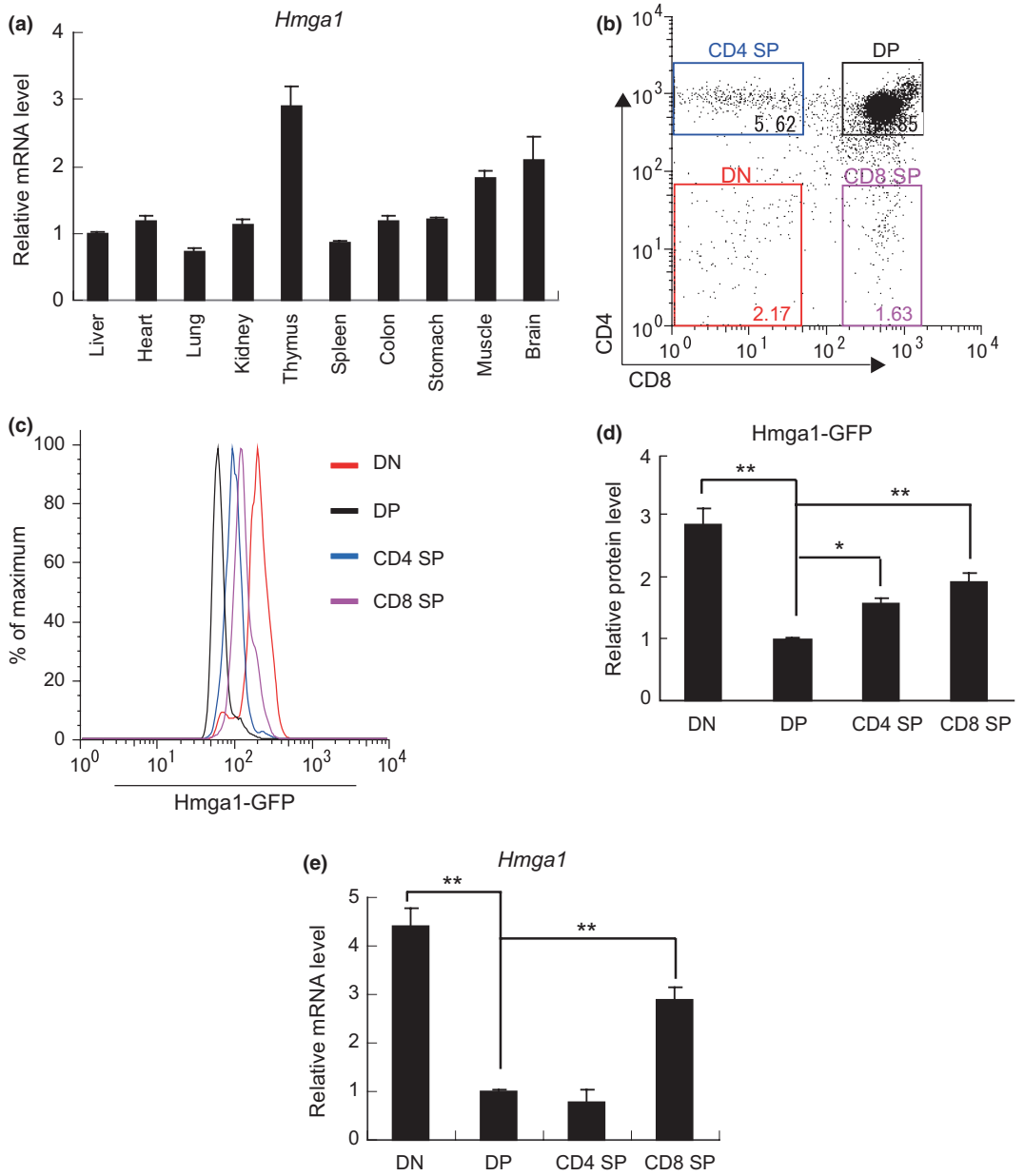

Fig. 1. Differential Hmga1 expression in T cell subpopulations from the mouse thymus. (a) Expression of *Hmga1* in tissues from wild-type mice. qRT-PCR analysis was performed and *Hmga1* expression in the liver was normalized to 1. Results are representative of more than three independent experiments. (b) The CD4/CD8 profiles in thymocytes from *Hmga1-green fluorescent protein* (*GFP*) knock-in (KI) mice. The numbers indicate the percentages of positive cells within the population. (c) Overlay of Hmga1-GFP levels in the subpopulations of thymocytes from *Hmga1-GFP* KI mice. (d) Quantitation of Hmga1-GFP levels in (c). The level of Hmga1-GFP in DP cells was normalized to 1. Values are given as means and standard deviations from more than six mice. (e) Quantitation of *Hmga1* mRNA levels in the subpopulations of thymocytes from WT mice. The level of *Hmga1* in DP cells was normalized to 1. Values are given as means and standard deviations from three independent experiments. Asterisks indicate statistically significant differences compared with that of DP cells (*$P < 0.05$, **$P < 0.01$). DN, CD4/CD8-double negative cells; DP, CD4/CD8-double positive cells.

doi: 10.1111/j.1349-7006.2011.02159.x

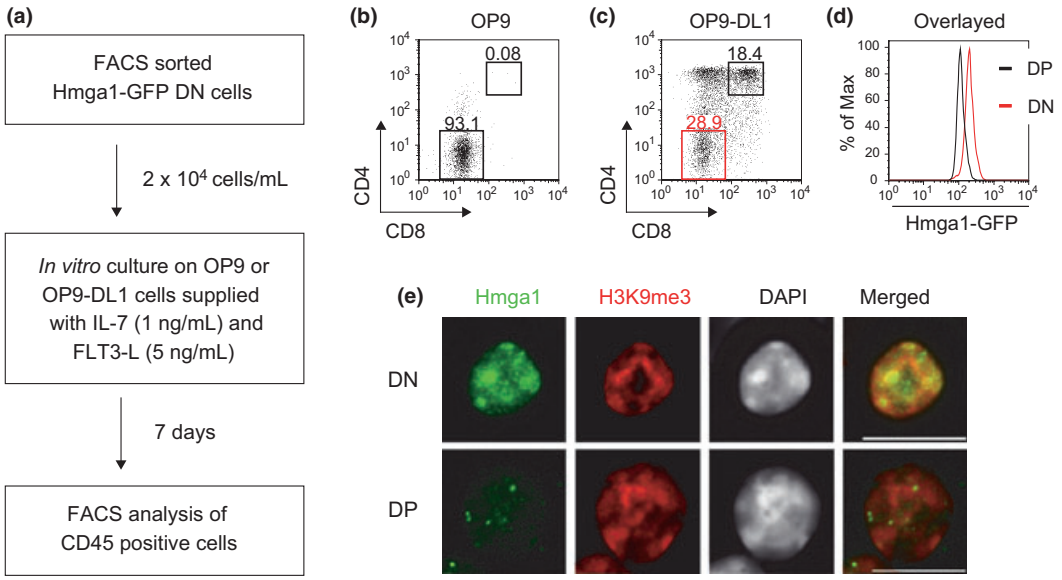

Fig. 2. Downregulation of Hmga1 during CD4/CD8-double negative cell (DN) to CD4/CD8-double positive cell (DP) differentiation of thymocytes. (a) Schematic procedure of *in vitro* differentiation of DN ($CD3^-$ $CD4^-$ $CD8^-$) cells from *Hmga1-green fluorescent protein* (*GFP*) knock-in mice. (b,c). Flow cytometry analyses of DN cells cultured on stromal cells OP9 (b) or OP9-DL1 that provided Notch-1 signaling (c) for 7 days. The numbers indicate the percentages of cells within the population. (d) Overlay of Hmga1-GFP levels in DN and DP subpopulations shown in (c). (e) Colocalization of highly expressed Hmga1 with H3K9me3-stained heterochromatin in DN cells from wild-type mice. An immunofluorescence analysis using anti-Hmga1 (green) and anti-trimethyl-histone H3 at lysine 9 (H3K9me3) antibodies (red) was performed using DN and DP cells. DNA was counterstained with DAPI. Scale bar represents 10 μm.

Biosystems, Foster City, CA, USA). For quantification, real-time PCR analysis was performed using a Power SYBR Green PCR Master Mix on an ABI Prism 7500 Sequence Detector (Applied Biosystems). The relative fold changes were quantified using the comparative threshold cycle method, and *β-actin* was used as an internal control. Primers are listed in Table S1.

Chromatin immunoprecipitation and quantitative PCR analysis. Chromatin immunoprecipitation (ChIP) was performed as described previously.[30] Briefly, EL4 cells (1.5×10^6) were cross-linked with 1% formaldehyde, and crude cell lysates were sonicated to generate DNA fragments of 200–500 bp. ChIP was performed with anti-HMGA1 (Active Motif, Carlsbad, CA, USA) and control IgG (Santa Cruz Biotechnology, CA, USA). DNA enrichment was determined by real-time PCR using a Power SYBR Green PCR Master Mix on an ABI Prism 7500 Sequence Detector. Statistical analysis was performed using Student's *t*-test and $P < 0.05$ was considered statically significant. Primers are listed in Table S2.

Plasmid construction and transfection. Mouse *Hmga1* shRNA and human *HMGA1* shRNA were cloned into pSIREN-Shuttle (Clontech Laboratories, Mountain View, CA, USA) and pSilencer-3.1-H1 (Ambion, Austin, TX, USA), respectively. ShRNA sequences were as follows: mouse *Hmga1*, 5′-AAACUGGAGAAGGAGGAAGAG TT-3′; and human *HMGA1*, 5′-AAGUGCCAACUCCGAAGAGACC TT-3′. Transfection was performed using the MicroPorator (Digital Bio, Tokyo, Japan) according to the manufacturer's instructions.

Western blot analysis. Cell Lysates were performed and separated, as described previously.[27] Primary antibodies were used against HMGA1 (Active Motif) and β-tubulin (Sigma).

Immunofluorescence analysis. After being attached to aminopropylsilane-coated glass slides using Cytospin (Thermo Scientific, Waltham, MA, USA), cells were treated with 0.5% Triton X-100 in cytoskeleton buffer,[31] fixed with 4% paraformaldehyde and post-fixed with 100% methanol. Primary antibodies were used against HMGA1 (Santa Cruz) and trimethyl-histone H3 at Lys 9 (H3K9me3) (Millipore, Billerica, MA, USA). The images were visualized with an Olympus IX171 microscope using Lumina Vision software (version 2.2; Mitani Corporation, Tokyo, Japan).

Results

Hmga1 is highly expressed in CD4/CD8-double negative cells and is transiently downregulated in CD4/CD8-double positive cells. Our initial analyses using bone marrow from $Hmga1^{GFP/+}$ heterozygous mice showed at least four subpopulations expressing distinct levels of Hmga1-GFP, which was dominantly expressed in immature hematopoietic cells (Fig. S1). We then analyzed Hmga1 expression in the thymus, because quantitative RT-PCR analyses showed that *Hmga1* is highly expressed in the thymus in comparison with other tissues (Fig. 1a). We divided the T cells into four stage-specific populations based on standard cell surface antigens, CD4 and CD8 (Fig. 1b). As shown in Figure 1c, Hmga1-GFP expression was high in DN cells, and was downregulated in DP cells. Quantitative analysis showed that the Hmga1-GFP level in DN cells was threefold higher compared with that of DP cells (Fig. 1d). Interestingly, Hmga1-GFP levels in CD4 SP and CD8 SP cells significantly increased compared with that of DP cells, which exemplifies an exceptional case of differentiated cells possessing higher Hmga1 expression. Similar Hmga1 expression data were obtained in wild-type control mice (Fig. 1e), suggesting the involvement of Hmga1 in T cell development.

Hmga1 expression decreases during CD4/CD8-double negative cell to CD4/CD8-double positive cell differentiation *in vitro*. To confirm whether Hmga1 was downregulated during differentiation from DN to DP stages, we performed an *in vitro* differentiation assay using DN cells sorted from the thymus of *Hmga1-GFP* mice (Fig. 2a). As a control, DN cells cultured on OP9 stromal cells were not differentiated to DP cells (Fig. 2b). DN cells cultured on OP9-DL1 cells, which support T cell differentiation by Notch-1 signaling, resulted in 18.4% of the population differentiating into DP cells (Fig. 2c). *In vitro* differentiated DP cells exhibited significantly lower Hmga1-GFP expression compared with that of DN cells (Fig. 2d), indicating that changes in Hmga1 expression are dependent on DN or DP stages.

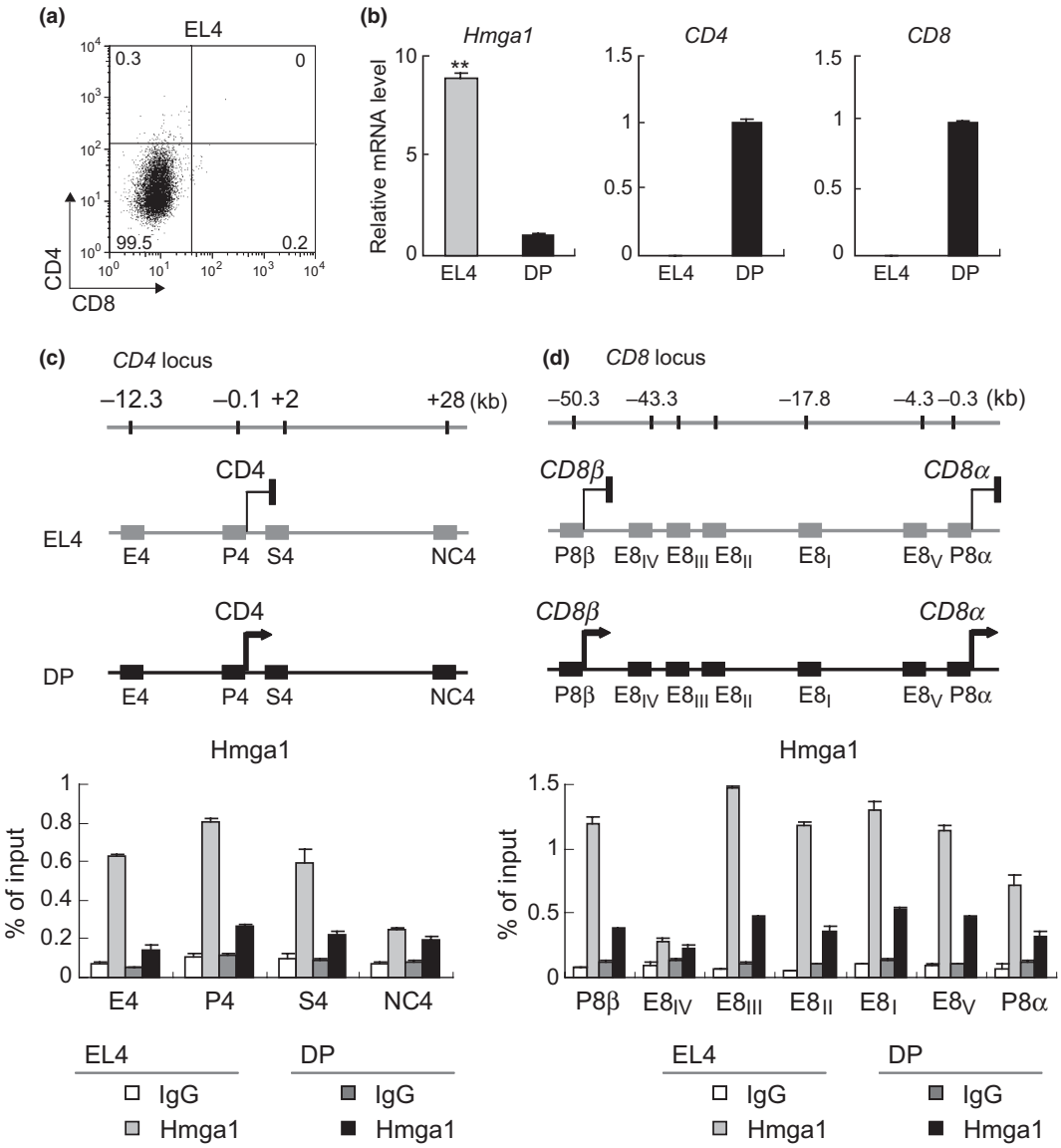

Fig. 3. Binding of Hmga1 to the *CD4/CD8* loci in CD4/CD8-double negative cell (DN)-type EL4 cells. (a) The DN stage of mouse thymoma EL4 cells. Flow cytometry analysis was performed using CD4 and CD8. The numbers indicate the percentage of cells within the population. (b) Expression status of *Hmga1*, *CD4* and *CD8* in EL4 and mouse CD4/CD8-double positive cell (DP) cells. The mRNA level in DP cells was normalized to 1. (c) Binding of Hmga1 to the *CD4* locus in EL4 cells. Chromatin immunoprecipitation-qPCR analysis was performed using EL4 and DP cells sorted by FACS (c,d). The *cis*-elements in the *CD4* locus and transcriptional status are indicated in EL4 and DP cells (upper panel). E4, enhancer; P4, promoter; S4, silencer; NC4, negative control, which is a region with high GC content. The numbers indicate the distance to the transcription start site of *CD4*. NC4 and the promoter region of the *Gapdh* gene (shown in Fig. 6e) were used as a negative control for Hmga1 binding to DNA. (d) Binding of Hmga1 to the *CD8* locus in DN cells. In the upper panel, promoters and stage-specific enhancers at the *CD8* locus and transcriptional status are indicated in EL4 and DP cells. P8β, promoter of *CD8β*; P8α, promoter of *CD8α*; $E8_I$, $E8_{II}$, $E8_{III}$, $E8_{IV}$, $E8_V$, stage-specific enhancers. The numbers indicate the distance to the transcription start site of *CD8α*.

Using an immunofluorescence analysis, we further investigated Hmga1 expression and localization in DN and DP cells (Fig. 2e). Highly expressed Hmga1 coexisted with trimethyl-histone H3 at lysine 9 (H3K9me3) and DAPI-stained heterochromatin in DN cells, while Hmga1 was significantly diminished in DP cells. Previous reports have shown that the silenced *CD4/CD8* loci are associated with the intranuclear position that is close to the heterochromatin in DN thymocytes.[32,33] Collectively, our data suggest that Hmga1 negatively regulates *CD4/CD8* expression in DN cells.

Hmga1 binds to the *CD4/CD8* loci in CD4/CD8-double negative cells, but not in CD4/CD8-double positive cells. To address whether Hmga1 directly represses *CD4/CD8* genes, we analyzed Hmga1 binding to the *CD4/CD8* loci using ChIP assays. Because DN cells generally have a very small population in the thymus, we chose mouse thymoma EL4 cells to obtain enough cells for the assay. We confirmed that EL4 cells are double negative for CD4/CD8 expression and highly expressed Hmga1 (Fig. 3a,b).

Hmga1 bound to the three *cis*-elements (E4 enhancer, P4 promoter and S4 silencer) in the *CD4* locus in EL4 cells (Fig. 3c), whereas no enrichment at the locus was detected in DP cells. With the exception of $E8_{IV}$, Hmga1 also bound to the stage-specific enhancers $E8_I$, $E8_{II}$, $E8_{III}$ and $E8_V$, as well as the *CD8B* (P8β) and *CD8A* (P8α) promoters in the *CD8* locus in EL4 cells (Fig. 3d). However, DP cells mostly showed decreased Hmga1 binding that was equivalent to the negative control levels, although Hmga1 to some extent bound to $E8_I$, $E8_{III}$ and $E8_V$,

doi: 10.1111/j.1349-7006.2011.02159.x

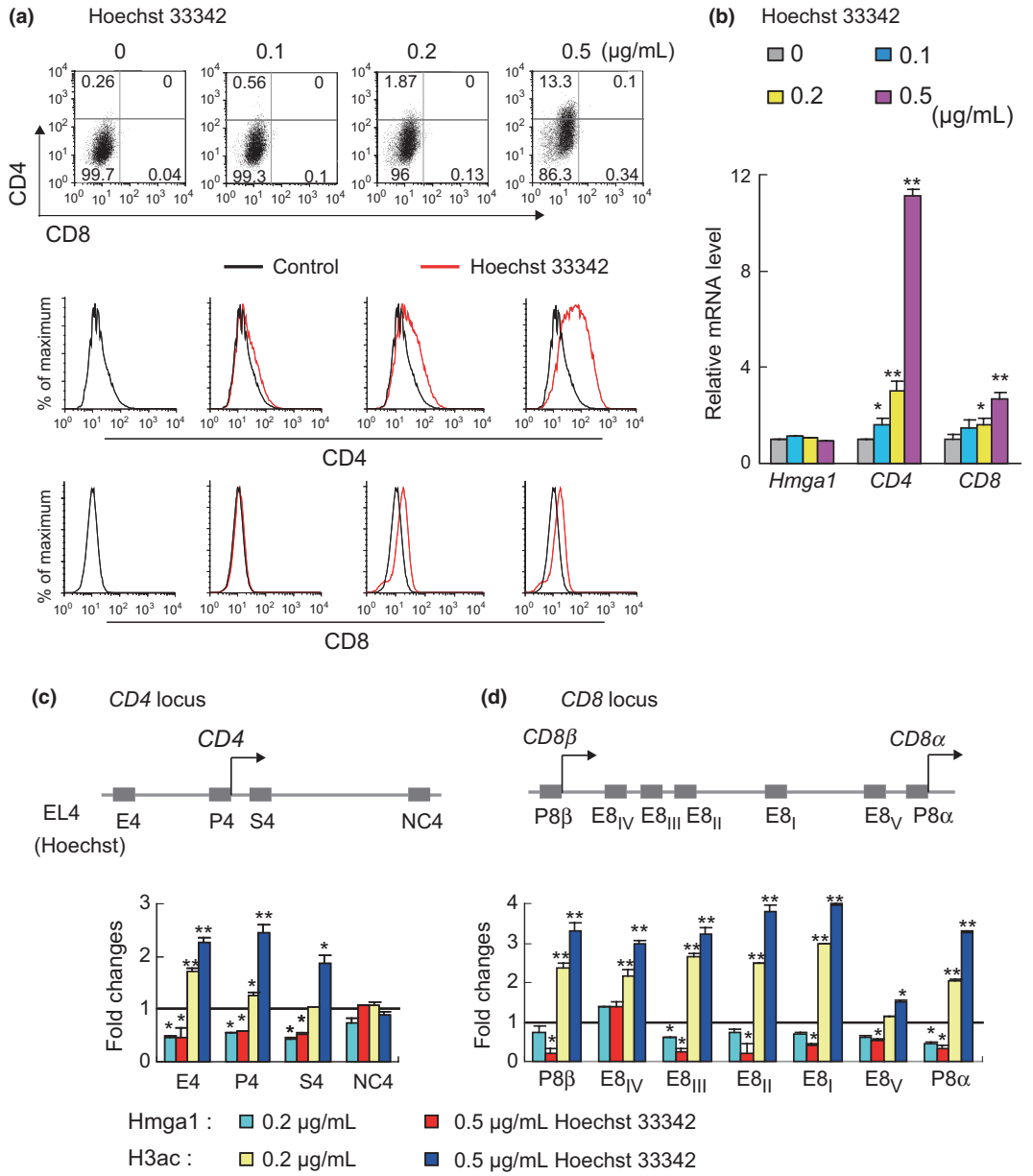

Fig. 4. Induction of CD4/CD8 expression by inhibiting Hmga1 binding to DNA. (a) Induction of CD4/CD8 expression in CD4/CD8-double negative cell-stage EL4 cells treated with Hoechst dyes. At 24 h after treatment with the indicated concentrations of Hoechst 33342 that inhibit Hmga1 binding to DNA. The numbers indicate the percentages of positive cells within the population (upper panel). Histograms are the overlay of CD4 or CD8 levels in the control (black) and Hoechst-treated cells (red). (b) Induction of *CD4/CD8* transcription in EL4 cells treated with Hoechst 33342. The mRNA level in control cells was normalized to 1. (c,d) Decreased Hmga1 binding to DNA at the *CD4/CD8* loci in EL4 cells treated with Hoechst 33342. Chromatin immunoprecipitation analyses of Hmga1 and acetylated histone H3 (H3ac) at the *CD4* (c) and *CD8* loci (d) were performed using EL4 cells treated with Hoechst 33342 for 24 h. Fold changes are shown as the relative enrichment by normalizing the value of untreated control cells to 1.

which contain AT-rich sequences (predicted from http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker) and the matrix attachment region.[34] These results suggest that Hmga1 is directly involved in the transcriptional regulation of *CD4/CD8* genes.

CD4/CD8 expression is induced by inhibition of Hmga1 binding to DNA. To investigate whether the binding of Hmga1 to DNA was required for silencing the *CD4/CD8* loci, we treated EL4 cells with Hoechst dyes (33342 or 33258), which are strong competitors for Hmga1-DNA interactions.[16,35] A dose-dependent increase of CD4 or CD8-positive cells was observed 24 h after Hoechst 33342 treatment (Fig. 4a). Under this condition, expression of *CD4/CD8* genes was significantly induced by Hoechst 33342 without affecting *Hmga1* expression (Fig. 4b). Similar results were obtained 24 h after Hoechst 33258 treatment (Fig. S2a). In addition, we observed Hmga1 dissociation from the heterochromatin foci after Hoechst 33342 treatment (Fig. S2b).

To elucidate whether the activation of *CD4/CD8* genes was caused by the loss of bound Hmga1 at these loci, ChIP analyses was performed. Hmga1 binding to *cis*-elements in the *CD4* locus was significantly decreased with Hoechst 33342 treatment (Fig. 4c). Similarly, Hoechst 33342 treatment (0.2 μg/mL) reduced Hmga1 binding to DNA at $E8_{III}$ and P8α, while the *cis*-elements, with the exception of $E8_{IV}$, exhibited significant decreases in Hmga1 binding after Hoechst 33342 treatment

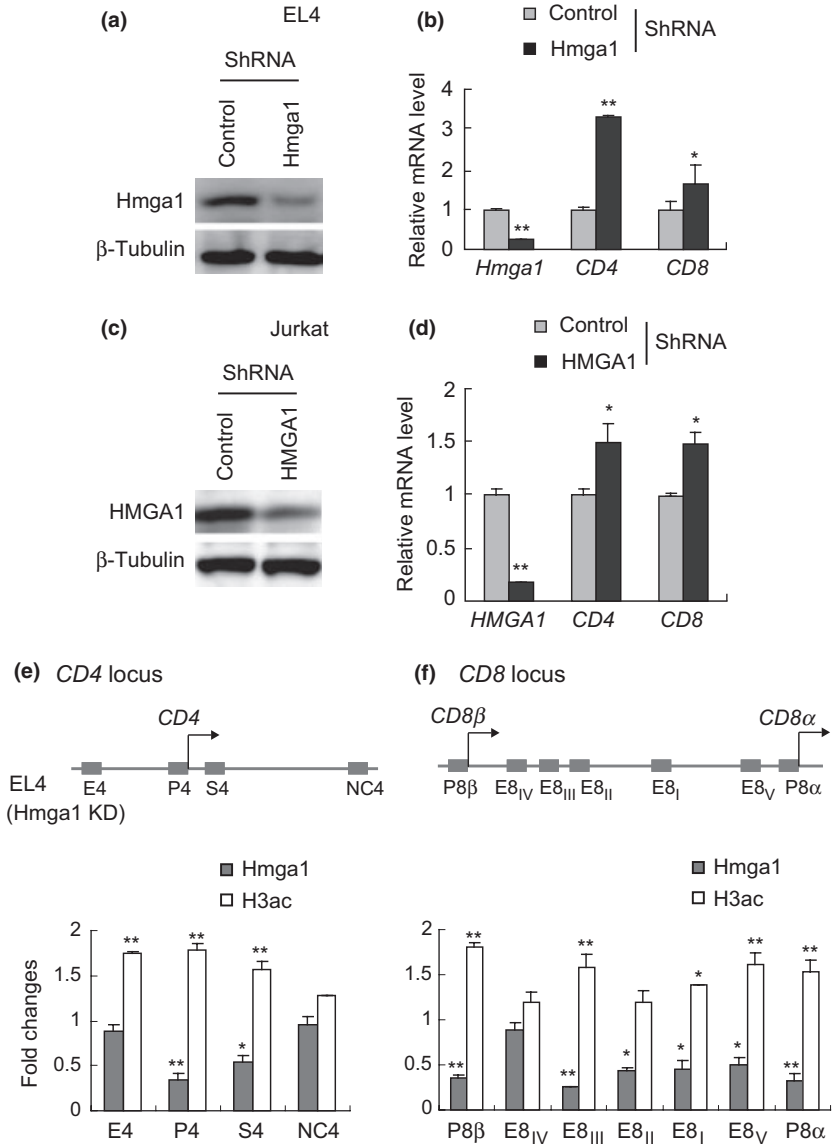

Fig. 5. Induction of *CD4/CD8* expression in Hmga1-depleted T cell lines. (a,c). shRNA-mediated Hmga1 knockdown. Western blot analyses showed that Hmga1 is selectively depleted in Hmga1-knockdown EL4 cells (a) and human Jurkat cells (c) 48 h after transfection. (b,d) Effect of *Hmga1* knockdown on *CD4* and *CD8* expression. qRT-PCR analyses of *Hmga1*, *CD4* and *CD8* were performed using EL4 cells (b) and Jurkat cells (d) 48 h after shRNA transfection. The mRNA level in control shRNA-transfected cells was normalized to 1. (e,f) Localization of Hmga1 and acetylated H3 in the *CD4/CD8* loci in Hmga1-knockdown EL4 cells. Chromatin immunoprecipitation analyses of Hmga1 and H3ac at the *CD4* (e) and *CD8* loci (f) was performed in EL4 cells 48 h after shRNA transfection.

(0.5 μg/mL) (Fig. 4d). In contrast to Hmga1 binding to DNA, acetylation of histone H3 (H3ac) was clearly augmented at the Hmga1 target sites. These results suggest that the decrease of Hmga1 binding to DNA is responsible for the induction of *CD4/CD8* expression.

CD4/CD8 expression is induced by Hmga1 knock down. Because Hoechst dyes are broad competitors of AT-rich binding proteins, we analyzed the effect of Hmga1 knockdown on *CD4/CD8* genes. We used a previously described shRNA against Hmga1,[25] and confirmed efficient reduction of Hmga1 at both protein and RNA levels in EL4 cells (Fig. 5a). Significant upregulation of *CD4/CD8* genes was observed in Hmga1-depleted cells compared with that of the control (Fig. 5b). An increase in *CD4/CD8* transcription was similarly detected in HMGA1-depleted human T leukemia Jurkat cells (Fig. 5c,d), suggesting that CD4/CD8 regulation by Hmga1 is not limited to specific cell lines. Based on ChIP assay results, DNA-bound Hmga1 decreased at P4 and S4 in the *CD4* locus, together with the increase of acetylated H3 (Fig. 5e). We also found a significant reduction in Hmga1 binding and increased H3ac at the *CD8* locus (Fig. 5f). These results suggest that Hmga1 negatively regulates *CD4/CD8* transcription through direct binding to *cis*-elements in the *CD4/CD8* loci.

Hmga1 suppresses cyclin-dependent kinase inhibitor genes *p21* and *p27* in T cell lines. During our study, we observed that Hmga1-depleted EL4 and Jurkat cells showed inhibited proliferation (see Fig. 5) compared with that of the control (Fig. 6a,b), suggesting that Hmga1 maintains cell growth in T cell lines. Flow cytometry analyses indicated an increase of G1 phase population and a decrease of both S and G2/M populations in the Hmga1-knockdown EL4 cells, compared to the control cells (Data S1, Fig. S3a). In addition, we found comparable expression levels of wild-type p53 protein and its responsive genes, such as *Bax*, *Bcl2* and *Mdm2*, in Hmga1-depleted and control

doi: 10.1111/j.1349-7006.2011.02159.x

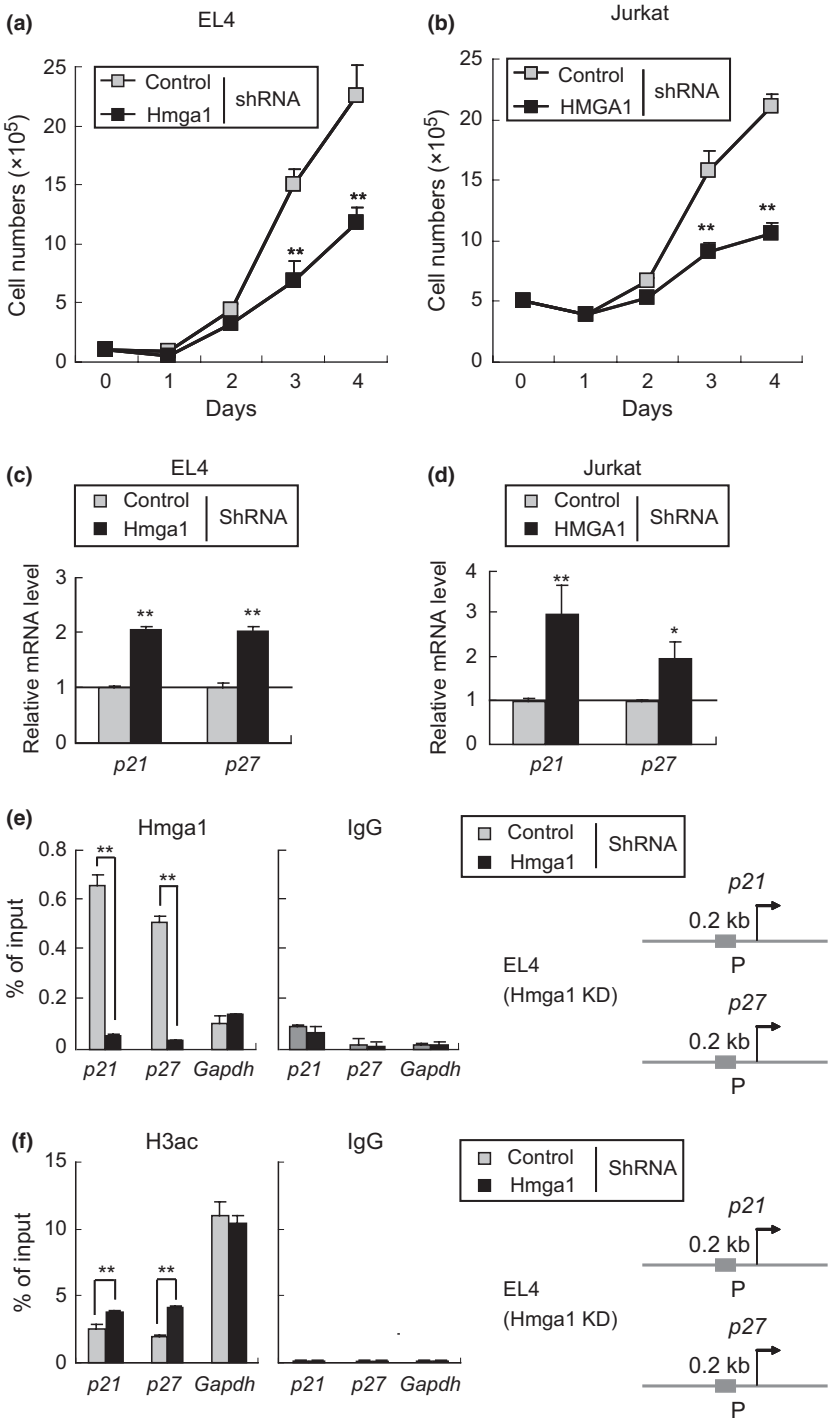

Fig. 6. Transcriptional repression of cyclin-dependent kinase inhibitor genes *p21* and *p27* in T cell lines. (a,b) Effect of *Hmga1* knockdown on cell proliferation. Cell numbers were determined on days 0, 1, 2, 3 and 4 after shRNA-mediated *Hmga1* knockdown in EL4 (a) and Jurkat cells (b). (c,d) Effect of *Hmga1* knockdown on *p21* and *p27* expression. qRT-PCR analyses of *p21* and *p27* genes were performed using Hmga1-knockdown EL4 (c) and Jurkat cells (d). The mRNA level in control shRNA-transfected cells was normalized to 1. (e,f) Localization of Hmga1 (e) and acetylated H3 (f) in the *p21* and *p27* gene promoters in Hmga1-depleted EL4 cells. Chromatin immunoprecipitation analysis was performed using control and Hmga1-knockdown EL4 cells. The GC-rich promoter region of the *Gapdh* gene was used as a negative control.

EL4 cells (Fig. S3b,c), suggesting the p53-independent role of Hmga1 in cell cycle regulation. We then evaluated the effect of Hmga1 on the expression of Cdk inhibitor genes *p21* and *p27*. Hmga1 knockdown resulted in upregulation of both *p21* and *p27* genes (Fig. 6c,d). Under this condition, DNA-bound Hmga1 decreased with the increase of acetylated H3 at the core promoter regions of these genes (Fig. 6e,f). Furthermore, in Hmga1-depleted cells, histone deacetylase Hdac1 dissociated from these core promoters (Fig. S3d), which was reported to transcriptionally inhibit these genes.[36] In fact, Hmga1 complexed with Hdac1 in EL4 cells (Data S1, Fig. S3e). These data suggest that the Hmga1–Hdac1 complex represses the expression of *p21* and *p27* genes to maintain cell proliferation.

Discussion

Although aberrant Hmga1 expression was reported to alter hematopoiesis and cause hematopoietic malignancies,[19–22,24] the molecular basis of these phenomena needs to be understood. The present study demonstrates distinct levels of Hmga1 expression in hematopoietic lineage cells and reveals the transcriptional silencing function of Hmga1 at the *CD4/CD8* loci during T cell differentiation. Our data indicate that: (i) Hmga1 has distinct expression during the differentiation of hematopoietic cells; (ii) Hmga1 is downregulated and dissociates from target genes and heterochromatin during DN to DP differentiation of T cells; (iii) Hmga1 represses *CD4/CD8* genes by directly binding

to *cis*-elements at these loci in DN-stage cells; and (iv) Hmga1 maintains cell proliferation most likely through direct inhibition of *p21* and *p27* genes in T leukemic cells. These findings suggest that Hmga1 is actively involved in hematopoietic differentiation and malignant phenotypes.

Although Hmga1 expression gradually decreases during erythrocytic maturation (Fig. S1), we found that Hmga1 is downregulated during DN to DP differentiation, followed by upregulation in CD4 SP and CD8 SP cells in the thymus. This observation suggests that Hmga1 has unique expression patterns during T cell development. The development of T cell lymphoma in Hmga1 transgenic mice[20,21] and B cell lymphoma in Hmga1 knockout mice[19] further supports the biological relevance of Hmga1 during hematopoietic differentiation. Despite HMGA1 overexpression in human B cell leukemia, high Hmga1 expression was observed to be persistently maintained in B cell lineages. Further study is required to resolve this issue. In addition, HMGA1 overexpression has been reported in leukemic cells in patients with megakaryoblastic, myeloblastic and lymphoblastic leukemia.[20,22] Our study has shed light on differential expression of Hmga1 in hematopoietic lineage cells, including progenitors.

Previous studies show that silencing of the *CD4/CD8* loci is associated with heterochromatin foci in DN thymocytes.[32,33] We found that Hmga1 significantly binds to the *CD4/CD8* loci and is co-localized with heterochromatin regions in DN T cells, whereas Hmga1 markedly decreases in DP cells. It has been reported that HMGA1 directly interacts with core histones, resulting in histone-bound HMGA1 as a chromatin docking complex for chromatin dynamics.[7,15,37] Hmga1 dissociation from heterochromatin regions coincides with *CD4/CD8* induction and increased levels of acetylated H3, even in the wide enhancer/promoter regions, probably due to the spread of hyperacetylation to neighboring sites, suggesting the Hmga1-mediated chromatin dynamics at the *CD4/CD8* loci.

At the DN stage, the intronic silencer (S4 in the present study) actively represses *CD4* transcription, but is dispensable for keeping *CD4* silenced in CD8 SP cells. The silencing activity of S4 must be reversible in DN cells, which can be ready for differentiation, and the *CD4* gene is stably silenced in CD8 SP cells.[38,39] Because HMGA1 binding can induce changes in the rotational setting of DNA on the surface of nucleosomes, Hmga1 in DN cells provides such a chromatin docking site for long-range remodeling. Indeed, mutations of BAF57 or Brg, two subunits of the SWI/SNF-like chromatin-remodeling BAF complex, both impaired *CD4* silencing in T cell-specific expressed transgenic mice.[40] We observed a significant decrease of Hmga1 binding at S4 in Hmga1-depleted DN T cells, and detected high Hmga1 expression in DN cells as well as CD8 SP cells. Therefore, it is of interest to clarify whether Hmga1 more selectively targets the *CD4* locus together with the chromatin-remodeling complex. Unlike *CD4* expression, *CD8* expression is enforced mainly by a series of developmental stage-specific enhancers ($E8_I$–$E8_V$).[39,41] Decreased DNA-bound Hmga1 at these enhancers indicates an architectural function of Hmga1 in the *CD8* locus. Hmga1 expression is recovered in CD8 SP cells, although whether this recovery acts on the constitutive *CD4* silencer or provides an Hmga1-DNA scaffold for stage-specific transcription factor accessibility at the *CD8* locus needs to be elucidated.

Our finding that the loss of Hmga1 function in T leukemic cells impaired proliferation is supported by previous reports on other cancer types.[23,25,27,42] As p53 is mutated in Jurkat cells,[43] the significant induction of *p21* and *p27* by HMGA1 knockdown further suggests a unique function of HMGA1 in leukemic cells. Previous studies have reported that the stage of thymocyte differentiation is closely related to the treatment outcome of T-ALL.[44–46] Early thymocyte malignancies, which are at the DN stage and negative for CD3, CD4 and CD8, represent approximately 34% of childhood T-ALL and have worse response to remission induction, worse survival and higher relative hazard rates than mature phenotypes of T-ALL.[44–46] The induction of CD4 positive cells and the reduction of cell proliferation in Hmga1-inhibited leukemic cells indicate that Hmga1 is a potential attractive target for the treatment of T-ALL. Finally, our previous study shows that the *Hmga1-GFP* knock-in mouse is a useful model to investigate the importance of Hmga1 protein in gastric cancers by cross-breeding with a stomach-specific inflammation mouse.[25] Our present results indicate that Hmga1 has a crucial function in hematopoietic lineage cells. In addition, the *Hmga1-GFP* knock-in mouse will be useful for investigating Hmga1 functions *in vivo*.

Acknowledgments

We thank Dr. N. Asou, Dr. K. Kuwahara, Dr. H. Sakamoto and Ms. M. Aoki (Kumamoto University) for providing Jurkat cells, EL4 cells, OP9/OP9-DL1 cells, and cell sorting support, respectively. This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and from the Global Center of Excellence (COE) ''Cell Fate Regulation Research and Education Unit,'' Kumamoto University (to M.N.). Y.X. was a Research Associate of the Global COE.

Disclosure Statement

The authors have no conflict of interest.

References

1 Georgopoulos K. Haematopoietic cell-fate decisions, chromatin regulation and ikaros. *Nat Rev Immunol* 2002; **2**: 162–73.
2 Cedar H, Bergman Y. Immunology. Epigenetics of haematopoietic cell development. *Nat Rev Immunol* 2011; **11**: 478–88.
3 Orkin SH. Diversification of haematopoietic stem cells to specific lineages. *Nat Rev Genet* 2000; **1**: 57–64.
4 Krangel MS. T cell development: better living through chromatin. *Nat Immunol* 2007; **8**: 687–94.
5 Ho L, Crabtree GR. Chromatin remodelling during development. *Nature* 2010; **463**: 474–84.
6 Catez F, Hock R. Binding and interplay of HMG proteins on chromatin: lessons from live cell imaging. *Biochim Biophys Acta* 2010; **1799**: 15–27.
7 Reeves R. Nuclear functions of the HMG proteins. *Biochim Biophys Acta* 2010; **1799**: 3–14.
8 Giancotti V, Berlingieri MT, DiFiore PP, Fusco A, Vecchio G, Crane-Robinson C. Changes in nuclear proteins on transformation of rat epithelial thyroid cells by a murine sarcoma retrovirus. *Cancer Res* 1985; **45**: 6051–7.
9 Reeves R, Nissen MS. The A.T-DNA-binding domain of mammalian high mobility group I chromosomal proteins. A novel peptide motif for recognizing DNA structure. *J Biol Chem* 1990; **265**: 8573–82.
10 Reeves R. Molecular biology of HMGA proteins: hubs of nuclear function. *Gene* 2001; **277**: 63–81.
11 Fashena SJ, Reeves R, Ruddle NH. A poly(dA-dT) upstream activating sequence binds high-mobility group I protein and contributes to lymphotoxin (tumor necrosis factor-beta) gene regulation. *Mol Cell Biol* 1992; **12**: 894–903.
12 Thanos D, Maniatis T. The high mobility group protein HMG I(Y) is required for NF-kappa B-dependent virus induction of the human IFN-beta gene. *Cell* 1992; **27**: 777–89.
13 Thanos D, Maniatis T. Virus induction of human IFN beta gene expression requires the assembly of an enhanceosome. *Cell* 1995; **83**: 1091–100.
14 John S, Reeves RB, Lin JX *et al.* Regulation of cell-type-specific interleukin-2 receptor alpha-chain gene expression: potential role of physical interactions between Elf-1, HMG-I(Y), and NF-kappa B family proteins. *Mol Cell Biol* 1995; **15**: 1786–96.

doi: 10.1111/j.1349-7006.2011.02159.x

15 Reeves R, Wolffe AP. Substrate structure influences binding of the non-histone protein HMG-I(Y) to free nucleosomal DNA. *Biochemistry* 1996; **35**: 5063–74.
16 Amirand C, Viari A, Ballini JP *et al.* Three distinct sub-nuclear populations of HMG-I protein of different properties revealed by co-localization image analysis. *J Cell Sci* 1998; **111**: 3551–61.
17 Brocher J, Vogel B, Hock R. HMGA1 down-regulation is crucial for chromatin composition and a gene expression profile permitting myogenic differentiation. *BMC Cell Biol* 2010; **11**: 64.
18 Chiappetta G, Avantaggiato V, Visconti R *et al.* High level expression of the HMGI (Y) gene during embryonic development. *Oncogene* 1996; **13**: 2439–46.
19 Fedele M, Fidanza V, Battista S *et al.* Haploinsufficiency of the Hmga1 gene causes cardiac hypertrophy and myelo-lymphoproliferative disorders in mice. *Cancer Res* 2006; **66**: 2536–43.
20 Xu Y, Sumter TF, Bhattacharya R *et al.* The HMG-I oncogene causes highly penetrant, aggressive lymphoid malignancy in transgenic mice and is overexpressed in human leukemia. *Cancer Res* 2004; **64**: 3371–5.
21 Fedele M, Pentimalli F, Baldassarre G *et al.* Transgenic mice overexpressing the wild-type form of the HMGA1 gene develop mixed growth hormone/prolactin cell pituitary adenomas and natural killer cell lymphomas. *Oncogene* 2005; **24**: 3427–35.
22 Battista S, Pentimalli F, Baldassarre G *et al.* Loss of Hmga1 gene function affects embryonic stem cell lympho-hematopoietic differentiation. *FASEB J* 2003; **17**: 1496–8.
23 Wood LJ, Mukherjee M, Dolde C. EHMG-I/Y, a new c-Myc target gene and potential oncogene. *Mol Cell Biol* 2000; **20**: 5490–502.
24 Pierantoni GM, Agosti V, Fedele M *et al.* High-mobility group A1 proteins are overexpressed in human leukaemias. *Biochem J* 2003; **372**: 145–50.
25 Akaboshi S, Watanabe S, Hino Y *et al.* HMGA1 is induced by Wnt/β-catenin pathway and maintains cell proliferation in gastric cancer. *Am J Pathol* 2009; **175**: 1675–85.
26 Fedele M, Fusco A. HMGA and cancer. *Biochim Biophys Acta* 2010; **1799**: 48–54.
27 Ueda Y, Watanabe S, Tei S, Saitoh N, Kuratsu J, Nakao M. High mobility group protein HMGA1 inhibits retinoblastoma protein-mediated cellular G0 arrest. *Cancer Sci* 2007; **98**: 1893–901.
28 Pierantoni GM, Rinaldo C, Esposito F, Mottolese M, Soddu S, Fusco A. High Mobility Group A1 (HMGA1) proteins interact with p53 and inhibit its apoptotic activity. *Cell Death Differ* 2006; **13**: 1554–63.
29 Hatta Y, Koeffler HP. Role of tumor suppressor genes in the development of adult T cell leukemia/lymphoma (ATLL). *Leukemia* 2002; **16**: 1069–85.
30 Watanabe S, Ueda Y, Akaboshi S, Hino Y, Sekita Y, Nakao M. HMGA2 maintains oncogenic RAS-induced epithelial-mesenchymal transition in human pancreatic cancer cells. *Am J Pathol* 2009; **174**: 854–68.
31 Aoto T, Saitoh N, Sakamoto Y, Watanabe S, Nakao M. Polycomb group protein-associated chromatin is reproduced in post-mitotic G1 phase and is required for S phase progression. *J Biol Chem* 2008; **283**: 18905–15.
32 Delaire S, Huang YH, Chan SW, Robey EA. Dynamic repositioning of CD4 and CD8 genes during T cell development. *J Exp Med* 2004; **200**: 1427–35.
33 Merkenschlager M, Amoils S, Roldan E *et al.* Centromeric repositioning of coreceptor loci predicts their stable silencing and the CD4/CD8 lineage choice. *J Exp Med* 2004; **200**: 1437–44.
34 Kieffer LJ, Greally JM, Landres I *et al.* Identification of a candidate regulatory region in the human CD8 gene complex by colocalization of DNase I hypersensitive sites and matrix attachment regions which bind SATB1 and GATA-3. *J Immunol* 2002; **168**: 3915–22.
35 Huth JR, Bewley CA, Nissen MS *et al.* The solution structure of an HMG-I(Y)-DNA complex defines a new architectural minor groove binding motif. *Nat Struct Biol* 1997; **4**: 657–65.
36 Sengupta S, Shimamoto A, Koshiji M *et al.* Tumor suppressor p53 represses transcription of RECQ4 helicase. *Oncogene* 2005; **24**: 1738–48.
37 Sgarra R, Zammitti S, Lo Sardo A *et al.* HMGA molecular network: from transcriptional regulation to chromatin remodeling. *Biochim Biophys Acta* 2010; **1799**: 37–47.
38 Collins A, Littman D, Taniuchi I. RUNX proteins in transcription factor networks that regulate T-cell lineage choice. *Nat Rev Immunol* 2009; **9**: 106–15.
39 Taniuchi I, Osato M, Egawa T *et al.* Differential requirements for Runx proteins in CD4 repression and epigenetic silencing during T lymphocyte development. *Cell* 2002; **111**: 621–33.
40 Chi TH, Wan M, Zhao K *et al.* Reciprocal regulation of CD4/CD8 expression by SWI/SNF-like BAF complexes. *Nature* 2002; **418**: 195–9.
41 Kioussis D, Ellmeier W. Chromatin and CD4, CD8A and CD8B gene expression during thymic differentiation. *Nat Rev Immunol* 2002; **2**: 909–19.
42 Fusco A, Fedele M. Roles of HMGA proteins in cancer. *Nat Rev Cancer* 2007; **7**: 899–910.
43 Cheng J, Haas M. Frequent mutations in the p53 tumor suppressor gene in human leukemia T-cell lines. *Mol Cell Biol* 1990; **10**: 5502–9.
44 Pui CH, Carroll WL, Meshinchi S, Arceci RJ. Biology, risk stratification, and therapy of pediatric acute leukemias: an update. *J Clin Oncol* 2011; **29**: 551–65.
45 Crist WM, Shuster JJ, Falletta J *et al.* Clincial feathres and outcome in childhood T-cell leukemia-lymphoma according to stage of thymocyte differentiation: a Pediatric Oncology Group Study. *Blood* 1988; **72**: 1891–7.
46 Uchun FM, Gaynon PS, Sense MG *et al.* Clinical features and treatment outcome of childhood T-lineage acute lymphoblastic leukemia according to the apparent maturational stage of T-lineage leukemic blasts: a children's cancer group study. *J Clin Oncol* 1997; **15**: 2214–21.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Hmga1 expression in bone marrow-derived hematopoietic cells.

Fig. S2. CD4/CD8 expression is induced by inhibiting Hmga1 binding to DNA.

Fig. S3. Cooperation of Hmga1 and Hdac1 in EL4 cells.

Table S1. Primers for quantitative RT-PCR.

Table S2. Primers for quantitative PCR.

Data S1. Materials and Methods.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.