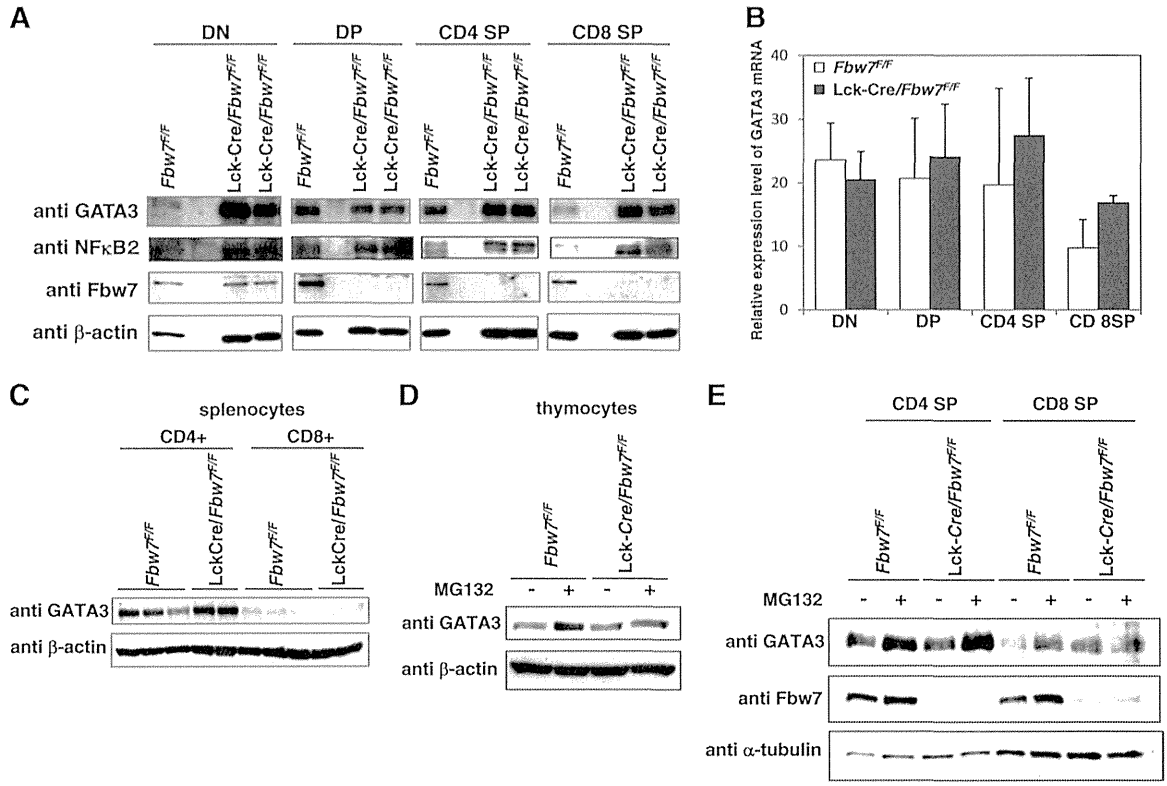

FIG 3 Loss of Fbw7 stabilizes GATA3 protein in mouse thymocytes. (A) Representative immunoblot analysis of Fbw7 and its target proteins in the subsets of thymocytes from *Fbw7*$^{flox/flox}$ or Lck-Cre/*Fbw7*$^{flox/flox}$ mice. DN, DP, CD4 SP, and CD8 SP cells were purified from *Fbw7*$^{flox/flox}$ or Lck-Cre/*Fbw7*$^{flox/flox}$ mice at 8 weeks of age using flow cytometry and lysed. The lysates were subjected to immunoblot analysis with the indicated antibodies. (B) qRT-PCR analysis of GATA3 expression in the sorted FACS fractions obtained in panel A. The amount of transcripts was normalized against that of GAPDH as an internal standard. Data are means ± SD of values from three *Fbw7*$^{flox/flox}$ and three Lck-Cre/*Fbw7*$^{flox/flox}$ mice. (C) Purification of CD8$^+$ and CD4$^+$ T cells from mouse splenocytes from *Fbw7*$^{flox/flox}$ or Lck-Cre/*Fbw7*$^{flox/flox}$ mice at 8 to 9 weeks of age was performed with CD8$^+$ positive selection and CD4$^+$ negative selection by the magnetic bead method, respectively. Immunoblot analysis of whole lysates from each purified splenic T-cell subset was performed with the indicated antibodies. (D) T-cell subsets obtained from *Fbw7*$^{flox/flox}$ or Lck-Cre/*Fbw7*$^{flox/flox}$ mice at 8 weeks of age were cultured in RPMI 1640 medium for 2 h and incubated with 20 μM MG132 for 4 h. Cells were lysed and subjected to immunoblot analysis with the indicated antibodies. (E) Whole thymocytes obtained from *Fbw7*$^{flox/flox}$ or Lck-Cre/*Fbw7*$^{flox/flox}$ mice at 8 weeks of age were cultured in RPMI 1640 medium for 4 h and incubated with 20 μM MG132 for 5 h. Cells were lysed and subjected to immunoblot analysis with the indicated antibodies.

started at DN2 (29). Therefore, Fbw7 expression can be manipulated after the transition to the DN2 stage in Lck-Cre/*Fbw7*$^{flox/flox}$ mice. Although Fbw7 was expressed in the DP subset of the controls, Fbw7 elimination did not result in increased GATA3 in DP cells of Lck-Cre/*Fbw7*$^{flox/flox}$ mice (Fig. 3A). To more closely evaluate GATA3 protein levels, relative expression levels of GATA3 mRNA during T-cell development were estimated by qRT-PCR. In contrast to protein levels, GATA3 mRNA expression was not significantly influenced by the genetic status of Fbw7 (Fig. 3B). Consequently, we speculated that Fbw7-mediated GATA3 degradation occurs at the DN stage but not at the DP stage. Although CD8 SP subsets in the thymus of the Lck-Cre/*Fbw7*$^{flox/flox}$ mice retained GATA3 expression, CD8$^+$ T cells in the spleen did not express GATA3 (Fig. 3C).

We demonstrated the involvement of the Fbw7 and proteasome-mediated degradation system in the regulation of GATA3 protein in mouse thymocytes. GATA3 protein accumulated in control thymocytes treated with the proteasome inhibitor MG132, whereas it did not accumulate in MG132-treated Lck-Cre/*Fbw7*$^{flox/flox}$ mouse thymocytes (Fig. 3D). Meanwhile, GATA3 levels in the absence of MG132 were equivalent in Lck-Cre/*Fbw7*$^{flox/flox}$ and *Fbw7*$^{flox/flox}$ thymocytes (Fig. 3D). Because approximately 80% of the thymocyte population consisted of the DP subset, in which we did not observe Fbw7-mediated GATA3 turnover, the influence of Fbw7 genotype on GATA3 levels would not be apparent in the entire thymocyte population. Increasing GATA3 protein in CD4 SP and CD8 SP subsets of Lck-Cre/*Fbw7*$^{flox/flox}$ mice proposes a role for Fbw7 in regulating GATA3 protein levels in both of these subsets (Fig. 3A). Nevertheless, other E3 ligases might have a partial contribution to GATA3 turnover in these cells because GATA3 accumulation under MG132 treatment was also observed in the CD4 SP subset of Lck-Cre/*Fbw7*$^{flox/flox}$ mice (Fig. 3E). Taking these observations together, we propose that the significant survival of the CD8 SP lineage is caused by the accumulation of GATA3 in Fbw7-depleted mice.

We also investigated levels of NF-κB2, another substrate of Fbw7 known to accumulate during T-cell development in the thymic subsets of Lck-Cre/*Fbw7*$^{flox/flox}$ mice (5). Changes in NF-κB2 protein levels were observed during the entire T-cell development stage of Lck-Cre/*Fbw7*$^{flox/flox}$ mice, showing a distinct pattern from changes in GATA3 levels (Fig. 3A). This implies that Fbw7-mediated GATA3 stability is regulated by signaling pathways distinct from those of NF-κB2.

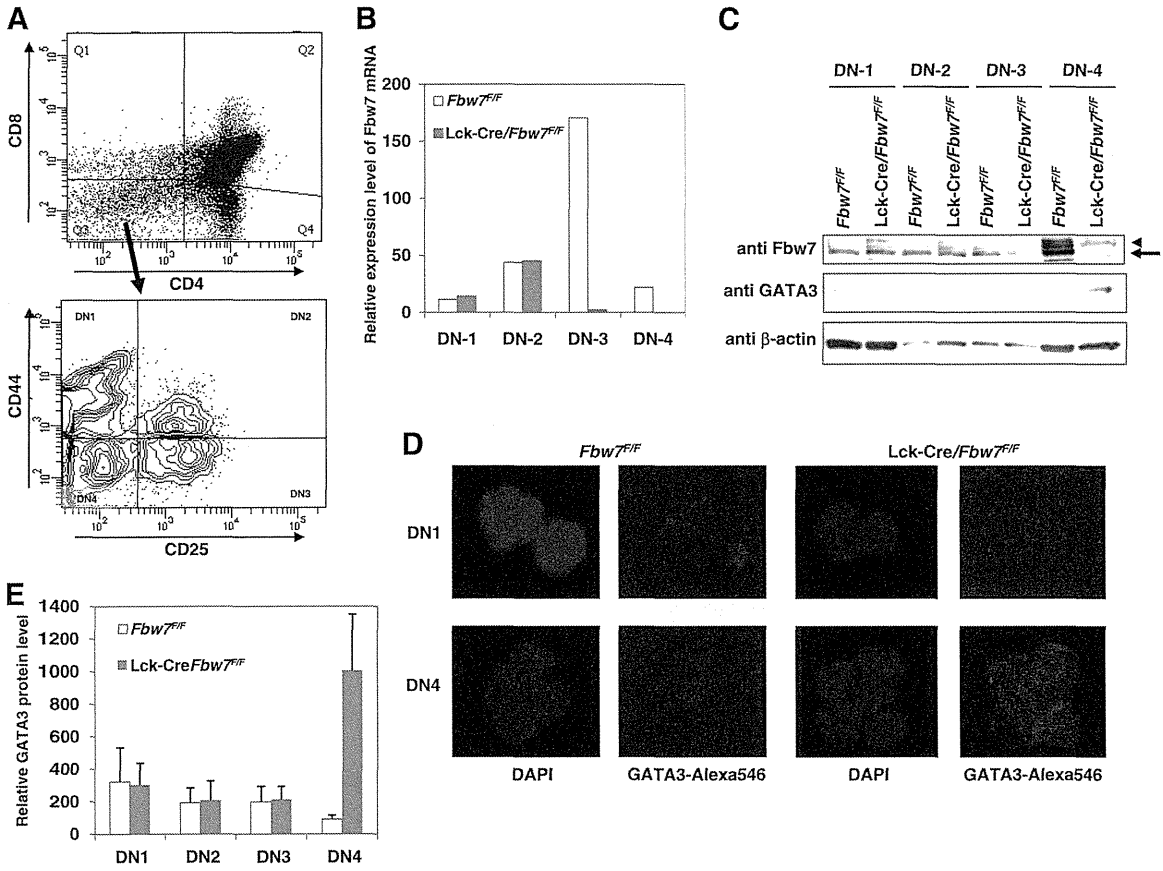

FIG 4 GATA3 protein is stabilized in the DN4 subset of Lck-Cre/*Fbw7*$^{flox/flox}$ mice. (A) Thymocytes from *Fbw7*$^{flox/flox}$ or Lck-Cre/*Fbw7*$^{flox/flox}$ mice at 9 to 10 weeks of age were incubated with CD4, CD8, CD25, and CD44 for sorting cells from DN1 to DN4 lineages. A representative gating strategy is shown. The sorted FACS fractions obtained in panel A were subjected to qRT-PCR analysis of Fbw7 expression (B), immunoblot analysis (C), and immunocytochemical staining using Alexa Fluor 546 conjugated with anti-GATA3 antibody (D and E). DAPI staining was also examined for detection of the nuclear location (D and E). In panel C, the arrow and arrowhead indicate Fbw7 and nonspecific signal, respectively.

To clarify the correlation of accumulation of GATA3 with depletion of Fbw7 in Lck-Cre/*Fbw7*$^{flox/flox}$ mice, we performed additional analysis focusing on subpopulations from DN1 to DN4, which were sorted using anti-CD44 and anti-CD25 antibodies, in addition to anti-CD4 and anti-CD8 antibodies (Fig. 4A). We initially observed the elimination of Fbw7 expression at the DN3 and DN4 stages in Lck-Cre/*Fbw7*$^{flox/flox}$ mice by comparing RNA expression levels to those of control mice (Fig. 4B). It was in accord with the quantitative transition of Fbw7 protein. As shown in Fig. 4C, the depletion of Fbw7 protein in Lck-Cre/*Fbw7*$^{flox/flox}$ mice started at DN3 and was completed by DN4. Moreover, we found that GATA3 protein was increased in DN4 (Fig. 4C), which was confirmed by immunocytochemical analysis (Fig. 4D and E). These results suggest that Fbw7 participates in the degradation of GATA3 in DN4 cells.

Fbw7 binds to, ubiquitylates, and degrades GATA3 in a Thr-156-dependent manner. The accumulation in the thymic subsets of Lck-Cre/*Fbw7*$^{flox/flox}$ mice predicts GATA3 as a novel target for Fbw7 ubiquitin ligase. Fbw7 often interacts with its substrates by binding the phosphorylated CPD in its target proteins (Fig. 5A). We searched the amino acid sequence of GATA3 and noticed two CPD sequences arranged in tandem in both human and mouse GATA3 proteins (Fig. 5A, left). To determine whether Fbw7 interacts with GATA3 protein and whether phosphorylation of CPD is required for recognition by Fbw7 in cultured cells, we prepared a human wild-type (WT) GATA3 expression plasmid and three GATA mutants with amino acid substitutions (T156A/S162A, T156A, and S162A), in which a Thr and/or Ser residue was replaced by Ala. The Myc-tagged WT or mutant GATA3 and FLAG-tagged Fbw7 expression plasmids were cotransfected into HEK293 cells. Cell extracts were subjected to immunoprecipitation (IP) to evaluate the binding between Fbw7 and GATA3. WT GATA3 successfully coimmunoprecipitated Fbw7 (Fig. 5B). Although the S162A mutant retained binding at levels similar to those of the WT, the T156A/S162A and T156A GATA3 mutants completely lost Fbw7 binding ability (Fig. 5B). This result suggests that Thr-156, but not Ser-162, in GATA3 is required for recognition by Fbw7.

We next examined whether Fbw7 promotes ubiquitylation of GATA3 in HEK293 cells by denatured IP analysis. A strongly enhanced ubiquitylation signal was detected on WT GATA3 in the presence of Fbw7 (Fig. 5C). Notably, Fbw7-mediated ubiquitylation of both T156A/S162A and T156A mutants was markedly reduced, while the S162A mutant was successfully ubiquitylated (Fig. 5C). Similar results were obtained in HeLa cells (Fig. 5D).

To address the possibility that the SCF^{Fbw7} ubiquitin ligase targets GATA3 for degradation, the effect of Fbw7 expression on the turnover of exogenous GATA3 was investigated in HeLa cells.

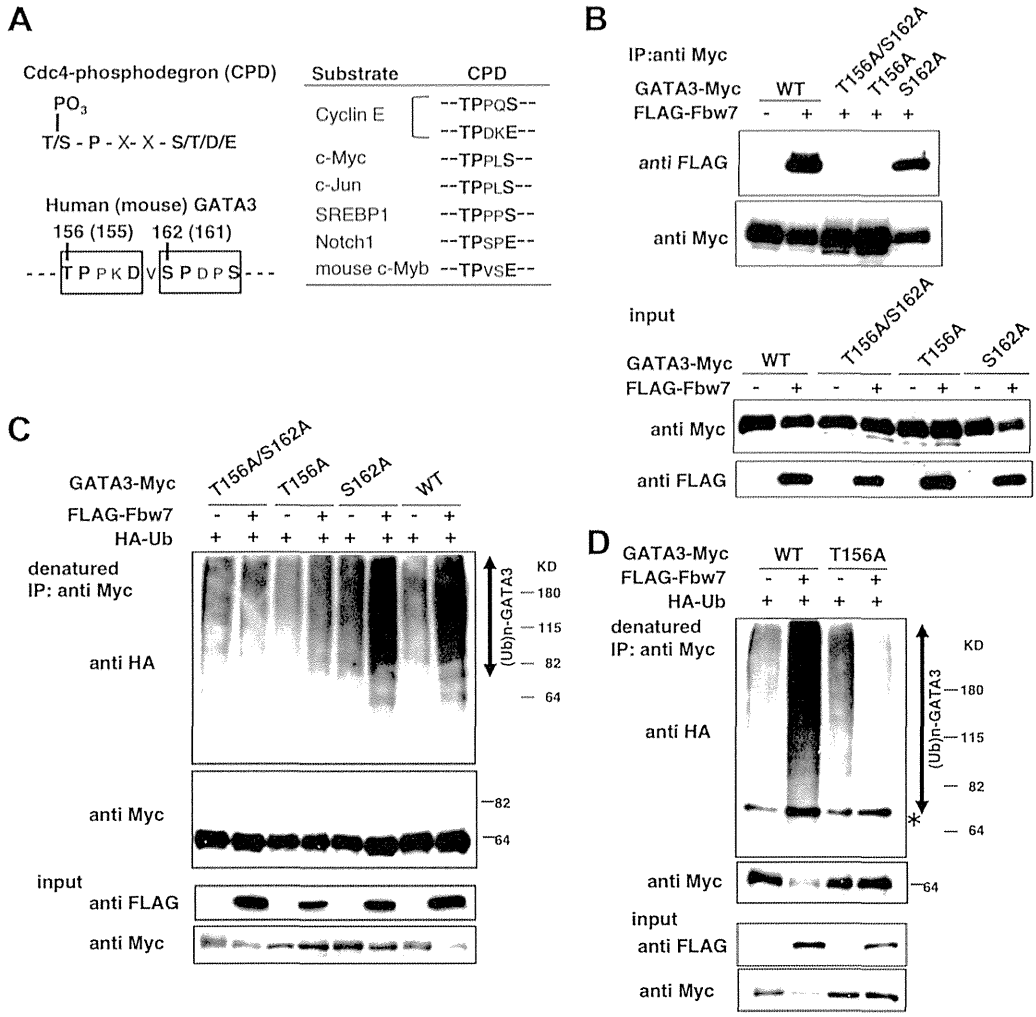

FIG 5 Fbw7 promotes ubiquitylation of GATA3 in a Thr-156-dependent manner. (A) Sequence alignment of known Fbw7 substrates containing the Cdc4 phosphodegron (CPD) consensus sequence for recognition by Fbw7. Conserved residues within the CPD are shown in bold. Two putative CPD sites (open box) in human and mouse GATA3 are indicated. Numbered residues indicate putative phosphorylation sites. (B) Fbw7 binding to GATA3 is dependent on Thr-156 in GATA3 *in vivo*. HEK293 cells were transfected with Myc-tagged wild-type (WT) or mutant GATA3 along with FLAG-Fbw7, as indicated, and incubated with 20 μM MG132 for 5 h. Cell lysates were immunoprecipitated (IP) with antibodies against Myc, followed by immunoblotting for FLAG and Myc (top panels). Immunoblot analysis of input from each transfection confirmed expression levels of WT GATA3, mutant GATA3, and Fbw7 (bottom panels). (C) HEK293 cells were transfected with Myc-tagged WT or mutant GATA3, FLAG-Fbw7, and HA-ubiquitin as indicated. Cells were incubated with 20 μM MG132 for 5 h, lysed, and denatured with sample buffer containing SDS and 2-mercaptoethanol to dissociate proteins associated with GATA3. Myc-tagged GATA3 was immunoprecipitated and analyzed by immunoblotting with the indicated antibodies (top panels). Whole-cell extracts (input) were subjected to immunoblotting to confirm protein expression (bottom panels). (D) The same experiment as described in panel A was performed in HeLa cells.

Coexpression of Fbw7 significantly facilitated the degradation of WT GATA3 (Fig. 6 left). In contrast, the T156A mutant was relatively stable compared with WT GATA3 in the absence of Fbw7, and its level did not change with Fbw7 (Fig. 6). These results suggest that modification of GATA3 on Thr-156 is one of the key events for recognition by SCF^{Fbw7}, which mediates GATA3 ubiquitylation and degradation. Moreover, owing to degradation of WT GATA3 by coexpressed Fbw7, protein levels of WT GATA3 in the presence of HA-Fbw7 were significantly reduced in comparison with levels in the absence of HA-Fbw7, even at the first measurement of this assay (0.1 h).

CDK2 phosphorylates Thr-156 in GATA3. We speculated that like other substrates of Fbw7, regulation of GATA3 by Fbw7 would be mediated by phosphorylation of Thr-156 in the CPD. To evaluate whether Thr-156 of GATA3 was phosphorylated in intact cells, a phospho-specific antibody (anti-P-T156-GATA3) that recognizes phosphorylated Thr-156 was prepared. Myc-tagged WT GATA3 expressed in HEK293 cells treated with both phosphatase and proteasome inhibitors was detected by the anti-P-T156-GATA3 antibody, but no signal was detected using the GATA3 T156A mutant (Fig. 7A). These data suggest that GATA3 Thr-156 is phosphorylated *in vivo*.

Fbw7-mediated degradation of substrate is often triggered by the activation of GSK3 (1, 4, 5, 30, 31). GSK3 phosphorylates Ser and Thr residues, after an initial "priming phosphorylation" of a Ser or Thr located four amino acids C-terminal to the site of GSK phosphorylation (32). If Asp and Glu can be considered to mimic this priming phosphorylation, the residues surrounding Thr-156

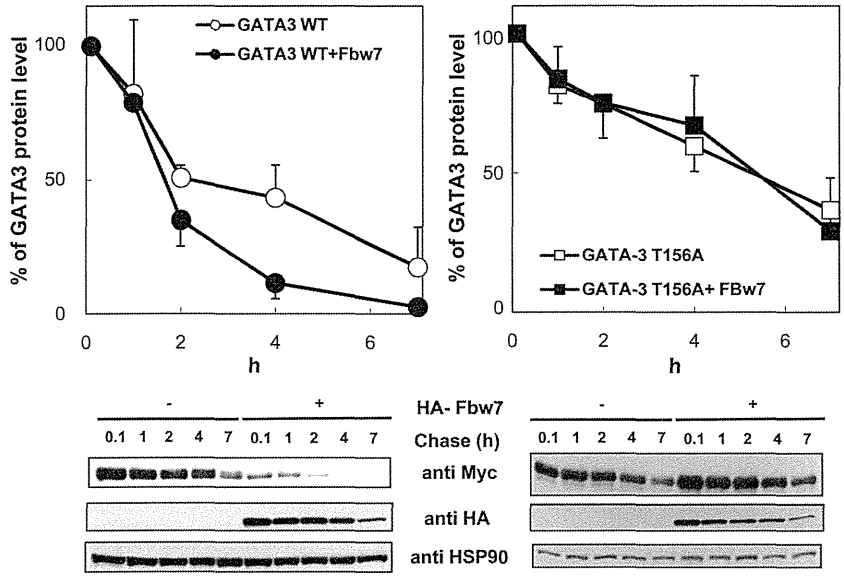

FIG 6 Fbw7 promotes degradation of GATA3 in a Thr-156-dependent manner. HeLa cells were transfected with Myc-tagged WT (left) or T156A GATA3 (right) in the absence or presence of HA-Fbw7 and treated with 12.5 μg/ml cycloheximide to inhibit protein synthesis for the indicated times. GATA3 levels were analyzed at various time points by immunoblotting (bottom panels). The percentage of GATA3 at each time point was quantitated by image analysis and normalized against HSP90 (top panels). GATA3 protein levels were calculated as the means ± SDs from three independent experiments. Immunoblots show data from one representative experiment.

in GATA3 can be classified as a GSK3 phosphorylation consensus motif (Fig. 5A, left). To examine whether GSK3 phosphorylates Thr-156 of GATA3 *in vitro*, we prepared two types of synthetic peptides (amino acids [aa] 150 to 161 of GATA3) for the WT and the Thr-156 mutant sequence in which Thr was replaced with Ala (T156A). Unexpectedly, there was no detectable incorporation of ^{32}P in the WT or T156A peptide, indicating that GSK3 does not phosphorylate either peptide (Fig. 7B, top). We examined other Ser/Thr kinases and found that cyclin E/CDK2 and cyclin A/CDK2, but not cyclin B1/CDK1, specifically phosphorylated WT GATA3 peptide (Fig. 7B, top). Their kinase activity was confirmed using the S11 peptide, which is a confirmed substrate for CDK1, CDK2, and CDK4/CDK 6 (Fig. 7B, bottom) (22).

To further confirm the specific phosphorylation on Thr-156, GST fusion recombinant GATA3 proteins, WT and T156A mutant, were examined as substrates. Reduced CDK2-mediated phosphorylation of the T156A mutant compared with WT GATA3 was confirmed by immunoblotting with anti-phospho-Thr and anti-P-T156-GATA3 antibodies (Fig. 7C and D). A previous study reported that GATA3 is also phosphorylated by p38 MAPK (33, 34). Consistent with this report, p38 strongly phosphorylated both recombinant GATA3 proteins (Fig. 7C) although it did not exhibit kinase activity toward Thr-156 in GATA3 (Fig. 7B, top, and D).

Physiological CDK2, which is activated during G_2/M phase, works on Thr-156 of GATA3 and regulates its stability. We further investigated physiological effects on GATA3 of CDK2. Reduction of CDK2 by siRNA inhibited phosphorylation on Thr-156, providing evidence that CDK2 is involved in CPD phosphorylation of GATA3 *in vivo* (Fig. 8A). To verify the requirement of phosphorylation of GATA3 for binding to Fbw7, we tested the binding ability of phosphorylated GATA3 to Fbw7 *in vitro*. WT GATA3 phosphorylated by CDK2 was able to bind Fbw7, while the T156A GATA3 mutant did not, regardless of the phosphorylating kinase (Fig. 8B). These results suggest that CDK2-mediated phosphorylation of Thr-156 is essential for binding of GATA3 to Fbw7.

Cyclin E/CDK2 plays a critical role in the G_1/S-phase transition (35). Cyclin A then replaces cyclin E to associate with CDK2, and this complex subsequently functions in S-phase progression and G_2/M transition (36). We conducted an *in vitro* phosphorylation assay using cell lysates prepared from G_1/S or G_2/M synchronized or nonsynchronized HeLa cells as the kinase source. Recombinant GATA3 was phosphorylated only in the presence of G_2/M lysate even though both cyclin A and cyclin E protein levels in the lysate were decreased (Fig. 8C). To verify the responsible kinase in the G_2/M lysate, we tested the effects of a CDK2 inhibitor (CVT313) and competitor (p27) on kinase activity. Both p27 and CVT313 inhibited the kinase activity on Thr-156 in a dose-dependent manner (Fig. 8D).

Phosphorylation at Thr-156 in GATA3 is executed in HUT78 cells during G_2/M phase and in thymocytes of mice. Cell cycle-dependent phosphorylation of CPD in endogenous GATA3 was also confirmed in the HUT78 cell line, which is a T-cell lymphoma line. Phosphorylated Thr-156 was detected in G_2/M arrested cells but not in nonsynchronized cells, which contained a G_2/M population of only approximately 15% (Fig. 8E). We further investigated the property of Thr-156-phosphorylated GATA3. MG132 treatment resulted in an accumulation of Thr-156-phosphorylated GATA3 in G_2/M (Fig. 8F). This implies that phosphorylation of GATA3 at Thr-156 induces its proteasome-dependent degradation during G_2/M. These results suggest that cyclin A/CDK2 regulates GATA3 stability through the phosphorylation of the CPD during G_2/M phase in cultured cells. GATA3 Thr-156 phosphorylation was also detected in mouse thymocytes (Fig. 8G). Furthermore, ICC analysis using anti-phospho-Thr-156 antibody

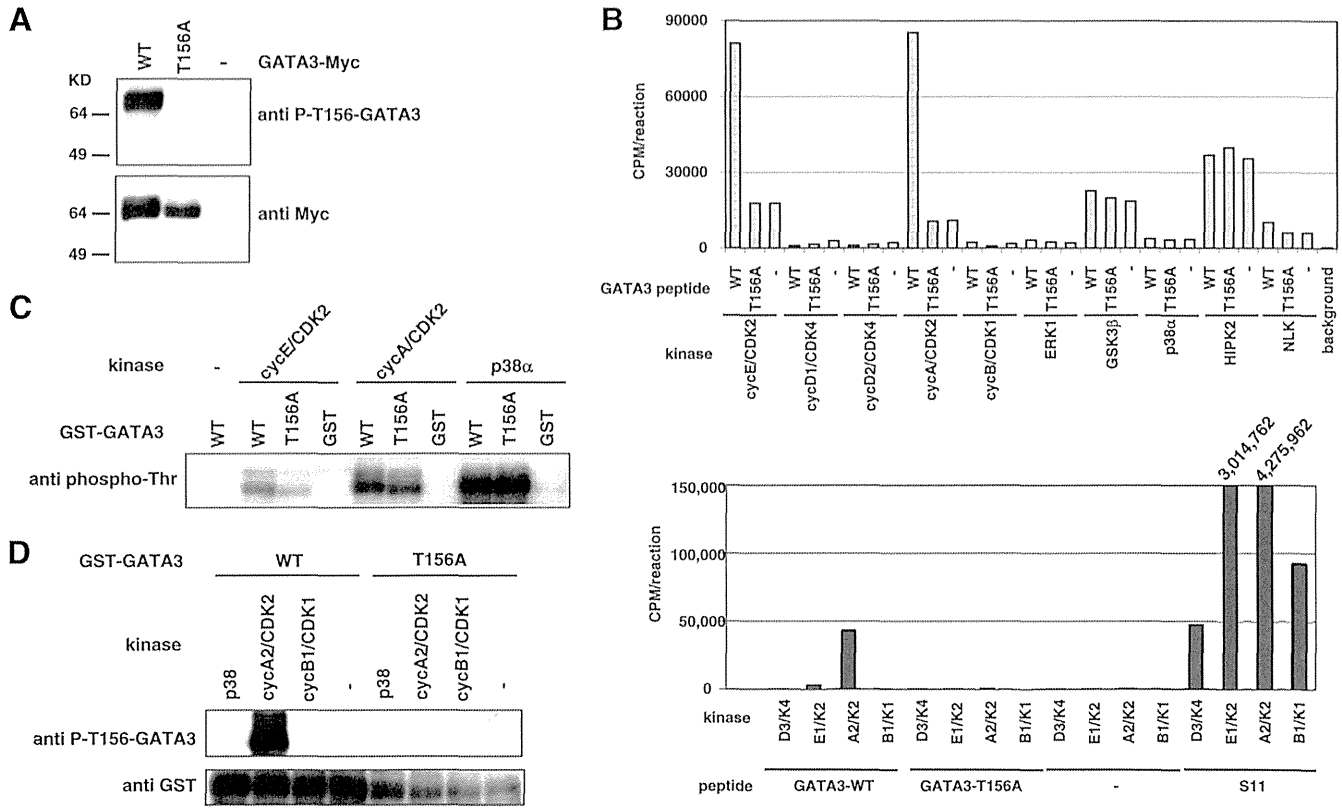

FIG 7 Thr-156 of GATA3 is phosphorylated by CDK2. (A) Transiently expressed GATA3 is phosphorylated at Thr-156 in HEK293 cells. HEK293 cells were transfected with WT or T156A GATA3 and treated with 20 μM MG132 and 20 nM okadaic acid for 5 h to inhibit proteolysis and dephosphorylation of GATA3. Cell lysates were prepared with lysis buffer containing phosphatase inhibitors and protease inhibitors and subjected to immunoblot analysis using phospho-T156-GATA3 or Myc antibodies. (B) CDK2 phosphorylates GATA3 peptide in a Thr-156-dependent manner *in vitro*. A synthetic peptide corresponding to aa 150 to 161 of WT or T156A GATA3 was incubated with [γ-^{32}P]ATP and the indicated kinases at 30°C for 30 min (top panel). S11 peptide (KAPLTPKKAK) is efficiently phosphorylated by various CDKs (22). To confirm the activities of the CDKs used in our experiments, we performed *in vitro* kinase assays using the S11 peptide as a positive control. Wild-type or T156A synthetic GATA3 peptide or S11 peptide was incubated with [γ-^{32}P]ATP along with the indicated CDKs at 30°C for 30 min (bottom panel). The peptides were trapped on P81 papers and monitored for radioactivity using a liquid scintillation counter. (C and D) Recombinant GATA3 is phosphorylated at Thr-156 by CDK2 *in vitro*. WT or T156A GST-fused GATA3 was expressed in *E. coli* and affinity purified using glutathione-Sepharose 4B. GST alone was prepared in parallel as a control. The proteins were incubated with the kinases as indicated in reaction buffer at 30°C for 30 min. Reaction products were subjected to immunoblot analysis with the indicated antibodies.

detected phosphorylated Thr-156 in GATA3 in the DN subset of thymocytes (data not shown). We found that phosphorylation of Thr-156 in GATA3 in DP subsets was observed at an equal level relative to that in DN subsets (DN/DP, 1:1.16) (data not shown). Because GATA3 accumulates in the DN but not in the DP subset in Lck-Cre/*Fbw7*$^{flox/flox}$ mice (Fig. 3A), Thr-156 phosphorylation of GATA3 may participate in Fbw7-mediated degradation of GATA3 in the DN subset. In contrast, an unknown mechanism that suppresses Fbw7-mediated degradation of GATA3 may be present in the DP lineage, while Thr-156 phosphorylation of GATA3 was observed.

DISCUSSION

Half of GATA3-overexpressing CD2-GATA3 Tg mice developed thymic lymphoblastoid tumors (6). This may be consistent with a previous study showing that half of Lck-Cre/*Fbw7*$^{flox/flox}$ mice developed thymic lymphoma (3). We confirmed the accumulation of GATA3 protein at the DN stage in Lck-Cre/*Fbw7*$^{flox/flox}$ mice. Based on the expression pattern of CD2, which starts at a late DN stage, like Lck, overexpression of GATA3 must occur at this point in CD2-GATA3 Tg mice. We speculate that the induction of GATA3 in the thymus of CD2-GATA3 Tg mice and the accumulation of GATA3 in the thymus of Lck-Cre/*Fbw7*$^{flox/flox}$ mice begin at the same phase. The increased abundance of GATA3 late in the DN stage may disturb accurate progression from DN to DP and may result in transformed cells, which have been characterized as CD4$^+$ CD8$^+$ in both Lck-Cre/*Fbw7*$^{flox/flox}$ and CD2-GATA3 Tg mice (3, 37). This may imply that an appropriate amount of GATA3 is essential for the favorable development of T cells not only at the SP phase but also at an earlier phase. We indicated that depletion of Fbw7 protein in Lck-Cre/*Fbw7*$^{flox/flox}$ mice started at DN3 and was completed at DN4 and that GATA3 accumulated at DN4 in mice. We further investigated whether GATA3 accumulation at DN4 affected the proportion of DN4 subpopulations. The proportion in Lck-Cre/*Fbw7*$^{flox/flox}$ mice was not significantly different from that in *Fbw7*$^{flox/flox}$ mice (data not shown). Previous studies suggested that development from DN2 to DN3 might be affected by the deregulation of GATA3 levels. Overexpression of GATA3 in mouse fetal liver progenitors blocked the development of DN2 and DN3 cells in fetal thymus organ culture or in an OP9-DL1 coculture system (14, 38). Xu et al. (39) reported that E2A repressed GATA3 expression at the DN2 stage. In that study,

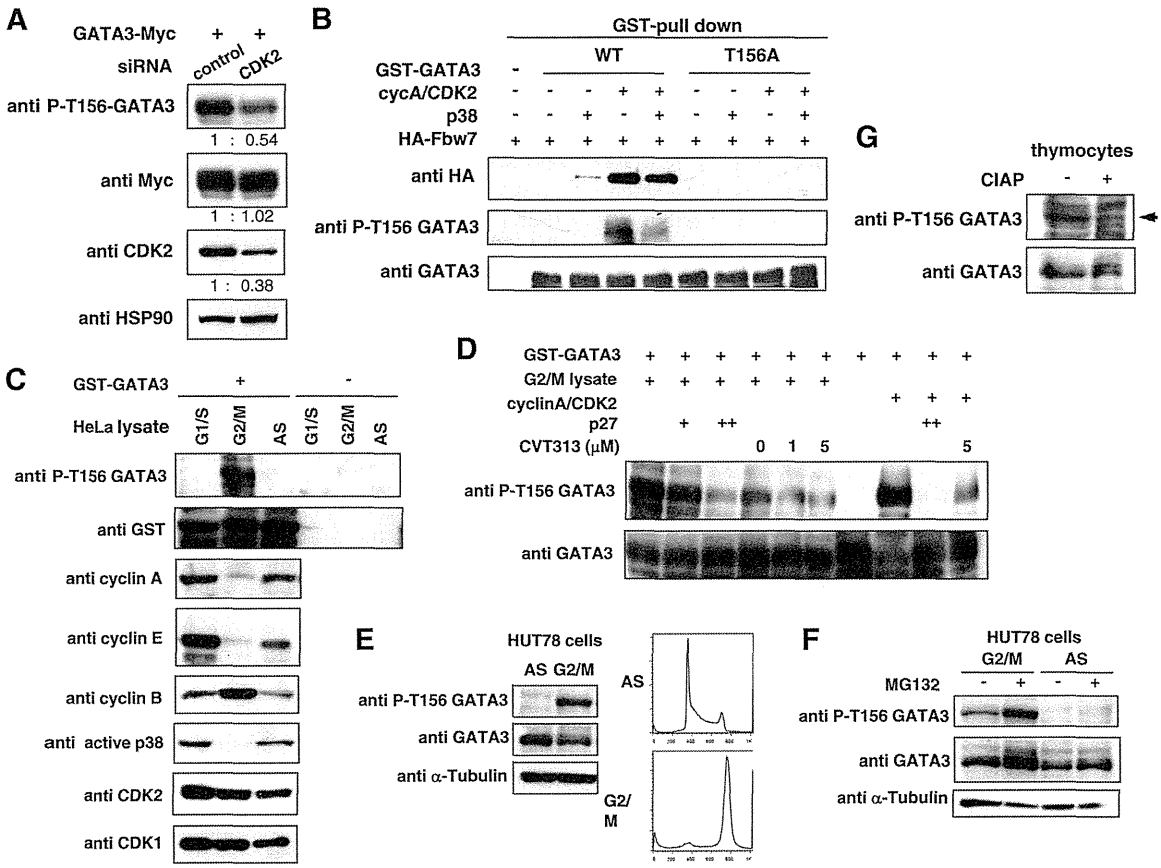

FIG 8 Phosphorylation at Thr-156 in GATA3 by CDK2 is required for association of Fbw7 and is executed in HUT78 cells during G_2/M phase and in thymocytes of mice. (A) Depletion of CDK2 reduced phosphorylation of Thr-156 in GATA3 in HEK293 cells. HEK293 cells were transfected with a WT GATA3 expression plasmid and CDK2 or a control siRNA. After 43 h of transfection, cells were treated with okadaic acid and MG132 for 5 h. Cell lysates were prepared and subjected to immunoblot analysis with the indicated antibodies. The numbers reflect the ratio of the levels of the indicated proteins between the CDK2 siRNA-transfected cells and control cells. (B) GATA3 binding to Fbw7 *in vitro* is Thr-156 phosphorylation dependent. Purified WT or T156A GST-GATA3 was incubated with the indicated kinases in reaction buffer at 30°C for 30 min. Lysates from HEK293 cells transfected with HA-Fbw7 were immunoprecipitated with HA antibody, and the immunocomplexes containing Fbw7 were incubated with phosphorylated GST-GATA3 *in vitro* as indicated. To analyze GATA3 and Fbw7 binding, the GST fusion protein complexes were precipitated using glutathione-Sepharose beads and subjected to immunoblotting with the indicated antibodies. (C and D) Phosphorylation of recombinant GATA3 *in vitro*. Reaction products were then subjected to immunoblot analysis with the indicated antibodies. (C) Phosphorylation of Thr-156 in GATA3 in G_2/M-arrested cells. GST-GATA3 was incubated with lysate prepared from either cells arrested in G_1/S or G_2/M phase or nonsynchronized (AS) HeLa cells. (D) Effects of CDK2 inhibition on Thr-156 phosphorylation of GATA3 in G_2/M cell lysate. The responsible kinase in G_2/M-phase cell lysate is CDK2. GST-GATA3 was incubated with the indicated kinase sources in the absence or presence of CDK2 inhibitor (CVT313) or competitor (p27). (E and F) Thr-156 phosphorylation of endogenous GATA3 in G_2/M phase in T-cell lymphoma HUT78 cells. G_2/M-arrested and asynchronous (AS) HUT78 cells were prepared as indicated in Materials and Methods. Cell lysates were prepared and subjected to immunoblot analysis with the indicated antibodies. (F) G_2/M-arrested and asynchronous cells were treated with or without MG132 for 5 h before harvest. Cell lysates were prepared and subjected to immunoblot analysis with the indicated antibodies. (G) Cell lysate from whole thymocytes obtained from an *Fbw7*$^{flox/flox}$ mouse at 6 weeks of age was incubated with or without calf intestinal alkaline phosphatase (CIAP) at 37°C for 30 min and subjected to immunoblot analysis with the indicated antibodies.

increased GATA3 in E2A$^{-/-}$ DN2 lineages prevented T-cell differentiation to the DN3 stage and caused an aberrant proliferation of DN2 cells. Fbw7 may be involved in development of the DN2-DN3 stage by repression of GATA3 levels. In our study using Lck-Cre/*Fbw7*$^{flox/flox}$ mice, the effects of Fbw7 depletion were focused on the DN4 stage. To evaluate the contribution of Fbw7 on GATA3 turnover in earlier DN subpopulations, Scl-Cre or Mx1-Cre driver Fbw7 knockout mice, in which depletion of Fbw7 is expected throughout the DN stages, may be useful. However, some confusion may occur when these mice are used because, in addition to GATA3, other Fbw7 targets such as Notch play roles in DN development. Data regarding GATA3-targeting genes, which are active in the DN lineage, have not been reported to date. Consequently, further observations will be required in future studies to identify phenotypes which might be obtained as a result of accumulation of GATA3 in Fbw7-depleted mice.

Aberrant expression of GATA3 was reported in classical Hodgkin lymphoma (cHL) (40). A pathological feature of cHL is the occurrence of a small number of the typical Hodgkin and Reed/Sternberg (HRS) tumor cells among a mixed cellular infiltrate. Whereas HRS cells are derived from germinal center B cells, they ectopically express GATA3. GATA3 contributes to cytokine signaling in HRS cells, which presumably has an essential role in cHL pathogenesis (40). Anomalous GATA3 expression in HRS cells is stimulated by the deregulated activity of NF-κB and Notch1, which bind directly to the GATA3 promoter (40). Intriguingly, both NF-κB and Notch1 are substrates of Fbw7 (5, 41). Therefore, once Fbw7 deficiency occurs in cHL, NF-κB and Notch1 accumu-

late and subsequently induce GATA3 expression. Further, the repressed degradation of GATA3 would enhance the malignant signal. Finally, the defect of Fbw7 in cHL may promote the development of aggressive disease caused by excess GATA3.

Although CD8 SP subsets in the thymus of the Lck-Cre/*Fbw7*$^{flox/flox}$ mice retained GATA3 expression, CD8$^+$ T cells in the spleen did not express GATA3 (Fig. 3C). The GATA3-independent pathway may be responsible for the reduced frequency of CD8$^+$ T cells in the spleen of Fbw7-deficient mice. Nonetheless, the decrease in a percentage of CD8$^+$ T cells in splenocytes of the Lck-Cre/*Fbw7*$^{flox/flox}$ mice compared with control mice was confirmed. This is consistent with results from CD2-GATA3 Tg mice showing that exogenous expression of GATA3 was not detected in splenocytes (37). We observed a significant reduction of CCR7 expression in the CD8 SP cells from Lck-Cre/*Fbw7*$^{flox/flox}$ mice compared with *Fbw7*$^{flox/flox}$ mice. Because of inadequate GATA3 levels, proper cell differentiation and exit from the thymus might be disturbed, and/or apoptosis of immature subsets might be induced in CD8 SP subsets of Lck-Cre/*Fbw7*$^{flox/flox}$ mice. Ultimately, only the normal CD8 SP subpopulation that expresses appropriate GATA3 protein levels may succeed in translocation toward peripheral organs. We detected a decrease in not only CD8$^+$ T cells but also CD4$^+$ T cells in splenocytes of the Lck-Cre/*Fbw7*$^{flox/flox}$ mice compared with control levels. A previous study showed that the CD4$^+$ T-cell subset in the spleen of CD2-GATA3 Tg mice advanced the Th2-committed phenotype although its rate was comparable to that of control mice (37). Accordingly, we speculate that some aberration, from excess GATA3 during differentiation and maturation in the thymus, persisted throughout development of CD4$^+$ T cells in the spleen. Nevertheless, similar to results in CD2-GATA3 Tg mice, in Lck-Cre/*Fbw7*$^{flox/flox}$ mice there was no significant effect on CD4 SP cells in the thymus. This might suggest that CD8 SP cells were more sensitive to changes in GATA3 than CD4 SP cells. In our experiments, GATA3 protein was markedly decreased in the CD8 SP subset after maturation of the DP lineage in control mice. In contrast, deficiency of Fbw7 resulted in accumulation of GATA3 protein in CD8 SP subsets, suggesting that Fbw7-mediated degradation plays a key role in regulating GATA3 protein levels in the subsets. Because the forced expression of GATA3 induces apoptosis and inhibits the final maturation of CD8 SP T cells, it is suggested that reduction of GATA3 is required for the satisfactory development of the CD8 SP lineage after positive selection. The proportion of CD8 SP cells in the thymic cells is dependent on the extent of apoptosis and proliferation during differentiation. It is speculated that enhanced apoptosis of CD8 SP cells in Lck-Cre/*Fbw7*$^{flox/flox}$ mice decreased their percentage in thymocytes. Consequently, GATA3 accumulation caused by the depletion of Fbw7 might induce this decrease in cell populations. However, the same explanation cannot be applied to the reduction of CD4 SP cells, because increased GATA3 was not observed to influence viability of CD4 SP cells. Indeed, it might be an effect of the accumulation of other Fbw7 substrates. Onoyama et al. also speculated that DP thymocytes had lost the ability to undergo positive selection or that the proliferation or survival of SP cells was impaired in Fbw7-deficient mice (3). Nevertheless, they suggested that the participating molecules remained to be elucidated.

In a reference database for gene expression analysis (RefExA), expression of GATA3 is observed not only in the thymus and spleen but also in salivary gland, breast, skin, bladder, kidney, placenta, and blood. RefExA also demonstrated the expression of Fbw7 in brain, breast, skin, heart, adrenal gland, intestine, stomach, and testis, in addition to thymus. Therefore, GATA3 may be regulated by Fbw7-mediated degradation in numerous tissues that express both GATA3 and Fbw7. This possibility should be investigated in the future using a tissue-specific conditional knockout system.

Many substrates of Fbw7 contain a CPD sequence, which is often phosphorylated by GSK3 (1, 4, 5, 30, 31). Although we initially predicted that the CPD in GATA3 is phosphorylated by GSK3, our data indicate that the targeting kinase is CDK2, not GSK3. Consistent with these data, we previously showed that S/T-P-X-K/R, which corresponds to the CPD sequence in GATA3, is the consensus targeting sequence of cyclin A/E-CDK2 (22). Here, we propose GATA3 as the first target of CDK2-mediated phosphorylation for degradation by Fbw7. These observations suggest that regulatory signals in addition to GSK3 participate in regulating Fbw7 substrates. Levels of these proteins, which have various functions in the development of T cells, will be maintained at the appropriate stage for proper differentiation, maturation, and survival of T cells. Because GSK3-independent phosphorylation of CPD in GATA3 is a unique Fbw7 targeting pathway, we predict that GATA3 degradation should be distinct from the other Fbw7 targets in T cells. MAPK-associated phosphorylation of GATA3 is related to the so-called emergent signals in later stages of T-cell development, while the CDK2-associated GATA3 degradation system mediated by Fbw7 may be required in early lineages for constitutive precise differentiation.

We showed that phosphorylation of Thr-156, which was an essential modification for binding of Fbw7, was mediated by CDK2. It is consistent that the phosphorylated level of intrinsic GATA3 varies during G_2/M phase in T-cell lymphoma HUT78 cells. We also detected the phosphorylation at Thr-156 of GATA3 in thymocytes of mouse. Furthermore, we clarified that Fbw7 participates in GATA3 degradation in the DN4 lineage. Because CDK2 activity is present during the G_1/S border to G_2/M stages in proliferating cells, it was speculated that CDK2-dependent phosphorylation of GATA3 and its degradation by Fbw7 in G_2/M occur in proliferating DN4 cells and T-cell lymphomas such as HUT78 cells. Our data indicated that Fbw7 also participates in GATA3 degradation in CD8 SP cells. Because cell proliferation is not present in CD8 SP cells, cell cycle-independent CDK2 activity may participate in the phosphorylation of Thr-156. Alternatively, it is possible that unknown kinases and/or other mechanisms are involved in Fbw7-mediated degradation of GATA3 in CD8 SP cells.

Finally, we propose that control of GATA3 levels by Fbw7 contributes to the fine-tuning of T-cell development.

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Anti-Cancer Agent-Induced Nephrotoxicity

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Abstract: Patients with cancer are frequently exposed to risk of renal injuries associated with disease-related or iatrogenic causes. Nephrotoxicity is a potential adverse effect of anti-cancer agents and may result in a variety of functional abnormalities, including glomerular or tubular dysfunction, hypertension and disturbance of the renal endocrine function. In this review article, we comprehensively discuss the incidence, clinical presentation, prevention and management of anti-cancer agent-induced renal dysfunction. We focus on both relatively new anti-cancer agents (bevacizumab, gefitinib, gemcitabine, imatinib, rituximab and trastuzumab) and traditional agents (cisplatin, methotrexate, ifosfamide and taxanes) to cover a selection of the most frequently used anti-cancer agents. Increased understanding of the mechanism of renal injury by these agents is considered to be important for developing novel anti-cancer agents that have far fewer adverse effects on kidneys.

Keywords: Acute kidney injury, anti-cancer agents, cisplatin, chemotherapy, electrolyte abnormality, nephrotoxicity.

INTRODUCTION

Patients with cancer are frequently at risk for renal dysfunction due to disease-related or iatrogenic causes [1]. The most clinically important factors are extracellular volume depletion secondary to vomiting, diarrhea or appetite loss. Moreover, patients with advanced prostate cancer are exposed to risk for post-renal obstructive nephropathy. Tumor lysis syndrome is another cause of renal injury in certain cancer patients. Generally, geriatric cancer patients are at a high risk for renal dysfunction following administration of anti-cancer agents because aging is accompanied by a steady decline in renal function and geriatric patients often have complications, including hypertension, diabetes mellitus and heart failure that make the kidneys more vulnerable to injury [2, 3].

Nephrotoxicity is an inherent adverse effect of certain anti-cancer agents [4, 5]. Nephrotoxic agents can cause renal injury *via* intrarenal vasoconstriction, damage to vasculature, direct tubular toxicity and intratubular obstruction [5]. The vulnerability of the kidney to nephrotoxic agents is attributed to several functional properties, including a rich blood supply, high levels of toxicant delivery, high tubular reabsorption capacity *via* specific transporters leading to high intracellular concentrations in tubular cells and the capacity to concentrate toxins to high levels within the medullary interstitium *via* countercurrent mechanisms. In addition, the kidney is an important site for xenobiotic metabolism and may transform relatively harmless compounds into toxic metabolites. Finally, the kidneys have a high metabolic rate, and the heavy workload of renal cells results in increased sensitivity to toxicants and vasoactive agents [4-6].

Recently, the increased number of novel anti-cancer agents has broadened the range of anti-cancer treatments, but these novel agents also have nephrotoxic potential [6]. In this review article, we discuss the incidence, clinical presentation, prevention and management of anti-cancer agent-induced renal dysfunction. We focus on both relatively new and classical agents to cover a selection of the most frequently used anti-cancer agents.

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CISPLATIN

Cisplatin [(*SP*-4-2)-Diamminedichloroplatinum, CDDP] is an inorganic platinum chemotherapeutic drug that is widely used to treat various solid organ cancers (Fig. **1A**) [7]. Although CDDP can also result in ototoxicity, gastrotoxicity, neurotoxicity and myelosuppression, the main dose-limiting adverse effect is nephrotoxicity [8, 9]. Despite intense efforts to discover less-toxic but equally effective alternatives, CDDP continues to be used.

CDDP is cleared by both glomerular filtration and tubular secretion in the kidney [10, 11]. CDDP concentrations in the kidney exceed those in blood, suggesting an active accumulation of drug by renal parenchymal cells. Previous studies using kidney slices [10], cultured renal epithelial cells [12] and isolated proximal tubular segments [13] have provided evidence for basolateral-to-apical transport of CDDP (Fig. **1B**). Recent studies have identified that organic cation transporter 2 (OCT2), a membrane transporter, is responsible for the basolateral transport of CDDP into cells [14, 15]. CDDP was also shown to inhibit the uptake of other OCT2 substrates, which is consistent with the view that these substrates share a common transport pathway. Likewise, cimetidine, another OCT2 substrate, reduces renal CDDP uptake and nephrotoxicity [14, 16]. Two recent observations point to an important role for OCT2 in mediating renal CDDP uptake and toxicity. First, knockout of the *OCT2* gene significantly reduces urinary CDDP excretion and nephrotoxicity [16, 17]. Second, a nonsynonymous single-nucleotide polymorphism (SNP) in the *OCT2* gene (rs316019) is associated with reduced CDDP-induced nephrotoxicity [17]. On a related note, multi-drug and toxin extrusion 1 (MATE1) is suggested to be responsible for the final step of urinary excretion of cationic drugs, such as CDDP [18, 19]. MATE1 exists in renal brush-border membranes and mediates the exchange of organic cations like CDDP with hydrogen ions (Fig. **1B**). Knockout of the *MATE1* gene results in renal accumulation of CDDP and significantly worsens nephrotoxicity [20]. Therefore, MATE1 might also play an important role in the development of CDDP-induced nephrotoxicity.

CDDP-induced nephrotoxicity can present in a number of ways (Table **1**). The most serious presentation is acute kidney injury (AKI). The incidence of AKI is in the range of 20-30% of patients even if these patients were treated with saline hydration and diuresis [21]. Typically, renal insufficiency begins several days

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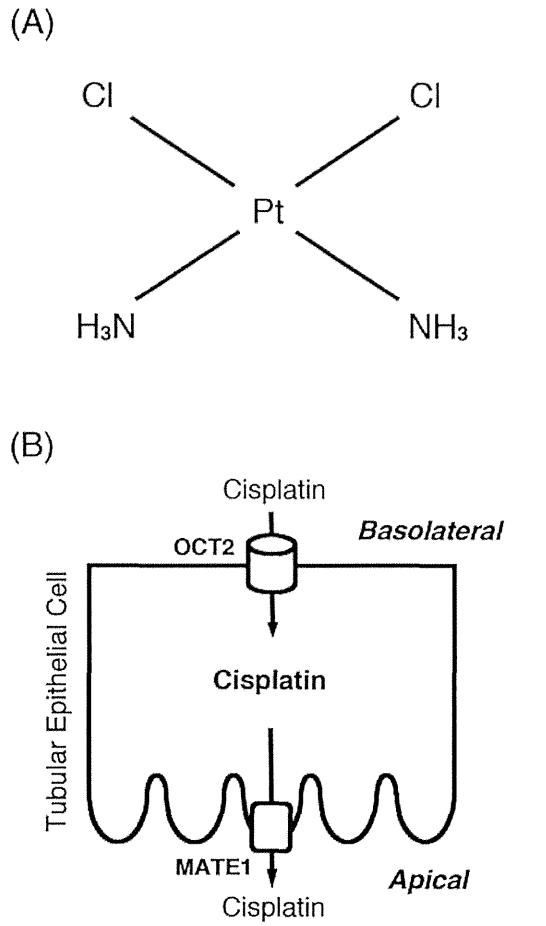

Fig. (1). Structure and transport of cisplatin (**A**) Cisplatin chemical structure. Cisplatin [cisplatinum or *cis*-diamminedichloroplatinum (II), CDDP] is a chemotherapy drug classified as an alkylating agent. (**B**) Basolateral-to-apical cisplatin transport. Organic cation transporter 2 (OCT2), a membrane transporter, is responsible for the basolateral transportation of cisplatin into tubular epithelial cells. Conversely, multi-drug and toxin extrusion 1 (MATE1) is expressed in renal brush-border membranes and mediates the urinary excretion of cisplatin from tubular epithelial cells.

after CDDP administration. Urine output is usually preserved (non-oliguric), but the urine may contain glucose and small amounts of protein, which are indicative of proximal tubular dysfunction. Recovery of renal function usually occurs over a period of 2-4 weeks, although more protracted courses and lack of recovery have been reported. Progressive nephrotoxicity can result with successive treatment courses, despite preventative measures [22].

Electrolyte abnormalities, including hypomagnesemia, hypokalemia, hypocalcemia, hypouricemia, hyponatremia and Fanconi syndrome, can occur after CDDP administration due to renal wasting and can persist for years, despite therapy cessation (Table **1**). Of these abnormalities, hypomagnesemia is the most common adverse effect, with an incidence rate as high as 90% [23]. CDDP-induced hypokalemia and hypocalcemia are the result of increased losses and altered renal processing in the presence of hypomagnesemia.

A number of risk factors for CDDP-induced nephrotoxicity have been identified. Nephrotoxicity increases with the frequency of administration and the cumulative dose of CDDP [24, 25]. Other patient variables, including female sex, older age, smoking and hypoalbuminemia, have been found to be associated with increased risk of nephrotoxicity [25, 26]. In general, pre-existing renal dysfunction increases the risk for AKI, but there are limited data on the incidence of nephrotoxicity in populations with chronic kidney disease (CKD) because many trials exclude patients with renal insufficiency [27]. At present, clinical studies have not identified any impacts of diabetic conditions on nephrotoxicity [28].

Table 1. Renal manifestations of cisplatin nephrotoxicity (modified from Miller *et al.* [101]).

Acute kidney injury (20-30%)
Hypomagnesemia (40-100%)
Hypocalcemia
Hypokalemia
Fanconi syndrome
Hyponatremia (renal salt wasting)
Distal renal tubular acidosis
Thrombotic microangiopathy
Transient proteinuria
Erythropoietin deficiency
Chronic renal failure

Volume expansion with sodium chloride has been the primary means for reducing CDDP-induced nephrotoxicity [29]. Although many hydration regimens include the use of either mannitol or furosemide, there is no good evidence that these drugs provide any additional benefit [30]. In fact, one trial reported greater nephrotoxicity in patients who received saline plus mannitol compared with saline alone [30]. Hypertonic saline (3%) has also been advocated; however, its use is limited by inconsistent findings of renoprotection and an increased risk of adverse effects relative to isotonic saline (0.9%) [31, 32]. Therefore, recent clinical guidelines recommend prehydration with 0.9% saline and the avoidance of diuretics [33].

Amifostine [2-(3-aminopropylamino) ethylsulfanyl phosphonic acid] is a pharmaceutical antidote to CDDP that is approved by the U.S. Food and Drug Administration (FDA) for reducing cumulative nephrotoxicity due to repeated CDDP dosing in patients with advanced ovarian cancer [33]. However, the data regarding the use of amifostine is limited just for the treatment of ovarian cancer.

Unfortunately, even with aggressive hydration, renal toxicity still occurs [21]. This has encouraged the development of more effective preventive strategies.

GEMCITABINE-ASSOCIATED THROMBOTIC MICRO-ANGIOPATHY

Gemcitabine [4-amino-1-(3,3-difluoro-4-hydroxy-5-[hydroxymethyl] tetrahydrofuran-2-yl)-1H-pyrimidin-2-one, Gemzar®; Eli Lilly and Company, Indianapolis, IN, USA] is a pyrimidine analog that is used to treat several malignancies, including pancreatic cancer, non-small cell lung cancer and biliary cancer. Gemcitabine-associated thrombotic microangiopathy (TMA) has been reported to be relatively rare, with an estimated incidence rate of 0.015 to 1.4 % [34, 35]. However, the indications for gemcitabine are expanding, making comprehensive characterization of this complication increasingly important. There are no gold standard laboratory values that define TMA, but the clinical triad of renal failure, thrombocytopenia and microangiopathic hemolytic anemia (MAHA) is considered the hallmark of TMA syndromes [36]. Clinically, new or exacerbated hypertension is a prominent feature and precedes the diagnosis by several weeks [37]. There is no clear-cut relationship between cumulative gemcitabine dose and TMA

risk, but most patients have been receiving gemcitabine for at least 3-5 months before TMA onset [38].

There is currently no consensus on the optimal prevention and treatment for chemotherapy-induced TMA. Therefore, immediate discontinuation of gemcitabine is the first step. In addition to supportive treatment including anti-hypertensive therapy and dialysis according to individual clinical presentation and severity, some authors recommend the use of plasma exchange [39, 40]. Humphreys *et al.* [37] reported the 5 patients who developed TMA after gemcitabine treatment and were undergone 5-10 sessions of plasma exchange. Of the 5 patients, one patient died due to progressive disease, one patient required transient hemodialysis, two patients developed end-stage renal failure and one patient developed CKD.

IFOSFAMIDE

Ifosfamide [*N*-3-bis (2-chloroethyl)-1,3,2-oxazaphosphinan-2-amide-2-oxide] is an alkylating agent of the nitrogen mustard group used to treat various malignancies, including sarcomas and lymphomas [5, 41, 42]. Nephrotoxicity can be caused by the agent itself or by a metabolite, chloroacetaldehyde [43]. Ifosfamide mainly causes proximal tubular dysfunction termed Fanconi syndrome and chronic renal insufficiency, but rarely causes AKI [44, 45]. Therefore, hypokalemia, hypouricemia, hypophosphatemia, metabolic acidosis, renal glycosuria and aminoaciduria due to renal wasting are characteristic features of ifosfamide-induced nephrotoxicity [5]. In a study of 120 patients who received a median cumulative ifosfamide dose of 30 g/m^2, evidence of proximal tubular dysfunction was observed in 66% of patients after treatment, and 7% of patients had Fanconi syndrome [46]. Dekkers *et al.* have also reported long-term nephrotoxicity due to ifosfamide [47]; high-dose ifosfamide treatment in childhood was significantly associated with persistent tubular damage and lower estimated glomerular filtration rate (GFR) in adult survivors.

Cumulative ifosfamide doses exceeding 45 g/m^2 and patient age are the two greatest risk factors for nephrotoxicity development [48, 49]. Previous or concurrent administration of cisplatin (CDDP) also increases the risk for nephrotoxicity. In fact, increased cumulative CDDP dose has been associated with increased severity of ifosfamide-induced renal dysfunction [49].

Treatment of ifosfamide-induced nephrotoxicity is supportive, with replacement of electrolytes as needed to prevent electrolyte imbalances and their consequences. Other than aggressive hydration, there are no specific therapies to protect against the development of ifosfamide-induced nephrotoxicity.

IMATINIB

Imatinib mesylate [4-(4-Methylpiperazin-1-ylmethyl)-*N*- (4-methyl-3- [4-pyridin-3-ylpyrimidin-2-ylamino] phenyl) benzamide monomethanesulfonate, Glivec®; Novartis, Basel, Switzerland] is the first tyrosine kinase inhibitor to be successfully used in clinical practice. Imatinib is now the first-line therapy for patients with chronic myeloid leukemia (CML) [50, 51] and is remarkably efficient for treating patients with gastrointestinal stromal tumors (GIST) [52] and several myeloproliferative diseases, such as idiopathic hypereosinophilic syndrome (HES) [53].

The characteristic genetic abnormality of CML is the Philadelphia chromosome [54], which results from a reciprocal translocation between the long arms of chromosomes 9 and 22 [55]. The molecular consequence of this translocation is the generation of the fusion protein BCR-ABL, a constitutively active tyrosine kinase, which is present in virtually all patients with CML. Imatinib was originally designed to target BCR-ABL, although many off-target kinases, including platelet-derived growth factor receptor (PDGF-R) and c-KIT (CD117), are also affected [56].

Several case reports suggest that imatinib can cause acute tubular necrosis [57-59], which may be related to PDGF-R inhibition [60]. PDGF-β expression has been reported in proximal tubules, mesangial cells and interstitial cells in the kidney. It has also been shown that the PDGF-β/PDGF-R axis plays an important role in renal tubular regeneration after acute tubular necrosis [61]. Thus, imatinib may interfere in tubular repair processes by inhibiting PDGF-R. Recently, Marcolino *et al.* reported that 7% of CML patients developed AKI, and 12% of those patients developed chronic renal insufficiency after imatinib treatment [62].

Other case reports also suggest that imatinib can cause TMA in patients with idiopathic HES [63] and Fanconi syndrome in patients with CML [64, 65], although those molecular mechanisms remain to be clarified.

There is currently no consensus on the optimal prevention and treatment for imatinib-induced nephrotoxicity. Therefore, its treatment is supportive according to individual clinical presentation and severity. Further studies are needed to clarify this issue.

METHOTREXATE

The dihydrofolate reductase inhibitor methotrexate [(*S*)-2-(4-[([2,4-diaminopteridin-6-yl] methyl) methylamino] benzamido) pentanedioic acid, MTX] is a widely used anti-cancer agent, and the administration of high-dose MTX followed by leucovorin rescue has become an essential component in the treatment of several cancers [66]. MTX is also used in non-cancerous conditions, such as rheumatoid arthritis. Acute tubular necrosis subsequent to crystallization of MTX and its metabolite, 7-hydroxy-MTX, within renal tubules is the underlying mechanism of MTX-induced nephrotoxicity [67]. The overall incidence rate of AKI in MTX-treated patients is approximately 1.8% (range, 0-12%), and renal injury is generally reversible [5]. Because MTX is mainly excreted *via* the kidneys, renal dysfunction prolongs MTX exposure, which in turn increases the risk of other MTX-associated toxicities, including myelosuppression, mucositis, hepatitis and dermatitis.

MTX solubility is pH-dependent. Volume depletion and acidic urine are risk factors of nephrotoxicity [68]. Therefore, aggressive hydration plus urinary alkalinization are recommended for preventing MTX precipitation within renal tubules and promoting MTX excretion into the urine. Infusion with 40-50 meq/L sodium bicarbonate solution should be initiated at least 12 hours before MTX administration and should be continued for up to 72 hours [4].

Recently, carboxypeptidase-G2 (CPDG2, Voraxaze®; BTG International Ltd, London, UK), a recombinant bacterial enzyme that rapidly hydrolyzes MTX to inactive metabolites, has become available for the treatment of nephrotoxicity induced by high-dose MTX. CPDG2 administration is well tolerated and results in rapid reductions of plasma MTX concentrations. The early administration of CPDG2 in addition to leucovorin may be beneficial for patients with MTX-induced nephrotoxicity and elevated plasma MTX concentrations [69, 70].

It is also noteworthy that significant MTX clearance can be achieved with high-flux dialyzers and that plasma MTX levels can be successfully lowered in patients with MTX-induced AKI by charcoal hemoperfusion and sequential hemodialysis [71].

RITUXIMAB

Rituximab (Rituxan®/MabThera®; Roche, Basel, Switzerland) is a humanized mouse chimeric monoclonal antibody against CD20 antigen that directly targets B cells and has recently been used to treat several tumors, including B-cell non-Hodgkin lymphoma, B-cell chronic lymphocytic leukemia (CLL) and Waldenström's macroglobulinemia [72-74]. In some patients with Waldenström's macroglobulinemia, a flare phenomenon of immunoglobulin M

has been reported after rituximab treatment [75, 76]. This reaction is usually transient, asymptomatic and resolvable. However, several patients with Waldenström's macroglobulinemia have reportedly developed AKI during rituximab treatment [77, 78]. Shaikh *et al.* [77] described a case of membranoproliferative glomerulonephritis due to type I cryoglobulinemia that was resolved with plasmapheresis. However, Izzedine *et al.* [78] reported a case of acute tubular necrosis without the presence of cryoglobulinemia. Therefore, rituximab-induced nephrotoxicity is mainly limited to patients with Waldenström's macroglobulinemia, and the pathological findings remain inconsistent.

TRASTUZUMAB

Human epidermal growth factor receptor 2 (*HER2*)/*neu* is a growth factor receptor gene that is amplified in approximately 20-25% of breast cancers, and its corresponding encoded protein, HER2, is also detected at abnormally high levels in these malignant cells [79, 80]. Its main function is to mediate cell growth, differentiation and survival, thereby promoting more aggressive tumor behavior. Studies have shown that women whose tumors exhibit either amplification of the *HER2/neu* gene or overexpression of HER2 have more aggressive breast cancers that are associated with significantly shortened disease-free and overall survival compared with women whose tumors do not exhibit them [81].

Trastuzumab (Herceptin®; Roche) is a humanized monoclonal antibody that targets the extracellular domain of HER2 and has been demonstrated to have significant survival benefit in patients with HER2-positive breast cancer [82]. To date, its nephrotoxicity has not been reported [83].

VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) BLOCKADE AND NEPHROTOXICITY

Tumor angiogenesis mediated by VEGF plays a critical role in tumor growth, invasion and metastasis. Therefore, anti-VEGF agents are important components in the treatment of many solid tumors. Of these, bevacizumab (Avastin®; Roche) is a humanized monoclonal antibody to the VEGF ligand that is the first FDA-approved agent, and its indication is growing. However, adverse effects have become apparent. Two of the most common adverse effects are hypertension (3-36% incidence) and proteinuria (21-64% incidence) [84]. Nephrotic-range proteinuria also occurs in 1-2% of bevacizumab-treated patients [84].

VEGF blockade induces endothelial dysfunction, which results in the inhibition of VEGF-dependent vasodilatory pathways, such as nitric oxide (NO) and prostacyclin (prostaglandin I_2), and the possible up-regulation of vasoconstrictive pathways, such as endothelin-1. Together with the loss of microvascular capillary density through capillary rarefaction, these mechanisms cause systemic vasoconstriction, resulting in increased afterload and hypertension. Endothelial dysfunction also resets the pressure-natriuresis relationship, resulting in inadequate renal sodium retention followed by increased blood pressure. VEGF blockade may also block VEGFR-3, the VEGF receptor expressed on endothelial cells, resulting in decreased lymphangiogenesis and reduced capacity of the lymphatic network to buffer sodium and extracellular fluid volume. Both of these mechanisms contribute to volume overload and exacerbate hypertension [85, 86].

The most severe renal lesion induced by VEGF blockade is TMA, which is characterized by profound endothelial swelling known as endotheliosis and may be associated with proteinuria. Although endotheliosis is a consistent finding of all TMAs, the degree and prominence of endothelial swelling observed in bevacizumab-treated patients is characteristic and is most similar to TMA seen in preeclampsia [87, 88]. Recently, Eremina *et al.* demonstrated that local reduction of VEGF was sufficient to induce TMA by using conditional knockout mice with a deleted VEGF ligand in the renal podocytes [89]. Their results indicate that the loss of VEGF disrupts paracrine signaling from podocytes to glomerular endothelial cells expressing VEGFR-2 and induces glomerular endothelial injuries and finally causes TMA [89].

Although the management of proteinuria has not been tested in prospective trials, it is recommended to examine the presence of proteinuria and renal function before and during the anti-VEGF therapy. The therapy should be interrupted if nephrotic syndrome becomes apparent.

OTHER ANTI-CANCER AGENTS

Gefitinib

Gefitinib [*N*- (3-chloro-4-fluoro-phenyl)-7-methoxy-6- (3-morpholin-4-ylpropoxy) quinazolin-4-amine, Iressa®; AstraZeneca, London, UK] is an oral epidermal growth factor receptor (EGFR)-associated tyrosine kinase inhibitor. Many studies reported that gefitinib could significantly improve the progression-free survival and quality of life in patients, and suggested that it can be used at any stage of therapy for non-small cell lung cancer (NSCLC) with *EGFR* mutations [90-92].

The common adverse effects of gefitinib include diarrhea, rash, acne, dry skin, nausea and vomiting. Interstitial pulmonary disease is a rare but potentially fetal complication [93, 94]. The nephrotoxicity by gefitinib is also very rare, although Kumasaka *et al.* [95] reported a case of nephrotic syndrome and Wan *et al.* [96] reported another case of AKI after the treatment. Both cases improved after the discontinuation of gefitinib.

Taxanes Including Paclitaxel and Docetaxel

Taxanes are di-terpenes produced by the plants of the genus *Taxus* (yews). Paclitaxel (Taxol®; Bristol-Myers Squibb, New York, NY, USA) and docetaxel (Taxotere®; Sanofi, Paris, France) belong to the Taxane family because of their chemical structures contain a common three phenols ring. The mechanism of action by taxanes is the inhibition of microtubule dynamics that promote microtubule polymerization and inhibit depolymerization, which results in cell cycle arrest in G_2 and M phase, leading to cell death [97-99].

The most frequent dose-limiting toxicities of paclitaxel and docetaxel include myelosuppression, hypersensitivity reactions, neuropathy and musculoskeletal effects [99]. In contrast, taxanes are supposed to be relatively safe for kidneys, although Takimoto *et al.* [100] recently reported that docetaxel can cause acute tubular nephrotoxicity in NSCLC patients.

CONCLUSION

The successful treatment of malignant diseases is actually limited by the nephrotoxicity of anti-cancer agents, although physicians are facing the dilemma that the discontinuation of anti-cancer treatment increases the potential for disease progression and relapse. Therefore, increasing our understanding of the mechanisms on renal injuries by anti-cancer agents is important for developing novel agents that have far fewer adverse effects on kidneys.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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Genes to Cells

YB-1 promotes transcription of *cyclin D1* in human non-small-cell lung cancers

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Cyclin D1, an oncogenic G1 cyclin, and YB-1, a transcription factor involved in cell growth, are both over-expressed in several human cancers. In human lung cancer, the functional association between YB-1 and cyclin D1 has never been elucidated. In this study, we show YB-1 is involved in the transcription of *cyclin D1* in human lung cancer. Depletion of endogenous YB-1 by siRNA inhibited progression of G1 phase and down-regulated both the protein and mRNA levels of cyclin D1 in human lung cancer cells. Forced over-expression of YB-1 with a cyclin D1 reporter plasmid increased luciferase activity, and ChIP assay results showed YB-1 bound to the *cyclin D1* promoter. Moreover, the amount of *YB-1* mRNA positively correlated with *cyclin D1* mRNA levels in clinical non-small-cell lung cancer (NSCLC) specimens. Immunohistochemical analysis also indicated YB-1 expression correlated with cyclin D1 expression in NSCLC specimens. In addition, most of the cases expressing both cyclin D1 and CDC6, another molecule controlled by YB-1, had co-existing YB-1 over-expression. Together, our results suggest that aberrant expression of both cyclin D1 and CDC6 by YB-1 over-expression may collaboratively participate in lung carcinogenesis.

Introduction

Lung cancer is the leading cause of cancer death worldwide, including in Japan (Jemal *et al.* 2011). In particular, non-small-cell lung cancer (NSCLC) accounts for 85% of human lung cancers. Despite significant progress in NSCLC treatment, such as chemotherapy, radiotherapy, and surgery, the prognosis for patients with NSCLC has been improved only minimally and the 5-year survival rate remains at 15% (Molina *et al.* 2008). Recent advances in the molecular characterization of NSCLC have enabled the identification of numerous cell growth and proliferation pathways that are disrupted in these tumors. These findings have provided insights into the mechanisms of tumor development in various histologic subtypes of NSCLC and have pointed toward targeted treatment strategies (Sanders & Albitar 2010). Li Ding *et al.* identified several mutated genes in NSCLC, including tumor suppressor and tyrosine kinase genes that may function as proto-oncogenes. Furthermore, the authors found a significant excess of mutations and copy number alterations in genes from

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the MAPK, p53, Wnt, cell cycle, and PI3K mTOR signaling pathways, suggesting that these pathways are linked to the disease development and progression (Okudela *et al.* 2007; Ding *et al.* 2008). Many of the genes and proteins involved in lung cancer pathogenesis can be categorized into three major pathways: cell cycle regulation, apoptosis, and angiogenesis. The molecules in these three pathways have been also investigated from the standpoint of their influence on the clinical outcome of NSCLC (Singhal *et al.* 2005).

Cyclins and their associated cyclin-dependent kinases (CDK) are the central machinery that control cell cycle progression. In G1 phase, once the Rb protein is phosphorylated by the cyclin D1/CDK complex, E2F is released, allowing transcription of other CDKs, cyclins, and S phase proteins, thereby promoting the transition from G1 to S phase of the cell cycle. During S phase, cyclin D1 is phosphorylated by glycogen synthase kinase 3β, which promotes nuclear export and ubiquitination of cyclin D1 by the SCF^{Fbox4}-αB crystalline complex, leading to proteasome-dependent degradation of cyclin D1. Other F-box proteins, such as Fbxw8, Fbox31, and Skp2, have also been reported as E3 ligases for cyclin D1, but knockout mouse analyses of these genes indicated that the contribution of these reported E3s toward degradation of cyclin D1 were not significant (Kanie *et al.* 2012). Therefore, rather than the ubiquitin proteasome-mediated degradation system, transcriptional regulation may be more important in controlling cyclin D1 expression.

Induction of cyclin D1 in the G1 phase depends on several growth factors, such as EGF and IGF, and several hormones, including estrogen (17β-estradiol:E2) and angiotensin II, until the restriction point (Klein & Assoian 2008; Witzel *et al.* 2010). Until the point of irreversible transition from G1 to S phase, cyclin D1 expression is tightly regulated at the level of transcriptional activation. Several transcriptional factors, including TCF/LEF, CREB, NF-κB, AP-1, and SP1, have been found to transactivate the *cyclin D1* promoter, and some transcriptional suppressors, such as Tob1 and Jumonji, have been reported to down-regulate *cyclin D1* gene promoter activity (Guttridge *et al.* 1999; Lee *et al.* 1999; Shtutman *et al.* 1999; Bakiri *et al.* 2000; Nagata *et al.* 2001; Boulon *et al.* 2002; Klein & Assoian 2008; Witzel *et al.* 2010). Over-expression of cyclin D1 is thought to enhance cell cycle progression from G1 to S phase and increase cell proliferation. The cyclin/CDK kinase complexes also target substrates that play important roles in centrosome duplication, mitochondrial function, cell growth, cell adhesion and motility, and cytoskeletal modeling. Therefore, elucidation of the transcriptional regulation of *cyclin D1* is essential to understand its role in the tumorigenesis of NSCLC (Musgrove *et al.* 2011).

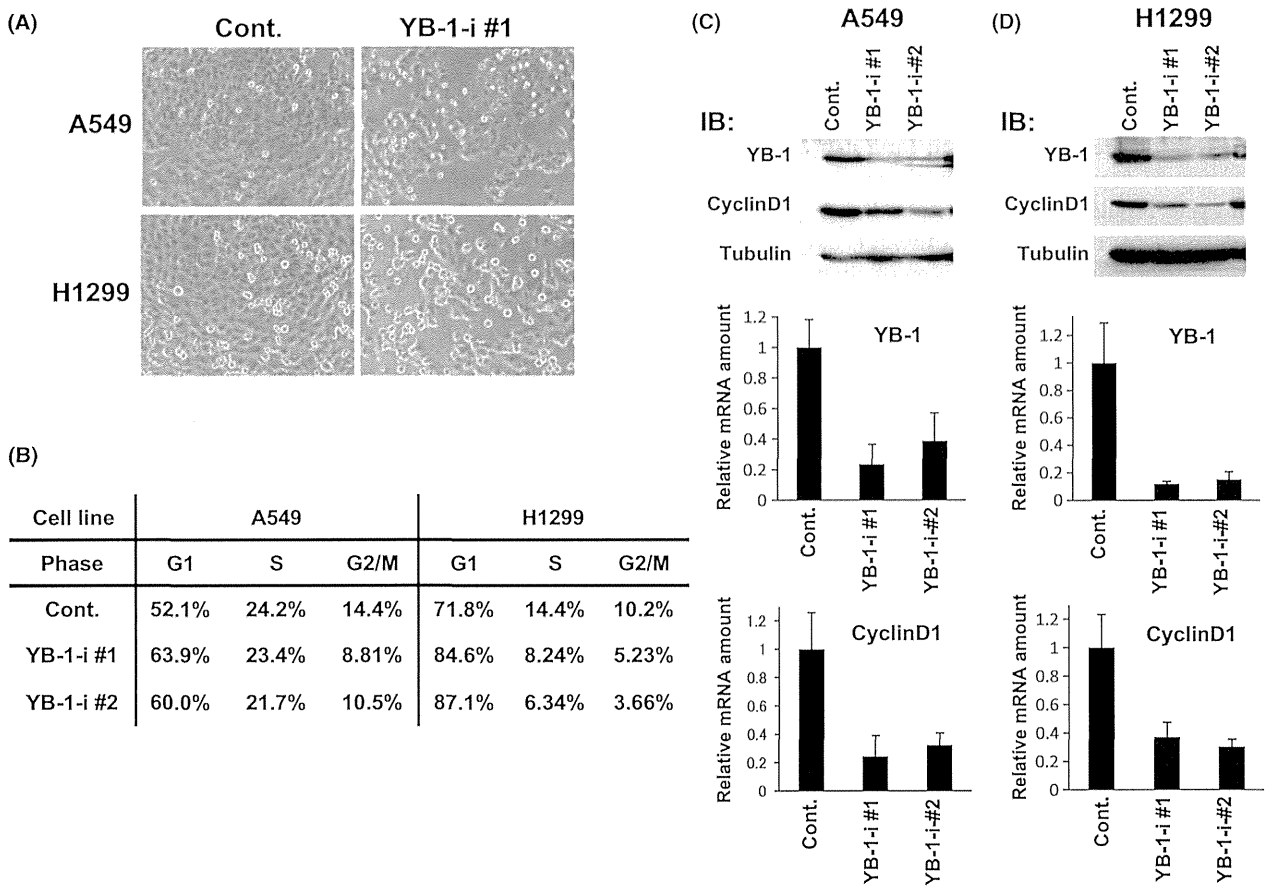
The Y-box-binding protein 1 (YB-1) is a member of the cold-shock domain protein superfamily that binds to an inverted CCAAT box, named the Y-box sequence, in the promoter regions of target genes. YB-1 is a multifunctional protein and regulates translation and transcription in the nucleus and cytoplasm. YB-1 has been reported to be a negative prognostic factor for several cancers, including breast, ovarian, and lung cancers and synovial sarcoma (Kohno *et al.* 2003). YB-1 has also been shown to up-regulate the transcription of cell-cycle-related molecules, including *cyclin A*, *cyclin B* (Jurchott *et al.* 2003), and *CDC6* (Basaki *et al.* 2010). However, we recently showed that YB-1 binds and represses the CDK inhibitor $p16^{ink4}$ gene (Kotake *et al.* 2013). This implies that YB-1 participates in cell cycle progression both via positive and negative regulatory pathways, that is, functioning as both an accelerator and a brake.

In lung cancer, increased expressions of not only YB-1 but also cyclin D1 were found independently, but the correlated expression and functional relationship between YB-1 and cyclin D1 have never been addressed in lung cancer (Eliseeva *et al.* 2011; Lasham *et al.* 2013). In the present study, we investigated whether YB-1 controls cyclin D1 expression in human lung cancers, with particular focus on our identification of several Y-boxes in the *cyclin D1* promoter. Moreover, we also investigated the correlation of CDC6, another target of YB-1, with expression of cyclin D1 and YB-1 in clinical samples of NSCLC.

Results

Effects of YB-1 knockdown in lung cancer cell lines

Based on the reports suggesting that YB-1 promotes cell cycle progression, we first evaluated whether the cell cycle of human lung cancer cell lines was positively regulated by YB-1. We investigated the effects of YB-1 knockdown on cellular proliferation of two lung cancer cell lines (A549 and H1299) by transfecting cells with YB-1 siRNA (YB-1-i #1) for 48 h. The proliferation of A549 and H1299 cells was suppressed to 41.9% and 45.7%, respectively, by YB-1 depletion (Fig. 1A), suggesting that YB-1 participates in the cell proliferation of these lung cancer cells. We next examined effects of YB-1 depletion on the cell cycle. A549 and H1299 cells were transfected with YB-1 siRNA (YB-1-i #1 or



(B)

Cell line	A549			H1299		
Phase	G1	S	G2/M	G1	S	G2/M
Cont.	52.1%	24.2%	14.4%	71.8%	14.4%	10.2%
YB-1-i #1	63.9%	23.4%	8.81%	84.6%	8.24%	5.23%
YB-1-i #2	60.0%	21.7%	10.5%	87.1%	6.34%	3.66%

Figure 1 Effect of YB-1 knockdown on cell proliferation, and expression of cyclin D1 protein and mRNA in non-small-cell lung cancer cells. (A) The cell proliferations of A549 and H1299 cells were observed by phase-contrast microscopy 48 h after treatment with YB-1 siRNA (right panels) or control siRNA (left panels). (B) A549 cells and H1299 cells were analyzed by flow cytometry 72 h post-transfection with two kinds of YB-1 siRNAs or control siRNA. The proportions of cell fractions in the different cell cycle phases are shown. (C) A549 cells and (D) H1299 cells were incubated with either YB-1 or control siRNA for 72 h, and lysates and total RNA were prepared. (Top panels) Western blot analysis for YB-1 and cyclin D1 proteins. α-tubulin served as a loading control. (Middle and bottom panels) Middle panel shows YB-1 relative mRNA amount levels, and bottom panel shows cyclin D1 relative mRNA amount levels as measured by qRT-PCR. Columns represent the mean of three independent experiments, and bars indicate SD.

YB-1-i #2) for 72 h and analyzed using flow cytometry. There was an increase in the proportion of cells in G1 phase (from 52.1% to 63.9% in A549 cells and from 71.8% to 87.1% in H1299 cells) and a decrease in the cells in S and G2/M phases, compared to control-siRNA-treated cells (Fig. 1B, Fig. S2 in Supporting Information). This result suggested that YB-1 might participate in the G1/S transition in lung cancer cells.

Effects of YB-1 knockdown on expression of cyclin D1 in NSCLC

Because cyclin D1 is involved in G1 progression, and as cyclin D1 contains several Y-box sequences in its promoter (see following section), we investigated whether expression of cyclin D1 was affected by YB-1 depletion in various lung cancer cell lines. Depletion of YB-1 by both YB-1-i #1 and #2 siRNA suppressed the protein levels of cyclin D1 in A549 and H1299 cells (Fig. 1C,D). Moreover, expression of cyclin D1 mRNA was also decreased in the two lung cancer cell lines treated with YB-1 siRNA compared with cells treated with control siRNA (Fig. 1C,D). The main histological findings in human lung cancers are adenocarcinoma and squamous cell carcinoma, so other types of human lung cancer cell lines (ABC1: adenocarcinoma cell line, EBC1: squamous cell carcinoma cell line) were also investigated.

As with A549 and H1299 cells, cyclin D1 protein and mRNA expression were also decreased by depletion of YB-1 in ABC1 and EBC1 cells (Fig. S3 in Supporting Information). These results suggested that YB-1 is a positive transcriptional regulator of the *cyclin D1* gene in NSCLC.

YB-1 enhanced transcriptional activity of the *cyclin D1* promoter

YB-1 was previously found to bind to the inverted CAAT box sequences, named the Y-box sequences, in the promoter region of target genes. We searched the promoter region of the human *cyclin D1* gene and confirmed several Y-box sequences, as shown in Fig. 3A. To elucidate whether YB-1 positively regulates the promoter activity of *cyclin D1*, luciferase assays were conducted using a *cyclin D1* reporter plasmid (Fig. 2A). Luciferase activity in cells cotransfected with the YB-1 expression vector was almost twofold higher than that in cells transfected with empty vector in both human embryonic kidney cells (HEK293) and A549 cells (Fig. 2B). Furthermore, depletion of YB-1 in A549 cells suppressed the *cyclin*

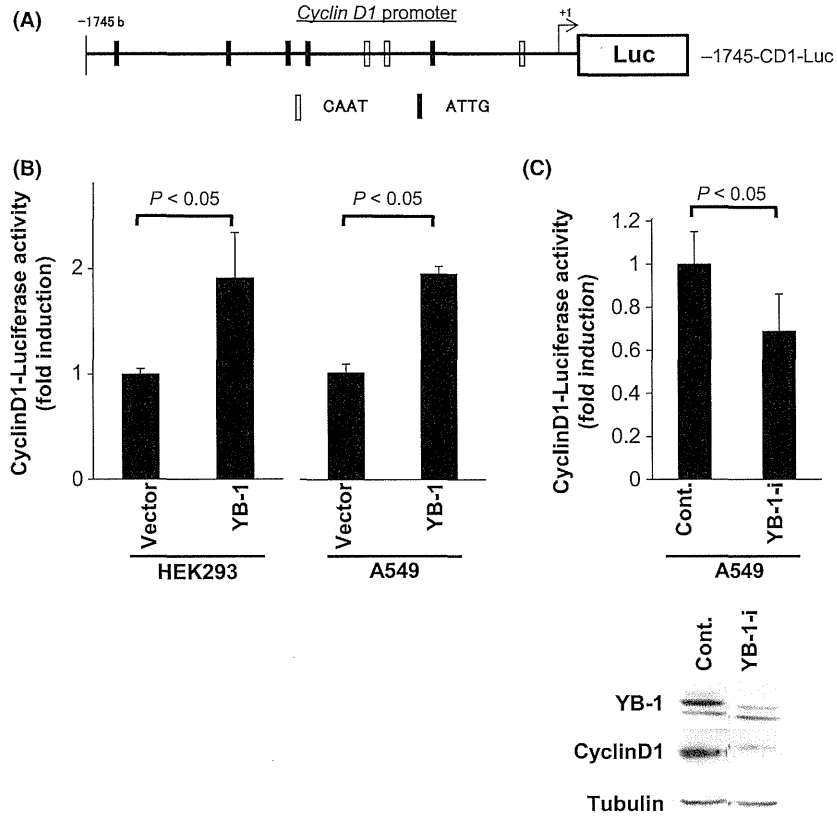


Figure 2 YB-1 enhanced the transcriptional activity of cyclin D1. (A) Schematic representation of the *cyclin D1* 5′ promoter construct in the pSPLUC vector. Potential YB-1-binding sites (Y-boxes), ATTG (black box) or CAAT (open box), are shown. (B) Luciferase assays using the *cyclin D1* reporter in HEK293 and A549 cells. Cells were transfected in Opti-MEM medium with the cyclin D1 promoter luciferase reporter plasmid, CMV β-gal plasmid, and either pcDNA3.1-YB-1 plasmid or pcDNA3 empty vector. After 48 h of incubation, lysates were prepared and luciferase activities were evaluated. Luciferase activity was normalized to β-gal activity, which was assayed in parallel. Luciferase activities of the pcDNA3-YB-1 transfected sample were presented as the relative ratio to pcDNA3 empty vector-transfected sample. Data represent the mean from three experiments. (C) Luciferase assays using the *cyclin D1* reporter in A549 cells. A549 cells were transfected in Opti-MEM medium with the cyclin D1 promoter luciferase reporter plasmid, CMVβ-gal plasmid, and either YB-1 or control siRNA. (Top panel) After 48 h incubation, lysates were prepared. Luciferase activities were normalized to parallel assays for β-gal activities. Luciferase activities of the YB-1 siRNA-transfected sample were presented as the relative ratio to control siRNA-transfected sample. Data represent the mean from three experiments. (Bottom panel) Lysates from the luciferase assay were analyzed by Western blot analysis.