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Aberrant upregulation of the endogenous PP2A inhibitor CIP2A is vital for myeloma cell growth and survival

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The serine/threonine kinase TAK1 is constitutively overexpressed and auto-phosphorylated in multiple myeloma (MM) cells. Protein phosphatase 2A (PP2A) is a major serine/threonine phosphatase which dephosphorylates proteins phosphorylated by various serine/threonine kinases to regulate multiple cellular functions. We recently reported that the serine/threonine kinase TGF-β-activated kinase-1 (TAK1) is highly expressed and auto-phosphorylated to mediate critical growth and survival signaling in MM cells. We demonstrate here that regulation of PP2A activity inversely affects the phosphorylation levels of TAK1 in MM cells, and that MM cells aberrantly overexpress cancerous inhibitor of PP2A (CIP2A), an endogenous inhibitor for PP2A. *CIP2A* gene silencing as well as treatment with the CIP2A inhibitor TD52 potently induced MM cell death along with suppression of TAK1 expression in MM cells. These results suggest the critical role of PP2A inactivation via CIP2A upregulation in TAK1 phosphorylation and its protein expression and thereby MM cell growth and survival, posing the CIP2A-PP2A axis as an important therapeutic target.

Key words: multiple myeloma, TAK1, CIP2A, PP2A

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Introduction

Multiple myeloma (MM) is characterized by preferential accumulation of tumor cells in bone marrow and devastating bone destruction. MM cells induce osteoclastic bone resorption, while inhibiting osteoblastic bone formation to cause extensive bone destruction with rapid bone loss [1–3]. Alongside of bone destruction, the bone marrow microenvironment is altered in MM, which underlies the unique pathophysiology of MM [4, 5]. MM cells grow and evolve through their constant crosstalk with the surrounding microenvironment, and emerging evidence indicates that angiogenesis and immunosuppression frequently occur simultaneously in response to this crosstalk. Accordingly, strategies combining anti-angiogenic therapy and immunotherapy seem to have the potential to tip the balance of the tumor microenvironment and improve treatment response [6].

Cancer cells are generally accepted to have the imbalance between kinases and phosphatases that enhances tumorigenesis [7, 8]. Protein phosphatase 2A (PP2A) is a major serine/threonine phosphatase which dephosphorylate proteins phosphorylated by various serine/threonine kinases to regulate multiple signal transduction pathways [9, 10]. We recently

reported that serine/threonine kinase TGF-β-activated kinase-1 (TAK1) is highly expressed and phosphorylated to mediate critical growth and survival signaling in MM cells but not in normal quiescent hematopoietic cells [11]. Because the serine/threonine kinase TAK1 is auto-phosphorylated by itself [12], we hypothesized that aberrant suppression of PP2A activity may upregulate and sustain the TAK1 phosphorylation state in MM cells to enhance their growth and survival. We therefore aimed to clarify the underlying mechanisms for potent enhancement of TAK1 activation and thereby growth and survival potential in MM cells with special reference to PP2A. We demonstrate here that MM cells aberrantly overexpress cancerous inhibitor of PP2A (CIP2A), an endogenous inhibitor for PP2A, and that inhibition of CIP2A is able to markedly induce MM cell death. Therefore, aberrantly upregulated CIP2A is vital for MM cell growth and survival, and serves as a novel therapeutic target for MM to restore PP2A activity and thereby mitigate phosphorylation by serine/threonine kinases.

Materials and Methods

Ethics

Written informed consent was observed in all procedures involving human samples from healthy donors and patients with MM in accordance with the Declaration of Helsinki and a protocol approved by the Institutional Review Board for human protection at Tokushima University (Permission number: 2638-4).

Reagents

The following reagents were purchased from the indicated manufacturers: rabbit antibodies against PP2A C subunit, phosphorylated TAK1, TAK1, PIM2, phosphorylated 4E-BP1, MYC, Sp1, Akt, and phosphorylated Akt, horseradish peroxidase (HRP)–anti-rabbit immunoglobulin G (IgG), HRP-anti-mouse IgG and okadaic acid from Cell Signaling Technology (Beverly, MA, USA); mouse anti-β-actin antibody from Sigma-Aldrich (St. Louis, MO, USA); mouse antibodies against CIP2A and CD138 from Santa Cruz Biotechnology Inc. (Dallas, TX, USA); PP2A activator SMAP (DT-061) and CIP2A inhibitor TD52 from Selleck Chemicals (Houston, TX, USA).

Cells and culture

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy volunteers with Ficoll density gradient centrifugation (Ficoll-Paque PLUS, Cytiva, Marlborough, MA, USA) as previously described [13–15]. MM cells were purified from bone marrow mononuclear cells from patients with MM by positive selection using anti-CD138 microbeads and Miltenyi magnetic cell sorting system (Miltenyi Biotec, Auburn, CA) in accordance with the manufacturer's instruction. The MM cell line INA-6 was kindly provided by Renate Burger (University of Kiel, Kiel, Germany). RPMI 8226, MM.1S and U266 MM cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA). JJN3 MM cell lines were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). KMS-11 MM cell lines were obtained from Japanese Collection of Research Bioresources cell bank (Osaka, Japan). Cells were cultured in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham MA, USA), penicillin G at 50 µg/mL and streptomycin at 50 µg/mL.

Western blotting

Cells were collected and lysed in RIPA lysis buffer (Santa Cruz). To collect cytosolic and nuclear extracts, cells were lysed in NE-PER extraction reagent (Thermo Fisher Scientific) according to the manufacturer's protocol. Western blot analysis was performed with equal protein amounts of cell lysate, as described previously [16]. The cell lysates were electrophoresed on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to polyvinylidene difluoride (PVDF) membranes (Millpore, Billerica, MA, USA). The membranes were blocked with 3% bovine serum albumin (FUJIFILM Wako Chemicals, Osaka, Japan) or 5% nonfat dry milk in tris (hydroxymethyl) aminomethane–buffered saline with 0.01% Tween 20 (TBS-T) for 1 hour at room temperature and incubated for 16 hours at 4°C with primary antibodies. After washing, secondary HRP–conjugated antibody was added, and the membranes were then developed with the enhanced chemiluminescence plus western blotting detection system (Cytiva). The band intensities were quantitated using ImageJ Gel Analysis program (NIH Image J System).

Cell viability

To estimate apoptotic cell death, cells were stained with an annexinV-FITC and propidium iodide labeling kit (MEBCYTO Apoptosis Kit; MBL, Nagano, Japan) and analyzed in flow cytometry.

Immunohistochemistry

PBMCs isolated from healthy donors were spun down to make cell pellets in 15-mL centrifugation tubes, and the cell pellets were fixed with paraformaldehyde (PFA) and embedded in paraffin. Bone marrow biopsy samples from MM patients were fixed in neutral-buffered formalin, and then decalcified by EDTA. The bone marrow samples were then embedded in paraffin. PBMCs were spun down in 15-mL conical centrifuge tubes, and then cellular pelettes were fixed with in neutral-buffered formalin and embedded in paraffin. Three µm-thick

serial sections were prepared. After deparaffinization, the sections were incubated with anti-CIP2A (Santa Cruz), CD138 (Santa Cruz) or isotype control IgG (Santa Cruz) overnight at 4°C. A HRP-streptavidin detection system (Agilent, Santa Clara, CA, USA) was used to detect their immunoreactivity.

Immunoprecipitation phosphatase assay

PP2A phosphatase activity were measured with an Immunoprecipitation Phosphatase Assay Kit (Millipore), following the manufacturer's protocol. MM cells were treated with or without LLZ at 5 μM for 13 hours. Then cell lysates were collected and measured with an Immunoprecipitation Phosphatase Assay Kit. To avoid variability due to differences in the amounts of immunoprecipitated protein between samples, the phosphatase activities were normalized to the immunoprecipitated PP2A that was detected and quantified by immunoblot analysis for each treatment group.

Transfection

pLKO-based plasmids for short hairpin RNAs (shRNAs) were purchased from Sigma-Aldrich, and *luciferase* shRNA was used as a negative control for shRNA experiments. The human *CIP2A* cDNA, which was obtained from the pcDNA3.1/CIP2A plasmid (kindly provided by Dr. Westermarck, Addgene plasmid #119287), ligated into the EcoRI site of pMSCV-neo retroviral expression vector (Takara Bio) using Gibson Assembly Master Mix (NEW ENGLAND BioLabs, MA, USA) according to the manufacturer's instructions. The vector without insertion of cDNA were used as a control vector (Empty).

Lentiviral and retroviral production was carried out using 293T cells as described previously [17, 18]. In brief, pLKO-based plasmids were transfected into 293T cells in combination with pCMV-dvpr and VSV-G for lentiviral packaging, and pMSCV plasmid with pMD-MLV and VSV-G for retroviral packaging, using TransIT-LT1 Transfection Reagent (Mirus Bio, WI, USA). Virus containing media were then harvested according to previous methods. MM cells were cultured with the virus containing media in the presence of polybrene (Santa-Cruz) for 5 hr. After 24 hr, shRNAs- or *CIP2A* cDNA-induced MM cells were selected using 1 mg/mL puromycin (Sigma-Aldrich) for 48 hours or 400 μg/mL G418 (FUJIFILM Wako Chemicals) for at least 7 days, respectively. The selected cells were subjected to following experiments.

Statistical analysis

Statistical analysis was performed using Student's *t*-test or oneway analysis of variance (ANOVA). All statistics were performed using the Statistical Package for Social Sciences (SPSS 13.0 for Windows; Chicago, IL, USA). $P < 0.05$ was considered as a significant difference.

Results

Regulation of TAK1 phosphorylation in MM cells by PP2A activity

The serine/threonine phosphatase PP2A was constitutively expressed at a protein level in all MM cell lines tested (Fig. 1A, left). Treatment with the PP2A inhibitor okadaic acid was able to further reduce PP2A activity in MM cells while the PP2A agonist SMAP (DT-061) restored it (Fig. 1A, right). We previously reported that the serine/threonine kinase TAK1 is constitutively overexpressed and auto-phosphorylated in MM cells [11]. Consistent with PP2A-mediated dephosphorylation of serine/threonine residues phosphorylated by serine/threonine kinases, treatment with the PP2A inhibitor okadaic acid (Fig. 1B) dose-dependently enhanced TAK1 phosphorylation levels, but the PP2A activator SMAP (DT-061) (Fig. 1C) suppressed them in MM cell lines. These results suggest that PP2A activity regulates TAK1 phosphorylation levels in MM cells.

Induction of apoptosis with TAK1 reduction in MM cells by PP2A activation

Because TAK1 phosphorylation has been demonstrated to activate a wide range of intracellular signaling pathways critical for MM growth and survival, including those mediated by NF-κB and extracellular signal-regulated kinase (ERK) and PIM2 [11], and because PP2A activation mitigates TAK1 phosphorylation in MM cells (Fig. 1C), we next examined whether PP2A activation affects MM cell viability. Treatment with the PP2A activator SMAP induced MM cell apoptosis at 24 hours (Fig. 2A). Interestingly, SMAP at 10 μM markedly reduced TAK1 protein levels at 24 hours (Fig. 2B). These results suggest that PP2A activity is critical in regulation of TAK1 expression and phosphorylation and thereby MM cell viability.

Aberrant CIP2A upregulation in MM cells

PP2A activity is controlled by endogenous inhibitory factors, including CIP2A, I2PP2A and PME-1 [19]. Among these, high CIP2A expression has been reported to correlate with poor prognosis in patients with various human cancer types, including MM [9, 20–26]. We therefore looked at the expression of CIP2A in MM cells. CIP2A was overexpressed in all MM cells tested compared to normal PBMCs (Fig. 3A). Aberrant CIP2A expression was further confirmed in MM cells in bone marrow samples from patients with MM (Fig. 3B). CD138-positive MM cells expressed CIP2A at varied levels while marginally in normal PBMCs. Therefore, MM cells are suggested to aberrantly overexpress CIP2A.

Effects of CIP2A gene silencing on MM cell survival

Because the PP2A activator SMAP suppressed TAK1 phos-

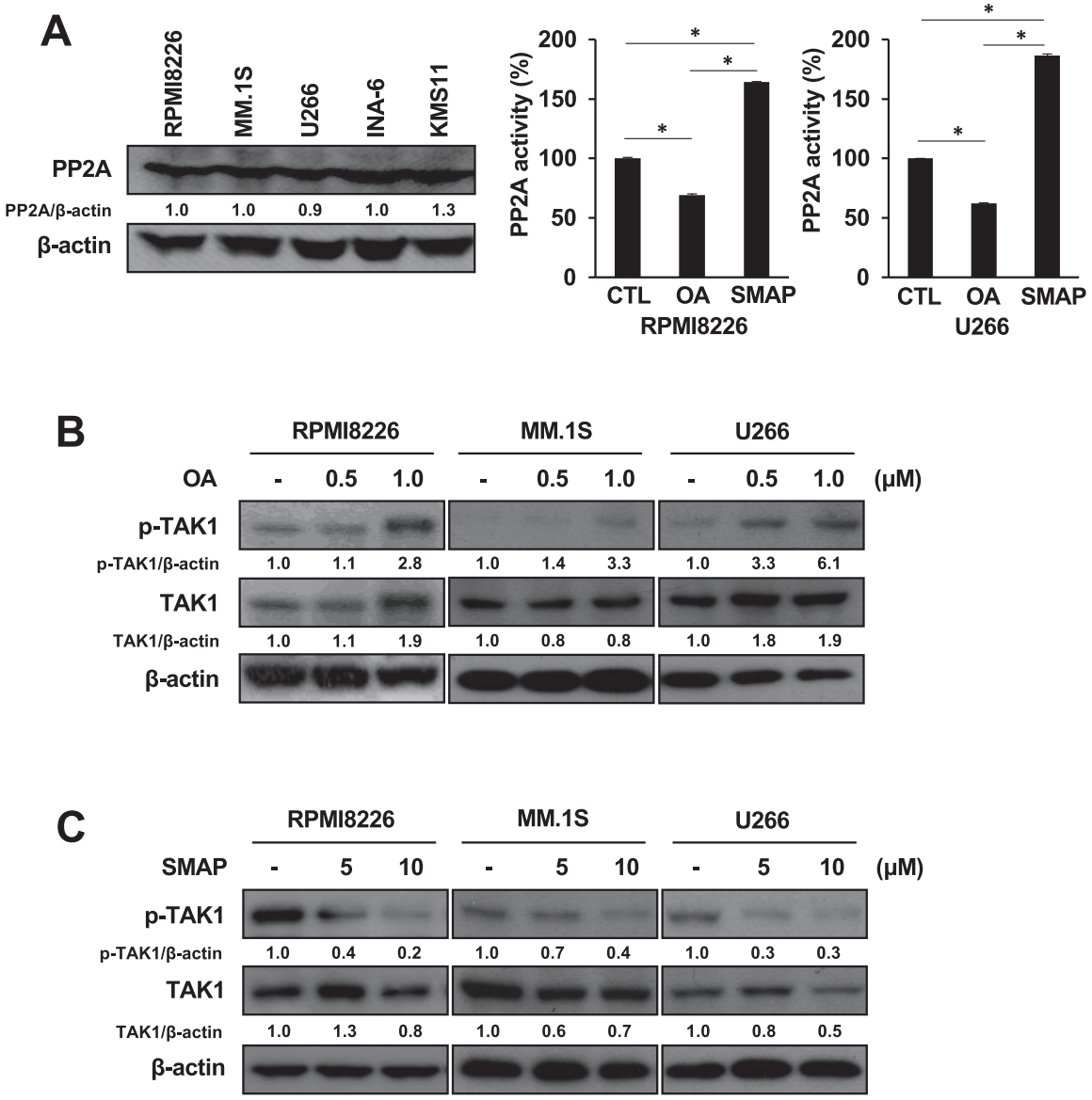

Figure 1. Regulation of TAK1 phosphorylation in MM cells by PP2A activity. (A) PP2A protein levels were determined in the indicated MM cell lines by western blotting analysis (left). β-actin was used as a loading control. PP2A activity was measured in MM cells in the presence or absence of okadaic acid (OA) or SMAP for 3 hours (right). The results are shown as mean ± SD (n = 3). *$P < 0.05$. (B, C) MM cells were treated for 3 hours with or without OA after starved for 3 hours (B) or SMAP (C) without the starvation at the indicated concentrations. Cell lysates were then collected, and TAK1 expression and phosphorylation were examined by western blotting analysis. Relative changes of the band intensities standardized by respective loading controls are indicated.

phorylation in MM cells (Fig. 1C) and induced MM cell apoptosis (Fig. 2A), and because the PP2A inhibitor CIP2A was aberrantly overexpressed in MM cells (Fig. 3), we next investigated the effects of suppression of CIP2A in MM cell survival. *CIP2A* gene silencing induced MM cell apoptosis (Fig. 4A), indicating a critical role of CIP2A in MM cell survival. We previously demonstrated that the TAK1-PIM2-MYC and TAK1-Sp1 pathways play critical roles in MM cell growth and survival [11, 27]. *CIP2A* gene silencing reduced TAK1 protein levels in parallel with the reduction of survival mediators downstream of TAK1, including PIM2, MYC and Sp1, and the phosphorylation of the PIM2 substrate 4E-BP1 in MM cells (Fig. 4B). These results suggest the critical role of CIP2A in TAK1-mediated growth and survival signaling in MM cells.

Effects of the CIP2A inhibitor TD52 on MM cell survival

To further elucidate the role of CIP2A in MM cell growth and survival, we next examined the effects of the erlotinib derivative TD52 which is a commercially available CIP2A inhibitor. TD52 has been reported to exert anti-proliferative and apoptotic effects on breast cancer cell lines at the concentrations 2–10 μM [28]. MM cells underwent apoptosis in the

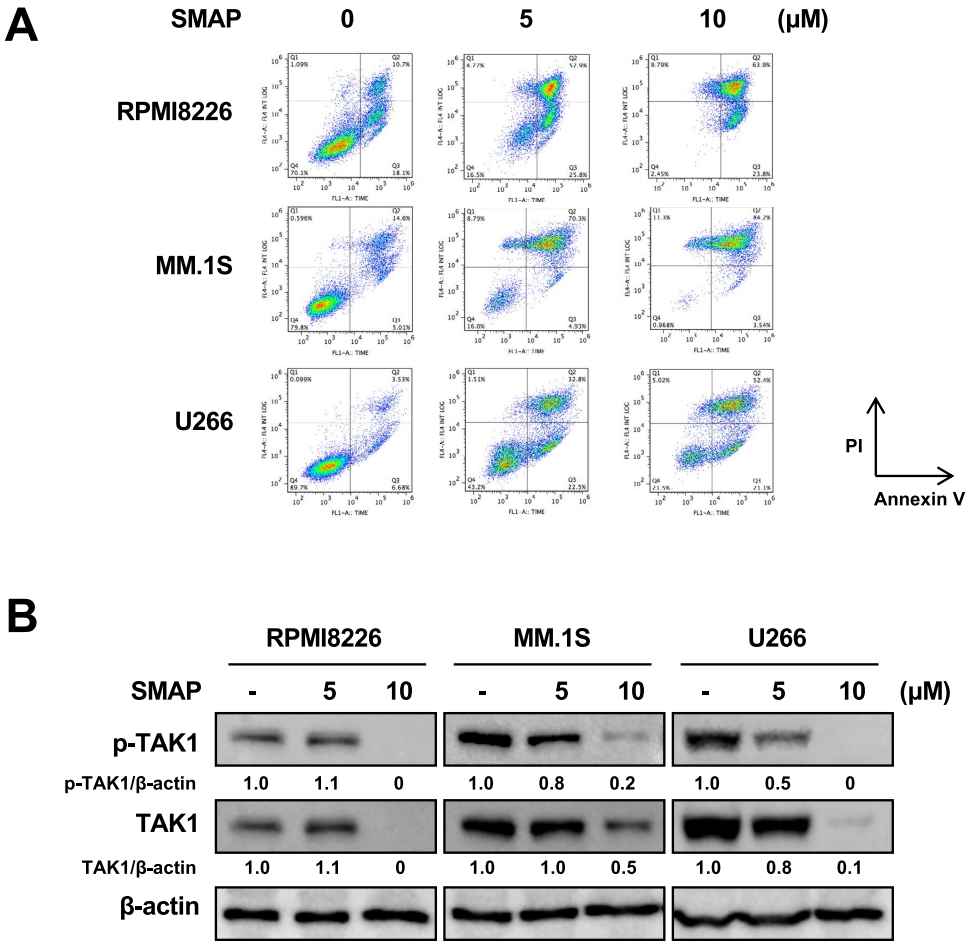

Figure 2. Induction of apoptosis with TAK1 reduction in MM cells by PP2A activation. (A) MM cells were treated for 24 hours with SMAP at the indicated concentrations. The induction of apoptosis was analyzed in MM cells, using annexinV and propidium iodide (PI) dual staining. (B) The indicated MM cell lines were cultured for 24 hours in the presence or absence of SMAP at the indicated concentrations. Cell lysates were then collected, and protein levels of phosphorylated TAK1 and TAK1 were analyzed by western blotting analysis. β-actin was used as a loading control. Relative changes of the band intensities standardized by respective loading controls are indicated.

presence of TD52 at 10 μM is (Fig. 5A), and reduced TAK1 protein levels along with the reduction of Akt phsopshorylation and protein levels of survival mediators downstream of TAK1, including PIM2 and MYC (Fig. 5B). TD52 is suggested to exert apoptotic effects on MM cells via regulating the CIP2A-PP2A axis and thereby the activity of critical mediators for MM growth and survival.

Discussion

Disruption of the balance between protein kinases and protein phosphatases causes various diseases, including cancers [29]. PP2A, a major serine/threonine phosphatase, is regarded as an important tumor suppressor and often inactivated in cancers cells. We previously reported that TAK1 overexpression and phosphorylation are vital for MM cell growth and survival. We demonstrated here that phosphorylation levels of the serine/threonine TAK1 is inversely regulated by PP2A activity in MM cells, and that MM cells aberrantly overexpress CIP2A to inactivate PP2A activity. *CIP2A* gene silencing as well as treatment with the CIP2A inhibitor TD52 induced MM cell death along with suppression of TAK1 protein levels in MM cells, suggesting the critical role of CIP2A upregulation in keeping TAK1 protein levels high and thereby enhancing MM cell growth and survival.

Although treatment with the PP2A activator SMAP suppressed phosphorylation levels of TAK1 in MM cells at 3 hours in Figure 1C, it rather reduced the levels of TAK1 protein itself at 24 hours in Figure 2B. Together with MM cell apoptosis by SMAP at 24 hours in Figure 2A, these results suggest that PP2A activity is critical in regulation of TAK1 expression and phosphorylation and thereby MM cell viability. Consistently, substantial reduction of TAK1 levels was observed in MM cells under CIP2A gene silencing as well as after incubation with the CIP2A inhibitor TD52 for 24 hours in Figures 4B and 5B. These results collectively suggest that CIP2A overexpression

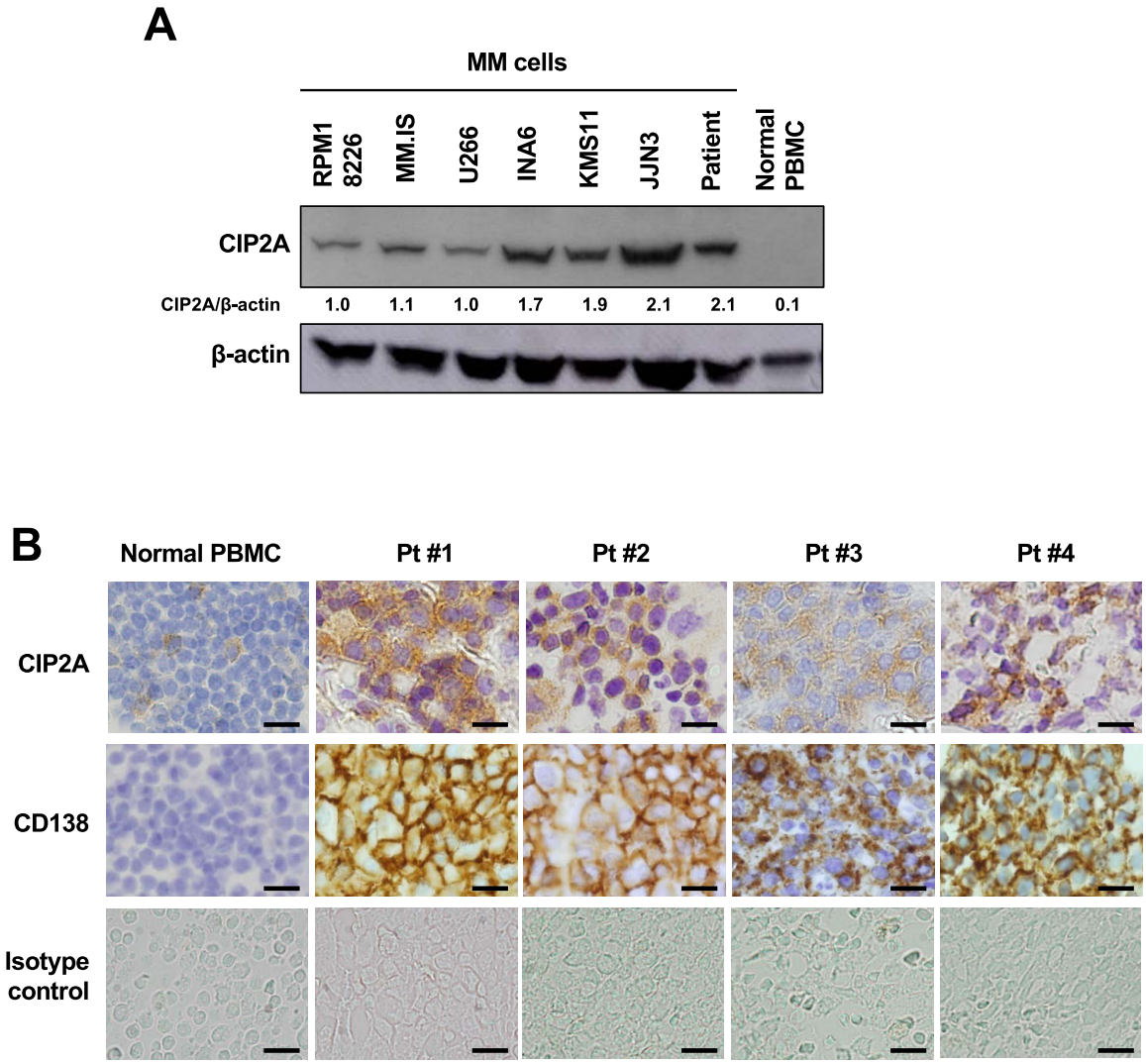

Figure 3. Aberrant CIP2A upregulation in MM cells. (A) Protein levels of the endogenous PP2A inhibitors CIP2A were examined by western blotting analysis in the indicated MM cell lines, primary MM cells and peripheral blood mononuclear cells (PBMCs). β-actin was used as a loading control. Relative changes of the band intensities standardized by respective loading controls are indicated. (B) CIP2A expression was immunohistochemically analyzed in primary CD138-positive MM cells. PBMCs from healthy donor and bone marrow biopsy samples from patients with MM were stained with antibodies against CIP2A or CD138, or isotype control IgG. Scale bar, 25 μm.

inhibits PP2A activity and thereby upregulates TAK1 phosphorylation in MM cells, and that TAK1 phosphorylation upregulated by CIP2A is required to maintain its protein levels. We plan to further clarify the connection between TAK1 phosphorylation and its protein expression and degradation in MM cells. PP2A activity can be controlled by different endogenous inhibitory factors, including I2PP2A and PME-1 as well as CIP2A. Although CIP2A appears to play a critical role in TAK1 phosphorylation and survival in MM cells, these endogenous inhibitory factors other than CIP2A are suggested to affect TAK1 expression and phosphorylation. Precise roles of I2PP2A and PME-1 as well as CIP2A in the regulation of PP2A activity and TAK1 expression and phosphorylation remain to be further deciphered in MM cells.

MYC has been well studied as a CIP2A-targeted PP2A substrate in cancers [30]. Aberrant expression of CIP2A increases the phosphorylation of MYC at serine 62 to increase MYC stability and function, and the positive-feedforward loop between CIP2A and MYC augments the activity of MYC in cancer cells [31, 32]. Among others, MM is regarded as a tumor to be addicted to MYC [33]. In MM cells, silencing of *CIP2A* gene substantially reduced MYC at a protein level, suggesting a critical role of CIP2A in MYC upregulation in MM cells. Besides MYC, various CIP2A-targeted PP2A substrates have been demonstrated in cancers, including Akt, mTORC1, E2F1and Plk1 [34–37]. Therefore, a number of factors may participate in CIP2A-induced growth and survival of MM cells.

There are multiple pathways to reduce PP2A activity in MM cells, including somatic mutations and posttranslational modifications with increased expression of PP2A-inhibitory

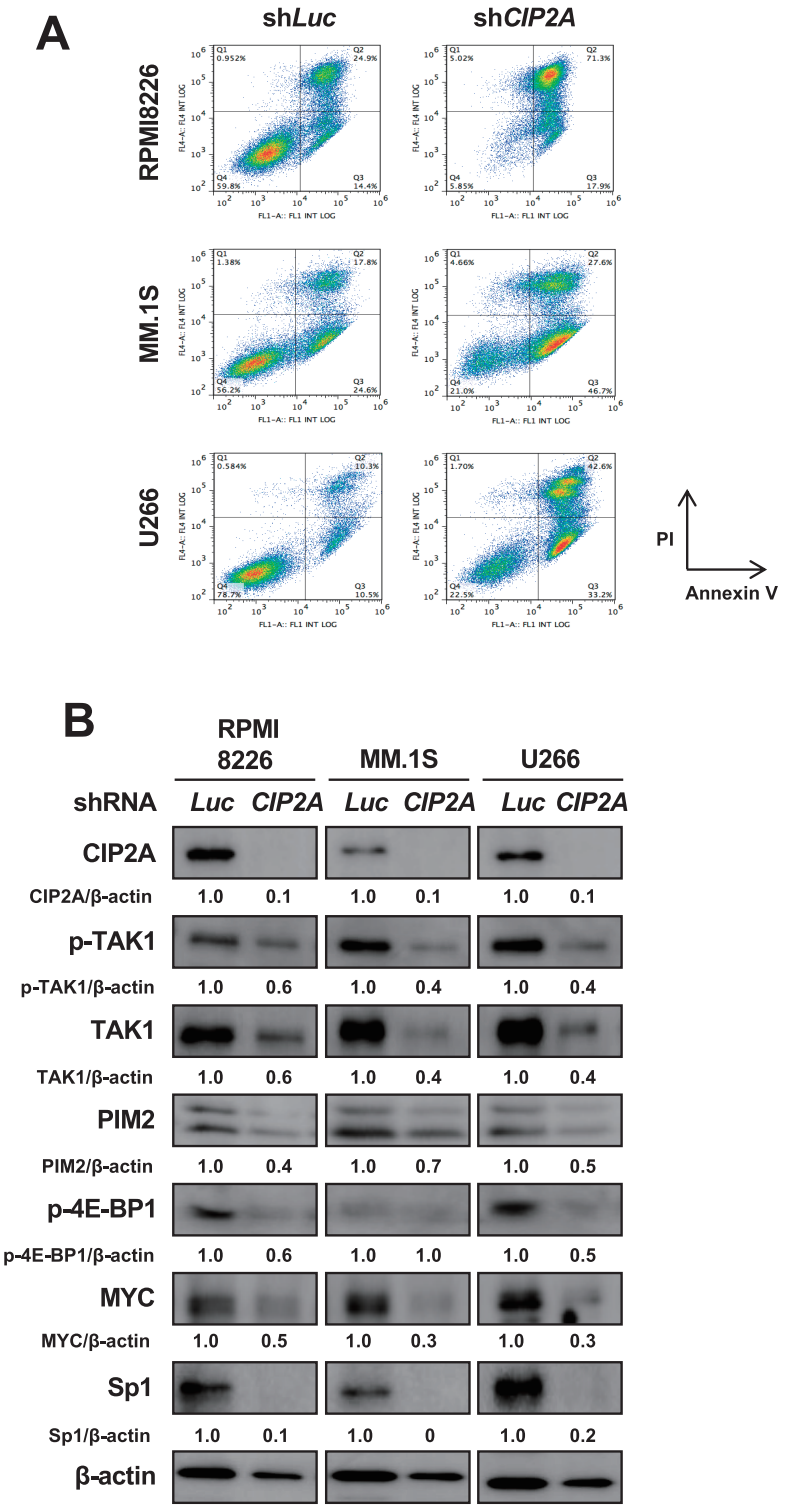

Figure 4. Effects of CIP2A gene silencing on MM cell survival. The indicated MM cell lines were transduced with scrambled (sh*Luc*) or human *CIP2A* shRNA and cultured for 13 hours. Apoptotic cell death was analyzed with annexinV and propidium iodide (PI) dual staining (A). Cell lysates were then collected, and protein levels of the indicated factors were analyzed by western blotting analysis (B). β-actin was used as a loading control. Relative changes of the band intensities standardized by respective loading controls are compared.

proteins such as CIP2A. According to the present study, CIP2A overexpression appears to be among major factors responsible for downregulating PP2A activity in MM cells. However, We found that primary CD138-positive MM cells as well as MM cell lines expressed CIP2A at varied levels. Although high CIP2A levels in MM cells has been reported to be associated with shorter progression-free survial and poor response in MM patients treated with bortezomib plus dexamethasone, the

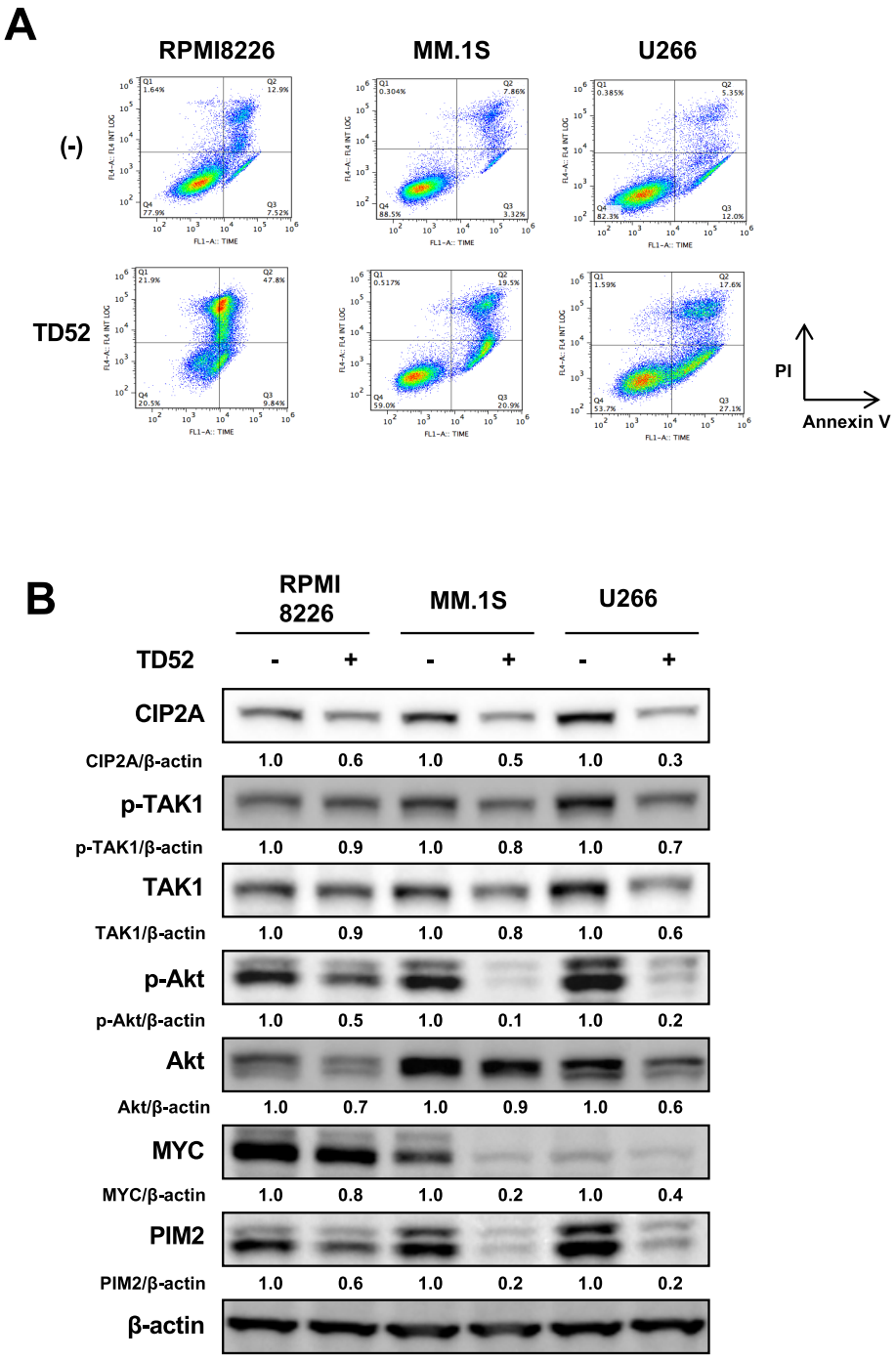

Figure 5. Effects of the CIP2A inhibitor TD52 on MM cell survival. The indicated MM cell lines were cultured for 24 hours in the presence or absence of TD52 at 10 μM. Apoptotic cell death was analyzed with annexinV and propidium iodide (PI) dual staining (A). Cell lysates were then collected, and protein levels of the indicated factors were analyzed by western blotting analysis (B). β-actin was used as a loading control. Relative changes of the band intensities standardized by respective loading controls are compared. The ratios in the samples without TD52 were set as 1.0 for the indicated cell line.

impact of the CIP2A expression levels in MM cells on drug resistance and dismal survival prognosis should be further studied.

The CIP2A inhibitor TD52 indirectly reduces CIP2A activity by disturbing binding of the transcription factor ELK1 to the *CIP2A* gene promoter [28, 38]. Treatment with TD52 induced apoptosis in MM cells although CIP2A protein levels were partly reduced (Fig. 5A), suggesting a partial role of ELK1 in aberrant overexpression of CIP2A in MM cells. Because the importance of CIP2A in MM tumor progression and drug resistance is highly implied, we need to further elucidate the complex transcriptional regulatory mechanisms for CIP2A

upregulation in MM cells.

The present study collectively demonstrates that MM cells aberrantly express CIP2A to inactivate PP2A and thereby potentiate TAK1 expression and its downstream survival signaling pathways, posing the CIP2A-PP2A axis as an important therapeutic target.

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Author Contributions

Conceptualization, S.S., J.T., T.H. and M.A.; methodology, S.S., J.T., T.H., A.O., Y.I., Y.S., I.E., K.T., E.T. and M.A.; investigation, S.S., J.T., T.H., M.H., H.T., A.O., A.S., Y.I., K.T., H.Y., Y.H., M.O., K.S., T.H., R.S., T.M,; formal analysis, S.S., J.T., T.H., M.H., H.T. and A.O.; re-sources, J.T., T.H., M.H., H.T., K.T. and M.A.; data curation, S.S., J.T., T.H., and M.A.; visualiza-tion, S.S., J.T., T.H., K.T. and M.A.; supervision, J.T., T.H., Y.S., I.E., T.M, E.T. and M.A.; project administration, J.T., T.H., T.M, E.T. and M.A.; writing—original draft preparation, S.S., J.T. and T.H.; and writing—review and editing, J.T. and M.A. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

M.A. received research funding from Chugai Pharmaceutical, Sanofi K.K., Pfizer Seiyaku K.K., Kyowa Hakko Kirin, MSD K.K., Astellas Pharma, Takeda Pharmaceutical, Teijin Pharma, and Ono Pharmaceutical, and honoraria from Daiichi Sankyo Company and Janssen Pharmaceutical K.K. The other authors declare no competing financial interests.

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