Rhein exhibits antitumorigenic effects by interfering with the interaction between prolyl isomerase Pin1 and c-Jun

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Abstract. The Pin1 protein (or peptidyl-prolyl cis/trans isomerase) specifically catalyzes the *cis/trans* isomerization of phosphorylated serine/threonine-proline (Ser/Thr-Pro) bonds and plays an important role in many cellular events through the effects of conformational change in the function of c-Jun, its biological substrate. Pin1 expression is involved in essential cellular pathways that mediate cell proliferation, cell cycle progression, tumorigenesis and apoptosis by altering their stability and function, and it is overexpressed in various types of tumors. Pin1 phosphorylation has been regarded as a marker of Pin1 isomerase activity, and the phosphorylation of Ser/Thr-Pro on protein substrates is prerequisite for its binding activity with Pin1 and subsequent isomerization. Since phosphorylation of proteins on Ser/Thr-Pro is a key regulatory mechanism in the control of cell proliferation and transformation, Pin1 has become an attractive molecule in cancer research. Many inhibitors of Pin1 have been discovered, including several classes of both designed inhibitors and natural products. Anthraquinone compounds possess antitumor properties and have therefore been applied in human and veterinary therapeutics as active substances in medicinal products. Among the anthraquinones, rhein (4,5-dihydroxy-9,10-dioxoanthracene-2-carboxylic acid) is a monomeric anthraquinone derivative found mainly in plants in the Polygonaceae family, such as rhubarb and *Polygonum cuspidatum*. Recent studies have shown that rhein has numerous pharmacological activities, including antitumor effects. Here, we demonstrated the antitumorigenic effect of rhein using cell proliferation assay, anchorage-independent cell transformation, pull-down assay, luciferase promoter activity, fluorescence-activated cell sorting and western blot analysis. The rhein/Pin1 association was found to play a regulatory role in cell proliferation and neoplastic cell transformation and the binding of phosphorylated c-Jun (Ser73) with Pin1 was markedly decreased and inhibited activator protein 1 or NF-κB reporter activity by rhein. Overall, our findings and the accompanying biochemical data demonstrated the antitumorigenic effect of rhein through its interference in Pin1/c-Jun interaction and suggest the possible use of rhein in suppressing the tumor-promoting effects of Pin1. Therefore, rhein may have practical implications for cancer prevention or therapy.

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Introduction

Rhein (4,5-dihydroxy-9,10-dioxoanthracene-2-carboxylic acid) is a primary anthraquinone (Fig. 1A) found in the roots of rhubarb, a traditional Chinese herb, and has been shown to exhibit various anticancer effects (1-3). Recently, *in vitro* and *in vivo* studies have shown that rhein inhibits the growth of many types of cancer cells, such as those found in human oral cancer (4-6), adenocarcinoma (7), lung cancer (8), breast cancer (8), hepatocellular carcinoma (9), and cervical cancer (10). Major compounds of therapeutic importance in rhubarb are derivatives of anthraquinone, and were found to inhibit multiple signal transduction pathways, including activator protein 1 (AP-1) and nuclear factor-κB (NF-κB) (11,12). Although little is known in regards to the effect of rhein on tumorigenesis, suppression of these signals is believed to contribute to its anticancer activities as these pathways are critical in carcinogenesis.

The peptidyl-prolyl cis/trans isomerase Pin1 catalyzes *cis/trans* isomerization in peptide bonds of phosphorylated serine/threonine-proline (Ser/Thr-Pro) motifs (13,14). Pin1 binds specifically to a wide variety of proteins that harbor WW domains [one of the smallest protein modules, composed of only 40 amino acids, which mediates specific protein-protein interactions with short proline-rich or proline-containing motifs (15)]. Thus, Pin1 plays a central role as a post-phosphorylation regulator in fine-tuning protein functions (16-18).

It isomerizes the peptide bond of specific phosphorylated serine or threonine residues that precede proline in several of the proteins involved in various cellular events, including mitosis, transcription, differentiation, and the response to DNA damage (14,19). The Ser/Thr-Pro motifs appear to be exclusive phosphorylation sites for many key protein kinases involved in the control of cell growth and transformation (20,21). Pin1 is most likely required for the full activation of multiple signal transduction pathways, including AP-1 and NF-κB (14,22,23). Furthermore, Pin1 plays a key role in oncogenic signaling pathways (24,25) and is abundant in various tumors (23,26). Notably, inhibition of Pin1 in cancer cells triggers apoptosis or suppresses the transformed phenotype (27,28). Rhein was reported to inhibit the activation of HER-2/Neu and its downstream signaling pathway in human breast cancer cells (29). Pin1 is also a downstream effector of oncogenic Neu/Ras signaling (30).

Therefore, we hypothesized that rhein may directly target Pin1 to suppress critical oncogenic signaling pathways and neoplastic cell transformation.

Materials and methods

Reagents and antibodies. The checkmate mammalian two-hybrid system, including expression vectors and the luciferase reporter vector, was obtained from Promega (Madison, WI, USA). Cell culture media and other supplements were purchased from Life Technologies (Rockville, MD, USA). Specific antibodies for use in the immunoblotting, immunoprecipitation, and immunocytochemical analyses were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA), Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), Upstate Biotechnology (Boston, MA, USA), and GE Healthcare (Piscataway, NJ, USA). For the transfection experiments, jetPEI transfection reagent was purchased from Qbiogene (Carlsbad, CA, USA). Rhein was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell viability assay. $Pin1^{+/+}$, $Pin1^{-/-}$, Neu/Pin1 knockout (KO) and Neu/Pin1 wild-type (WT) mouse embryonic fibroblast (MEF) cells, which were originally developed by Fujimori *et al* (31) and Ryo *et al* (27), were obtained from Dr Kun Ping Lu (Harvard Medical School, Boston, MA, USA). K1735 C10 and K1735 M2 melanoma cells were kindly provided by Zigang Dong (University of Minnesota, Austin, MN, USA). JB6 CI41 and human embryonic kidney 293T cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells from the mouse epidermal cell line JB6 CI41 were seeded in a 96-well microtiter plate ($2x10^3$/100 μl) and were incubated for 24 or 48 h with rhein (10-60 μM). Viability was estimated using the CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega) according to the manufacturer's instructions. The assay solution was added to each well, and absorbance (492 nm, 690 nm background) was read using a 96-well plate reader.

siRNA Pin1 plasmid construction. The mU6Pro-Pin1 plasmid was constructed using the mU6Pro vector (32). The inserted sequence was: 5'-TTTGGTCAGGAGAGGAAGACTTTTTC AAGAGAAAAGTCTTCCTCTCCTGACTTTTT-3'. The siRNA sequence targeted against mRNA Pin1 was GUCAGG AGAGGAAGACUUU.

Construction of wild-type (WT) or mutant Pin1 expression vectors, and establishing stable cells. The coding sequences of Pin1 were obtained after polymerase chain reaction (PCR) amplification (30 cycles, with annealing temperature at 65˚C) of cDNA derived from reverse transcription of the total RNA of Neu/Pin1 in WT MEFs. The amplified PCR products were ligated into the pcDNA4.0-HisMaxC plasmid (Invitrogen, Grand Island, NY, USA) to produce an expression vector for 6x-His-tagged and Xpress-tagged Pin1. Transformants of Pin1 were obtained by transfecting 1 μg of pcDNA4.0-HisMaxC, the pcDNA4.0-HisMaxC/Pin1 vector into Neu/Pin1 KO and K1735 M2 cells using jetPEI and were then maintained by adding Zeocin to the culture medium. Either the pU6pro vector control or pU6pro vector containing siPin1 was transfected into the JB6 CI41 cells.

Cell culture and transfection. $Pin1^{-/-}$ and $Pin1^{+/+}$ MEFs and 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). JB6 CI41 cells were grown in 5% FBS-MEM. Cells were cultured at 37˚C in a 5% CO_2 incubator. Either the pU6pro vector control or pU6pro vector containing siPin1 was transfected into the JB6 CI41 cells. The resulting stable transfectants were isolated by growing them in the selective MEM containing G418. The JB6 CI41 cells engineered to express Pin1 at low levels are designated as JB6 CI41/siPin1.

Anchorage-independent transformation assay. Using soft agar assays, we evaluated the functional activity of Pin1 in cells stimulated with 12-*O*-tetradecanoylphorbol-13-acetate (TPA): JB6 CI41, JB6 CI41/siMock, K1735 C10/siMock, K1735 C10/siPin1, K1735 M2/Mock and K1735 M2/siPin1. The cells at a density of $8x10^3$ cells were treated with TPA (20 ng/ml) in 1 ml of 0.3% basal medium Eagle's (BME) agar over 3 ml of 0.5% BME agar containing 10% FBS. The cultures were maintained in a 5% CO_2 incubator at 37˚C for 14 days, and the cell colonies were counted using a microscope and Image-Pro Plus computer software (Media Cybernetics, Warrendale, PA, USA).

GST/Pin1 pull-down assay. To investigate the interaction of Pin1 and c-Jun, a glutathione *S*-transferase (GST) pull-down assay was performed, as described elsewhere (33). The pcDNA3.1/c-Jun plasmid was kindly provided by Dr Michael J. Birrer (National Cancer Institute, Bethesda, MD, USA). For expression of c-Jun, the c-Jun plasmid (pcDNA3.1/c-Jun) was transfected into 293T cells. For the GST/Pin1 pull-down assay, 4 mg of GST and GST/Pin1 full-length proteins were collected on glutathione-sepharose beads (Amersham Biosciences, Little Chalfont, UK) and were treated with rhein (0, 20, 40, 60 and 80 μM). The bound proteins were denatured in sample buffer, separated, and analyzed by immunoblotting with anti-phospho-c-Jun (pc-Jun) (Ser73).

Luciferase assay. Transient transfection was conducted using jetPEI, and activity of the firefly and *Renilla* luciferases was measured according to the manufacturer's instructions

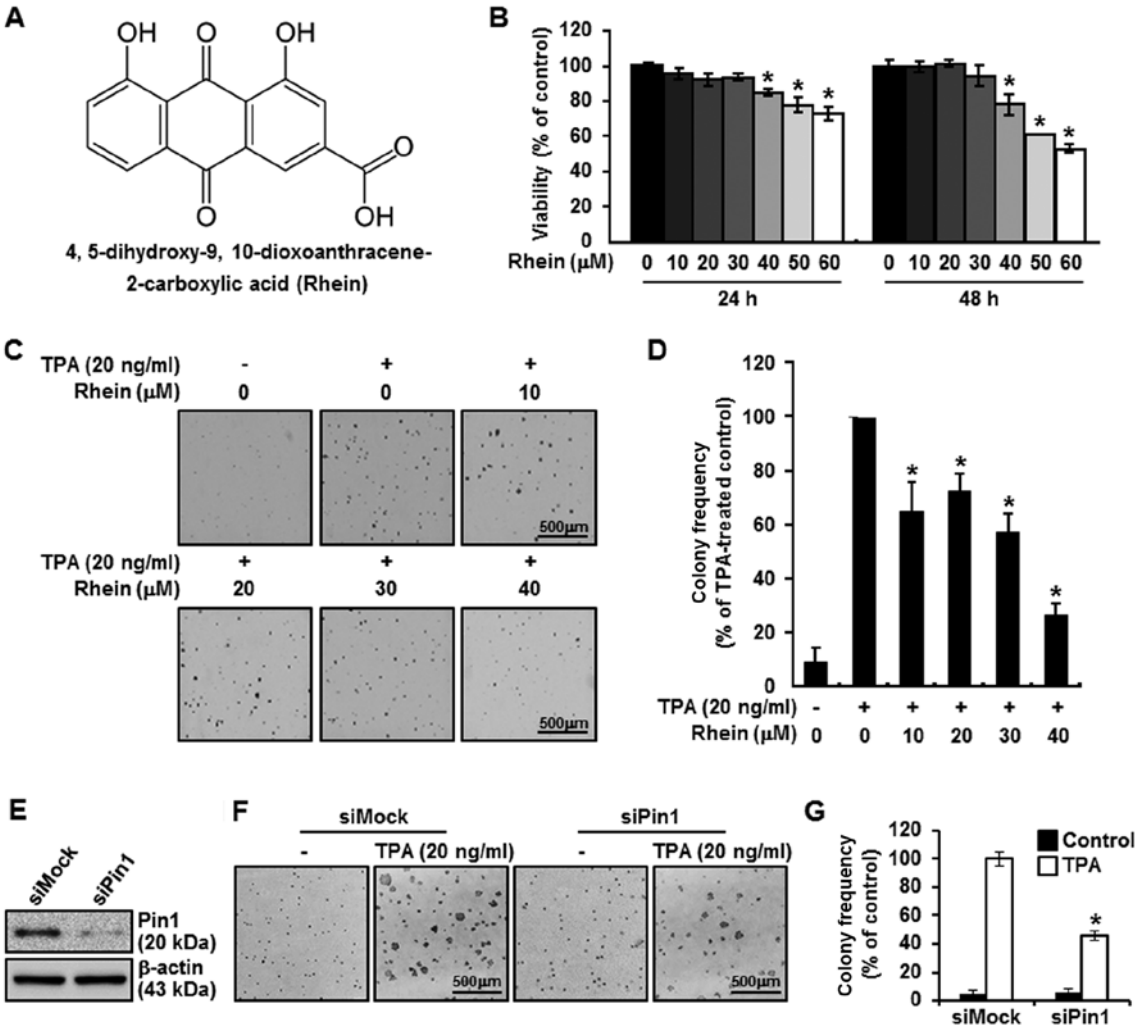

Figure 1. The effect of rhein on cell viability and tumorigenesis of JB6 CI41 cells. (A) Chemical structure of rhein. (B) Cell viability in rhein-treated JB6 CI41 cells and DMSO-treated controls was detected using the CellTiter 96 AQueous One Solution Cell Proliferation Assay kit. Data are presented as mean percentage levels ± SD. Student's t-tests revealed significant differences between rhein-treated and vehicle-treated controls (n=3; $^{*}P<0.05$). (C) Rhein inhibited TPA-induced colony formation in JB6 CI41 cells. (D) The numbers of colonies counted were expressed as means ± SD (n=3; $^{*}P<0.0001$ vs. control cells). (E) Downregulation of endogenous Pin1 protein levels in JB6 CI41 cells following Pin1 silencing. Expression of Pin1 was detected in JB6 CI41/siMock and JB6 CI41/siPin1 cells by western blot analysis using anti-Pin1. (F) Pin1-silencing effects on anchorage-independent growth of JB6 CI41 cells. (G) The counted colonies are expressed as means ± SD (n=3; $^{*}P<0.05$) vs. TPA-induced cells.

(Promega). Pin1 WT and Pin1 KO MEFs ($1x10^4$ cells/well) were cotransfected with the AP-1 or NF-κB reporter plasmid, cultured for 24 h, and then treated with rhein (10-60 μM). The cells were incubated for 1 h before TPA was added and were then cultured for another 24 h. Firefly and *Renilla* luciferase activities were measured using the Luminoskan Ascent plate reader (1450 MicroBeta TriLux; Perkin-Elmer, Waltham, MA, USA). Relative luciferase activity was calculated as a percentage of the activity of TPA-induced AP-1 or NF-κB luciferase, as compared with untreated controls, and transfection efficiency was normalized against *Renilla* luciferase activity.

Cell cycle and apoptosis analyses. Cell cycle distribution was analyzed by flow cytometry, as previously described (34). After treatment with rhein for 24 h, the cells were analyzed with a Beckman Coulter Epics XL-MCL flow cytometer (Beckman Coulter, Miami, FL, USA) using MultiCycle software (Phoenix Flow Systems, San Diego, CA, USA), which indicated the proportions of cells in the G_1/G_0, S, and G_2/M phases. Rates of apoptosis were determined using Annexin V-based immunofluorescence. Cells ($1x10^5$) were plated in 60-mm culture dishes at concentrations determined to yield G_1/G_0, S and G_2/M within 24 h and were treated with either DMSO or rhein (0, 30 and 60 μM) for 24 h. The cells were then double-labeled with Annexin V and propidium iodide (PI) and analyzed by flow cytometry.

Immunoblotting. Cell lysates including proteins were resolved onto SDS-PAGE, and the protein of interest was visualized as a sharp band by means of a specific antibody raised against antigen. In brief, the protein samples were subjected to SDS-PAGE and were subsequently transferred to membranes, which were blocked with 5% skim milk and then probed with the indicated antibodies (1:1,000). After the incubation of blots with horseradish peroxidase (HRP)-conjugated secondary antibody (1:5,000), the bands of interest were visualized using a chemiluminescence detection kit according to the

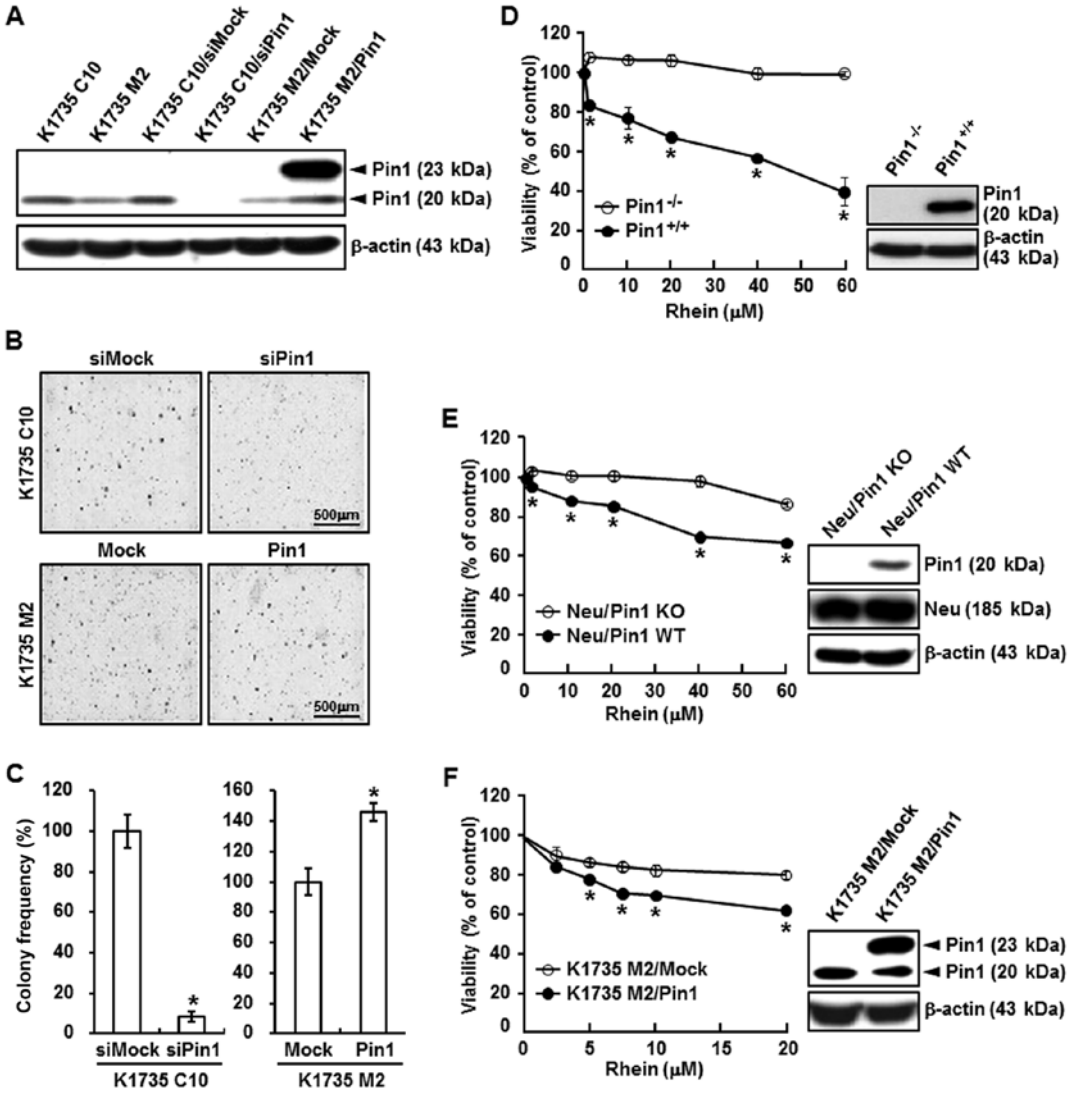

Figure 2. Inhibitory effects of rhein and Pin1 in mediating tumorigenesis and cell growth. (A) The expression of Pin1 in K1735 C10, K1735 M2, K1735 C10/siMock, K1735 C10/siPin1, K1735 M2/Mock, and K1735 M2/Pin1 cells. (B and C) Functional activity of Pin1 was evaluated by soft agar assays in K1735 C10/siMock, K1735 C10/siPin1, K1735 M2/Mock, and K1735 M2/Pin1 cells. (D) Effects of various concentrations of rhein (10-60 μM) on the growth of $Pin1^{-/-}$ and $Pin1^{+/+}$ mouse embryonic fibroblasts (MEFs). (E) Effect of various concentrations of rhein (10-60 μM) on the growth of Neu/Pin1 KO and Neu/Pin1 WT MEFs. (F) Effects of various concentrations of rhein (5-20 μM) on the growth of K1735 M2/Mock and K1735 M2/Pin1 cells. Cells were treated with various doses of rhein for different times, and growth was then estimated by means of the CellTiter 96 AQueous One Solution Cell Proliferation Assay kit. Data are expressed as means ± SD (n=3; *P<0.05).

manufacturer's instructions (GE Healthcare, Piscataway, NJ, USA).

Statistical analysis. Results are presented as mean ± SD of at least 3 independent experiments performed in triplicates. Data were analyzed for statistical significance using a one-way analysis of variance. A P-value <0.05 was considered to indicate a statistically significant result.

Results

Rhein suppresses the viability of JB6 CI41 cells. The effects of rhein on the viability of JB6 CI41 cells were verified by results of the proliferation assay (Fig. 1B). First, we confirmed that the concentration of rhein required to significantly inhibit viability was reached in the JB6 CI41 cells and ranged from 40 to 60 μM. Fig. 1B shows that the viability of the JB6 CI41 cells was decreased at 24 and 48 h in a dose-dependent as well as a time-dependent manner.

Biological function of rhein and Pin1 in mediating tumorigenic effects of TPA. We investigated whether rhein affected colony formation via Pin1 in an anchorage-independent cell transformation assay. Rhein exhibited an inhibitory effect on JB6 CI41 cell transformation in a dose-dependent manner (Fig. 1C and D). Next, to gain insight into how Pin1 expression levels are associated with tumorigenesis, a soft agar assay was performed using cell lines that were either subjected or not subjected to siRNA silencing. The siRNAs were utilized to effectively diminish Pin1 expression, and siPin1 cells exhibited decreased Pin1 expression of more than 50%, as compared with the decrease achieved in cells with mock treatment (Fig. 1E). Subsequently, stimulation with TPA was used to induce tumorigenesis in the siRNA-transfected JB6

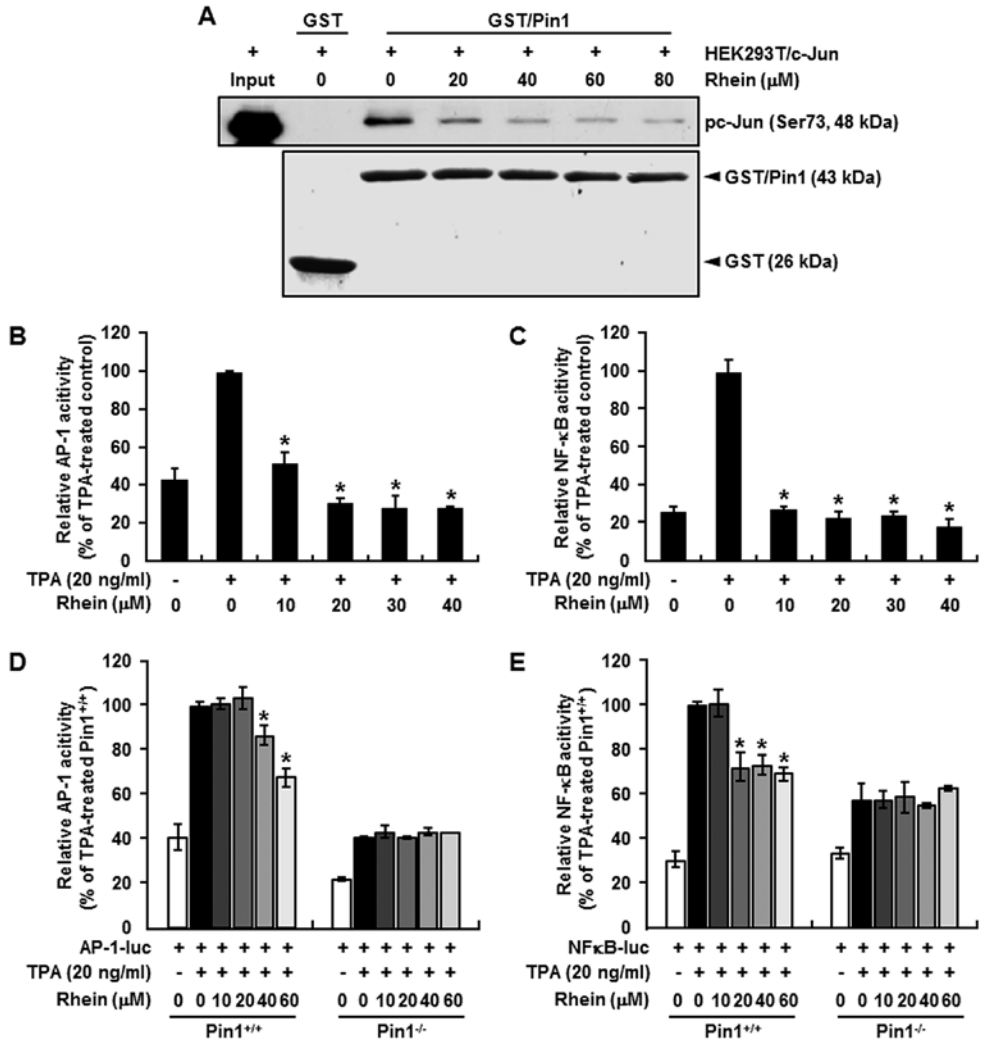

Figure 3. Rhein inhibits the interaction of Pin1 with active c-Jun and attenuates TPA-induced AP-1 and NF-κB promoter activities. (A) The effects of rhein on GST/Pin1 protein and active c-Jun binding affinity. Lysates from 293T cells transfected with c-Jun were treated with different concentrations of rhein and then subjected to a pull-down assay with GST and GST/Pin1, followed by immunoblotting with an antibody to detect phosphorylation of c-Jun (Ser73). Purified GST and GST/Pin1 abundance was visualized by immunoblotting with an antibody against GST (A, bottom). The effects of rhein on (B and D) AP-1 or (C and E) NF-κB reporter gene activity. For the reporter gene assay, JB6 CI41, Pin1$^{+/+}$ and Pin1$^{-/-}$ MEFs were transfected with a plasmid mixture containing the AP-1 or NF-κB luciferase reporter gene (100 ng) and the pRL-SV40 gene (10 ng) for normalization. At 24 h after transfection, cells were treated with various concentrations of rhein (10, 20, 40 and 60 μM) for 1 h before treatment with 20 ng/ml of TPA for an additional 48 h. Data are shown as means ± SD (n=6; *P<0.005).

CI41 cells. Mock-transfected cells produced more foci in the soft agar assay than did the Pin1 siRNA-transfected cells, thus expressing quantitatively that anchorage-independent potency in the Pin1-silenced JB6 CI41 cells declined to ~46.1% when compared with the mock-treated group (Fig. 1F). The decrease in the number of colonies of JB6 CI41 cells subjected to Pin1 silencing was statistically significant compared with that in the mock group (Fig. 1G).

Impact of rhein on Pin1 activity. First, we showed that Pin1 is required for tumorigenesis in the K1735 mouse melanoma cell lines derived from the same parental lines: a non-metastatic cell line (K1735 C10) and a metastatic cell line (K1735 M2). In the first set of experiments, expression of Pin1 was evaluated by western blot assays in lysates of K1735 C10, K1735 M2, K1735 C10/siMock, K1735 C10/siPin1, K1735 M2/Mock, and K1735 M2/Pin1 cells (Fig. 2A). Pin1 had an inhibitory effect on K1735 C10/siPin1 cell transformation but enhanced K1735 M2/Pin1 cell transformation (Fig. 2B and C). Next, to study the effect of rhein on cell proliferation, we treated Pin1 WT (Pin1$^{+/+}$) and Pin1 KO (Pin1$^{-/-}$) MEFs (Fig. 2D, right) with rhein. Rhein was administered at various concentrations in Neu/Pin1 KO MEFs (overexpression of Neu oncogene but not Pin1), Neu/Pin1 WT MEFs (overexpression of Neu oncogene and Pin1) (Fig. 2E, right), and K1735 M2/Mock, and K1735 M2/Pin1 (overexpression of Pin1) cells (Fig. 2F, right). Proliferation was assessed using a cell viability assay, as described in Materials and methods. Treatment of these cells with rhein inhibited the proliferation of the Pin1$^{+/+}$ MEFs, Neu/Pin1 WT MEFs, and K1735 M2 Pin1 MEFs but had no effect on Pin1$^{-/-}$ MEFs, Neu/Pin1 KO MEFs, and K1735 M2/Mock cells (Fig. 2D-F, left). These results showed that the Pin1-deficient cells were resistent to rhein treatment and Pin1 is essential for tumorigenesis.

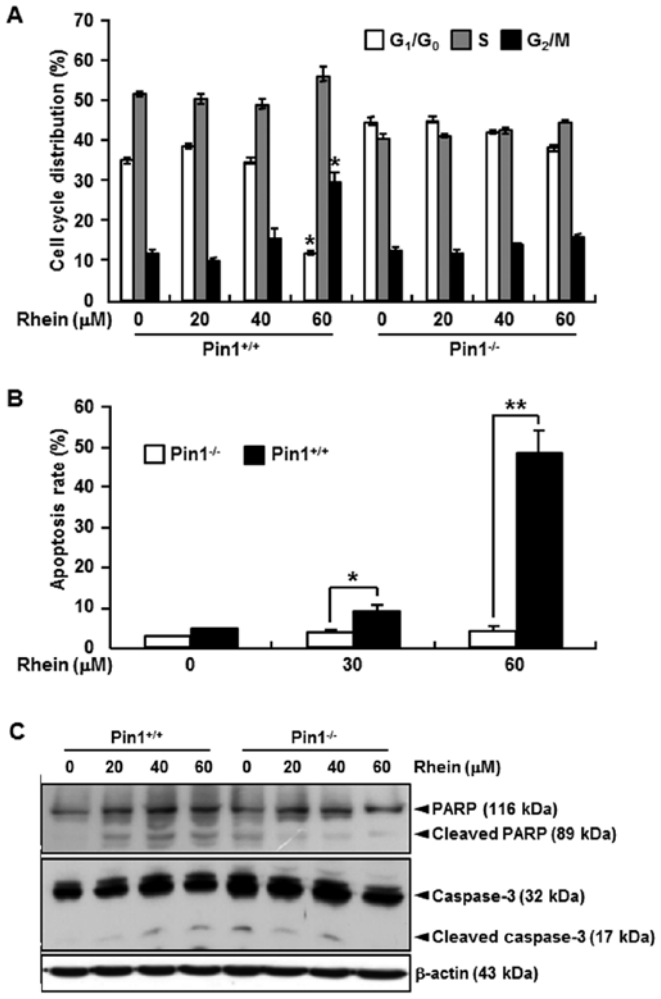

Figure 4. Effect of rhein on the cell cycle and apoptosis. (A) Effects of rhein on the cell cycle in $Pin1^{+/+}$ and $Pin1^{-/-}$ MEFs. Cells were cultured for 24 h with various doses of rhein (20, 40 and 60 μM). Cells were stained with PI and subjected to analysis by flow cytometry to assess cell distribution at each phase of the cell cycle, as described in the Materials and methods section. All experiments were carried out in duplicate and yielded similar results. Data are shown as means ± SD. (B) Effect of rhein to induce apoptosis in $Pin1^{+/+}$ and $Pin1^{-/-}$ MEFs. $Pin1^{+/+}$ or $Pin1^{-/-}$ MEFs were treated with various doses of rhein (30 and 60 μM) for 24 h. Cells were then labeled with Annexin V and PI and analyzed by flow cytometry. Data are shown as means ± SD (n=3; $^{*}P<0.01$; $^{**}P<0.005$). (C) Rhein regulates anti-apoptotic marker protein expression. Cells were treated with various doses of rhein (20, 40 and 60 μM) for 24 h and then harvested. Immunoblotting was carried out with specific antibodies to caspase-3, cleaved caspase-3, poly(ADP-ribose) polymerase (PARP), and cleaved PARP. β-actin was used as a control to verify equal loading of protein.

Inhibitory effect of rhein on binding between Pin1 and c-Jun. Formation of the Pin1/c-Jun complex is an important regulator of c-Jun stabilization, and Pin1 has been shown to bind phosphorylated c-Jun and potentiate its transcriptional activity toward cyclin D1 (35). We therefore used a GST/Pin1 pull-down assay to determine the effect of increasing concentrations of rhein on the formation of the Pin1/c-Jun complex in cell lysates prepared from 293T cells that were transfected with c-Jun. Pin1 was phosphorylated on Ser73, as indicated by western blot analysis. No binding was observed between GST alone and pc-Jun (Ser73), but binding did occur between pc-Jun (Ser73) and the GST/Pin1. Most importantly, the binding of pc-Jun (Ser73) with Pin1 was markedly decreased by rhein (Fig. 3A).

Inhibitory effect of rhein on AP-1 and NF-κB promoter activities. Rhein has been reported to inhibit AP-1 and NF-κB activity (12,36,37), and Pin1 is required for the full activation of signal transduction pathways, including AP-1 and NF-κB (18,22). Therefore, we hypothesized that the inhibitory effect of rhein may effectively suppress AP-1 and NF-κB activity through Pin1. We examined the effect of rhein on AP-1 and NF-κB activity or AP-1 and NF-κB promoter activity in $Pin1^{-/-}$ and $Pin1^{+/+}$ MEFs. The results indicated that rhein significantly suppressed AP-1 (Fig. 3B and D) and NF-κB (Fig. 3C and E) reporter activity in $Pin1^{+/+}$ cells but had little effect on these activities in the $Pin1^{-/-}$ cells.

Effect of rhein on cell cycle and apoptosis. We compared the effects of rhein on the cell cycle and apoptosis in $Pin1^{+/+}$ and $Pin1^{-/-}$ MEFs. Rhein induced the accumulation of $Pin1^{+/+}$ cells in the G_2/M-phase and induced apoptosis (Fig. 4A and B) and also regulated several pro- and anti-apoptotic marker genes (Fig. 4C). Rhein induced a higher rate of apoptosis in the $Pin1^{+/+}$ cells than that in the $Pin1^{-/-}$ cells (Fig. 4B).

Discussion

Pin1 can promote tumorigenesis by activating or stabilizing numerous oncoproteins and also by inactivating or destabilizing a number of tumor-suppressor genes (38,39). Since the phosphorylation of proteins on Ser/Thr-Pro is a key regulatory mechanism for the control of cell proliferation and transformation, effective inhibitors of Pin1 activation could offer a novel clinical strategy for the prevention or treatment of cancer. In a previous study, we demonstrated the biological effects of Pin1 on oncogenic signaling pathways and neoplastic cell transformation (32), and other studies have shown that inhibition of Pin1 in cancer cells triggers apoptosis or suppresses the transformed phenotype (27,28). Therefore, Pin1 has become an attractive molecule in cancer research, and many inhibitors of Pin1 have been discovered, including several classes of designed inhibitors and natural products (40).

Natural anthraquinones are distinctive in having considerable structural variety, a wide range of biological activities, and low levels of toxicity. Some of the naturally occurring anthraquinones currently identified include emodin, physcion, cascarin, catenarin and rhein (41). In this study, we demonstrated that the antitumorigenic effect of rhein occurs as a result of its interference in the interaction between Pin1 and c-Jun, suggesting a use for this anthraquinone in suppressing the tumor-promoting effects of Pin1. We showed that the rhein/Pin1 association plays a regulatory role in cell proliferation and neoplastic cell transformation (Figs. 1 and 2).

Pin1 has many reported protein targets, and an important Pin1 substrate is the phospho-Ser63/73-Pro motif in c-Jun. Consistent with our data, the binding of phosphorylated c-Jun (Ser73) with Pin1 was markedly decreased by rhein (Fig. 3A). It is possible that rhein also regulates Pin1-mediated tumorigenesis through other substrates, such as c-Jun.

Rhein has been reported to inhibit tumor promoter-induced cell transformation mediated by AP-1 and NF-κB

activation (36,42,43). Pin1 has also been shown to regulate NF-κB DNA binding and reporter activity after Pin1 recognition of its phosphorylated p65/RelA subunit (44). We found that rhein effectively inhibited AP-1 and NF-κB reporter activity in $Pin1^{+/+}$ MEFs but not in $Pin1^{-/-}$ MEFs (Fig. 3B-E). These data also support the hypothesis that rhein directly targets Pin1 and that this association can inhibit Pin1 tumor promoter activity.

Next, we compared the effects of rhein on the cell cycle and apoptosis in $Pin1^{+/+}$ and $Pin1^{-/-}$ MEFs. We found that rhein induced the accumulation of $Pin1^{+/+}$ MEFs in the G_2/M phase of the cell cycle (Fig. 4A), and apoptosis was markedly induced following rhein treatment in the $Pin1^{+/+}$ MEFs as compared with the $Pin1^{-/-}$ MEFs (Fig. 4B). Moreover, rhein-induced changes in the levels of various pro-apoptosis-associated and anti-apoptosis-associated proteins were more apparent in the $Pin1^{+/+}$ MEFs (Fig. 4C).

In conclusion, our findings and the accompanying biochemical data showed that rhein directly inhibits the tumor-promoting activity of Pin1 and may therefore have practical implications for cancer prevention or therapy.

Acknowledgements

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