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p53 Retards cell-growth and suppresses etoposide-induced apoptosis in Pin1-deficient mouse embryonic fibroblasts

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ABSTRACT

We studied the effects of Pin1, a regulatory molecule of the oncosuppressor p53, on both cell cycle arrest and apoptosis by treating primary mouse embryonic fibroblasts (MEFs) with etoposide. Etoposide induced G1 arrest in both wild-type and Pin1 null (*pin1*$^{-/-}$) MEFs, and G2/M arrest and apoptotic cell death in MEFs lacking either p53 only (*p53*$^{-/-}$) or both Pin1 and p53 (*pin1*$^{-/-}$*p53*$^{-/-}$). Both *pin1*$^{-/-}$ and *pin1*$^{-/-}$*p53*$^{-/-}$ MEFs were enhanced the release of cytochrome c from the mitochondria, which might induce apoptosis. In response to etoposide treatment, apoptotic cell death was displayed in pin1$^{-/-}$p53$^{-/-}$ MEFs but not in pin1$^{-/-}$ MEFs. These results suggest that p53 retards growth and suppresses etoposide-induced apoptosis in *pin1*$^{-/-}$ MEFs.

1. Introduction

The prolyl isomerase Pin1 is an enzyme capable of recognizing specific amino acid resides, namely, phosphorylated serine/threonine–proline (pS/pT–P), and catalyzing conformational changes in its substrates, thereby modulating their activity, stability, and subcellular localization. Pin1 regulates cell growth and apoptosis by catalyzing the isomerization of various molecules [1–4]. Mice lacking Pin1 display retinal hypoplasia and mammary gland impairment which are also the characteristic of cyclin D1-deficient mice [5], and the mouse embryonic fibroblasts (MEFs) lacking Pin1 (*pin1*$^{-/-}$ MEFs) fail to induce a transition from G0 to G1 [6]. The phenotypes of *pin1*$^{-/-}$ mice and MEFs show that the physiological function of Pin1 might be regulation of cell growth and development.

The protein p53 acts as a gatekeeper molecule by regulating DNA repair, cell cycle, and apoptosis, to protect cells from chromosome instability [7–9]. Loss of p53 function increases the susceptibility of a cell to malignant transformation [10]. The level of p53 protein is rapidly elevated, and the protein is activated in response to UV radiation, DNA-damaging chemicals, hypoxia, or activated oncogenes. High levels of activated p53 drive the transcription of a large number of genes that mediate its biological functions. Phosphorylation of p53 in response to stress is essential for its accumulation and activation, and it enables p53 to bind to DNA and activate transcription [11,12].

Pin1 is involved in controlling the accumulation and activation of p53 in cells exposed to DNA-damaging agents [13–15]. Pin1 might be a critical node of integration within the DNA damage-induced checkpoint pathway. We recently created mice lacking both Pin1 and p53 (*pin1*$^{-/-}$*p53*$^{-/-}$ mice), and found that these mice displayed thymic hyperplasia with up-regulation of the expression of the intracellular form of Notch-1, suggesting that both Pin1 and p53 might regulate the cleavage of Notch-1 by presenilin-1 [16]. It is clear that in the thymus, p53 is essential for the elimination, via apoptosis, of DNA-damaged cells, and it appears that loss of apoptotic pathways is critical to tumor formation in this cell type. The phenotypes of *pin1*$^{-/-}$*p53*$^{-/-}$ mice indicate that the physiological network between Pin1 and p53 might promote the elimination of DNA-damaged cells [13–16].

Primary MEFs provide an ideal model system for studying both cell cycle arrest and apoptotic functions related to p53. As anticancer agents are rarely selective for tumor cells, it is important to define their effects on both normal and neoplastic cells [17]. The MEFs expressing adenovirus and activated Ras undergo p53-dependent apoptosis, and this p53-dependent apoptotic pathway plays an important role in the response of mouse tumors to chemotherapy [18]. Ding et al. [19] show that p53 itself causes caspase activation in the cell-free extracts from E1A/ras-transformed,

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but not from normal fibroblasts, through a mechanism that is independent of cytochrome c release from mitochondria. These results indicate that p53 induces apoptosis through caspase activation that is not resulted from mitochondrial permeability but oncogene products, in response to cellular stress.

In the present study, we used primary MEFs to examine the interactions between Pin1 and p53 during apoptotic events following exposure to etoposide, an inhibitor of topoisomerase II. The *pin1*$^{-/-}$ and *pin1*$^{-/-}$*p53*$^{-/-}$ MEFs showed the release of cytochrome c from the mitochondria into the cytoplasm as well as growth arrest, but *pin1*$^{-/-}$ MEFs did not undergo apoptosis. The absence of p53 expression increased etoposide-induced apoptotic cell death. However, *p53*$^{-/-}$ MEFs displayed apoptotic cell death without an increase in mitochondrial permeability, following treatment with etoposide. Our observations thus indicate that p53 retards cell-growth and suppresses etoposide-induced apoptosis at the process after mitochondrial permeability, depended on Pin1 expression in primary MEFs. Our results suggest a major role of Pin1 and/or p53 on control of apoptosis after cytochrome c release.

2. Materials and methods

2.1. Preparation of MEFs and cell culture

Primary cultured MEFs were obtained from 14 days post coitus (dpc) embryos created by mating *pin1*$^{+/-}$ and *p53*$^{+/-}$ mice. Our study was approved by the Animal Use and Care Committees of both Showa University and Tohoku University. Pin1$^{-/-}$ mice were previously generated by Fujimori et al. [6], and bred in collaboration with Dr. Tony Hunter. Mice lacking p53 (p53 KO mice) were generated by Livingstone et al., and were provided by DuPont Central & Research Development (Wilmington, DE) [20]. Both mouse strains were maintained on a C57BL/6J background. The *p53*$^{+/-}$ mice were mated with *pin1*$^{+/-}$ mice to obtain *pin1*$^{+/-}$*p53+/-* offspring, which were intercrossed. Embryos carrying the nine possible genotypes (*wild-type*, *p53*$^{+/-}$, *p53*$^{-/-}$, *pin1*$^{+/-}$, *pin1*$^{+/-}$*p53*$^{+/-}$, *pin1*$^{+/-}$*p53*$^{-/-}$, *pin1*$^{-/-}$, *pin1*$^{-/-}$*p53*$^{+/-}$, *and pin1*$^{-/-}$*p53*$^{-/-}$) were generated [16]. For genotyping the Pin1 locus, the primers WILD1.2A (5′ AAG GGA TTA GAA GCA AGA TTC G 3′), 2L (5′ AGC ACC CGA TCC TGT TCT GCA A 3′), and Start2 (5′ CAG AGG CCA CTT GTG TA 3′) were used [5,17]. For genotyping the p53 locus, primers X7 (5′ TAT ACT CAG AGC CGG CCT 3′), X6.5 (5′ ACA GCG TGG TGG TAC CTT AT 3′), and Neo19 (5′ CAT TCA GGA CAT AGC GTT GG 3′) were used [16,20]. In this study, we used the primary MEFs obtained by passage 4. All investigations were conducted according to the principles of the Declaration of Helsinki.

2.2. Cell viability analysis

To analyze induction of cell death after treatment with etoposide, growing cells were treated with 3 μM or 30 μM etoposide for 48 h. After treatment, MEFs were harvested, and viability was measured by staining the cells with trypan blue dye, and then counting unstained cells by using a hemocytometer.

2.3. Cell cycle analysis

To analyze the DNA damage response in MEFs, etoposide-treated cells were harvested and resuspended in staining solution containing 0.1% Triton X100, 50 μg/mL RNase A, and 0.5 μg/mL propidium iodide in PBS. The cells were then placed in a fluorescence-activated cell analyzer (FACS-Calibur, Becton Dickinson, Franklin Lakes, NJ), and counted using FACS-Calibur software. To estimate the number of S-phase cells actively replicating their DNA, we administered a pulse of bromodeoxyuridine during the last 4 h of etoposide-treatment, in a manner similar to that used by Kastan et al. [17].

2.4. Total cell lysate preparation and immunoblot analysis

Total MEF lysates were prepared with NP40 lysis buffer [21]. Protein concentrations of the total lysates were determined by the Bradford method (protein assay; Bio-Rad). Twenty microgram of protein amount of total lysates were subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE), and then transferred to Immobilon-P membranes (Millipore, Billerica, MA). The membranes were probed with polyclonal antibodies against p53, pSer15-p53 (Cell Signaling, Danvers, MA), Pin1, or p21^{Cip1} (Santa Cruz Biotechnology, Santa Cruz, CA). Proteins were visualized by ECL-plus (Invitrogen, Carlsbad, CA).

2.5. Preparation of mitochondrial and cytosolic MEF fractions

Mitochondrial and cytosolic MEF fractions were prepared in mitochondrial fraction buffer [22]. Briefly, homogenized MEFs were centrifuged at 3000×*g*, 4 °C, for 10 min to remove the nuclei. The supernatants were further centrifuged at 10,000×*g*, 4 °C, for 15 min. Resultant pellets were resuspended in mitochondrial fraction buffer and used as the mitochondrial fraction. The remaining supernatants were ultracentrifuged at 100,000×*g*, 4 °C, for 60 min, and then used as the cytosolic fraction. Both fractions were probed for cytochrome c by using immunoblotting analysis.

3. Results and discussion

3.1. Effects of pin1 ablation on proliferation of MEFs are dependent upon p53 expression

We compared the proliferative properties of MEFs among different MEF genotypes, namely, *pin1*$^{+/-}$, *pin1*$^{-/-}$, *pin1*$^{+/-}$*p53*$^{-/-}$, and *pin1*$^{-/-}$*p53*$^{-/-}$. Growth rates of *p53*$^{-/-}$ MEFs were higher compared to *pin1*$^{+/-}$ and *pin1*$^{-/-}$ MEFs (Fig. 1A). Because Pin1-deficient mice phenotypically resemble Cyclin D1-deficient mice, and it was reported that Pin1 induced the stabilization of Cyclin D1 through its isomerization [5], we assumed that ablation of Pin1 might decrease the growth rates of MEFs by down-regulating Cyclin D1. However, *pin1*$^{-/-}$*p53*$^{-/-}$ MEFs displayed a higher increase in growth rates than *pin1*$^{+/-}$*p53*$^{-/-}$ MEFs. It was suggested that Pin1 might have substrates that regulate cellular proliferation independent of p53. We previously reported that in the absence of serum, *pin1*$^{-/-}$ MEFs proved significantly deficient in their ability to re-enter the cell cycle from G0 arrest [6]. Therefore, we examined the effects of serum depletion on *pin1*$^{-/-}$*p53*$^{-/-}$ MEFs. To synchronize cells in G0 for cell cycle arrest assays, MEFs were plated, placed into medium containing 0.1% fetal calf serum (FCS) for 4 days, and subsequently, switched to medium containing 10% FCS to stimulate cell cycle re-entry. Unlike the *pin1*$^{-/-}$ MEFs, *pin1*$^{-/-}$*p53*$^{-/-}$ MEFs re-entered the cell cycle in the presence of 10% FCS (Fig. 1B). These results suggested that *pin1*$^{-/-}$ MEFs displayed p53-dependent growth properties under both asynchronous and synchronous conditions.

We utilized two-dimensional FACS analysis in which cells were sorted on the basis of DNA content, measured by propidium iodide, and active replication, measured by bromodeoxyuridine incorporation into cellular DNA during the S phase of the cell cycle. All the examined serum-starved MEFs showed G1 arrest (Supplementary Fig. S1). Serum-stimulation for 24 h with 10% FCS facilitated a robust entry of all examined MEF genotypes into S phase. After 24 h, G2/M phase-cells ratio was increased in Pin1-null MEFs; that is, wild-type versus *pin1*$^{-/-}$ was 10% and 17%, and *p53*$^{-/-}$ versus

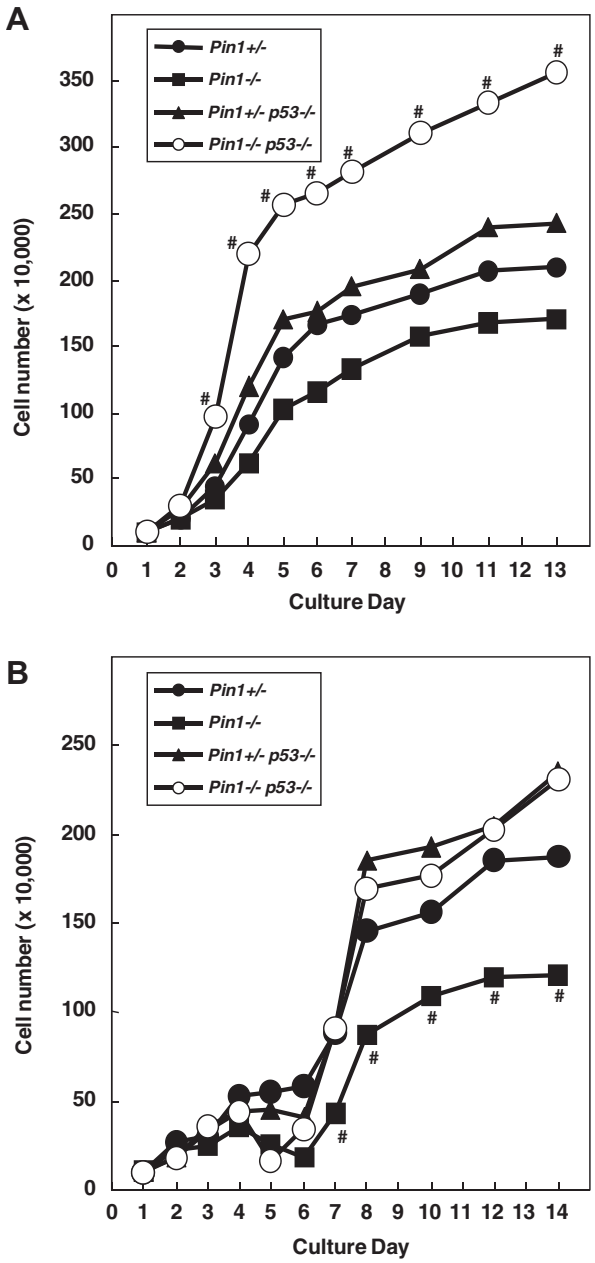

Fig. 1. Effect of Pin1 on p53-dependent MEF proliferation. (A) Proliferation curve of asynchronized MEF cultures that were analyzed for 13 days. The *pin1*$^{-/-}$*p53*$^{-/-}$ MEFs (open circle) showed the highest cell cycle progression rate and decrease in contact inhibition among the tested genotypes. (B) Proliferation curve of synchronized MEF cultures that were analyzed for 14 days. Cells were synchronized using medium containing 0.1% fetal calf serum (FCS) for 4 days, and then switched to complete culture medium containing 10% FCS. Closed circle, *pin1*$^{+/-}$ as control cells; Square, *pin1*$^{-/-}$ and Triangle, *pin1*$^{+/-}$*p53*$^{-/-}$. # indicates the significance at the $p \leqslant 0.05$ level (Student's *t*-test) between *pin1*$^{+/-}$ and *pin1*$^{-/-}$*p53*$^{-/-}$ MEFs.

pin1$^{-/-}$*p53*$^{-/-}$ was 11% and 19%, respectively (Supplemental Fig. S1). Cell cycle progression in *pin1*$^{-/-}$ MEFs was slower than in *pin1*$^{-/-}$*p53*$^{-/-}$ MEFs, which were not arrested. Thus, Pin1 might be the crucial factor that regulates the progression of MEFs into mitosis.

3.2. Etoposide-induced apoptosis in MEFs lacking p53

Topoisomerase II functions during both DNA replication and transcription. The topoisomerase II inhibitor, etoposide is an anti-tumor drug that has been widely used to couple DNA damage to cell cycle arrest and apoptosis [23,24]. A previous study has shown that etoposide treatment induced p53 stabilization in various cell culture systems [25]. Etoposide stabilizes the complex formed by topoisomerase II and the 5′-cleaved ends in DNA, thus forming stable protein-linked DNA double-strand breaks; cells are apparently able to recognize such DNA damage [23–25]. We examined the effects of etoposide treatment on the viability of wild-type, *pin1*$^{-/-}$, *p53*$^{-/-}$, and *pin1*$^{-/-}$*p53*$^{-/-}$ MEFs. Treatment with 3 μM etoposide induced lower viability of *p53*$^{-/-}$ (59%) and *pin1*$^{-/-}$*p53*$^{-/-}$ (63%) MEFs compared to wild-type (87%) and *pin1*$^{-/-}$ (96%) MEFs. The wild-type and *pin1*$^{-/-}$ MEFs were barely affected by exposure to 3 μM etoposide (Fig. 2A). Treatment with 30 μM etoposide induced lower viability in *pin1*$^{-/-}$ (40%), *p53*$^{-/-}$ (43%), and *pin1*$^{-/-}$*p53*$^{-/-}$ (28%) MEFs than in wild-type (71%) MEFs (Fig. 3A). Etoposide

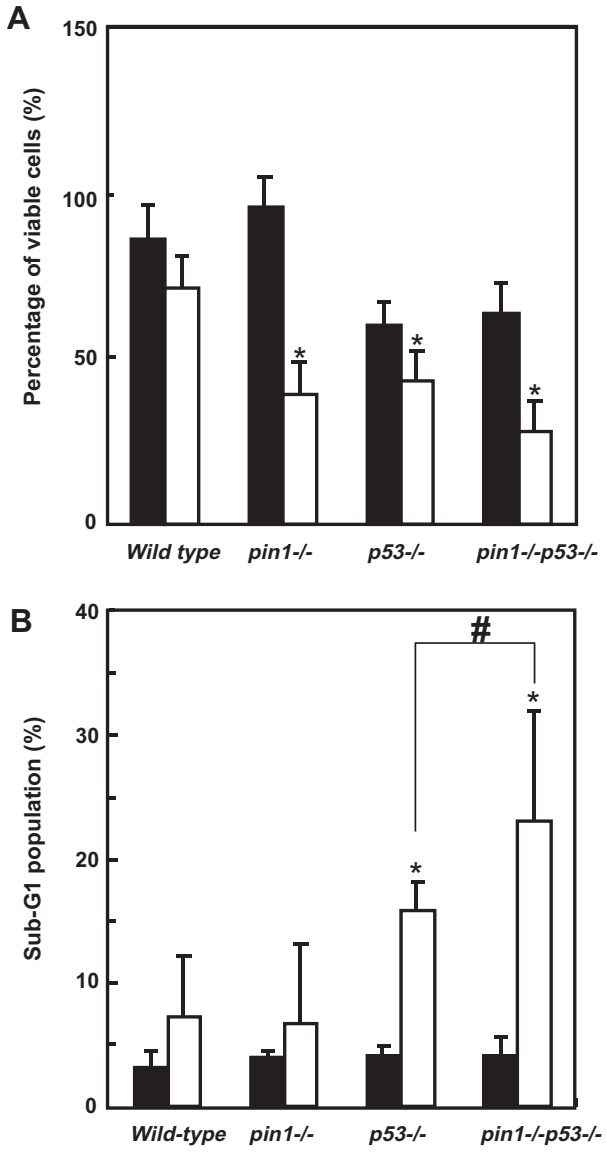

Fig. 2. Primary MEFs lacking the pin1 and p53 genes show lower viability in response to etoposide treatment. (A) The percentage of viable wild-type, *pin1*$^{-/-}$, *pin1*$^{+/-}$*p53*$^{-/-}$, and *pin1*$^{-/-}$*p53*$^{-/-}$ MEFs in response to 3 μM or 30 μM etoposide treatment for 48 h, as measured by the number of trypan blue-unstained cells, and compared as a ratio to initial cell numbers, which were set at 100%. (B) The percentage of sub-G1 populations was estimated in the propidium iodide-stained MEFs after 48-h etoposide treatment. Closed bar, 3 μM etoposide and open bar, 30 μM etoposide. ∗ indicates the significance at the $p < 0.05$ level (Student's *t*-test) toward wild-type, and # indicates the significance at the $p < 0.05$ level between *p53*$^{-/-}$ and *pin1*$^{-/-}$*p53*$^{-/-}$ MEFs.

tolerance in the *pin1*-deficient MEFs was lost at high concentrations of the drug. Moreover, the viability of p53 deficient cells was highly affected by 3 μM etoposide; otherwise, p53 deficiency—without etoposide treatment—induced efficient proliferation. We suspected that the decrease in MEF viability observed with etoposide treatment might be attributed to cell cycle arrest and/or apoptosis. When we used flow cytometry to analyze MEFs that had been treated with 3 μM etoposide and then stained with propidium iodide, we found that wild-type MEFs were arrested at the G2/M phase, but $pin1^{-/-}$ MEFs were not affected by etoposide treatment, and etoposide-treated $p53^{-/-}$ and $pin1^{-/-}p53^{-/-}$ MEFs were arrested at S phase. The sub-G1 population was not detected by 48 h in 3 μM etoposide-treated MEFs. Treatment with 30 μM etoposide in particular, stimulated the emergence of a sub-G1 population in $p53^{-/-}$ and $pin1^{-/-}p53^{-/-}$ MEFs (Fig. 2B). Etoposide may have decreased cell viability through the induction of apoptosis, which is regulated by p53, particularly at high concentrations. Interestingly, high etoposide concentrations also decreased the number of viable $pin1^{-/-}$ cells without inducing apoptosis.

3.3. High concentrations of etoposide induce G1 arrest in MEFs that is dependent upon the Pin1–p53 network

To accurately examine etoposide-induced MEF growth arrest, we quantified the percentages of asynchronized MEFs that had been stained with either BrdU or PI. As shown in the two-dimensional FACS histograms in Fig. 3, treatment for 24 h with 3 μM etoposide led to a partial G2/M arrest response in wild-type, $p53^{-/-}$ and $pin1^{-/-}p53^{-/-}$ MEFs. With 30 μM etoposide treatment, while wild-type MEFs displayed G2/M arrest, $pin1^{-/-}$ MEFs showed G1 arrest, suggesting that Pin1 might play a role in the regulation of mitosis. In addition, $p53^{-/-}$ MEFs displayed G2/M phase arrest more effectively than wild-type MEFs. The $pin1^{-/-}p53^{-/-}$ MEFs showed effective G2/M arrest, but not G1 arrest in response to both 3 μM and 30 μM etoposide. The 30 μM etoposide-treated $pin1^{-/-}p53^{-/-}$ MEFs displayed milder G2/M arrest (34–42%) compared to $p53^{-/-}$ MEFs (28–59%). Our results suggest that Pin1 could partially play a role in etoposide-induced G2/M arrest in p53-deficient cells. Furthermore, differences in the ratios of G1 phase-cells between the wild-type and p53-deficient MEFs ($p53^{-/-}$ and $pin1^{-/-}p53^{-/-}$) could be attributed to the differences in the progression rates from G1 to S phases among the MEF phenotypes. These differences could lead to higher proliferation rates in the $pin1^{-/-}p53^{-/-}$ MEFs compared to the wild-type cells (Fig. 1). Thus, the Pin1–p53 network might be required for exact G1 check point, and double loss of both Pin1 and p53 would increase MEF vulnerability to etoposide-induced DNA damage.

However, the $pin1^{-/-}p53^{-/-}$ MEFs showed a higher evasion of entry into the G1 phase in response to 30 μM etoposide compared with $p53^{-/-}$ MEFs. These results indicated that the proliferating cells with replicated DNA were arrested at the pre-mitotic (4*N*) stages and protected from apoptotic cell death, which is dependent

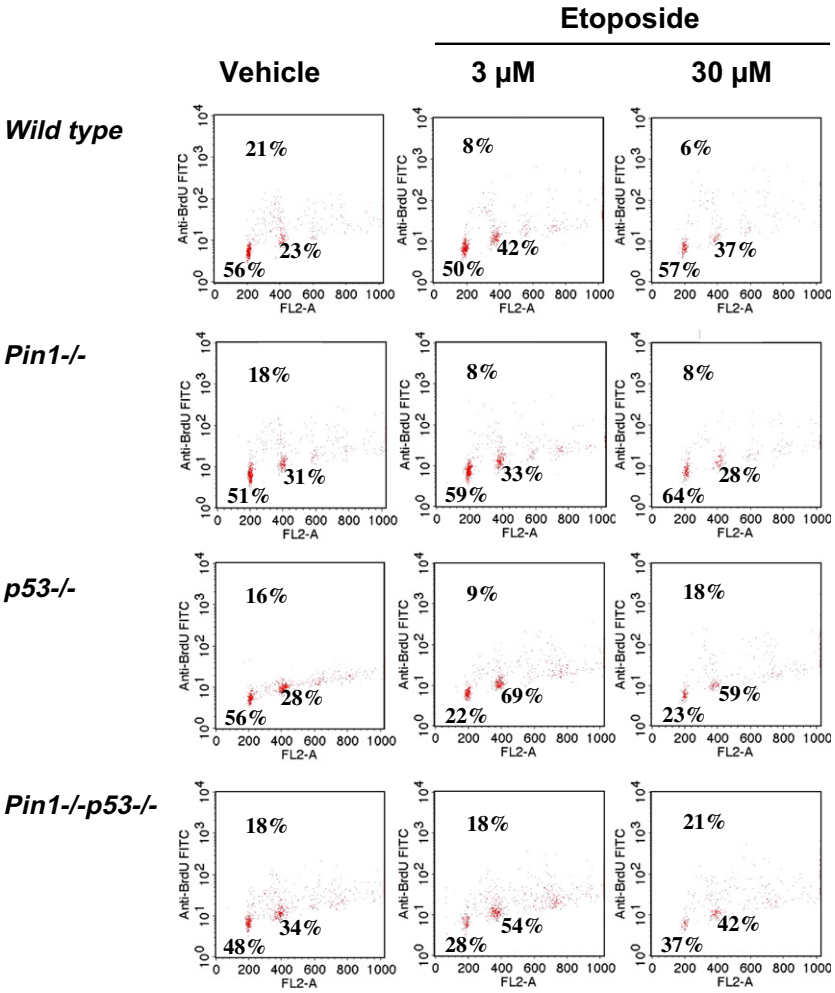

Fig. 3. Cell cycle analysis of etoposide-treated primary MEFs lacking pin1 and p53 genes. A two-dimensional FACS analysis of cells treated with vehicle (0.1% DMSO, Left), 3 μM etoposide for 48 h (Middle) or 30 μM etoposide for 48 h (Right). The cells were sorted for DNA content, measured by propidium iodide staining (FL2-A, Horizon), as well as active replication, measured by bromodeoxyuridine incorporation (anti-BrdU FITC, Vertical). (A) wild-type, (B) $pin1^{-/-}$, (C) $p53^{-/-}$, and (D) $pin1^{-/-}p53^{-/-}$ MEFs, respectively.

upon the Pin1–p53 network. Furthermore, this network might effectively arrest MEFs in the G1 phase of the cell cycle under etoposide-induced topoisomerase II-inhibited conditions.

3.4. Mitochondrial permeavilization and phosphorylated p53 in etoposide-treated MEFs

Multiple death signals affect the mitochondria during the progression of apoptosis [26]. We thus examined whether the mitochondrial pathway contributed to etoposide-induced apoptosis. Increased mitochondrial permeabilization releases cytochrome *c* into the cytoplasm, which is an essential pro-apoptotic factor for caspase activation in the mitochondrial apoptosis pathway [26]. Interestingly, cytochrome *c* was detected in cytoplasmic fractions from Pin1-deficient cells—*pin1*$^{-/-}$ and *pin1*$^{-/-}$*p53*$^{-/-}$ MEFs—but not from *p53*$^{-/-}$ cytoplasmic fractions. However, since apoptotic cell death in *p53*$^{-/-}$ MEFs was induced by 30 μM etoposide, the absence of Pin1, independent of p53 status, might facilitate the release of cytochrome *c* in response to etoposide treatment (Fig. 4A). These results indicated that Pin1 suppressed etoposide-induced release of cytochrome c. In addition, although both *pin1*$^{-/-}$ and *pin1*$^{-/-}$*p53*$^{-/-}$ MEFs showed a release of cytochrome c, the apoptotic levels in *pin1*$^{-/-}$ MEFs were less than in the *pin1*$^{-/-}$*p53*$^{-/-}$ MEFs. Thus, loss of Pin1 could induce mitochondrial permeability, but inhibit the apoptotic response in a p53-dependent manner. Since the *p53*$^{-/-}$ MEFs did not show a release of cytochrome c, the mitochondrial pathway was not involved in the apoptotic death of these cells. Actually, 30 μM etoposide resulted in a higher production of phosphorylated p53 in wild-type MEFs than the *pin1*$^{-/-}$ MEFs. Whereas etoposide-treated total *p53*$^{-/-}$ cell lysates had preserved p21^{Cip1} protein levels, the *pin1*$^{-/-}$*p53*$^{-/-}$ MEFs displayed low levels of p21^{Cip1} protein (Fig. 4B), suggesting that p21^{Cip1} levels were affected by a deficiency in p53, but not in Pin1. In contrast, p53 levels were affected by the absence of Pin1.

Mouse embryonic fibroblasts expressing the EIA adenovirus are generally sensitive to DNA-damaging chemotherapeutic agents. Furthermore, p53 itself was shown to cause caspase activation in cell-free extracts from E1A/ras-transformed, but not from normal, fibroblasts by a mechanism independent of transcription or the presence of cytochrome c [27]. It was suggested that DNA-damaging agents might cause oncogene-transformed MEFs to undergo p53-dependent apoptosis, and primary MEFs, to undergo p53-independent apoptosis. In our study, the number of apoptotic *pin1*$^{-/-}$*p53*$^{-/-}$ MEFs was higher than that of apoptotic *p53*$^{-/-}$ MEFs. The comparison between p53$^{-/-}$ and *pin1*$^{-/-}$*p53*$^{-/-}$ MEFs indicates that Pin1 functioned as a suppressor of apoptosis in *p53*$^{-/-}$ cells, in part. Mitochondrial permeability in etoposide-treated MEFs may be regulated by Pin1. In *p53*$^{-/-}$ cells, etoposide induced a p53-independent apoptotic pathway, and in the *pin1*$^{-/-}$*p53*$^{-/-}$ cells, etoposide induced both a p53-independent apoptotic pathway and mitochondrial permeability, which is normally suppressed in the presence of Pin1.

Our results suggest that Pin1 inactivation can facilitate apoptotic cell death in response to a DNA-damaging stressor, such as etoposide. Our observations also indicate that an etoposide-induced apoptotic pathway is suppressed by p53 expression. Interactions between Pin1 and p53 may control mitochondrial permeability and caspase activation in response to etoposide treatment. Our results reveal a major role of Pin1 and/or p53 to control apoptosis at the post-mitochondrial level or after cytochrome c release. In our current study, we have focused on the interaction between Pin1 and p53 as a potential target in tumor chemotherapy, and in the future, we would like to study the responses of MEFs to other available anti-cancer agents.

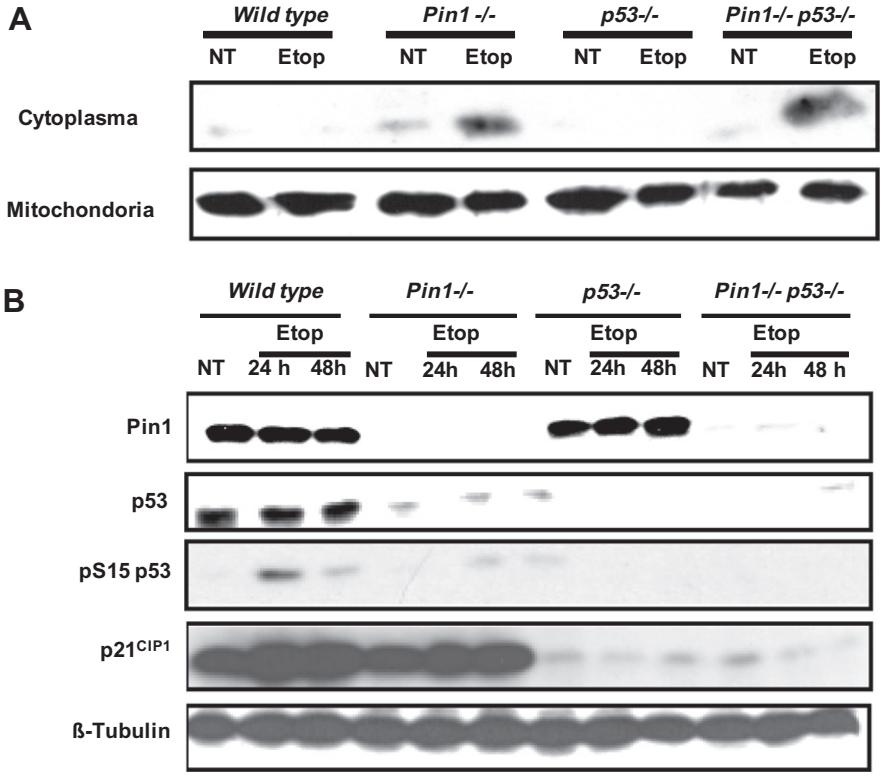

Fig. 4. Mitochondria permeabilization and phosphorylated p53 accumulation in etoposide-treated pin1$^{-/-}$ MEFs. (A) After wild-type, *pin1*$^{-/-}$, *p53*$^{-/-}$, and *pin1*$^{-/-}$*p53*$^{-/-}$ MEFs were treated with 30 μM etoposide for 6 h, cytoplasmic and mitochondrial fractions were prepared as mentioned in Section 2. Cytochrome c was detected using immunoblot analysis. NT; cells not treated with etoposide. (B) Total cell lysates were prepared from MEFs, and were analyzed with immunoblotting. Protein levels of p53 phosphorylated at Ser15 (pS15-p53) were detected using a specific antibody against pS15-p53 (Cell Signaling).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.04.121.

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