Peroxiredoxin I plays a protective role against cisplatin cytotoxicity through mitogen activated kinase signals.

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Summary The anticancer agent *cis*-diamminedichloroplatinum (cisplatin) is a first-line chemotherapeutic agent for oral cancer. Cell exposure to cisplatin is associated with increased oxidative stress and post-translational changes in components of apoptosis pathways, including p38 Mitogen-activated protein kinase (MAPK), c-Jun-NH2-kinase (JNK), and extracellular signal-regulated kinase (ERK). Peroxiredoxin (Prx) I is an oxidative stress-inducible protein expressed in many tissues and important for reducing reactive oxygen species *in vivo;* however, whether Prx I helps protect cells from cisplatin injury is unknown. In this report, we examined the effects of Prx I on cell sensitivity to cisplatin-induced apoptosis. Mouse embryo fibroblasts (MEFs) derived from Prx I-deficient mice showed increased cisplatin-induced apoptosis compared with wild-type MEFs. Cisplatin treatment also led to increased activation of p38 MAPK and JNK, and reduced ERK phosphorylation in Prx I-deficient MEFs compared with wild-type MEFs. Furthermore, JNK- and ERK-specific inhibitors protected the Prx I-deficient MEFs from cisplatin-induced apoptosis, but Prx I-deficient MEFs remained more sensitive than wild-type MEFs when treated with a p38 MAPK-specific inhibitor. These findings indicate that Prx I modulates the cisplatin-evoked activation of MAPKs that lead to apoptosis, and Prx I may thus represent a useful target as a protective therapy against cisplatin cytotoxicity.

Introduction

Cisplatin is widely used as a first-line therapy against malignant tumors [1, 2], and cisplatin-based chemotherapy is the central component of several curative approaches for patients with head and neck cancer [3]. Despite its high efficacy, however, its usage is limited by its cytotoxicity, especially nephrotoxicity, which requires lowing its dosage or actively hydrating patients [4]. Thus, it is very important to discover mechanisms to prevent or attenuate cisplatin toxicity.

The molecular mechanisms underlying cisplatin toxicity were recently clarified. One is oxidative stress. Cisplatin treatment increases the level of reactive oxygen species (ROS), which trigger cisplatin-induced apoptosis [5]. Cisplatin induces ROS *in vitro* by decreasing the activity of antioxidant enzymes and depleting the intracellular concentration of GSH. Antioxidants help protect cells from cisplatin-induced apoptosis *in vitro*. *In vivo*, antioxidants reduce the degree of renal failure caused by cisplatin[1]. However, the potential benefits of using an endogenous antioxidant protein, Peroxiredoxin (Prx), to block Cisplatin-induced cytotoxicity have not been evaluated extensively.

Members of the Prx family catalyze the reduction of H_2O_2, alkyl hydroperoxides, and radicals via the reducing equivalents provided by thiol-containing proteins, such as thioredoxin [6-9]. There are currently six Prx family members, which share a common reactive Cys residue in the N-terminal region. Prx I is the major cytosolic Prx; it is ubiquitously expressed in various tissues [10].

We previously used a Prx I-deficient mouse to investigate the anti-oxidative activities of Prx I against ferric-nitrilotriacetate (Fe-NTA)-induced oxidative stress and its scavenging of radicals in the kidney and liver, using real-time electron paramagnetic resonance imaging. We detected greater damage in the liver and kidney of Prx I-deficient mice than wild-type mice, suggesting that the Prx I deficiency decreased the anti-oxidative activities in these organs [10]. In the present study, we found that Prx I-deficient mouse embryo fibroblast cells (MEFs) were more sensitive to cisplatin than wild-type MEFs. We then investigated the apoptotic signaling components in the Prx I-deficient MEFs, and showed that Prx I interfered with cisplatin-induced apoptosis by modulating the mitogen-activated protein kinase (MAPK)-associated pathways.

Materials and methods

Cell Culture and Treatment

Homozygous Prx I-deficient (Prx I (-/-)) (OmniBank, Lexicon Pharmaceuticals, Inc.) and wild-type (Prx I (+/+)) mice were treated as described previously [10]. Non-immortalized MEFs were generated from 13.5-day Prx I (-/-) and Prx I (+/+) embryos. The brain and dark-red (internal) organs were dissected away from the embryos, and the remaining tissue was finely minced. The cells were disaggregated using 0.25% trypsin, which was removed by centrifugation. The cells were cultured at 37 °C in 95% air 5% CO_2 as an adherent monolayer in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) containing penicillin (100 U/ml) and streptomycin (100 μg/ml). Cisplatin was purchased from Sigma (Sigma Aldrich Japan, K.K., Tokyo). The MAPK-specific inhibitors SB203580, SP600125, and PD98059 were purchased from Wako (Wako Pure Chemical Industries, Osaka).

Cytotoxicity assays

Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT) assay as described previously [11]. Cells (5 x 10^3/well) were seeded in 96-well plates and incubated at 37 °C overnight. Following exposure to increasing concentrations of cisplatin for 3 days at 37 °C, MTT was added, and the cells were incubated for 4 hours at 37 °C. The absorbance at 540 nm was determined using a Varioskan (ThermoFisher Scientific K.K., Yokohama). Cytotoxicity was expressed as the ratio of the absorbance of the cisplatin-treated cells to that of the untreated cells.

Flow Cytometry Analysis

The apoptosis in Prx I(-/-) and Prx I(+/+) MEFs was assessed using an Annexin V–FLUOS Staining Kit (Roche Diagnostics K.K., Tokyo) followed by flow cytometry. Cells were seeded at 1 x 10^5 cells per 35-mm dish, grown overnight in 10% FBS/DMEM, and then treated for 24, 48, or 72 hours with 5 μg/ml cisplatin. The resuspended cells were stained with a fluorescein isothiocyanate (FITC)-conjugated

Annexin V antibody and propidium iodide (PI), according to the instructions provided by the manufacturer. The cells (2 x 10^4 per sample) were then analyzed using a flow cytometer (FACS Calibur; Becton Dickinson co., Tokyo) equipped with a 488-nm argon laser and Cell Quest Pro software.

Western blot analysis

Proteins were electrophoretically resolved in polyacrylamide gels and transblotted onto PVDF membranes. The polyclonal antibodies against Prx I were described previously [12]. The antibodies against p-p38, p38, p-Erk1/2, Erk1/2, p-JNK, and JNK were purchased from Cell Signaling Technology (Cell Signaling Technology Japan, K.K., Tokyo), and the anti-actin antibody was purchased from Sigma Aldrich (Sigma Aldrich Japan, K.K., Tokyo). The protein-antibody complexes were detected using an ECL plus kit (GE Healthcare Bio-Sciences K.K., Tokyo). The intensity of the protein bands was determined using the ImageJ Analysis software 1.41o (Wayne Rasband, National Institutes of Health, Bethesda).

Statistical analysis

All data are presented as the mean ± SD. Differences among data were determined using Student's *t*-test (StatView, version 5.0, Abacus Concepts, Berkeley).

Results

Loss of Peroxiredoxin I alters the cells' sensitivity to cisplatin.

First, we examined the effect of Prx I expression on the cells' sensitivity to cisplatin. For these studies, we used Prx I (-/-) and Prx I (+/+) MEFs obtained from 13.5-day-old embryos. Prx I (+/+) and Prx I (-/-) MEFs were exposed to various concentrations of cisplatin for 72 hours, and cell survival was determined by the MTT assay. Fig. 1 shows the cell viability of cisplatin-treated Prx I (+/+) and Prx I (-/-) MEFs. Prx I (-/-) MEFs were more sensitive to cisplatin at concentrations of 5 μg/ml ($P<0.001$), and 10 μg/ml

(P<0.001) after 72 hours.

Enhanced sensitivity of Prx I-deficient cells to cisplatin is owing to apoptosis

We performed FACS analyses to confirm whether the measured increase in the sensitivity of the Prx I-deficient MEFs to cisplatin was owing to cisplatin-induced apoptosis. The upper panels in Fig. 2A show untreated and cisplatin-treated Prx I (+/+) MEFs. The lower panels show the results for Prx I (-/-) MEFs subjected to the same treatments. There were more dots in the lower-right quadrant for the cisplatin-treated Prx I (-/-) MEFs than for the similarly treated Prx I (+/+) MEFs, suggesting that the Prx I (-/-) MEFs were predisposed to undergo apoptosis. Fig. 2B shows the quantification of the FACS analysis. The percentage of cells that appeared in the upper- and lower-right quadrants was used as the apoptosis ratio. Twenty-four hours after cisplatin exposure, 26.1% of the Prx I (-/-) cells were apoptotic, compared with only 17.5% of the Prx I (+/+) cells, a significant difference ($P < 0.01$). The proportion of apoptotic cells increased with time in culture for both groups of cisplatin-treated cells. After 48 hours of cisplatin exposure, 74.3% of the Prx I (-/-) cells were apoptotic, a significant increase compared with the 30.1% of Prx I (+/+) cells that were apoptotic ($P < 0.001$). After 72 hours of cisplatin exposure, 89.1% of the Prx I (-/-) cells were apoptotic, compared with only 43.5% of the Prx I (+/+) cells ($P < 0.001$). These results indicate that the Prx I (-/-) MEFs were more susceptible to cisplatin-induced apoptosis than the Prx I (+/+) MEFs.

Differential mitogen-activated protein kinase activation by cisplatin in Prx I-deficient cells

Previous reports indicate that the apoptotic signaling induced by cisplatin is conducted through MAPKs. There are three major pathways involving MAPKs: the c-Jun-N-terminal kinase (JNK), p38 MAP kinase, and extracellular signal-regulated kinase (ERK) pathways [13]. We therefore examined the effect of Prx I loss on the activation of MAPKs following exposure to cisplatin.

As shown in Fig. 3A, exposure to cisplatin induced greater p38 MAPK

phosphorylation in the Prx I (-/-) MEFs than the control Prx (+/+) MEFs, while the total p38 MAPK protein level remained similar between the two groups. Fig. 3B shows the ratio of the phosphorylated p38 MAPK to p38 MAPK. The basal level of p38 MAPK phosphorylation in the Prx I (-/-) MEFs was lower than in the Prx I (+/+) MEFs, and the p38 MAPK activation in the Prx I (-/-) MEFs was greater than in the Prx I (+/+) MEFs at the 1- and 2-hour time points. JNK activation was also elevated in the Prx I (-/-) MEFs, and the ratio of phosphorylated JNK to JNK was significantly increased at the 2-hour time point (Fig. 3C, D). On the other hand, exposure to cisplatin induced ERK1/2 phosphorylation in the control Prx I (+/+) MEFs but not in the Prx I (-/-) MEFs (Fig. 3E, F). Thus, the loss of Prx I in cells is associated with increased cisplatin-induced p38 MAPK and JNK signaling, and reduced ERK signaling.

Cisplatin-induced apoptosis is attenuated by MAPK inhibitors

We therefore examined which MAPK activation was critical for cisplatin-induced apoptosis by using MAPK-specific inhibitors. We used the p38 MAPK inhibitor SB203580, the JNK inhibitor SP600125, and the ERK inhibitor PD98059, and examined the cisplatin-induced apoptosis in Prx I (-/-) and Prx I (+/+) MEFs by FACS.

Fig. 4A shows typical FACScalibur results from cultures treated with inhibitors, and Fig. 4B shows the quantification of the FACS analysis. The percentage of cells appearing in the upper- and lower-right quadrants was used as the apoptosis ratio. After cisplatin exposure, 65.6% of the Prx I (-/-) cells were in the upper- and lower-right quadrants, i.e. in the early and late stages of apoptosis, whereas only 48.1% of the Prx I (+/+) MEFs were apoptotic under the same conditions. SB203580 reduced the cisplatin-induced apoptosis in Prx I (-/-) cells (††$P < 0.01$), but did not significantly affect it in Prx I (+/+) MEFs. After treatment with cisplatin and 10 μM SB203580, apoptosis was observed in 51.7% of the Prx I (-/-) cells and 39.2% of the Prx I (+/+) cells, a significant difference (**$P<0.01$).

In the case of the JNK inhibitor, SP600125, the percentage of apoptotic SP600125-treated Prx I (-/-) and Prx I (+/+) cells was significantly reduced compared with cisplatin treatment alone (#$P < 0.05$, †††$P < 0.001$, respectively), and there was no difference in the apoptosis ratio between the Prx I (-/-) and Prx I (+/+) MEFs. These results indicate that SP600125 blocked the specific apoptotic signal that Prx I modulates.

Similar results were obtained using the ERK inhibitor PD98059. That is, the percentage of apoptotic PD98059-treated Prx I (-/-) and Prx I (+/+) cells was reduced significantly compared with cisplatin treatment alone (##P < 0.01, †††P < 0.001, respectively), and PD98059 also eliminated the difference in cisplatin-induced apoptosis between the Prx I (-/-) and Prx I (+/+) MEFs. These results indicate that the increased cisplatin-induced apoptosis in the Prx I (-/-) MEFs mainly depended on JNK and ERK signaling, whereas p38 MAPK may be activated independent of the apoptotic signaling that is modulated by Prx I.

Discussion

Our results showed that Prx I (-/-) MEFs were more sensitive to cisplatin than Prx I (+/+) MEFs, and this difference was due to increased apoptosis. MAPKs are critical components of the intracellular signaling networks that regulate gene expression in response to cisplatin, and MAPK activation determines the fate of cells in response to this drug [13]. The three major pathways involving MAPKs are the JNK, p38 kinase, and ERK pathways. We observed that cisplatin treatment enhanced the phosphorylation of p38, MAPK, and JNK, and suppressed the phosphorylation of ERK1/2 in Prx I (-/-) MEFs (Fig. 3). Furthermore, the JNK inhibitor SP600125 and ERK inhibitor PD98059 eliminated the difference in the apoptotic ratio between the Prx I (-/-) and Prx I (+/+) MEFs, but the p38 MAPK inhibitor SB203580 did not. These findings suggest that the apoptotic signals mediated by JNK were enhanced by cisplatin, whereas those mediated by ERK were reduced by it, in the Prx I (-/-) cells. On the other hand, although p38 MAPK was activated in the Prx I (-/-) cells, it is unclear that its activation was directly associated with the cisplatin-induced apoptosis. Our findings suggest that Prx I modulates the apoptosis signal negatively via JNK and positively via ERK, but that p38 MAPK activation may not be modulated by Prx I in cisplatin-induced apoptotic signaling.

Prx I was first cloned as an oxidative stress-induced protein [12, 14], and one of its functions is to eliminate hydrogen peroxide and hydroxyl radicals [8, 9, 15-18]. In this experiment, oxidative cisplatin treatment also induced marked Prx I expression within 8 hr (Supplemantary Fig. 1). Several reports have described substances that bind to Prx I, and among them are two candidate factors for cisplatin sensitivity: c-Abl and ASK1

(Apoptosis signal-regulating kinase 1).

PAG, the human counterpart of Prx I, binds to the SH3 domain of c-Abl and inhibits c-Abl's kinase activity and cytostatic function; it is also a physiological inhibitor of c-Abl[19 20]. c-Abl is activated in response to oxidative stress, and oxidative stress-induced apoptosis is attenuated in c-Abl-deficient fibroblasts, supporting a pro-apoptotic role for c-Abl in the oxidative stress response [21-23]. Furthermore, c-Abl functions upstream of JNK in response to cisplatin [24]. Considering these findings, we hypothesize that the cisplatin-induced apoptotic signal that enhances the activation of c-Abl is mediated by oxidative stress, and that Prx I may modulate the apoptotic signal by acting as a physiological inhibitor of c-Abl.

ASK1 is a MAP kinase kinase kinase (MAPKKK) that is thought to be a major player in cisplatin sensitivity. Thioredoxin (TRX), which interacts with ASK1, is a sensor for oxidative stress and helps to protect tissues from oxidative stress-associated damage [25]. In vitro, cisplatin activates ASK1, and subsequently, its downstream subgroups SEK1 (or MKK4), and MKK3/MKK6, which in turn activate JNK and p38 MAPK, prior to the onset of apoptosis [26]. Recently, Kim et al. reported that Prx I interacts with the TRX-binding domain of ASK1 and helps to inhibit ASK1-induced apoptosis [27]. Thus, we can hypothesize that one of the major sites of Prx I action in the cisplatin-induced apoptosis pathways is its interaction with ASK1.

In our study, ERK activation was suppressed in Prx I (-/-) MEFs, but apoptosis was enhanced in them. ERK can upregulate either apoptosis or cell survival by multiple mechanisms, including by increasing p53 and BAX activities, increasing caspase-3 and caspase-8 activities, decreasing Akt activity, and increasing TNF α production [28]. Therefore, if Prx I modulates a specific pathway associated with cisplatin-induced apoptotic signaling, the suppression of ERK phosphorylation in Prx I (-/-) cells could result in reduced apoptosis. Further studies are necessary to elucidate the upstream and downstream components of the cisplatin-induced apoptotic cascade and to define the cross-talk point of Prx I's action on the signaling pathways.

In conclusion, our study revealed that Prx I plays an anti-apoptotic role by reducing cisplatin-induced damage in MEFs. The mechanism underlying this phenomenon mainly involves modulating the apoptosis signal relayed through MAPKs. It is not yet clear what the main target of Prx I is in the apoptotic pathway; nevertheless, our results identify Prx I as an important factor in the protection of cells from cisplatin

damage. This finding highlights the potential usefulness of Prx I as either a protective factor that could ameliorate cisplatin-induced side effects, such as renal failure, or as an exacerbating factor in the resistance of malignant tumors associated with oral cancers to cisplatin.

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Legends to Figures

Figure 1. Prx I-deficient (Prx I (-/-)) MEFs are more sensitive than wild-type (Prx I (+/+)) MEFs to cisplatin treatment. Prx I (-/-) and Prx I (+/+) MEFs were seeded into 96-well plates and treated with different doses of cisplatin for 72 hours. Cell viability was assessed using the MTT colorimetric assay, as described in Materials and Methods. Prx I (-/-) and Prx I (+/+) MEFs were maintained in medium containing 10% FBS for 24 hours, followed by incubation with cisplatin (0, 2.5, 5.0, 10.0, and 25.0 μg/ml). Values are the means ± SD from five measurements. $P < 0.001$ (***) compared with Prx I (+/+) cells.

Figure 2. Enhanced apoptosis of Prx I (-/-) MEFs following treatment with cisplatin. A. Prx I (+/+) and Prx I (-/-) MEFs were cultured in medium containing 10% FBS for 24 hours, followed by incubation with 5.0 μg/ml cisplatin for 24, 48, or 72 hours. Cells were doubly stained for annexin V and PI, and were analyzed by FACS. The cells in the lower-right quadrant (annexin V-positive/PI-negative) were in early apoptosis, those cells in the lower-left quadrant (annexin V-negative /PI-negative) were alive, those in the upper-right quadrant (annexin V-positive/PI-positive) were in late apoptosis, and cells in the upper-left quadrant (annexin V-negative/PI-positive) were damaged. B. Prx I (-/-) MEF cells treated with cisplatin showed increased frequencies of apoptotic cells compared with the Prx I (+/+) MEF cells. The percentage of cells in the upper- and lower-right quadrants was used as the apoptosis ratio. Values are the means ± SD from five independent experiments. $P < 0.01$ (**), $P < 0.001$ (***) compared with Prx I (+/+) cells.

Figure 3. Cisplatin-induced activation of MAPKs in Prx I (+/+) and Prx I (-/-) MEFs. MEFs were treated with 50 μg/ml cisplatin for the indicated times. The cell proteins were separated by 10% SDS-PAGE, and analyzed by immunoblotting with anti-MAPK (A, p38 MAPK; C, JNK; and E, ERK1/2) antibodies. The fold increase in phosphorylated MAPK was determined from the band density of the phospho-MAPK/total MAPK. p-p38, phosphorylated p38; p38, total p38; actin, the loading control; Prx I, Prx I expression. B, Quantification of the p38 phosphorylation in

Prx I (+/+) and Prx I (-/-) MEFs. C and D, The phosphorylation and quantification of JNK. Panels E and F, the phosphorylation and quantification of ERK1/2. Values are expressed as the means ± SD of five independent experiments.

Figure 4. Effect of MAPK inhibitors on cisplatin-induced apoptosis. Prx I (-/-) and Prx I (+/+) MEFs were induced to undergo apoptosis by cisplatin (Cis; 50 μg/ml) in the absence or presence of MAPK-specific inhibitors. The inhibitors were added to the cells in DMEM medium 30 min before the addition of cisplatin. After 24 hours, the cells were stained with FITC-conjugated annexin V and PI. The percentage of annexin-V/PI-stained cells was determined by flow cytometry. A, Representative results of cisplatin-induced apoptosis in the presence of SB203580 (SB: p38 inhibitor), SP600125 (SP: JNK inhibitor), and PD98059 (PD: ERK inhibitor). B, Proportion of apoptotic cells in each treatment condition and in untreated control cells. The percentage of cells in the upper- and lower-right quadrants was used as the apoptosis ratio. Values are expressed as the means ± SD of five independent experiments. $P < 0.01$ (**), $P < 0.001$ (***) compared with Prx I (+/+) MEFs. $P < 0.05$ (†), $P < 0.01$ (††), $P < 0.001$ (†††) compared with cisplatin-treated Prx I (+/+) MEFs. $P < 0.05$ (#), $P < 0.01$ (##) compared with cisplatin-treated Prx I (-/-) MEFs.

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Legend to supplementary figures.

Supplementary figure 1. Cisplatin induces Prx I expression. Prx I (+/+) MEFs were cultured in medium containing 10% FBS for 24 hours, followed by incubation with 50 μg/ml cisplatin for 0, 8, or 16 hours. Cells were analyzed by immunoblotting with an anti-Prx I polyclonal antibody. Prx I expression was induced by cisplatin and peaked at 8 hr of treatment.

Supplementary figure 2. Effects of MAPK-specific inhibitors on Prx I (-/-) and Prx I (+/+) MEFs. Apoptosis was induced in Prx I (-/-) and Prx I (+/+) MEFs by cisplatin treatment (50 μg/ml) in the absence or presence of MAPK-specific inhibitors. The inhibitors were added to the cells in DMEM medium 30 min before the addition of cisplatin. After 3 hours, the cells were collected and analyzed by immunoblotting. The figure shows representative results of cisplatin-induced phosphorylation in the presence or absence of PD98059 (ERK inhibitor), SP600125 (JNK inhibitor), or SB203580 (p38 inhibitor). PD98059 and SP600125 inhibited the phosphorylation of ERK1/2 and JNK, respectively. SB203580 did not inhibit the phosphorylation of p38, because SB203580 has no significant effect on the activities of MAPKs, including p38 MAPK. [1]

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Figure

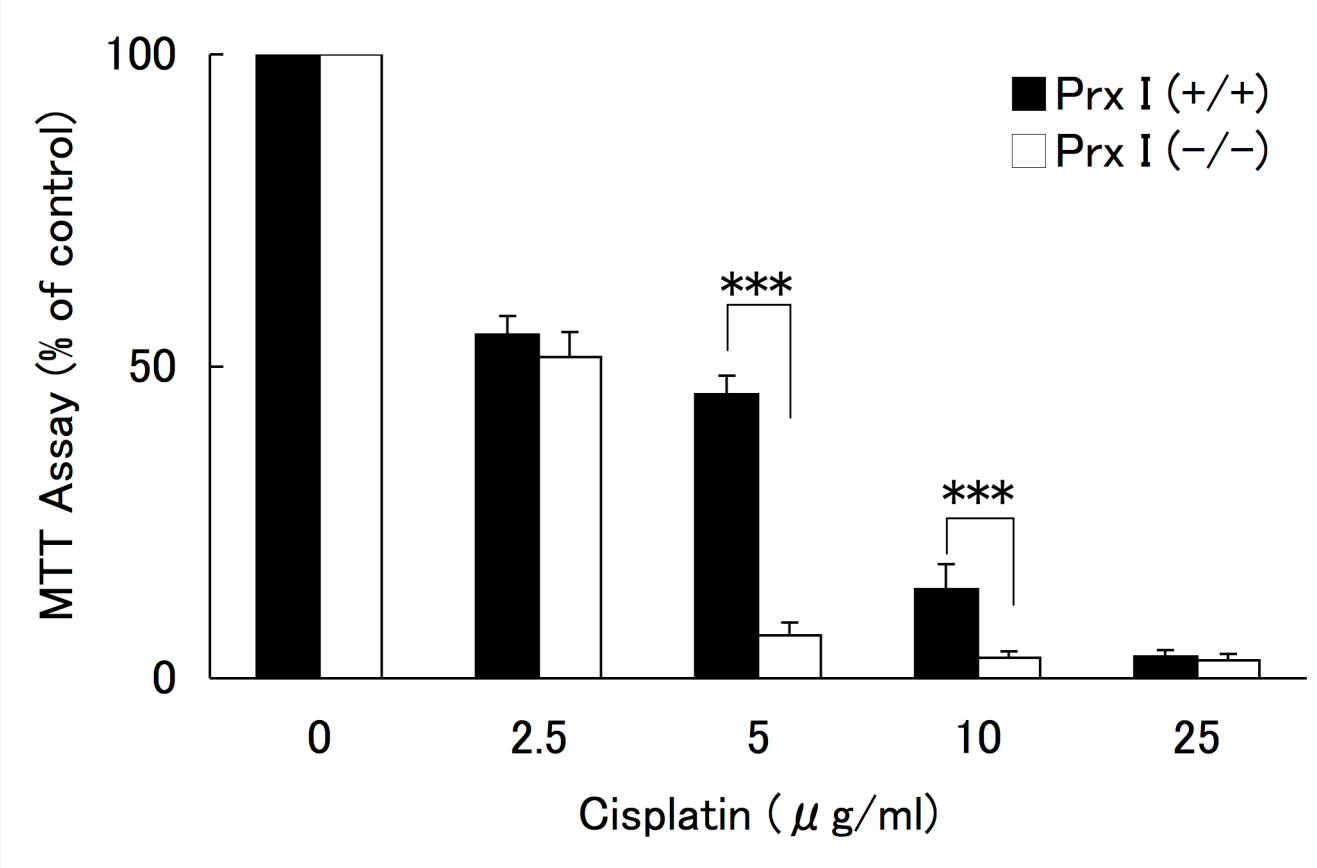


Figure 1 (Ma *et al.*)

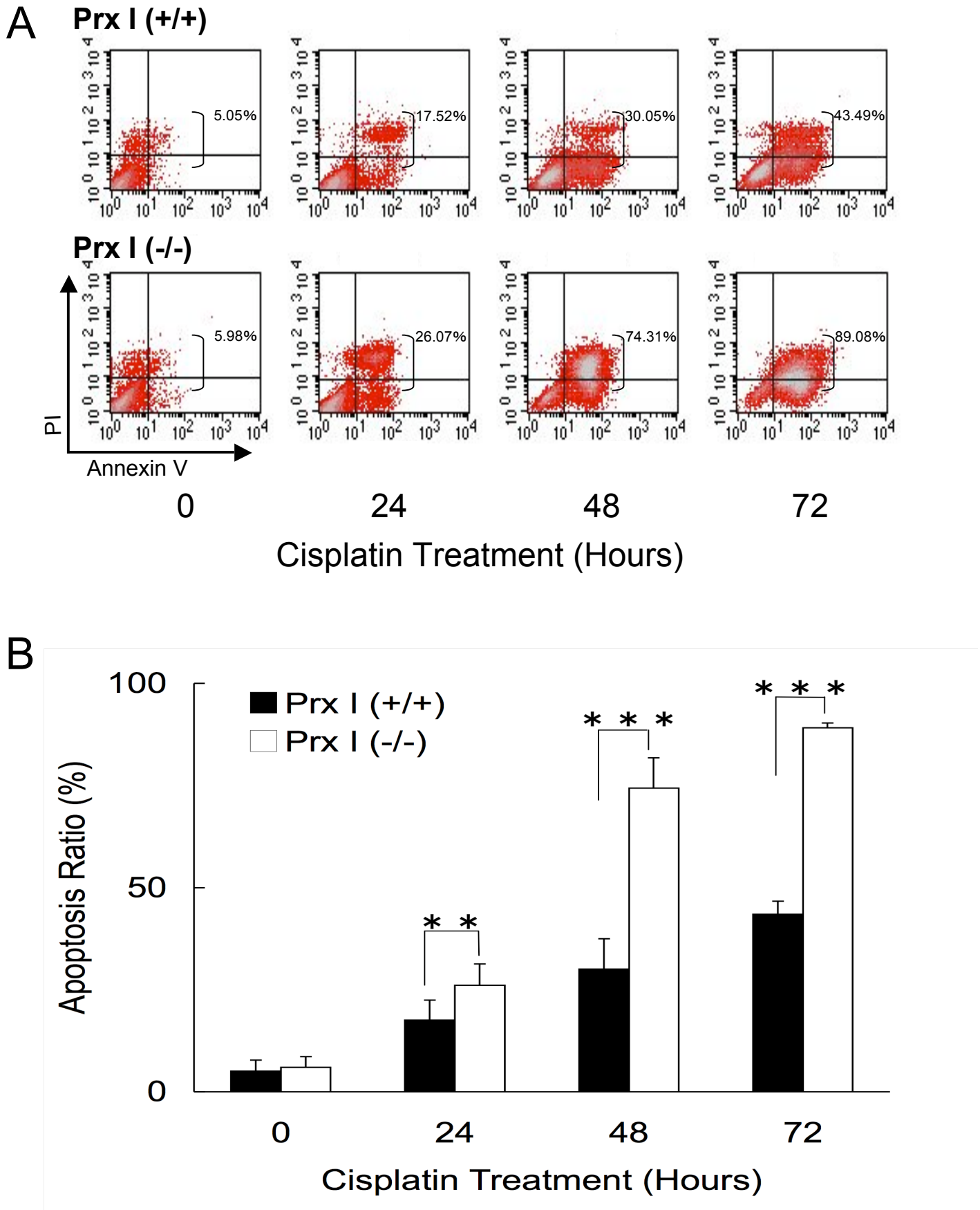
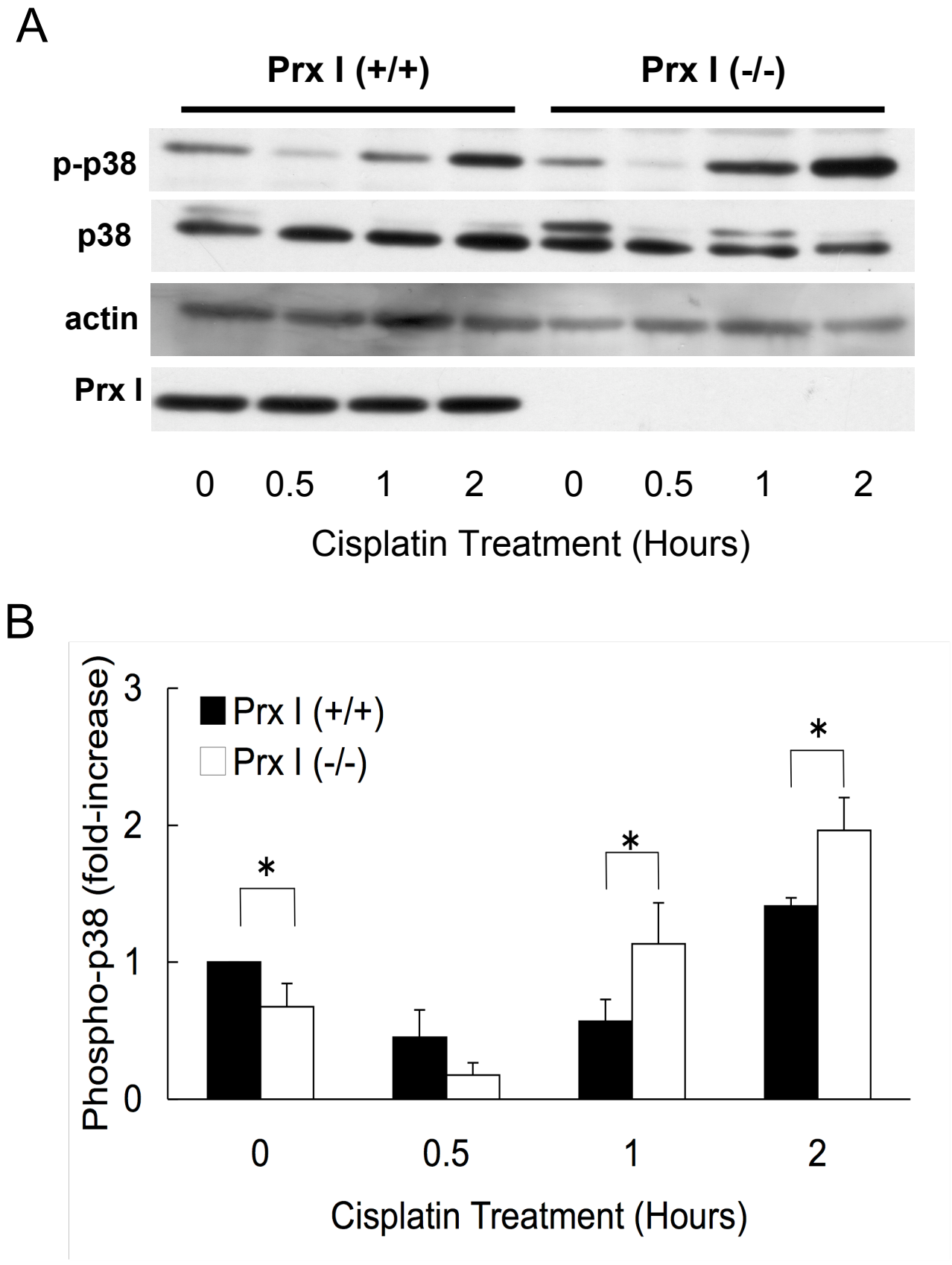


Figure 2 (Ma *et al.*)



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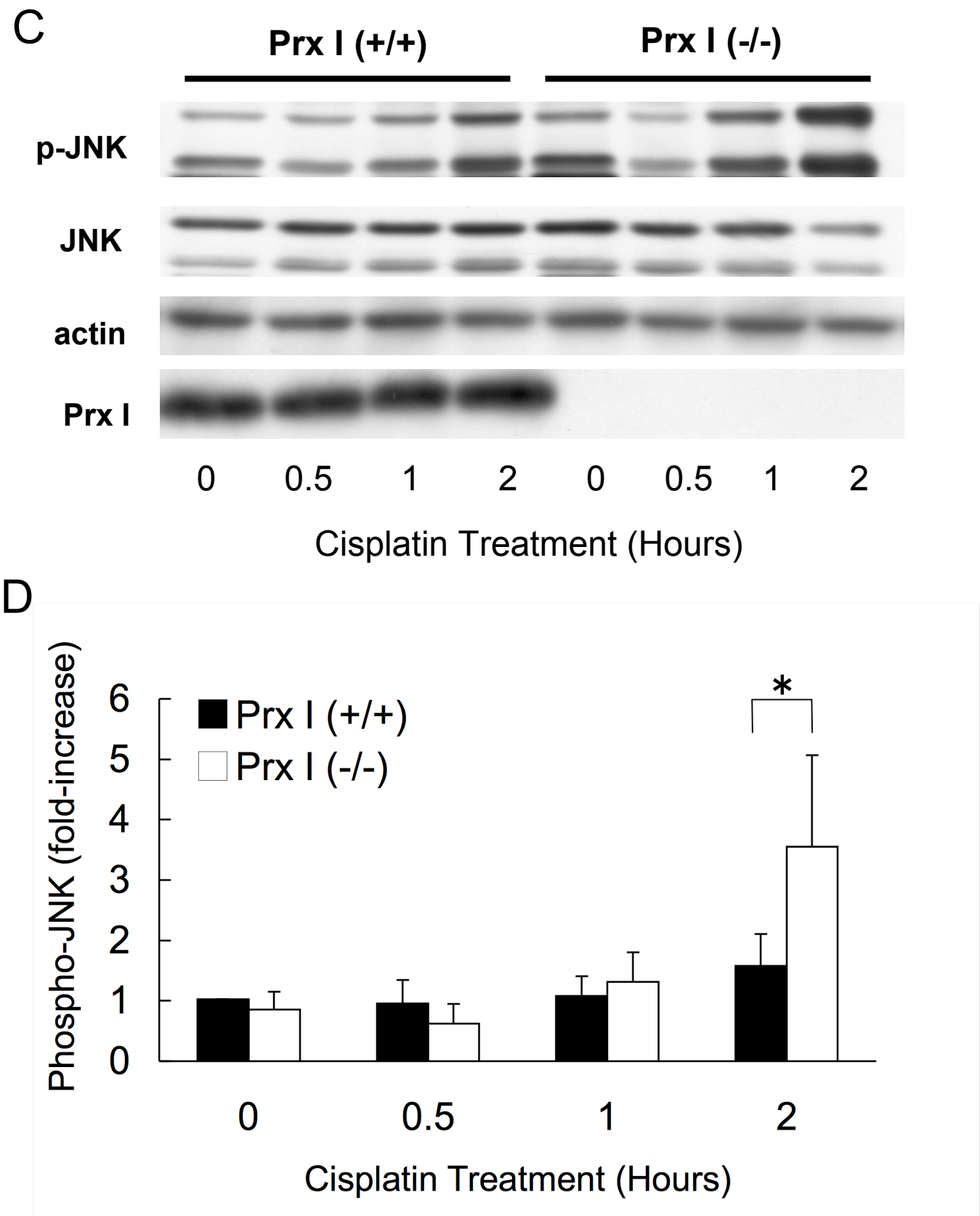

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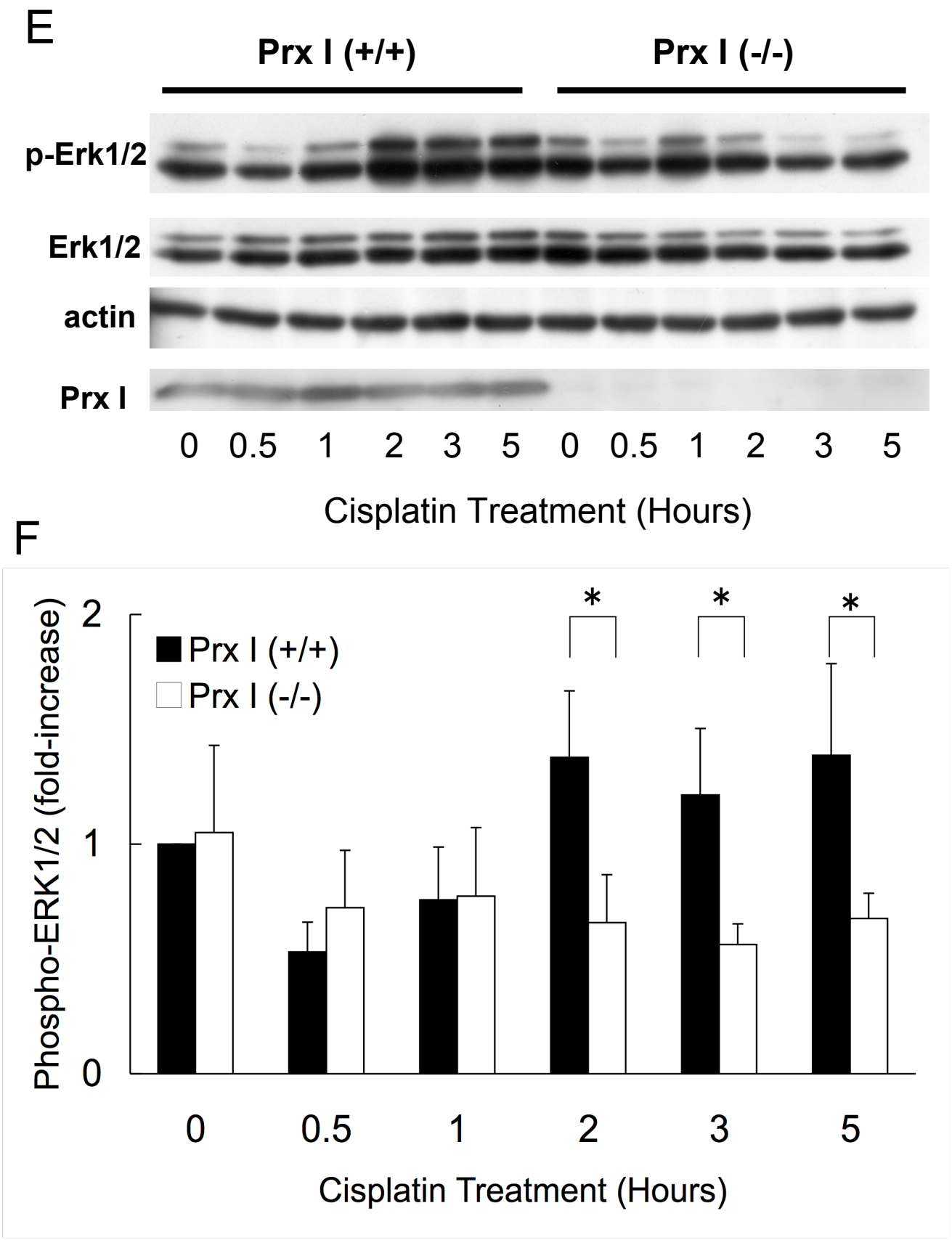
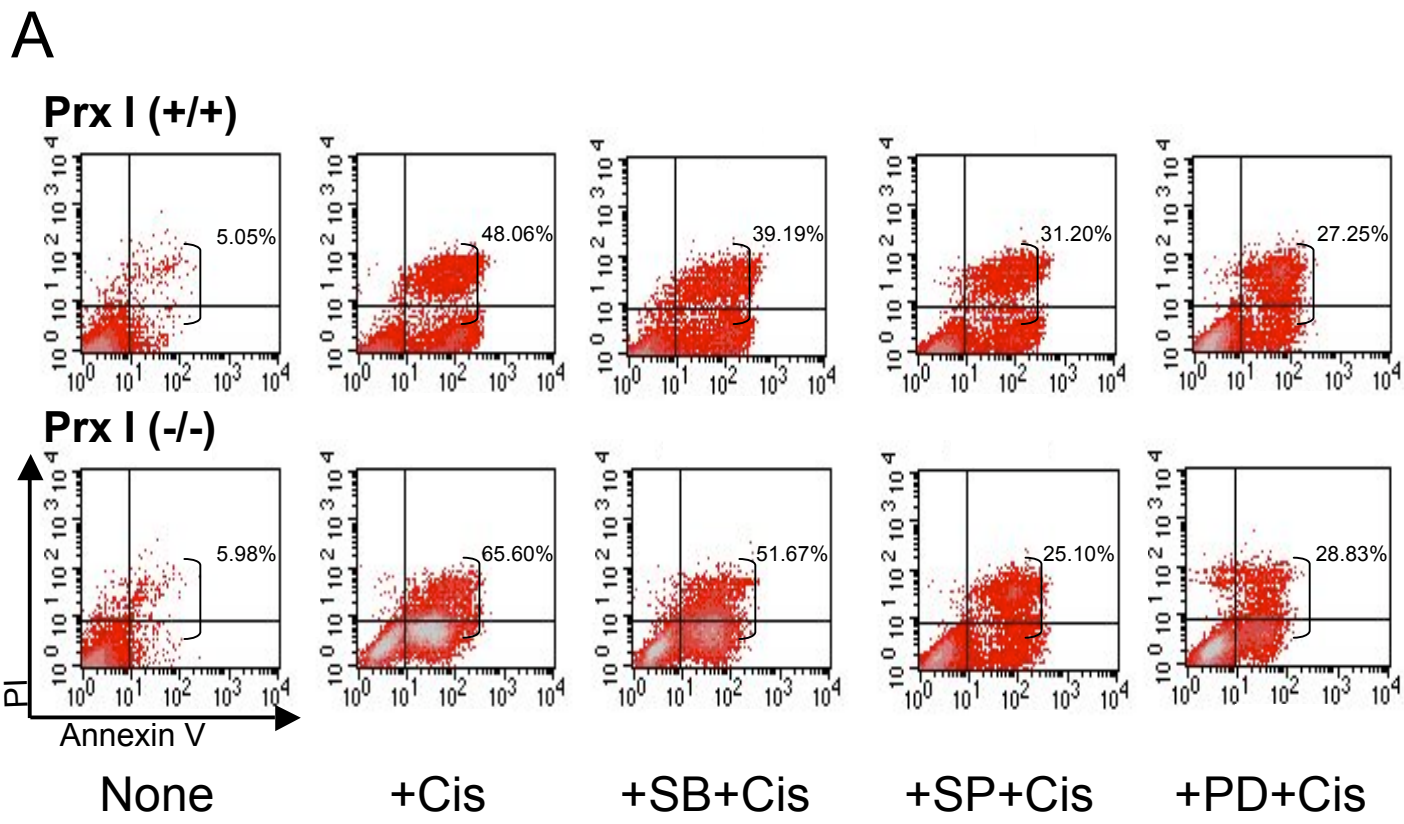


Figure 3 (Ma *et al.*)



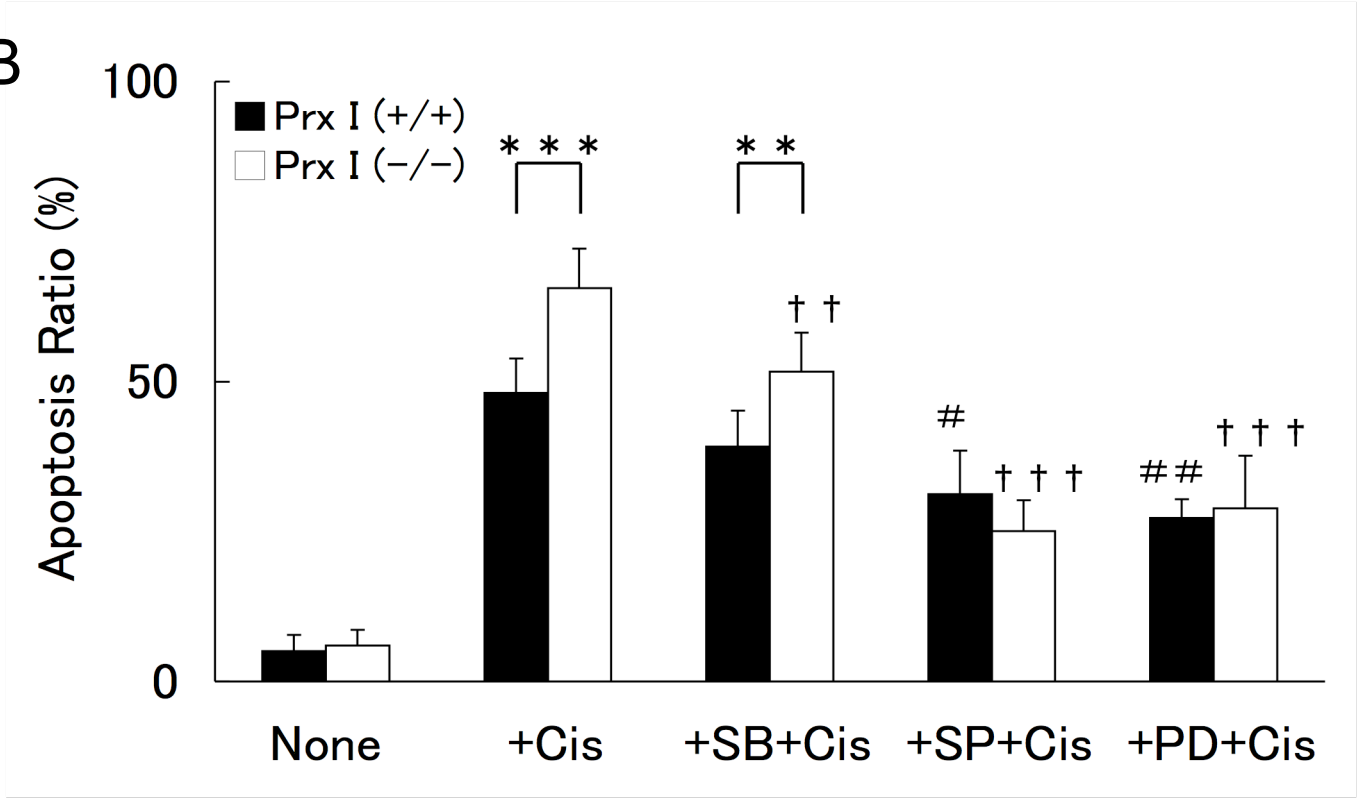


Figure 4 (Ma *et al.*)

Supplementary Data (online only)

Click here to download Supplementary Data (online only): SupplementaryFig.pdf

*** Conflict of Interest Statement**

Conflict of Interest statement: None declared.