TITLE: CYCLOHEXIMIDE PROTECTS HEPG2 CELLS FROM SERUM WITHDRAWAL INDUCED APOPTOSIS BY DECREASING P53 AND PHOSPHORYLATED P53 LEVELS

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Running title: CHX Prevents Serum Withdrawal Induced Apoptosis

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Number or pages: 32

Number of Tables: 0

Number of Figures: 7

Number of References: 34

Number of Words-Abstract: 250

-Introduction: 571

-Discussion: 1168

Section: Toxicology

Abbreviations: CHX, cycloheximide; ActD, Actinomycin D; ROS, reactive oxygen species; GSH, Reduced glutathione; PBS, phosphate-buffered saline; PP53, Phosphorylated P53; SW, serum withdrawal; PI, Propidium iodide; DCFDA, dichlorodihydrofluorecin diacetate; siRNA, small interfering RNA; MG115, N-CBZ-LEU-LEU-norvalinal.

ABSTRACT

Cycloheximide (CHX), an inhibitor of protein synthesis, has been reported to prevent cell death in a wide variety of cell types and produced by different apoptotic stimuli. However the mechanisms by which CHX protects cells from apoptosis are still unclear. In this study, we investigated whether P53 plays a role in the protection by CHX against serum withdrawal induced apoptosis. Deprivation of serum from the culture medium causes apoptosis in HepG2 cells and CHX dramatically protects cells from death. P53, P21 and Bax protein levels were elevated and cell cycle arrest was produced after serum withdrawal. CHX abolished this elevation of P53, P21 and Bax as well as the cell cycle arrest induced by serum deprivation. The P53 inhibitor pifithrin-α protects HepG2 cells against apoptosis induced by serum withdrawal. HepG2 cells expressing a dominant negative form of mutant P53 and Hep3B cells lacking P53 were resistant to serum withdrawal induced apoptosis. Lowering of P53 by siRNA protects HepG2 cells from serum withdrawal induced apoptosis. P53 phosphorylation was induced by serum withdrawal and other chemotherapeutic reagents such as actinomycin D, doxorubicin and etoposide. CHX decreases the levels of phosphorylated P53 (PP53) even in the presence of a proteasome inhibitor which maintains the total P53 levels, while it does not affect the dephosphorylation of PP53. These results suggest the possibility that kinases which phosphorylate P53 might be affected by CHX administration. In summary, CHX protects HepG2 cells from serum withdrawal induced apoptosis through inhibiting the synthesis of P53 and the phosphorylation of P53.

JPET Fast Forward. Published on September 13, 2006 as DOI: 10.1124/jpet.106.110007
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INTRODUCTION

Cycloheximide (CHX), a protein synthesis inhibitor, has been widely employed in studies of apoptosis. However, its effects on apoptosis vary from inhibition to enhancement depending on the apoptotic stimulators, e.g. CHX sensitizes cells to TNF-α or Fas induced apoptosis (Fulda et al., 2000), but it prevents rat hepatocytes from apoptosis induced by Transformation Growth Factor β (Sâanchez et al., 1997) and protects rat thymocytes and mouse lymphoid cell lines against apoptosis induced by glucocorticoids and a calcium ionophore (Wyllie et al., 1984). The mechanisms by which CHX regulates apoptosis are not clear and still under investigation. The hypotheses whereby CHX sensitizes cells to CD95-induced apoptosis include down-regulating FLIP and RIP expression (Fulda et al., 2000) or inhibiting the synthesis of anti-apoptotic proteins such as Bcl-2 or Bcl-xL. The standard view of the mechanism whereby CHX prevents cell death is that it inhibits the production of "killer proteins" by blocking protein synthesis. However, until now such 'killer proteins' have not been identified. Other mechanisms such as preventing cell death by activation of anti-apoptotic signaling pathways have also been considered (Mattson and Furukawa, 1997).

The activation of wild type P53, a major tumor suppressor protein, which is implicated in cell cycle control, DNA repair, and apoptosis (Levine, 1997) (Liebermann et al., 1995; Steller, 1995), has been reported to be involved in the process of apoptosis (Fritsche et al., 1993; Steller, 1995). An increase in P53 levels results in large part from stabilization of the P53 protein against degradation, and accumulation of P53 occurs because of this increased protein stability in the presence of ongoing translation (Fritsche et al., 1993). As a transcription factor, P53 protein enhances the transcription rate of several genes including $P21^{WAF1}$, $GADD^{45}$, and 14-3-3σ, which are genes involved in cell cycle arrest ((Kastan et al., 1992; el-Deiry et al., 1995; Hermeking et al., 1997), and Bax, CD95, Noxa, PUMA, and P53AIP1, which are related to p53-dependent apoptosis (Miyashita and Reed, 1995; Owen-Schaub et al., 1995; Polyak et al., 1997; Oda et al., 2000a; Oda et al., 2000b; Yu et al., 2001).

Serum, a mixture of hundreds of proteins, contains various factors needed for proliferation of cells in culture. The major functions of serum in culture media are to provide: (1) hormonal factors that stimulate cell growth and proliferation, and promote differentiated functions, (2) transport proteins that carry hormones (e.g.,

transcortin), minerals and trace elements (e.g., transferrin), and lipids (e.g., lipoproteins), (3) attachment and spreading factors (i.e., components of the extracellular matrix), and (4) stabilizing and detoxifying factors, needed to maintain pH or to inhibit proteases either directly, such as α-antitrypsin or α_2-macroglobulin, or indirectly, by acting as a nonspecific sink for proteases and other (toxic) molecules(van der Valk et al., 2004). Serum withdrawal can induce apoptosis in a variety of cells including HepG2 cells and it has been used as a model to investigate the regulation of apoptosis (Kang et al., 2003; Schamberger et al., 2005).

In the current study, we characterized serum withdrawal induced apoptosis and cell cycle arrest in HepG2 cells. P53, P21 and Bax protein levels were elevated after serum withdrawal. CHX efficiently blocked this cell death caused by serum withdrawal and it abolished the elevation of P53, P21 and Bax as well as the cell cycle arrest induced by serum deprivation. P53 as a candidate for being one of the “killer proteins” was investigated and the role of CHX in regulating P53 activation was evaluated.

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MATERIALS AND METHODS

Reagents- Propidium Iodide was purchased from Molecular Probes (Eugene, OR). Polyclonal antibodies raised in rabbit against P53 or P21were obtained from Santa Cruz (Santa Cruz, CA). Polyclonal antibody raised in rabbit against phosphorylated P53 on serine 15 was obtained from Calbiochem. Horseradish peroxidase conjugated to goat anti-rabbit IgG, MEM, and fetal bovine serum were purchased from Sigma (St. Louis, MO).

Cell Culture and Transfection- HepG2 and Hep3B cells were cultured in MEM containing 10% fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine in a humidified atmosphere in 5% CO_2 at 37 °C. Serum withdrawal medium refers to the above medium lacking the 10% fetal calf serum. For transfection, HepG2 cells were seeded onto 10-cm culture dishes and grown to 80% confluence. The expression plasmid vector pCMV-p53mt135 (Clontech), containing mutant p53 or empty vector pCMV-neo, were transfected into HepG2 cells using Effectene transfection reagent (Qiagen) according to the instructions provided by the manufacturer. Cells were selected using MEM containing 1 mg/ml G418. Two weeks after transfection, the surviving clones were isolated and grown to large scale. Stable cell lines with overexpression of mutant P53 (HepG2-mtp53) as well as cells transfected with empty vector (HepG2-neo) were selected and maintained in MEM containing 1 mg/ml G418. siRNA-p53 and siRNA-control (a nontargeting siRNA) (Santa Cruz Biotechnology) were transfected into HepG2 cells using Lipofectamine™ RNAiMAX reagent (Invitrogen) according to the manufacturer's protocol.

Trypan Blue Exclusion Assay- Cells were trypsinized and collected by centrifugation. An aliquot of collected cells was combined with Trypan Blue dye (Sigma) to a concentration of 0.04% (W/V) and analyzed microscopically on a hemocytometer. Blue cells were counted as non-viable and cells excluding dye were counted as viable. Cell viability was presented as percentage of viable cells. The cell viability data obtained from this assay is not affected by the change of cell proliferation due to serum withdrawal or the treatment of CHX, since equivalent total cells are being counted.

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Western Blotting- Cell lysates were prepared by sonicating cells followed by centrifugation. The protein concentration of the supernatant was measured (DC protein assay reagent, Bio-Rad), and 30 µg of denatured protein was resolved on 10% SDS-PAGE and electroblotted onto nitrocellulose membranes (Bio-Rad). The membrane was incubated with primary antibody followed by incubation with horseradish peroxidase conjugated secondary antibody. Detection by the chemiluminescence reaction was carried out for 1 min using the ECL kit (Amersham Biosciences) followed by exposure to Kodak X-Omat x-ray film (Eastman Kodak Co.).

Intracellular Measurement of Reactive Oxygen Species (ROS)- Fluorescence spectrophotometry was used to measure production of intracellular ROS with 2',7'-DCF-DA as the probe as previously described (Bai et al., 1999). Briefly, HepG2 cells were incubated in serum-free medium with or without 10 µM CHX for 0, 4, 8, 24, 48 and 72h followed by incubation with 5 µM DCF-DA in MEM for 30 min at 37 °C in the dark. The cells were washed in PBS, trypsinized, and resuspended in 3 ml of PBS, and the intensity of fluorescence was immediately read in a fluorescence spectrophotometer (PerkinElmer Life Sciences 650-10S, Hitachi, Ltd.) at 503 nm for excitation and at 529 nm for emission.

DNA Fragmentation Assay- The DNA fragmentation pattern (DNA ladder) was carried out by agarose gel electrophoresis. Cells (5×10^5) treated with various reagents were scraped and centrifuged at 1200 rpm for 10 min. The cell pellet was resuspended in 0.5 ml of lysis buffer consisting of 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 10 mM EDTA, 100 µg/ml proteinase K, and 0.5% SDS and incubated for 2 h at 50 °C. DNA was extracted with 0.5 ml of phenol, pH 8.0, followed by extraction with 0.5 ml of phenol/chloroform (1:1) and chloroform. The aqueous phase was precipitated with 2.0 volumes of ice-cold ethanol and 0.1 volume of 3 M sodium acetate, pH 5.2, at 20 °C overnight. The precipitates were collected by centrifugation at 13,000 × *g* for 10 min. The pellets were air-dried and resuspended in 50 µl of Tris/EDTA (10 mM Tris, 1 mM EDTA, pH 8.0) buffer supplemented with 100 µg/ml RNase A. DNA was loaded onto a 1.5% agarose gel containing ethidium bromide, electrophoresed in Tris acetate/EDTA buffer for 2 h at 50 V, and photographed under UV illumination.

Flow Cytometry for Cell Cycle Analysis- Cell cycle was analyzed by flow cytometry. 5×10^5 cells were seeded onto six-well plates and incubated with medium containing 10% serum or medium without serum in the

presence or absence of 10 μM CHX for 16 h. Cells were harvested by trypsinization, washed with PBS, followed by centrifugation at 2000 rpm for 10 min. The pellet of cells was resuspended in 80% ethanol and stored at 4 °C for overnight. Cells were washed twice with PBS. The pellet was resuspended in PBS containing 100 μg/ml RNase A, incubated at 37 °C for 30 min, stained with PI (50 μg/ml), and analyzed by flow cytometry for DNA analysis.

Statistics- Results are expressed as mean ± SD. One-way analysis of variance with subsequent post hoc comparisons by Scheffe was used for the DCFDA fluorescence data analysis of Fig.2A. A $p < 0.05$ was considered as statistically significant. Student's *t* test was used for analysis for all other data with $P < 0.05$ considered as the level of significance.

RESULTS

CHX protects HepG2 cells from apoptosis induced by serum deprivation- HepG2 cells were cultured in MEM with 10% FBS or without the 10% FBS (serum deprivation medium) and treated with or without CHX. Serum withdrawal caused severe cell death at 48 and 72 h. Morphology change of cells after 72 h serum withdrawal was visualized under the light microscope as shown in Fig. 1A. Cells cultured in serum withdrawal medium (-serum, -CHX) displayed shrinkage and nuclei condensation; however, cells cultured in serum withdrawal medium plus 10 μM CHX (-serum, +CHX) showed normal morphology. CHX at different concentrations (0.5, 1, 5, 10 and 40μM) protected HepG2 cells from cell death induced by serum deprivation (cell viability from 46% to 89% compared to < 20% viability in the absence of CHX). At a concentration of 10 μM, CHX provided maximal protection and did not cause significant toxicity to HepG2 cells (Fig. 1B a); therefore 10 μM CHX was used in the following experiments. Cell viabilities at different time points (0, 24, 48 and 72h) of treatment with or without 10 μM CHX in serum free medium were evaluated by trypan blue exclusion as shown in Fig. 1B b. Cell viability decreased in cells cultured in serum withdrawal medium for 48 h and 72 h (about 40% and 20% 0h control values, respectively). CHX effectively blocked serum withdrawal induced cell death (90% survival at 48h and 72 h) (Fig. 1B b). However, if CHX was administrated at 4, 8 or 24 h after serum deprivation, the cell viability at 72 h after serum withdrawal (< 20% in the absence of CHX) decreased to 80%, 60% or 40% respectively and no protective effect was observed if adding CHX at 36 h after serum deprivation. To evaluate whether serum deprivation induces apoptosis, the DNA was extracted from cells cultured in medium with 10% FBS or serum deprivation medium and treated with or without 10 μM CHX for 48 and 56 hours. Formation of a DNA ladder was observed in cells cultured in serum deprivation medium for 48 h and 56h, whereas no DNA ladder was detected in cells cultured in serum withdrawal medium plus CHX for 56 h. No DNA ladder was found in cells cultured in 10% FBS medium plus CHX for 56 h (Fig. 1C).

CHX does not decrease intracellular ROS production caused by serum deprivation- Serum withdrawal can induce reactive oxidative stress which is believed to play an important role in cell death (Satoh et al., 1996; King et al., 2003). Fluorescence spectrophotometry was used to measure intracellular ROS production with DCF-DA as the probe. Intracellular levels of ROS in cells increased about 4-fold after 24 h of serum

deprivation compared with cells incubated in medium containing 10% FBS (control). However, production of ROS in HepG2 cells cultured in serum-free medium plus CHX was even greater than that in cells cultured in serum-free medium without CHX at 8 and 24 h (Fig.2A). At 48 and 72 h of serum withdrawal, the DCF fluorescence was still maintained at a high level compared to control 0h values but there were no significant differences between cells treated with and without CHX (Fig. 2A). Trolox, an antioxidant chemical reagent, could protect against serum deprivation induced cell death validating that ROS plays an important role in the toxicity produced by serum withdrawal. Morphology changes of HepG2 cells after 48 h serum withdrawal with or without 50 μM trolox treatment was visualized under the light microscope as shown in Fig. 2B.

CHX could block P53 activation and cell cycle arrest induced by serum deprivation- Serum withdrawal induces P53 activation. Total P53, P21, Bax and especially phosphorylated (pSer-15) P53 levels were elevated at 8, 16 and 24 hours after serum withdrawal compared with control cells (Fig. 3A). In cells treated with serum withdrawal plus 10 μM CHX, total P53 levels were strongly decreased, phosphorylated P53 was not detected, and levels of P21 and Bax declined to control levels (Fig.3 A). Cell cycle analysis showed that serum withdrawal caused G_1-S cell cycle arrest. The cell population in the G_0-G_1 phase increased in cells cultured with serum deprivation medium for 16 h (74%) compared with cells cultured in 10% serum medium (58%). CHX abolished this increase induced by serum withdrawal (57% cells in G_0-G_1) (Fig.3B, a and b). Similar results of the cell population in the G_0-G_1 phase were observed at 24 h after serum deprivation as the percent cells in the G_0-G_1 phase were 76% in the absence of CHX and 56% in the presence of CHX (data not shown).

HepG2 cells treated with a P53 inhibitor or HepG2 cells expressing mutant P53 or Hep3B cells lacking P53 are more resistant to apoptosis induced by serum deprivation- Pifithrin-α, an effective P53 inhibitor has been widely used in studies with p53. It inhibits p53-mediated apoptosis and p53-dependent gene transcription such as p21, bax and mdm2 expression (Komarov et al., 1999; Liu et al., 2004; Murphy et al., 2004). In order to confirm the inhibitory effect of pifithrin-α on p53-dependent gene transcription, P21 and Bax and total P53 protein levels in HepG2 cells treated with 0, 30 or 60μM pifithrin-α for 24h were evaluated. The P21 and Bax protein level in HepG2 cells treated with 30 μM pifithrin-α was 50% lower

JPET Fast Forward. Published on September 13, 2006 as DOI: 10.1124/jpet.106.110007
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compared with cells treated without pifithrin-α, and was further lowered in cells treated with 60 μM pifithrin-α, although the total P53 levels was not significantly different in the absence or presence of pifithrin-α (Fig.4 Aa). Cells treated with 30 and 60 μM pifithrin-α were more resistant to serum withdrawal induced apoptosis. Cell viabilities were 60% and 80% respectively in cells cultured in serum-free medium plus 30 and 60 μM pifithrin-α for 48 h compared with 36% viable cells after treatment with serum-free medium plus DMSO (control) (Fig 4Ba). The morphological changes as shown in Fig. 4Ca confirm the protection by pifithrin-α against the serum withdrawal-induced apoptosis. To further evaluate the role of P53 in the toxicity induced by serum withdrawal, a HepG2 cell line overexpressing a dominant negative mutant P53 (hepG2-mtP53) was established by transfecting HepG2 cells with pCMV-p53mt135 plasmid. pCMV-p53mt135 expresses the p53mt135 mutant, which because of a conformational change, blocks normal P53 function. When p53mt135 and p53 are co-expressed, they form a mixed tetramer that is unable to interact with p53-binding sites; therefore, the downstream effects of p53 are blocked (Vogelstein and Kinzler, 1992). HepG2-mtP53 cells showed higher levels of P53 protein due to the expression of mutant P53 compared with control cells transfected with empty vector plasmid (HepG2-neo), however their P21 and Bax levels were lower than HepG2-neo cells because of the decrease in functional P53 as a result of the interaction of mutant P53 with normal P53 (Fig. 4Ab-lanes 1 and 2). Hep3B cells, another hepatoma cell line which lack normal P53 expression showed a very low P21 protein level. The Bax level in Hep3B cells while not as dramatically low as the level of P21, is about 30% lower than the Bax level in HepG2-neo cells (Fig.4Ab-lane 3). HepG2-mtP53 cells or Hep3B cells were resistant to serum withdrawal induced apoptosis compared with HepG2-neo cells. Cell viabilities were around 80% in HepG2-mtp53 cells and Hep3B cells compared with 38% in HepG2-neo control cells (Fig. 4Bb), and morphological changes as shown in Fig. 4Cb confirm the lack of toxicity in the HepG2-mtp53 and Hep3B cells subjected to serum withdrawal.

siRNA lowering of p53 protects HepG2 cells from serum withdrawal induced cell death- To further evaluate the role of P53 in the toxicity induced by serum deprivation, siRNA-p53 and siRNA-control (a nontargeting siRNA) were transfected into HepG2 cells. Twenty-four hours after transfection, cells

were cultured in medium with or without serum for an additional 24 h. P53 and PP53 as well as Bax levels were evaluated by Western blot analysis as shown in Fig.5A. The total P53 and Bax levels in HepG2 cells transfected with p53 siRNA dramatically decreased compared with cells transfected with control siRNA. Serum deprivation increased PP53 and Bax levels in HepG2 cells transfected with control siRNA but not in cells transfected with p53 siRNA (Fig. 5A). Cell viability evaluated after serum deprivation for 48 h was higher in cells transfected with siRNA-p53 compared with cells transfected with siRNA-control (62% vs 39%, $p<0.05$) (Fig. 5B).

CHX decreases P53 accumulation and P53 phosphorylation induced by ActD, doxorubicin and etoposide- In order to further study how CHX inhibits P53 activation and levels of P53, HepG2 cells were treated with therapeutic reagents 2.5μM Act D, 2.5μM doxorubicin and 25μM etoposide with or without 10μM CHX for 16 hours. Total P53 and PP53 (Ser-15) levels were evaluated by western blot. These three reagents induce total P53 and PP53 levels compared with control. CHX blocked this elevation of total P53 and PP53 (Fig.6), showing that lowering of P53 levels and activation of P53 to PP53 by CHX is not limited to only the serum withdrawal model.

The CHX decrease of P53 accumulation is related to P53 phosphorylation but not to PP53 dephosphorylation- P53 accumulation caused by DNA damaging reagents is mainly due to posttranslational modifications such as P53 phosphorylation as catalyzed by DNA kinases. Phosphorylated P53 is much more stable against proteasome-mediated degradation than non-phosphorylated P53. Once phosphorylated, PP53 can be dephosphorylated by phosphatases. CHX inhibition of P53 protein synthesis could be a major explanation for the decrease in total P53 levels by CHX. However CHX might also regulate P53 and PP53 levels by affecting the phosphorylation or dephosphorylation of P53 via acting on the DNA kinases or phosphatases responsible for these posttranslational modifications. In order to evaluate this, the proteasome inhibitor 10μM MG115 was used to prevent degradation of P53 and thus maintain the total P53 level (unlike results in Fig. 6 where total P53 levels declined in the presence of CHX). Two hours after treatment with MG115, the HepG2 cells were treated with 2.5μM ActD for 4 hour in order to induce PP53 accumulation, followed by treatment with 10μM CHX for

an additional 6 hour. This allows evaluation of the effect of CHX on already accumulated PP53 and P53. Fig. 7A shows that MG115 alone, as expected, increased P53 levels but did not cause an increase in PP53 levels. ActD in the presence of MG115 increased both total P53 and PP53 levels. However, 6 hours additional treatment with CHX did not lower PP53 levels. This suggests that a). CHX did not promote/activate P53 dephosphorylation (e.g. activation of PP53 phosphatases) so that once formed, PP53 levels are resistant to the CHX-mediated decline and b) once total P53 is stabilized against proteasome-dependent degradation, CHX no longer lowers this stabilized P53. In a second experiment, the HepG2 cells were again pretreated with 10μM MG115 for 2 h, and then treated with either 2.5μM ActD alone, or 10μM CHX alone, or both ActD+CHX for 6 hours. In the presence of MG115, total P53 was maintained at the same level in the absence or presence of CHX or CHX plus ActD (Fig.7B). Thus CHX does not lower P53 as long as the P53 cannot be degraded by the proteasome. ActD treatment induces PP53 formation. CHX plus ActD showed a decrease in the PP53 level compared with ActD alone although total P53 levels were similar in these two groups (Fig.7B). This suggests the possibility that CHX lowers the activity of DNA kinases, thereby decreasing the phosphorylation of P53, resulting in lowering of both total P53 and PP53 levels.

DISCUSSION

In the current study we observed that CHX protects HepG2 cells from apoptosis induced by serum withdrawal. This is in general agreement with previous reports in various cell culture models, e.g. CHX prevents apoptosis of cultured sympathetic neurons induced by withdrawal of nerve growth factor (Martin et al., 1988); prevents trophic factor deprivation-induced and glutamate-induced death of PC12 cells (Pittman et al., 1993; Serghini et al., 1994); protects cultured retinal ganglion cells against excitotoxicity (Dreyer et al., 1995). All these studies provided evidence in favor of a protein synthesis inhibition-based mechanism for the anti-apoptotic action of CHX. In a study of anti-apoptotic action of CHX and its inhibition of protein synthesis, Wyllie, et al. (Wyllie et al., 1984) reported that concentrations of CHX greater than 1μM protected rat thymocytes and mouse lymphoid cell lines against apoptosis induced by glucocorticoids and a calcium ionophore, and were effective in inhibiting protein synthesis by more than 50%. Martin at al (Martin et al., 1988) also reported that 3-10 μM CHX prevented apoptosis of cultured sympathetic neurons following withdrawal of nerve growth factor, and caused greater than 90% inhibition in overall protein synthesis. In our study, CHX concentrations from 5μM to 40 μM were found to effectively prevent HepG2 cells from apoptosis induced by serum withdrawal (Fig. 1B a). These reports suggest that the cell death process requires synthesis of "killer proteins" and CHX prevent cell death by inhibiting the synthesis of these killer proteins, although other mechanisms such as preventing cell death by activation of anti-apoptotic signaling pathways need to be considered (Mattson and Furukawa, 1997; Yu et al., 2003).

Serum deprivation induces oxidative stress in cells as demonstrated by an excess production of reactive oxygen species (ROS), such as superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical, and organic peroxides that can be inhibited by intracellular antioxidants (Satoh et al., 1996; King et al., 2003). We also found that trolox protects HepG2 cells from serum withdrawal induced cell death (Fig. 2B). CHX might increase resistance to oxidative insults as suggested by the work of Ratan et al. (Ratan et al., 1994) who showed that CHX can enhance cellular antioxidant pathways by shunting cysteine from global protein synthesis into the formation of the antioxidant glutathione. However, we did not detect any evidence that the administration of CHX decreases intracellular ROS (Fig. 2A). Conversely, we found that the ROS levels significantly increased

in cells treated with CHX compared with cells not treated with CHX at the time points of 8 and 24 h after serum withdrawal, which might be explained by CHX decreasing the synthesis of antioxidant enzymes, e.g. catalase and glutathione synthase. Sanchez, et al. (Sâanchez et al., 1997) also reported that CHX prevents apoptosis, ROS production and GSH depletion induced by TGF-β in fetal rat hepatocytes. However, they concluded that the CHX protection against oxidative stress induced by TGF-β in fetal hepatocytes is not related to an enhancement in the production of cellular antioxidants, such as glutathione, as it appeared, the relationship between TGF-β induced apoptosis and endogenous production of ROS was not causal, but rather the consequence of cell lysis. Our results indicate that the protection by CHX is not due to a decrease in ROS production after serum withdrawal.

P53 is implicated in cell cycle control, DNA repair, and apoptosis (Levine, 1997; Liebermann et al., 1995; Steller, 1995). It has been reported that CHX prevented apoptosis of HeLa cells induced by p53 (Yonish-Rouach et al., 1995). In the current study we found that serum withdrawal induces phosphorylation and accumulation of P53. CHX decreases total P53, PP53, P21 and Bax levels and blocks cell death (Fig. 3A). CHX also blocks the G1-S cell cycle arrest induced by serum withdrawal, most likely due to the decrease in P53 and P21 (Fig. 3B). The P53 inhibitor pifithrin-α protects HepG2 cells from serum withdrawal induced cell death. Hep3B cells lacking P53 and HepG2 cells expressing dominant negative P53 are more resistant to apoptosis induced by serum withdrawal than control cells (Fig. 4). Decreasing P53 levels by treatment with siRNA of p53 protects HepG2 cells from serum withdrawal induced apoptosis (Fig. 5). These data indicate that P53 plays an important role in the apoptosis caused by serum withdrawal in HepG2 cells, and that an important mechanism by which CHX prevents cell death is linked to the CHX-mediated decline in P53 and PP53 levels.

P53 accumulation caused by DNA damaging reagents is mainly due to posttranslational modifications such as P53 phosphorylation caused by DNA kinases. Phosphorylated P53 has a long half-life compared to non phosphorylated P53, due to its slower degradation, which then causes P53 accumulation. The phosphorylated P53 can also be dephosphorylated by phosphatases. CHX inhibition of P53 protein synthesis could be a major explanation for its decreasing total P53. However, it might also regulate P53 and PP53 levels by affecting the

phosphorylation or dephosphorylation of P53 via acting on DNA kinases or phosphatases. To study the effects of CHX on P53, ActD and the cancer therapeutic reagents doxorubicin and etoposide were used to induce P53. These reagents activate P53 and cause increases of both P53 and PP53. CHX effectively decreases P53 and PP53 levels induced by these reagents (Fig. 6). To determine the effects of CHX on phosphorylation or dephosphorylation of P53, the proteasome inhibitor MG115 was used to maintain the total P53 level at a constant level. MG115 increases the total P53 level but it does not increase the PP53 level. In the presence of MG115, ActD does not cause any further increase in total P53 levels over the levels found with MG115 alone, but it did increase the PP53 level. 4 hours after treatment with ActD to induce PP53, administration of CHX for 6 hrs does not affect the PP53 level compared with the no CHX treated levels (Fig. 7A). This suggests that CHX does not stimulate the dephosphorylation of PP53. However, in the presence of MG115 to maintain the total P53 levels, CHX, when added together with ActD, prevents the ActD mediated increase in PP53 levels (Fig. 7B). This suggests that CHX inhibits the phosphorylation of P53 and raises the possibility that kinases which phosphorylate P53 might be inhibited by CHX administration. ATM, a DNA kinase which catalyzes P53 phosphorylation was therefore evaluated. However there was no difference in content or activity of ATM in the presence and absence of CHX (Data not shown). Other kinases which phosphorylate P53 will require future investigation. These data demonstrate that the CHX decrease of P53 and PP53 levels might be mediated through inhibiting P53 protein synthesis and inhibiting the phosphorylation of P53. The latter will also decrease the total P53 levels via proteasome catalyzed degradation due to the longer half life of PP53 than P53.

In summary, these experiments indicate that CHX protects HepG2 cells from serum withdrawal induced apoptosis. “Killer protein” P53 is involved in the apoptosis induced by serum withdrawal. CHX decreases the P53 and PP53 level via inhibiting P53 protein synthesis and its phosphorylation, thereby preventing cell death.

ACKNOWLEDGMENTS

We thank Dr. James Manfredi, Mount Sinai Ruttenberg Cancer Center for many helpful suggestions and advice.

REFERENCES

Bai J, Rodriguez AM, Melendez JA and Cederbaum AI (1999) Overexpression of catalase in cytosolic or mitochondrial compartment protects HepG2 cells against oxidative injury. *J Biol Chem* **274**:26217-26224.

Dreyer EB, Zhang D and Lipton SA (1995) Transcriptional or translational inhibition blocks low dose NMDA-mediated cell death. *Neuroreport.* **6**:942-944.

el-Deiry WS, Tokino T, Waldman T, Oliner JD, Velculescu VE, Burrell M, Hill DE, Healy E, Rees JL, Hamilton SR and et al. (1995) Topological control of p21WAF1/CIP1 expression in normal and neoplastic tissues. *Cancer Res* **55**:2910-2919.

Fritsche M, Haessler C and Brandner G (1993) Induction of nuclear accumulation of the tumor-suppressor protein p53 by DNA-damaging agents. *Oncogene* **8**:307-318.

Fulda S, Meyer E and Debatin KM (2000) Metabolic inhibitors sensitize for CD95 (APO-1/Fas)-induced apoptosis by down-regulating Fas-associated death domain-like interleukin 1-converting enzyme inhibitory protein expression. *Cancer Res* **60**:3947-3956.

Hermeking H, Lengauer C, Polyak K, He TC, Zhang L, Thiagalingam S, Kinzler KW and Vogelstein B (1997) 14-3-3 sigma is a p53-regulated inhibitor of G2/M progression. *Mol Cell.* **1**:3-11.

Kang S, Song J, Kang H, Kim S, Lee Y and Park D (2003) Insulin can block apoptosis by decreasing oxidative stress via phosphatidylinositol 3-kinase- and extracellular signal-regulated protein kinase-dependent signaling pathways in HepG2 cells. *Eur J Endocrinol* **148**:147-155.

Kastan MB, Zhan Q, el-Deiry WS, Carrier F, Jacks T, Walsh WV, Plunkett BS, Vogelstein B and Fornace AJ, Jr. (1992) A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. *Cell* **71**:587-597.

King AR, Francis SE, Bridgeman CJ, Bird H, Whyte MK and Crossman DC (2003) A role for caspase-1 in serum withdrawal-induced apoptosis of endothelial cells. *Lab Invest* **83**:1497-1508.

Komarov PG, Komarova EA, Kondratov RV, Christov-Tselkov K, Coon JS, Chernov MV and Gudkov AV (1999) A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy. *Science* **285**:1733-1737.

Levine AJ (1997) p53, the cellular gatekeeper for growth and division. *Cell* **88**:323-331.

Liebermann DA, Hoffman B and Steinman RA (1995) Molecular controls of growth arrest and apoptosis: p53-dependent and independent pathways. *Oncogene* **11**:199-210.

Liu X, Chua CC, Gao J, Chen Z, Landy CL, Hamdy R and Chua BH (2004) Pifithrin-alpha protects against doxorubicin-induced apoptosis and acute cardiotoxicity in mice. *Am J Physiol Heart Circ Physiol* **286**:H933-939.

Martin DP, Schmidt RE, DiStefano PS, Lowry OH, Carter JG and Johnson EM, Jr. (1988) Inhibitors of protein synthesis and RNA synthesis prevent neuronal death caused by nerve growth factor deprivation. *J Cell Biol* **106**:829-844.

Mattson MP and Furukawa K (1997) Anti-apoptotic actions of cycloheximide: blockade of programmed cell death or induction of programmed cell life? *Apoptosis* **2**:257-264.

Miyashita T and Reed JC (1995) Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell* **80**:293-299.

Murphy PJ, Galigniana MD, Morishima Y, Harrell JM, Kwok RP, Ljungman M and Pratt WB (2004) Pifithrin-alpha inhibits p53 signaling after interaction of the tumor suppressor protein with hsp90 and its nuclear translocation. *J Biol Chem* **279**:30195-30201.

Oda E, Ohki R, Murasawa H, Nemoto J, Shibue T, Yamashita T, Tokino T, Taniguchi T and Tanaka N (2000a) Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science* **288**:1053-1058.

Oda K, Arakawa H, Tanaka T, Matsuda K, Tanikawa C, Mori T, Nishimori H, Tamai K, Tokino T, Nakamura Y and Taya Y (2000b) p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. *Cell* **102**:849-862.

Owen-Schaub LB, Zhang W, Cusack JC, Angelo LS, Santee SM, Fujiwara T, Roth JA, Deisseroth AB, Zhang WW, Kruzel E and et al. (1995) Wild-type human p53 and a temperature-sensitive mutant induce Fas/APO-1 expression. *Mol Cell Biol* **15**:3032-3040.

Pittman RN, Wang S, DiBenedetto AJ and Mills JC (1993) A system for characterizing cellular and molecular events in programmed neuronal cell death. *J Neurosci* **13**:3669-3680.

Polyak K, Xia Y, Zweier JL, Kinzler KW and Vogelstein B (1997) A model for p53-induced apoptosis. *Nature* **389**:300-305.

Ratan RR, Murphy TH and Baraban JM (1994) Macromolecular synthesis inhibitors prevent oxidative stress-induced apoptosis in embryonic cortical neurons by shunting cysteine from protein synthesis to glutathione. *J Neurosci* **14**:4385-4392.

Sâanchez A, Alvarez AM, Benito M and Fabregat I (1997) Cycloheximide prevents apoptosis, reactive oxygen species production, and glutathione depletion induced by transforming growth factor beta in fetal rat hepatocytes in primary culture. *Hepatology* **26**:935-943.

Satoh T, Sakai N, Enokido Y, Uchiyama Y and Hatanaka H (1996) Survival factor-insensitive generation of reactive oxygen species induced by serum deprivation in neuronal cells. *Brain Res* **733**:9-14.

Schamberger CJ, Gerner C and Cerni C (2005) Caspase-9 plays a marginal role in serum starvation-induced apoptosis. *Exp Cell Res* **302**:115-128.

Serghini R, Froissard P, Sola B and Duval D (1994) Cycloheximide and actinomycin D block the toxic effect of glutamic acid on PC12 cells. *Neuroreport* **6**:29-32.

Steller H (1995) Mechanisms and genes of cellular suicide. *Science* **267**:1445-1449.

van der Valk J, Mellor D, Brands R, Fischer R, Gruber F, Gstraunthaler G, Hellebrekers L, Hyllner J, Jonker FH, Prieto P, Thalen M and Baumans V (2004) The humane collection of fetal bovine serum and possibilities for serum-free cell and tissue culture. *Toxicol In Vitro* **18**:1-12.

Vogelstein B and Kinzler KW (1992) p53 function and dysfunction. *Cell* **70**:523-526.

Wyllie AH, Morris RG, Smith AL and Dunlop D (1984) Chromatin cleavage in apoptosis: association with condensed chromatin morphology and dependence on macromolecular synthesis. *J Pathol* **142**:67-77.

Yonish-Rouach E, Deguin V, Zaitchouk T, Breugnot C, Mishal Z, Jenkins JR and May E (1995) Transcriptional activation plays a role in the induction of apoptosis by transiently transfected wild-type p53. *Oncogene* **11**:2197-2205.

Yu AC, Yung HW, Hui MH, Lau LT, Chen XQ and Collins RA (2003) Cycloheximide and actinomycin D delay death and affect bcl-2, bax, and Ice gene expression in astrocytes under in vitro ischemia. *J Neurosci Res* **74**:318-325.

Yu J, Zhang L, Hwang PM, Kinzler KW and Vogelstein B (2001) PUMA induces the rapid apoptosis of colorectal cancer cells. *Mol Cell* **7**:673-682.

FOOTNOTES

These studies were supported by USPHS Grants AA03312 (Cederbaum, A.I) and AA015697 (Bai, J) from the National Institute on Alcohol Abuse and Alcoholism.

FIGURE LEGENDS

Fig. 1. *CHX protects HepG2 cells from apoptosis induced by serum deprivation*- HepG2 cells were cultured in MEM with 10% FBS or without FBS and treated with or without CHX. A. Morphology change of cells at 72 hours after culture in medium with or without serum in the presence or absence 10 μM CHX was visualized and photographed under the light microscope. B. Cell viability evaluated by trypan blue exclusion assay. a, Cells were cultured in serum free medium in the absence or presence of different concentrations CHX (0.5, 1, 5, 10 and 40 μM) for 72 h. b, Cells were cultured in serum free medium in the absence or presence of 10 μM CHX for different times (0, 24, 48 and 72h). Cell viability is presented as percentage of viable cells. Data are the mean of three independent experiments. C. DNA was extracted from cells cultured in MEM with 10% FBS or without FBS and treated with or without 10 μM CHX for 48 and 56 h, and electrophoresed on agarose gels, and stained with ethidium bromide and photographed under UV illumination .

Fig. 2. ***Serum deprivation induces intracellular ROS production and an antioxidant protects against cell death induced by serum deprivation*** **– A.** HepG2 cells were incubated in serum-free medium with or without 10 μM CHX for 0, 4, 8, 24, 48 and 72h followed by incubation with 5 μM DCF-DA in MEM for 30 min at 37 °C in the dark. The cells were washed in PBS, trypsinized, and resuspended in PBS, and the intensity of fluorescence was immediately read in a fluorescence spectrophotometer at 503 nm for excitation and at 529 nm for emission. The results are expressed as arbitrary units of the fluorescence intensity. Data are the mean ± SD of three independent experiments conducted in duplicate. ∗ $p<0.05$ vs. -CHX 8h; ∗∗ $p<0.001$ vs. –CHX 24h. B. HepG2 cells were incubated in serum-free medium with or without 50 μM trolox for 48. Morphology change of HepG2 cells was visualized under the light microscope.

Fig. 3. *CHX blocks P53 activation and cell cycle arrest induced by serum deprivation*- A. HepG2 cells were cultured in serum-free medium with or without 10μM CHX for 8, 16 and 24 hours. Cells cultured in 10% serum containing medium for the same time points were used as control. Cell lysates were prepared by sonicating cells followed by centrifugation. Total P53, phosphorylated (pSer-15) P53, P21 and Bax levels were evaluated by Western blot analysis using respective antibody against P53, phosphorylated (pSer-15) P53, P21 and Bax. B.

HepG2 cells were incubated with medium containing 10% serum or no serum in the presence or absence of 10 μM CHX for 16 h. Cells were harvested and prepared for cell cycle analysis as described in "Materials and Methods". a. Flow cytometry DNA histographs of cells treated with complete serum containing medium (control), serum containing medium plus CHX (CHX), serum free medium (SW) and serum free medium plus CHX. b. The percentage of cells in different stages of G_0 and G_1, S-G_2 and M were quantified and data are the mean of two independent experiments.

Fig. 4. *HepG2 cells treated with a P53 inhibitor or HepG2 cells expressing mutant P53 or Hep3B cells lacking P53 are more resistant to apoptosis induced by serum deprivation*- (A). P53, P21 and Bax levels in HepG2 cells treated with different concentrations of pifithrin-α (0, 30 and 60 μM) for 24 h (a) or in HepG2 cells expressing mutant P53 (HepG2-mtP53), control cells (HepG2-neo) or Hep3B cells (b) were evaluated by Western blot (B)**.** Cell viability was evaluated by trypan blue exclusion assay. a, HepG2 cells were cultured in medium containing 10%FBS or serum withdrawal medium in the presence of DMSO (control), or 30 or 60 μM pifithrin-α for 48 h. b, HepG2 cells transfected with dominant negative form of P53 plasmid (HepG2-mtp53) and empty vector plasmid (HepG2-neo) as well as Hep3B hepatoma cells lacking wild type P53 were cultured in medium containing 10%FBS or serum withdrawal medium for 48 h. Cell viability is presented as percentage of viable cells. Data are the mean ± SD of three independent experiments. ∗, $p< 0.05$ vs no serum control; ∗∗, $p<0.01$ vs. no serum control. (C). Morphology change of cells at 48 hours treatment was visualized and photographed under the light microscope. a, HepG2 cells were cultured in serum withdrawal medium in the presence of DMSO (control), or 30 or 60 μM pifithrin-α for 48 h. b, HepG2-neo, HepG2-mtp53 and Hep3B cells were cultured in serum withdrawal medium for 48 h.

Fig. 5. ***siRNA-p53 protects HepG2 cells from serum withdrawal induced apoptosis***- siRNA-p53 and siRNA-control (a nontargeting siRNA) were transfected into HepG2 cells using Lipofectamine™ RNAiMAX reagents. A. Twenty-four hours after transfection, cells were cultured in medium with or without serum for an additional 24 h. Cell lysates were prepared by sonicating cells followed by centrifugation. P53, PP53, Bax and β-actin were evaluated by Western blot analysis. These results represent a

similar pattern of two independent experiments. B. Cell viability was evaluated by trypan blue exclusion assay after culture in serum containing medium (control) or serum free medium (SW) for 48 h in cells transfected with siRNA-p53 or siRNA-control. Data are the mean ± SD of three independent experiments. ∗ $p<0.05$ vs. siRNA-control.

Fig. 6. ***CHX decreases P53 accumulation and P53 phosphorylation induced by ActD, doxorubicin and etoposide-*** HepG2 cells were treated with the therapeutic reagents 2.5μM Act D, 2.5μM doxorubicin or 25μM etoposide in the absence or presence of 10μM CHX for 16 hour. Cell lysates were prepared by sonicating cells followed by centrifugation. Total P53, phosphorylated (pSer-15) P53 and β-actin were evaluated by Western blot analysis using respective antibody against P53, phosphorylated (pSer-15) P53 and β-actin. This result represents a similar pattern of three independent experiments.

Fig. 7. ***CHX decrease of P53 accumulation is related to P53 phosphorylation but not to PP53 dephosphorylation-*** A***,*** Two hours after treatment with10μM MG115, HepG2 cells were treated with 2.5μM ActD for 4 hours, followed by treatment with 10μM CHX for an additional 6 hours. B, Two hours after treatment with 10μM MG115, HepG2 cells were treated with 2.5μM ActD alone or 10μM CHX alone or ActD plus CHX for 6 hrs. Cell lysates were prepared by sonicating cells followed by centrifugation. Total P53, phosphorylated (pSer-15) P53 and β-actin were evaluated by Western blot analysis using respective antibody against P53, phosphorylated (pSer-15) P53 and β-actin. These results represent a similar pattern of two independent experiments.

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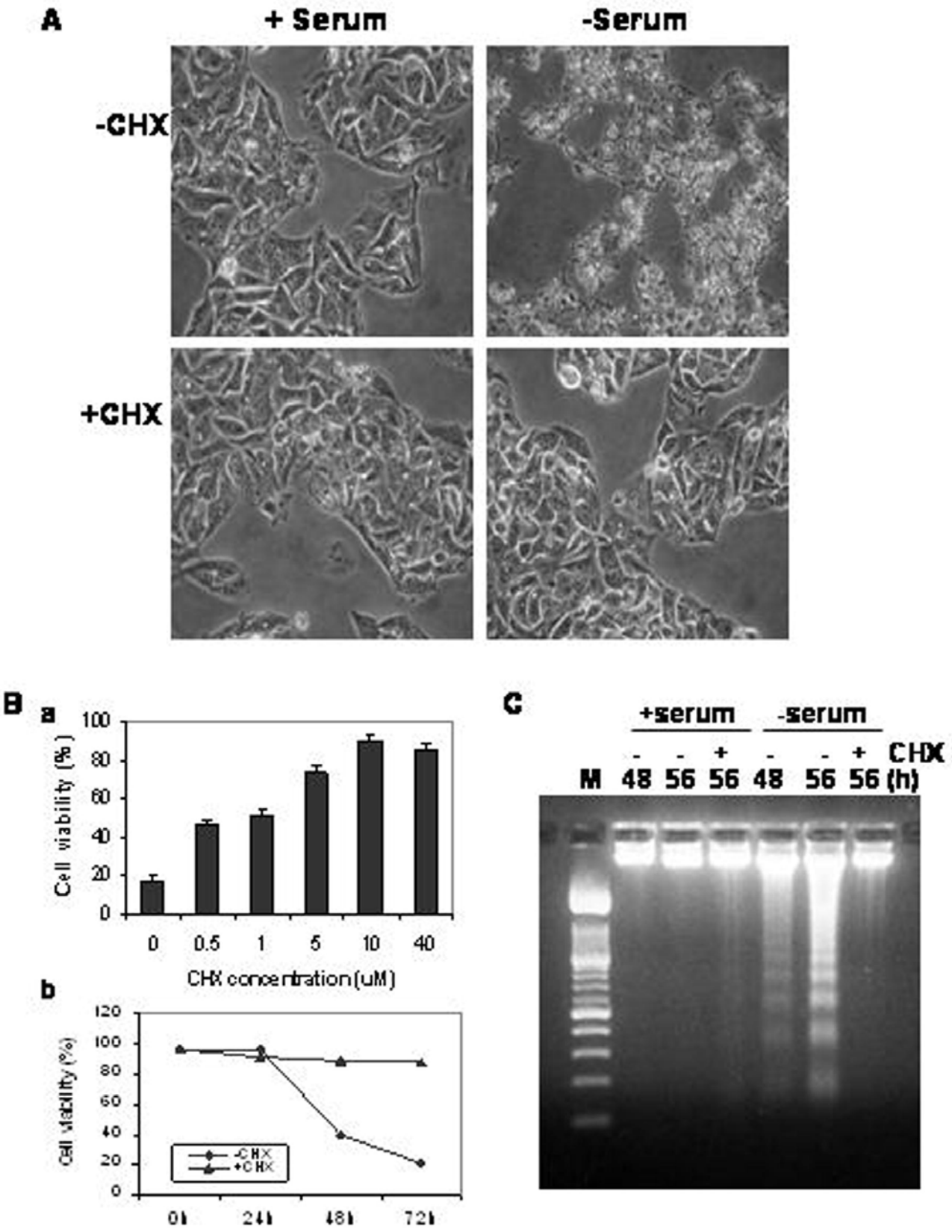

Fig. 1

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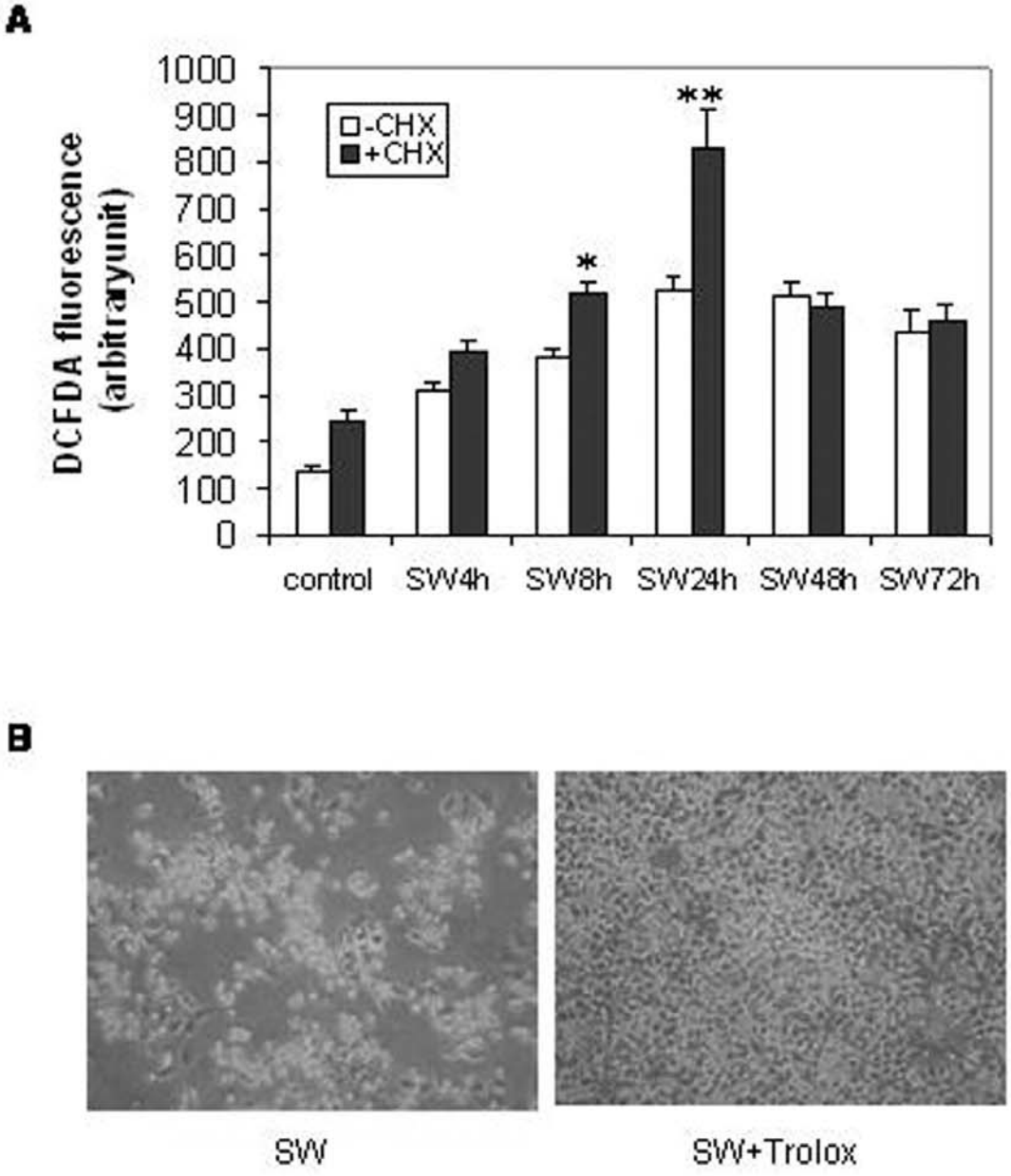

Fig.2

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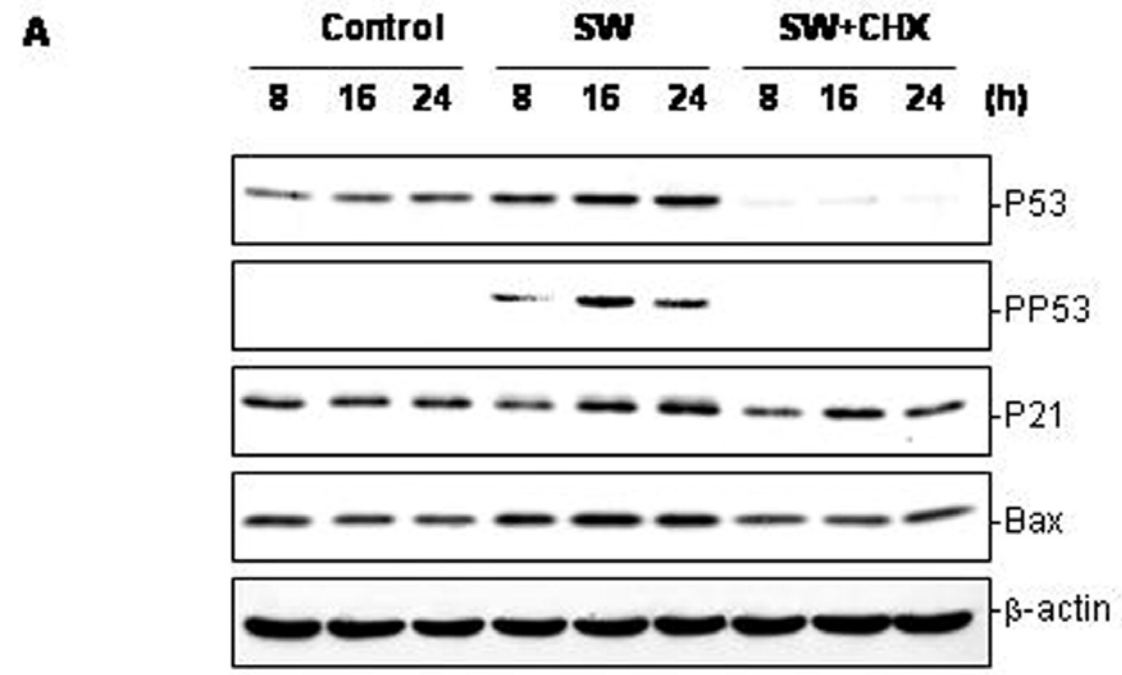

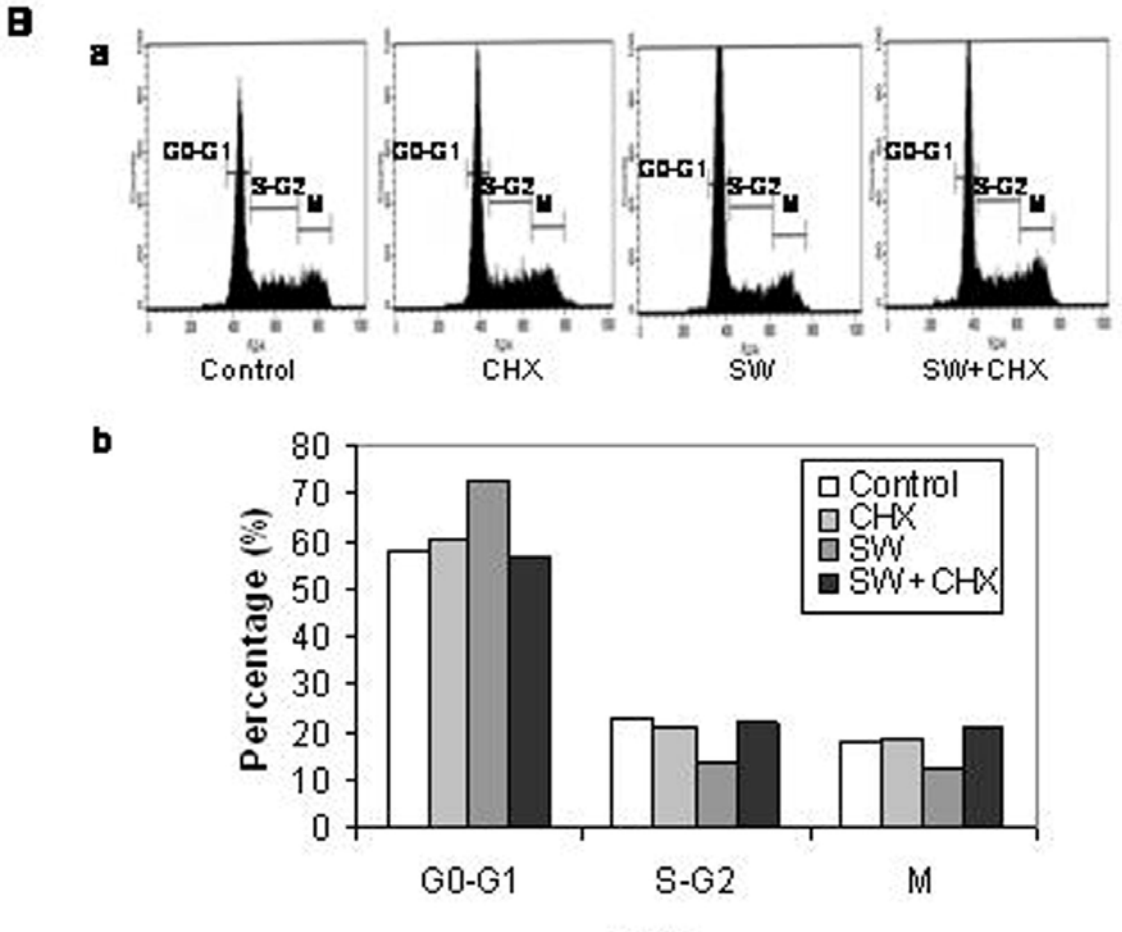

Fig.3

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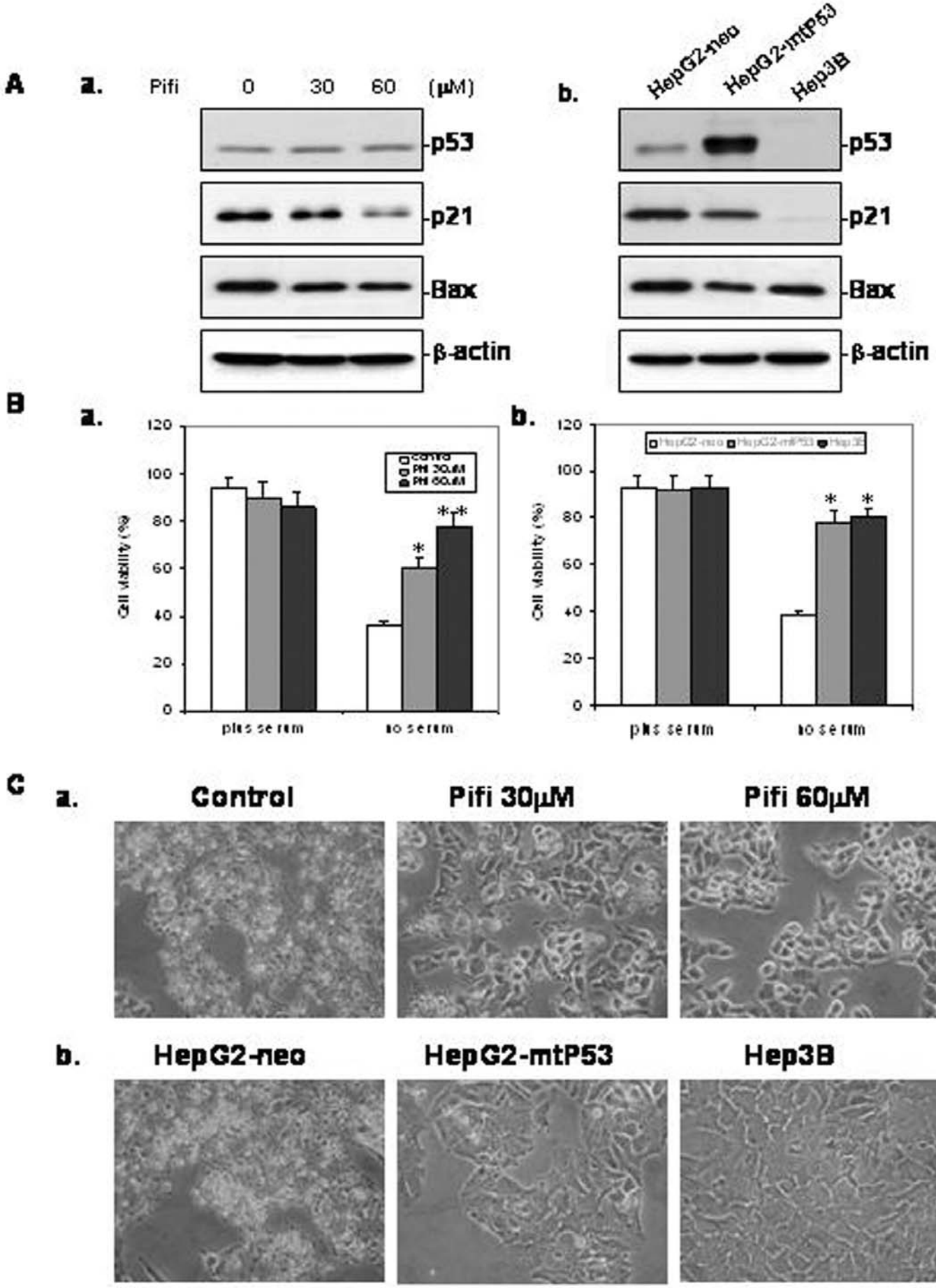

Fig.4

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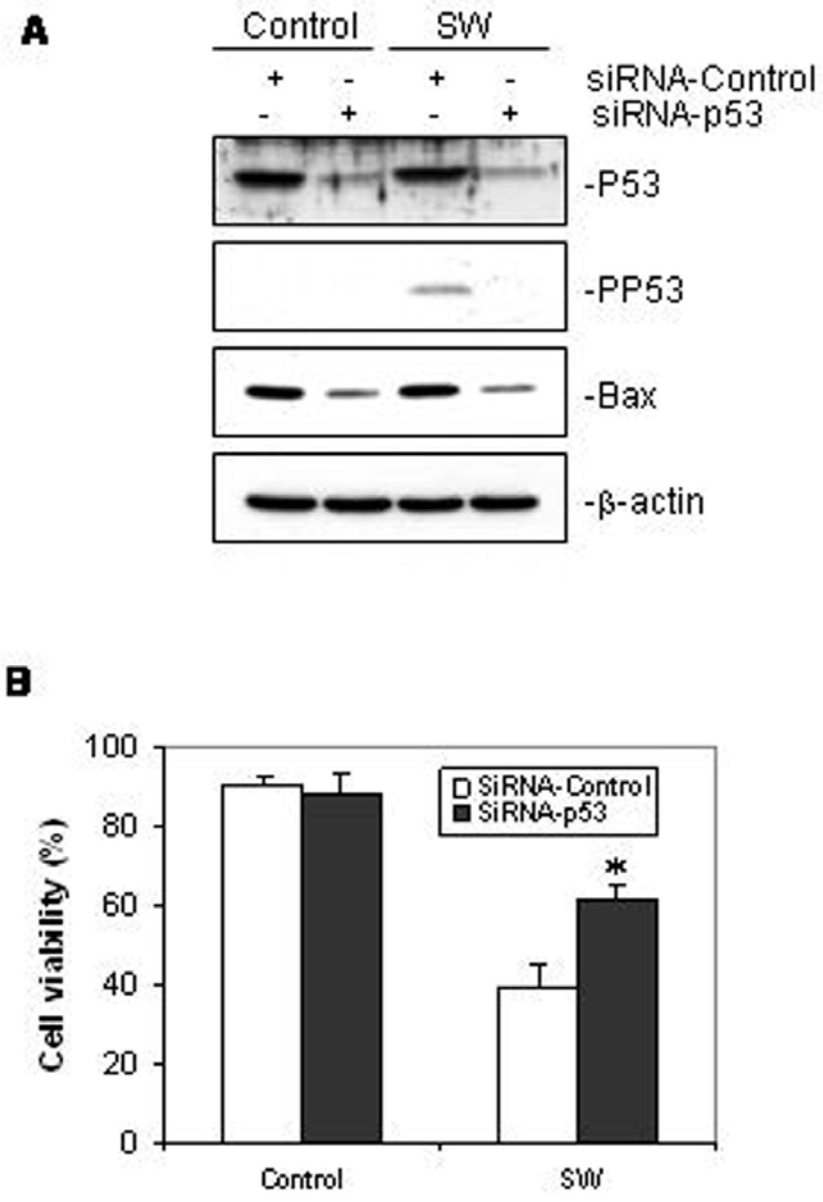

Fig. 5

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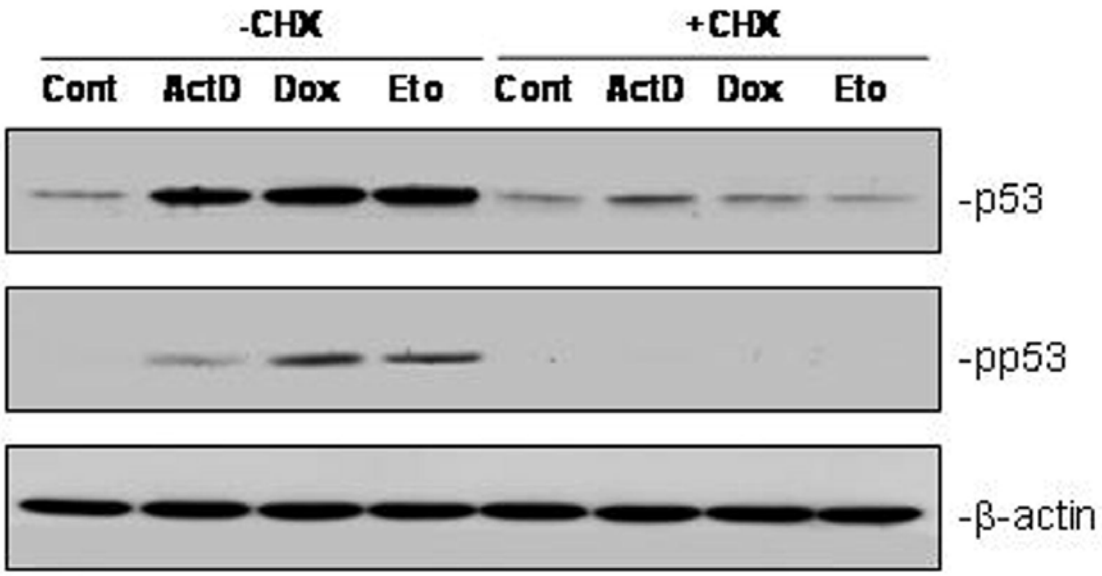

Fig.6

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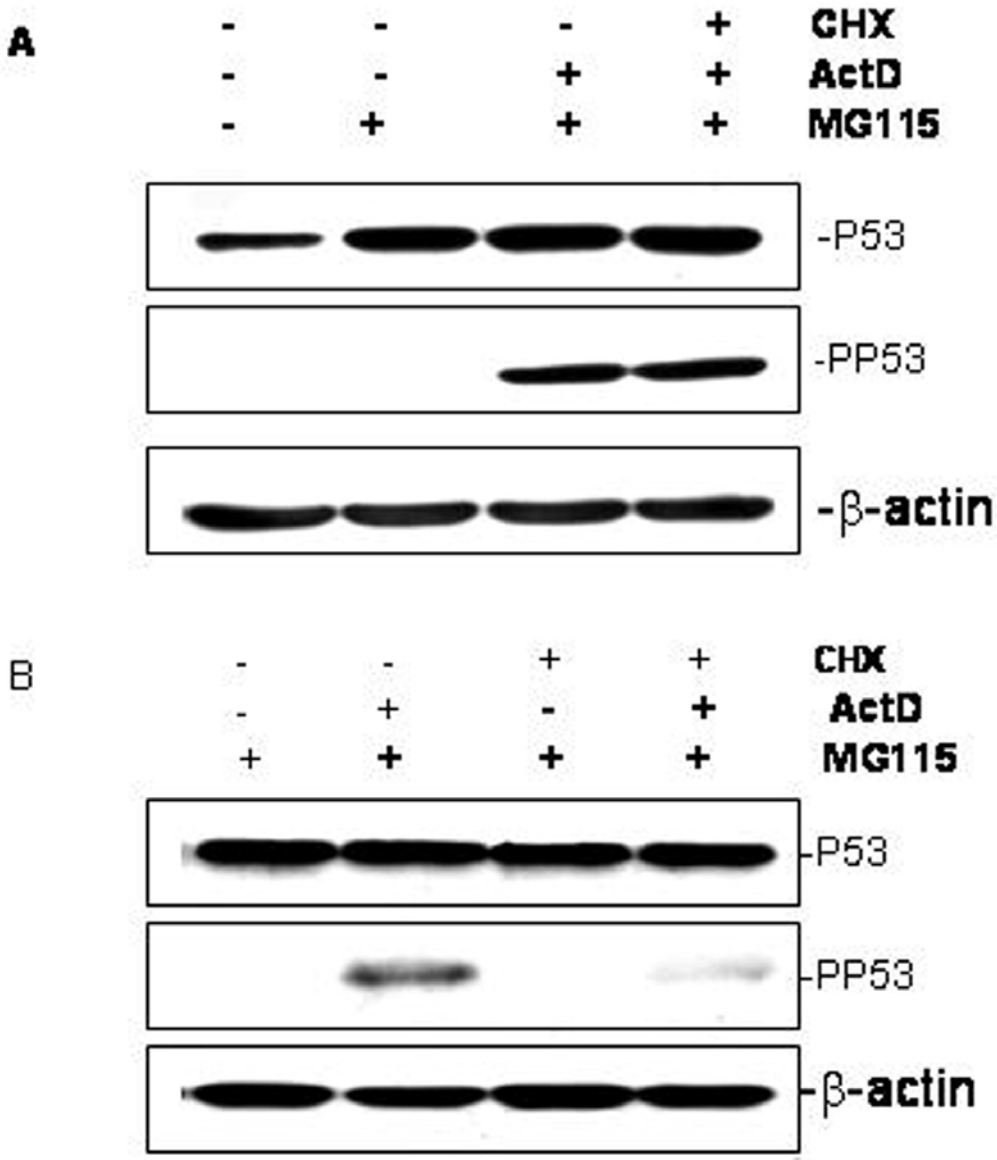

Fig. 7