

Mortalin–p53 interaction in cancer cells is stress dependent and constitutes a selective target for cancer therapy

W-J Lu¹, NP Lee¹, SC Kaul², F Lan³, RTP Poon¹, R Wadhwa^{*,2} and JM Luk^{*,1,4,5}

Stress protein mortalin is a multifunctional protein and is highly expressed in cancers. It has been shown to interact with tumor suppressor protein-p53 (both wild and mutant types) and inactivates its transcriptional activation and apoptotic functions in cancer cells. In the present study, we found that, unlike most of the cancer cells, HepG2 hepatoma lacked mortalin–p53 interaction. We demonstrate that the mortalin–p53 interaction exists in cancer cells that are either physiologically stressed (frequently associated with p53 mutations) or treated with stress-inducing chemicals. Targeting mortalin–p53 interaction with either mortalin small hairpin RNA or a chemical or peptide inhibitor could induce p53-mediated tumor cell-specific apoptosis in hepatocellular carcinoma; p53-null hepatoma or normal hepatocytes remain unaffected.

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The wild-type p53 is a key tumor suppressor protein that eliminates genetically unstable cells by inducing either cell cycle arrest or apoptosis through transcriptional regulation or direct interaction with apoptotic proteins.^{1,2} Functional inactivation of p53, a frequent event in cancer cells, occurs by three main mechanisms: (i) mutations, (ii) post-translational modifications and (iii) cytoplasmic sequestration^{3–5} by its binding proteins. Although several cellular proteins are shown to interact with p53, the mechanism of its inactivation still remains unclear. Furthermore, interaction of p53 with its binding partners is context dependent, and influenced by both intracellular and extracellular environment. Cellular stress response (intrinsic and extrinsic) has been shown to evoke p53 signaling⁶ through its modifications, including phosphorylation (at serine and/or threonine),⁷ acetylation,⁸ sumoylation,⁹ glycosylation,¹⁰ ribosylation¹¹ or ubiquitylation.¹² Furthermore, it has been shown that p53 protein interacts with several stress proteins, including Hsp40, Hsp70, Hsp84, Hsp90, DnaK, DnaJ and GrpE *in vivo*,^{13–15} that potentially modulate p53 activities. However, their roles in development and progression of cancer, physiologically a stressed condition, remain unclear.

Mortalin/mthsp70/GRP75/PBP74, a member of the heat shock protein (Hsp) 70 family, is enriched in human cancer cells.^{16–18} Overexpression of mortalin was sufficient to increase the malignancy of breast cancer cells in both *in vitro* and *in vivo* models. The underlying mechanism was shown to be the sequestration of wild-type p53 in the cytoplasm, leading

to inhibition of its transcriptional activation and control of centrosome duplication functions.^{19–22} A cationic inhibitor (MKT-077) of mortalin that releases p53 from mortalin–p53 complex was shown to cause activation of p53 and growth arrest of cancer cells.²³ Similarly, mortalin-binding p53 peptides caused nuclear translocation and activation of p53.¹⁹ Mortalin was identified as a marker for hepatocellular carcinoma (HCC) metastasis and recurrence by proteomics analysis of matched tumor and non-tumor tissues,¹⁸ suggesting that it contributes to HCC development and recurrence.

In the present study, we found that unlike most cancer cells, HepG2 hepatoma lacked mortalin–p53 interaction. Using HepG2 as a model, we demonstrate that the mortalin–p53 interaction that causes inactivation of p53-mediated apoptosis depends on the cellular stress level. Unstressed or weakly stressed cancer and normal cells lack mortalin–p53 interaction. Furthermore, mortalin small hairpin RNA (shRNA) could reactivate p53-mediated apoptosis selectively in cancer cells. These findings open the possibilities for clinical application of mortalin shRNA, mortalin–p53 binding antagonists or their combination with chemotherapeutic drugs in the HCC treatment.

Results

Human HepG2 cells lacked mortalin–p53 interaction and were resistant to mortalin shRNA-induced apoptosis. Mortalin-specific shRNA-2166 was designed

¹Department of Surgery, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong, China; ²National Institute of Advanced Industrial Science & Technology (AIST), Central 4, 1-1-1 Higashi, Tsukuba, Ibaraki 305 8562, Japan; ³Department of Medicine, Division of Cardiology, Stanford University School of Medicine, Stanford, CA 94305, USA; ⁴Department of Pharmacology and Department of Surgery, National University Health System, National University of Singapore, Singapore 117597, Singapore and ⁵Cancer Science Institute, National University of Singapore, Singapore 117597, Singapore

*Corresponding author: R Wadhwa, National Institute of Advanced Industrial Science & Technology (AIST), Central 4, 1-1-1 Higashi, Tsukuba, Ibaraki 305 8562, Japan. Tel: + 81 29 861 9464; Fax: + 81 29 861 2900; E-mail: renu-wadhwa@aist.go.jp or JM Luk, Department of Pharmacology, National University Health System, National University of Singapore, MD-11 #05-09, 10 Medical Drive, Singapore 117597, Singapore. Tel: + 65 6516 4516; Fax: + 65 6873 7690; E-mail: jmluk@nus.edu.sg

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Abbreviations: shRNA, small hairpin RNA; Hsp70, heat shock protein 70; HCC, hepatocellular carcinoma; GRP78, glucose-regulated protein 78; MKT-077, 1-ethyl-2-[[3-ethyl-5-(3-methylbenzothiazolin-2-ylidene)]-4-oxothiazolidin-2-ylidene]methyl]pyridinium chloride; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide

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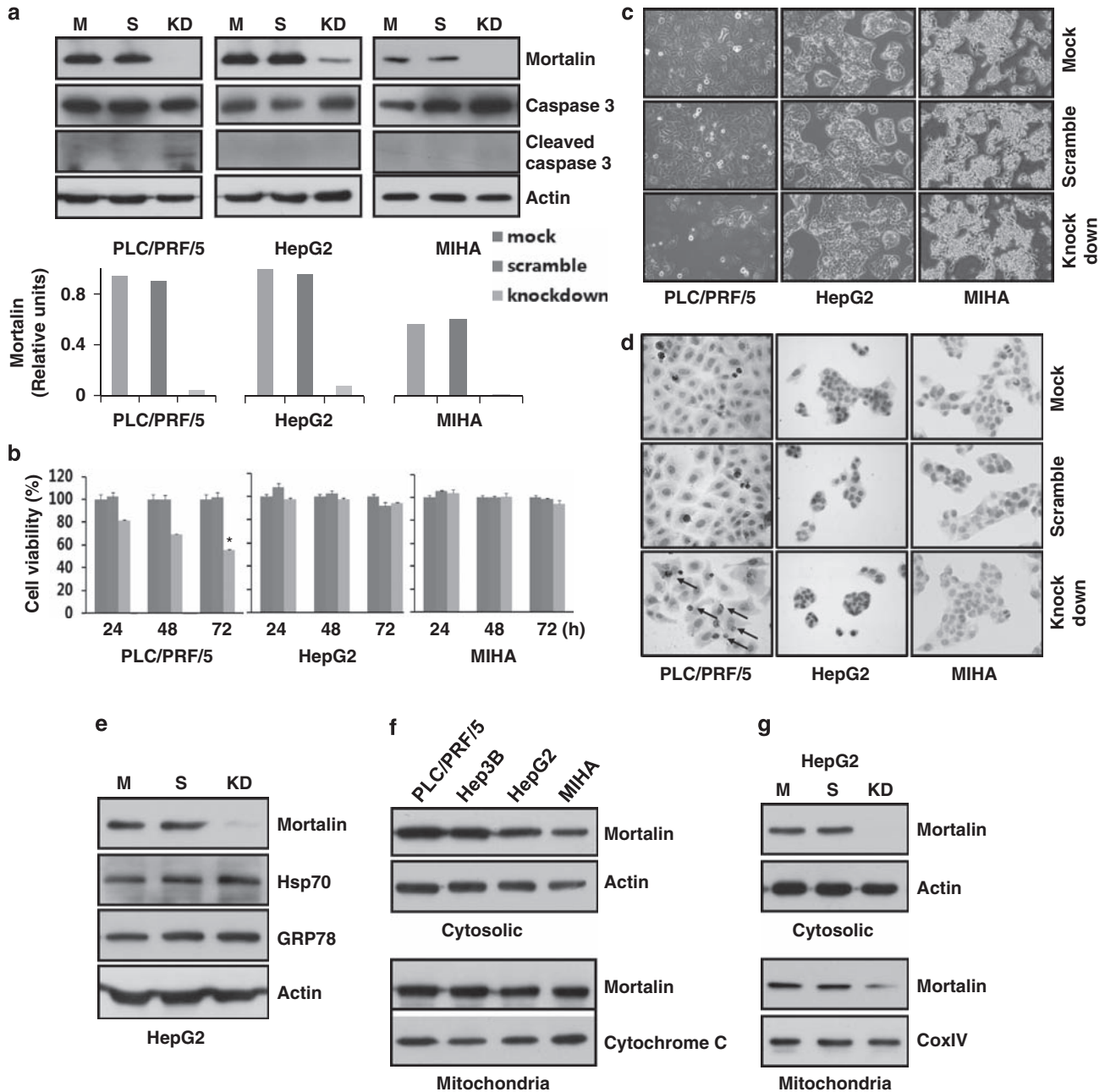


Figure 1 Mortalin silencing induces apoptosis in PLC/PRF/5, but not in MIHA and HepG2 cells. (a) Mortalin shRNA caused reduction in mortalin expression (KD) in all the three cell lines (examined at 72 h post transfection); empty vector transfected (Mock-M) and scrambled shRNA transfected (S) cells were used as control. (b) Viability (MTT assay) was significantly reduced in PLC/PRF/5 ($*P < 0.05$) at 72 h post-transfection of mortalin shRNA; MIHA cells and HepG2 did not show any change as compared with controls. (c) Phase-contrast images of control and mortalin shRNA transfected cells showing apoptosis in mortalin shRNA transfected PLC/PRF/5 cells only. (d) *In situ* TUNEL staining identified mortalin shRNA-induced apoptosis in PLC/PRF/5, but not in MIHA and HepG2 cells. Apoptotic PLC cells are marked with arrows. (e) Mortalin knockdown cells (KD) were examined for Hsp70 and glucose-regulated protein 78 (GRP78) by western blotting with specific antibodies. The mortalin shRNA-2166 was highly specific for mortalin and did not cause any reduction in Hsp70 and GRP78. (f) Presence of mortalin in the cytoplasmic and mitochondrial fractions in four cell lines was examined by western blotting with mortalin specific antibody. (g) Mortalin knockdown by shRNA-2166 caused reduction in mortalin in both cytosolic and mitochondrial fractions

and used to intervene mortalin-p53 interaction in cancer cells. HCC cell line PLC/PRF/5 (PLC) (mutant p53-249 serine mutation, the most common p53 mutation site in HCC with G:C to T:A transversion) transfected with shRNA-2166 showed mortalin suppression and dramatic reduction (almost 50%) in cell viability (Figures 1a and b). However, although immortalized human hepatocytes MIHA and HepG2 hepatoma

showed 70–90% knockdown of mortalin expression, their viability was not affected (Figures 1a and b). On the basis of the cell morphology and the rapidly reduced cell viability, it appeared that the shRNA-2166 led the cells to apoptosis, which was further confirmed by the presence of caspase cleavage and deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining (Figures 1a, c and d).

Of note, consistent with the cell viability data (Figure 1b), apoptosis was not observed in MIHA and HepG2 cells, and they were negative for both caspase cleavage and TUNEL staining (Figures 1a, c and d). We first confirmed that mortalin shRNA-2166 was specific and did not crossreact with other mRNAs (Hsp70, glucose-regulated protein 78) that show close homology to mortalin. As shown in Figure 1e, we confirmed that shRNA-2166 was specific to mortalin. Mortalin was earlier shown to sequester p53 in the cytoplasm and inactivate its transcriptional activities. On the basis of this finding, mortalin shRNA-2166-induced reduction in mortalin expression was expected to cause nuclear translocation and activation of p53, possibly resulting in apoptosis as seen in Figures 1a–d. We further examined whether mortalin was present in the cytosolic fractions of these cell lines. Similar to several other cell lines,^{24,25} all the four HCC lines used in this study showed mortalin in both the cytosolic and mitochondrial fractions (Figure 1f). Furthermore, shRNA-2166 caused reduction in both the cytosolic and mitochondrial fractions (Figure 1g).

In order to confirm that the apoptosis induced by mortalin shRNA was p53 dependent, we carried out p53 knockdown in combination with mortalin knockdown. As expected and shown in Figures 2a–c, the efficacy of mortalin silencing-induced apoptosis was significantly compromised by p53 knockdown and by treatment with a specific inhibitor (PFT- μ) of p53.²⁶ The data confirmed that the knockdown of mortalin-induced apoptosis was p53 dependent. In order to demonstrate that the effect was specific to mortalin shRNA, we also performed Hsp60 knockdown and found no apoptosis either in PLC or in HepG2 cells (Figures 2d–f). As PFT- μ was shown to be an inhibitor of Hsp70 (HSPA1A/B),²⁷ we confirmed that mortalin shRNA-2166 did not cause any effect on Hsp70 (Figure 1e). We also performed the knockdown of Hsp70 and of mortalin together. Hsp70 knockdown caused a low level of apoptosis (about 5–10% as compared with the 40–50% caused by mortalin knockdown). The effect of two knockdowns (mot-shRNA and Hsp70-shRNA) was synergistic, and the apoptosis caused by mortalin knockdown was rescued by PFT- μ (Figures 2g and h). These data demonstrated that the mortalin shRNA-induced apoptosis was p53 dependent. Hence, HepG2 cells that were resistant to the apoptotic activity of mortalin shRNA may lack mortalin–p53 interaction. We, therefore, examined the interaction of mortalin and p53 by co-immunoprecipitation assay. As seen in Figure 3a, mortalin immunocomplexes contained p53 in PLC, but not in MIHA and HepG2 cells; Hep3B (p53 null) cells were used as a control. Specificity of the mortalin antibody was confirmed by probing the mortalin immunocomplexes with anti-Hsp70 antibody. As shown in Figure 3b, mortalin antibody was specific and did not pull down any Hsp70. Double immunostaining was performed, and we found that the knockdown of mortalin by mortalin-specific shRNA-2166 led to nuclear translocation of p53 in PLC cells only. MIHA and HepG2 that lacked mortalin–p53 interaction did not show any change in subcellular localization of p53 (Figure 3c), suggesting that mortalin shRNA-induced apoptosis is dependent on mortalin–p53 interactions.

Cellular stress is a key determinant of mortalin–p53 interactions and apoptosis. On the basis of the above

data, we hypothesized that the interaction of mortalin and p53 may be stress dependent. Hence, we examined the stress-induced phosphorylation of p53 and its interaction with mortalin in a panel of HCC cells. The phosphorylation of p53 was detected at three (Ser 15, Ser 37, Ser 392) of the seven sites in five HCC cell lines (97H, 97L, H2P, H2M, PLC) (Figure 4a). Interestingly, these five cell lines underwent apoptosis by shRNA-2166 (Figure 1 and data not shown). Furthermore, silencing of mortalin enhanced p53 phosphorylation at one or more sites (Figure 4b). The two cell lines (HepG2, MIHA) that were not affected by silencing of mortalin showed a low level of stress status (lower level of p53 accumulation and phosphorylation).

In order to confirm further that the interaction of mortalin and p53 is stress dependent, we exposed HepG2 cells to low doses of cisplatin (a platinum-based chemotherapeutic drug to induce DNA cross-linking and damages). By cell cycle analysis, we confirmed that the low doses (1–2 μ g/ml) did not cause any growth arrest of cells (data not shown). As shown in Figure 5a, the levels of both mortalin and p53 were found to increase in response to the cisplatin treatment (>2 μ g/ml) (Figure 5a), and p53 was phosphorylated at Ser 15 (Figure 5c). Of note, consistent with our model, we found that cisplatin treatment induced the mortalin–p53 interaction in HCC cells (Figure 5b). Whereas mortalin immunocomplexes lacked p53 in control HepG2 cells, cisplatin-treated cells showed co-precipitation of p53 with mortalin (Figure 5b). Most interestingly, we found that the cisplatin-treated cells were sensitized to mortalin sh-2166-induced apoptosis mediated by cleavage of caspase 3 (Figures 5c and d). Cleaved caspase 3 was not detected in control (vector-transfected and cisplatin-treated) groups, demonstrating that the apoptosis was not directly induced by cisplatin at suboptimal dosage used in the present experiment. Instead, it was marked by an increased phosphorylation of p53 at Ser 15 and cleavage of caspase 3 (Figure 5c), as also detected in mortalin shRNA-responsive cell lines shown in Figures 1 and 3. These data demonstrated that the mortalin–p53 interaction occurs in stressed cancer cells, and may serve as an effective target for cancer therapy. In order to confirm that the cisplatin and mortalin shRNA-induced apoptosis (Figures 5c and d) was mediated by p53, we recruited p53 shRNA and p53 inhibitor to rescue the apoptosis in HepG2 cells. As shown in Figure 5e, the apoptosis induced by the combination of cisplatin and mortalin shRNA was significantly reduced (about 80%) either by p53 shRNA or by treatment with p53 inhibitor. In order to prove that the mortalin–p53 interaction was not restricted to cisplatin, we also recruited two other kinds of stress-inducing reagents—hydrogen peroxide (generating free radicals) and doxorubicin (cytotoxic anthracycline antibiotics). As shown in Figure 6, similar to cisplatin, both of these agents caused apoptosis of cells in combination with mortalin shRNA.

Targeting mortalin–p53 interactions reactivates p53-dependent apoptotic pathway in stressed cancer cells. We next targeted mortalin–p53 interaction by a small molecule (MKT-077, a cationic rhodacyanine dye) and a peptide (p53^{312–352}, carboxy-terminal amino-acid residues 312–352 of p53), previously shown to bind to mortalin and reactivate wild-type p53, leading to growth arrest.^{19, 23} When

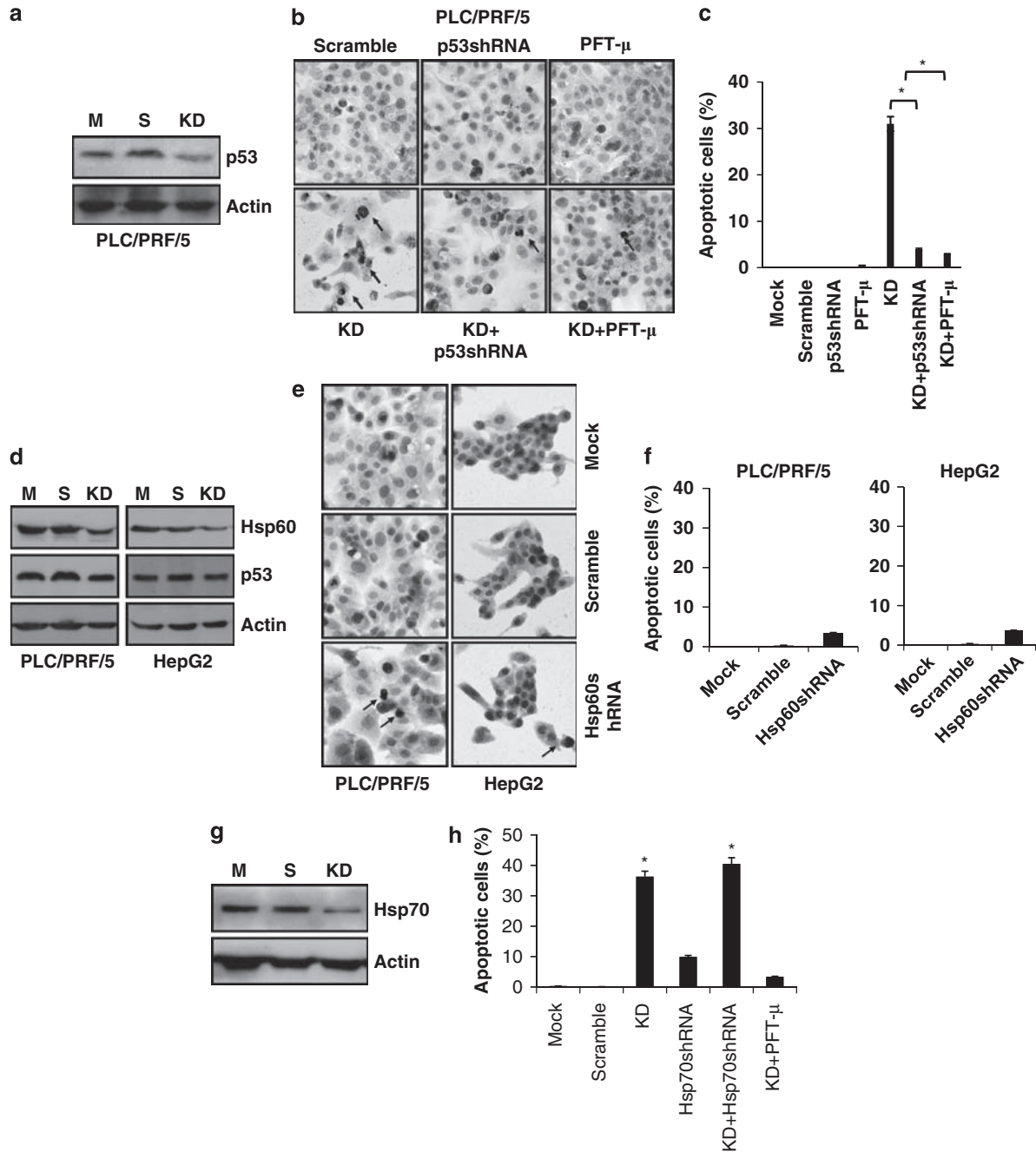


Figure 2 Mortalin silencing-induced apoptosis could be rescued by p53 shRNA and p53-specific inhibitor. (a) Western blot showing p53 knockdown with p53 shRNA in PLC/PRF/5 cells. (b) *In situ* TUNEL staining showing that mortalin knockdown (KD)-induced apoptosis was significantly compromised by treatment with p53 shRNA and p53-specific inhibitor (PFT-μ) in PLC/PRF/5 cells. (c) Quantitation of apoptotic cells is shown (* $P < 0.05$). (d) Western blot showing Hsp60 knockdown. (e) *In situ* TUNEL staining showing that Hsp60 knockdown did not induce apoptosis. (f) Quantitation of apoptotic cells is shown. (g) Cells transfected with Hsp70 shRNA showed reduction in Hsp70 protein. (h) Mortalin knockdown (KD) showed higher level of apoptosis as compared with the Hsp70 knockdown cells, and mortalin knockdown-induced apoptosis was recovered by co-treatment with PFT-μ cells (* $P < 0.05$). Arrows in b and e show the apoptotic cells

subjected to MKT-077 or p53^{312–352} peptide expression, PLC cells (high level of stress as indicated by mutant and phosphorylated p53) underwent apoptosis (Figure 7a). Even with 30–40% efficiency of transient transfections, approximately 12–30% apoptotic cells were observed (Figures 7a and c) at 24–48 h post-treatment or post-transfection, respectively. Consistent with the occurrence of

apoptosis, nuclear translocation and accumulation of p53 were observed (data not shown). In contrast to PLC cells, HepG2 cells that lacked mortalin-p53 interactions (Figure 3) did not show any apoptosis either with p53^{312–352} peptide or with MKT-077 inhibitor (Figures 7a and b). However, when these cells were co-treated with the low dose (2 μg/ml) of cisplatin (stress inducer) that induced mortalin-p53

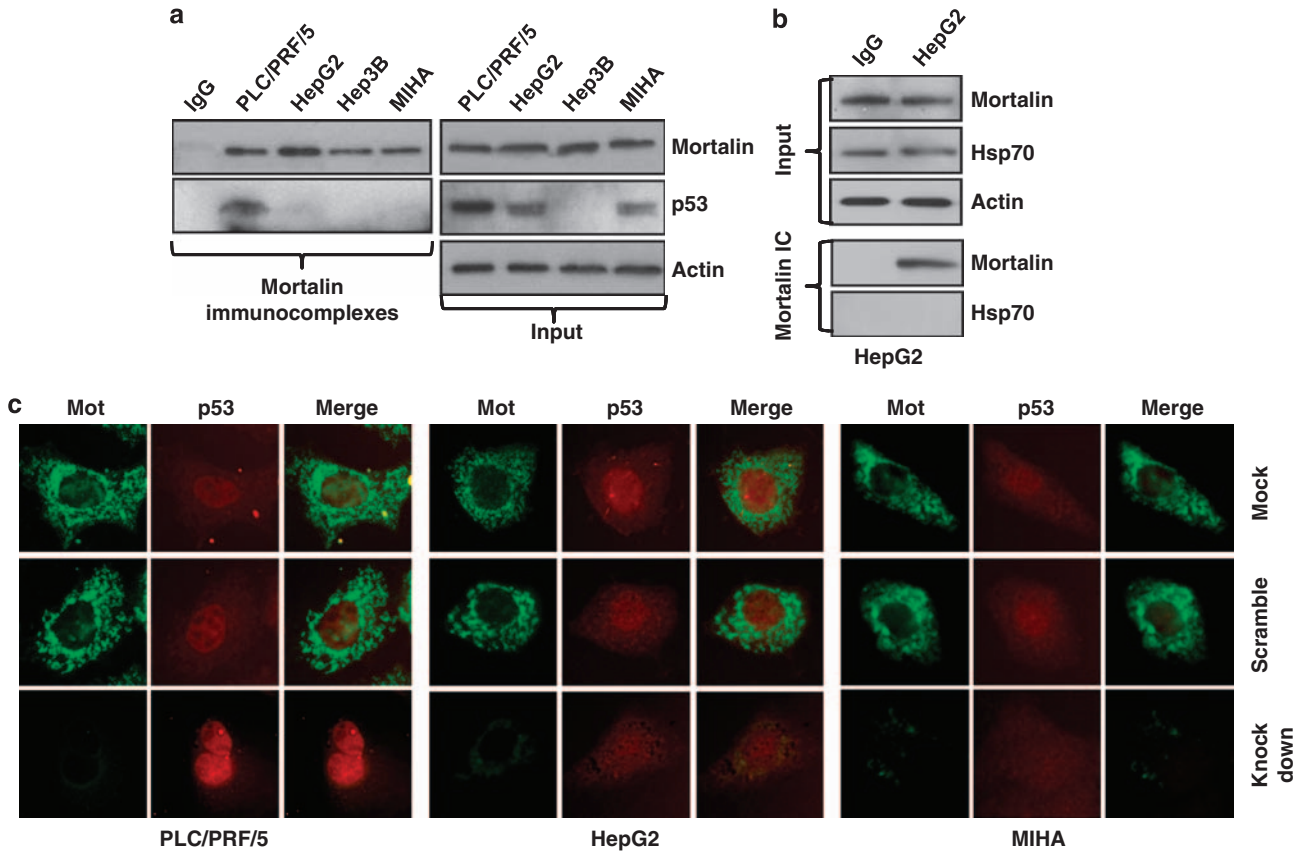


Figure 3 Mortalin-p53 interaction was detected only in PLC, but not in MIHA and HepG2 cells. (a) p53 was detected in mortalin immunocomplexes in PLC/PRF/5. HepG2, Hep3B and MIHA cells lacked this interaction. (b) Mortalin antibody used for immunoprecipitation was specific for mortalin and did not crossreact with Hsp70. (c) Mortalin (green) knockdown induced nuclear translocation of p53 (red) in PLC cells. HepG2 and MIHA cells did not show nuclear translocation of p53 after mortalin knockdown (examined at 72 h post-transfection)

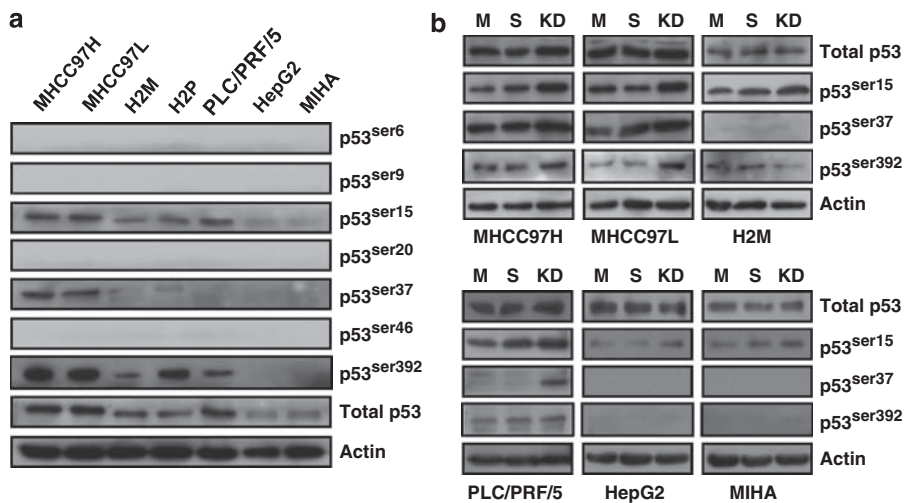


Figure 4 Mortalin shRNA-induced apoptosis depends on mortalin-p53 interaction that in turn depends on the level of cellular stress reflected by p53 phosphorylation. (a) p53 phosphorylation at seven common phosphorylation sites was detected in six HCC and one normal immortalized liver cell line, MIHA. Strong phosphorylation signals at three phosphorylation sites (Ser 15, Ser 37, Ser 392) were detected in 97H, 97L, H2M, H2P and PLC/PRF/5 as compared with HepG2 and MIHA cells, suggesting that the former four cell lines represent cases of higher physiological stress level. (b) Mortalin shRNA-2166 caused an increase in p53 phosphorylation at one or more sites in four HCC cell lines; HepG2 and MIHA did not show any change in p53 phosphorylation (examined at 72 h post-transfection)

interaction (Figure 5), apoptosis and growth arrest were induced. Whereas apoptotic index was less than 3% in HepG2 cells in response to MKT-077 treatment (24 h or

p53³¹²⁻³⁵² expression (48 h), it increased to approximately 11-13% and 21-24% by cisplatin treatment, respectively. Double immunostaining showed nuclear accumulation of p53

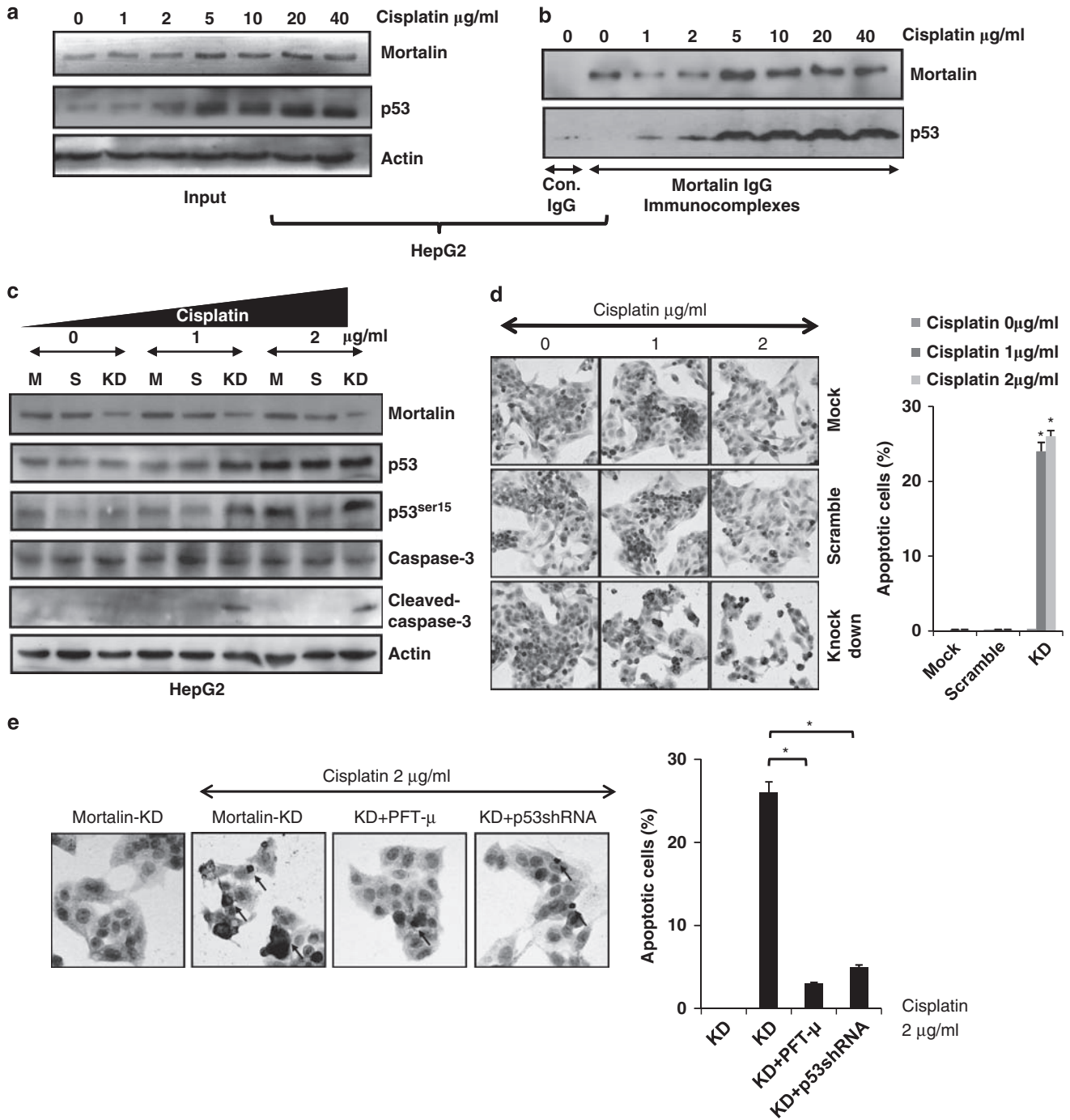


Figure 5 Mortalin-p53 interaction is induced by stress. (a) Exposure of HepG2 cells to cisplatin caused increase in p53 and mortalin. (b) Mortalin-p53 interaction was induced in HepG2 cells by cisplatin treatment (48 h). (c) HepG2 cells were sensitized to mortalin shRNA-induced apoptosis after cisplatin stress-induced mortalin-p53 interactions. Cisplatin-treated cells showed increase in p53-serine15 phosphorylation and caspase 3 cleavage when mortalin shRNA-2166 (KD). (d) *In situ* TUNEL staining of HepG2 cells corresponding to Figure 3c. Apoptosis was observed in cisplatin (1 and 2 $\mu\text{g/ml}$) and mortalin shRNA-2166 (knockdown) cells only. Quantitation of apoptotic cells is shown (* $P < 0.05$). (e) Apoptosis induced by mortalin knockdown (KD) and cisplatin treatment was rescued by either p53 knockdown with p53 shRNA or with a specific inhibitor of p53 (PFT- μ)

in HepG2 cells when treated with cisplatin and MKT-077 (targeting mortalin-p53 interaction) (Figure 8a). Similarly, cells transfected with mortalin-p53 binding antagonist peptide (p53³¹²⁻³⁵²) showed nuclear accumulation of p53 only when treated with cisplatin (Figure 8b).

These data were consistent with our model shown in Figure 9. It proposes that (panel a) normal, immortalized and non-malignant cancer cells have low p53 level that does not interact with mortalin. p53 freely translocates from cytoplasm to nucleus. Mortalin knockdown has no or minimal effect in this

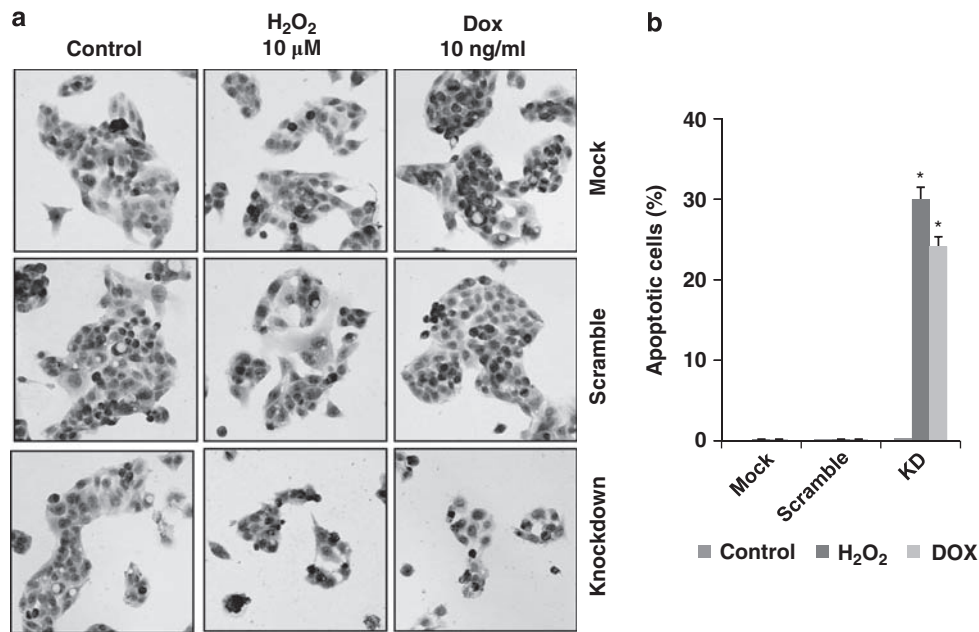


Figure 6 HepG2 cells were sensitized to mortalin knockdown-induced apoptosis by stress treatment. (a) *In situ* TUNEL staining showing both H₂O₂ (oxidative stress) and doxorubicin (DNA damage stress) treatment (48 h) caused apoptosis of HepG2 cells in combination with mortalin shRNA. (b) Quantitation of apoptotic cells is shown (**P* < 0.05)

category of cells; (panel b) mortalin-p53 interaction is induced by cellular stress. Cells exposed to genotoxic or malignancy stress have mortalin-p53 interaction, resulting in abrogation of apoptotic ability of p53. In these cells, mortalin knockdown causes nuclear translocation and activation of p53; finally, (panel c) malignant cancer cells are physiologically stressed and accumulate p53 (mutant) that is inactivated by its interaction with mortalin. In these cells, mortalin knockdown causes nuclear translocation of p53 leading to apoptotic death of cells. According to the proposed model and our present findings, mortalin-p53 interaction is selective for stressed cancer cells, and hence could serve as a safe target for cancer therapeutics. Mortalin shRNA could activate p53-dependent apoptosis selectively in cancer cells either by itself (PLC/PRF/5 cells, Figure 1) or in combination with stress-inducing agent (HepG2, Figures 5 and 6).

We next investigated the mechanism of mortalin shRNA-induced p53-dependent apoptosis. Hep3B (p53-null) cells transfected with either wild-type or mutant p53-expressing plasmids were examined for wild-type p53-specific reporter activity. We found that the silencing of mortalin resulted in increased transactivation function of wild-type p53. However, mutant p53 did not show any wild-type p53-transcriptional activity (Lu *et al*, data not shown). On the basis of these data, we concluded that the mutant p53-mediated apoptosis was independent of its transactivation function. We further investigated whether mortalin knockdown could cause an increase in p53-Bax interactions that may cause apoptosis. Hep3B cells transfected with either wild-type or mutant p53 were examined for p53-Bax and p53-Bcl-2 complexes. As shown in Figures 8c and d, we found that the interaction of p53 and Bax increased significantly in cells that were compromised for mortalin. The effect was observed for both wild-type

and mutant p53. In the same cells, p53-Bcl-2 interaction did not show significant change after mortalin silencing. Taken together, the study suggested that mortalin-p53 interaction abrogates p53-Bax complex formation that is required for the apoptotic function of p53 as suggested in earlier studies.²⁸ Thus, mortalin silencing by shRNA-2166 led to increased p53-Bax complex formation, resulting in apoptosis.

Discussion

p53-mediated apoptosis has been shown to serve as a tumor suppression mechanism to eliminate genetically unstable cells.²⁹ However, in most of the cancers including HCC, such apoptotic functions are often deregulated either by mutations or by binding to other modulating proteins. Mutant p53 is insufficient for binding to DNA and for transcription-activating function. It blocks endogenous wild-type p53 functions in a dominant-negative manner.^{30,31} Besides mutations, the cytoplasmic sequestration of p53 by its binding proteins has been shown to inactivate p53 functions in cancer cells. These include Parc,³ adenovirus E1B protein⁴ and small Hsp alphaB-crystallin.⁵ Similar to Parc,³ mortalin blocks nuclear translocation of p53.¹⁹ The strategies that can restore the tumor suppression and apoptotic function of p53 are promising for cancer treatment.

Heterogeneity of cancer cells is an established feature. It has been shown that cancer cell lines range from immortal, transformed, malignant and aggressively metastatic phenotypes. It has also been established that cancer cells are under a physiologically stressed condition that may vary from one cancer type to another and among different stages of cancer.²⁹ Most cancer cells exhibit high-level expression of Hsp70 family proteins that have crucial roles in tumor

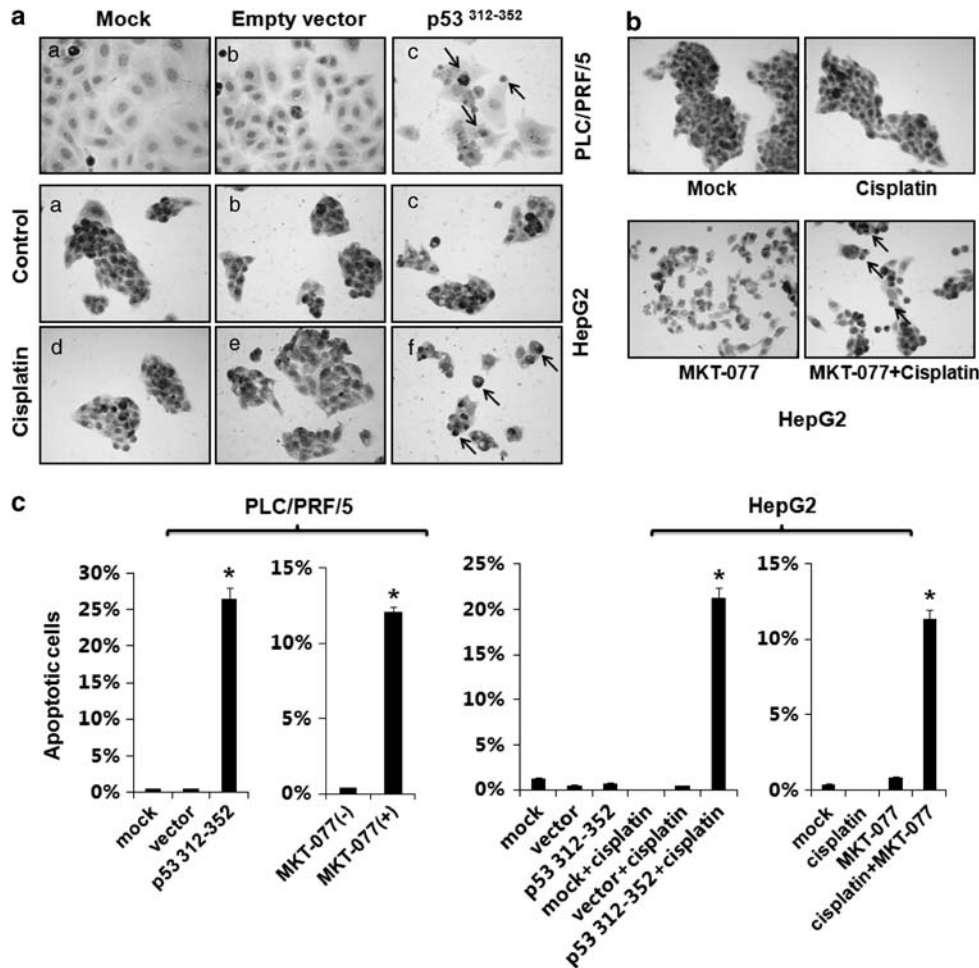


Figure 7 Reactivation of the apoptotic function of p53 by targeting mortalin-p53 interaction with and without stress. Mortalin-p53 interaction was targeted by mortalin-p53 binding antagonists, mortalin-binding peptide p53³¹²⁻³⁵² and cationic dye MKT-077. (a) PLC/PRF/5 cells underwent apoptosis in response to the expression of p53³¹²⁻³⁵² peptide. HepG2 cells (lacking mortalin-p53 interactions) were sensitized to p53³¹²⁻³⁵²-induced apoptosis by cisplatin treatment. (b) HepG2 cells showed no apoptosis in response to either cisplatin or MKT-077 treatment alone. Apoptosis was induced by co-treatment with cisplatin and MKT-077. (c) Quantitation of apoptotic cells was shown (**P* < 0.05)

progression. The level of stress proteins including Hsp70 and Hsp27, and response of p53-Mdm2 feedback loop have been suggested to serve as prognostic and therapeutic markers for cancer cells.³²⁻³⁴ Neutralization of Hsp70 through antisense cDNA transfection resulted in tumor death, independent of p53 status.³⁵ Mortalin-p53 interaction was shown to cause inactivation of both wild-type¹⁹⁻²³ and mutant p53 (Lu *et al*, data not shown) activities, including the deregulation of apoptosis in cancer cells by mechanisms involving formation of nuclear Bax/p53 complexes³⁶ and their crosstalk with nuclear chaperone, nucleophosmin.³⁷ Here, we showed that cancer cells under low stress did not have mortalin-p53 interaction and this was not due to their wild-type p53 status. Both the wild-type and mutant p53 interacted with mortalin in stressed cancer cells. This was validated by using genotoxic stressors such as cisplatin, doxorubicin and hydrogen peroxide. Furthermore, mortalin shRNA-induced apoptosis was exclusive to cancer cells that had phosphorylated p53 (an established marker and barcode of cellular stress).^{6,7} Cells exposed to stress showed mortalin-p53 interaction, and were sensitized to apoptosis by mortalin shRNA and mortalin-p53

binding antagonists, cationic dye (MKT-077) and p53 carboxyl-terminus peptide (p53³¹²⁻³⁵²) (Figure 6). The data demonstrated that the mortalin-p53 interaction is a unique phenotype to cancer cells that are under physiologically stressed conditions. It causes inactivation of transcription-dependent and -independent apoptotic functions of p53, and hence constitutes a selective target to reactivate the apoptotic activities of p53. Taken together, we showed for the first time that (i) mortalin binds to p53 and inactivates its apoptotic function in stressed cancer cells and (ii) anti-mortalin molecules, shRNA, mortalin-binding drug and peptides can rescue the apoptotic activity of (both wild-type and mutant) p53 in cancer cells. Thus, targeting of mortalin warrants further investigation in pre-clinical and clinical trial studies.

Materials and Methods

Cell lines and culture. Human HCC cell lines MHCC97H, MHCC97L, H2P, H2M, PLC/PRF/5, HepG2, Hep3B and an immortalized human hepatocyte liver cell line MIHA³⁸ were cultured in Dulbecco's modified Eagle's medium, a high-glucose medium (Invitrogen, Carlsbad, CA, USA), containing 10% heat-inactivated fetal bovine serum (Invitrogen) in a 5% CO₂ humidified atmosphere at 37 °C.

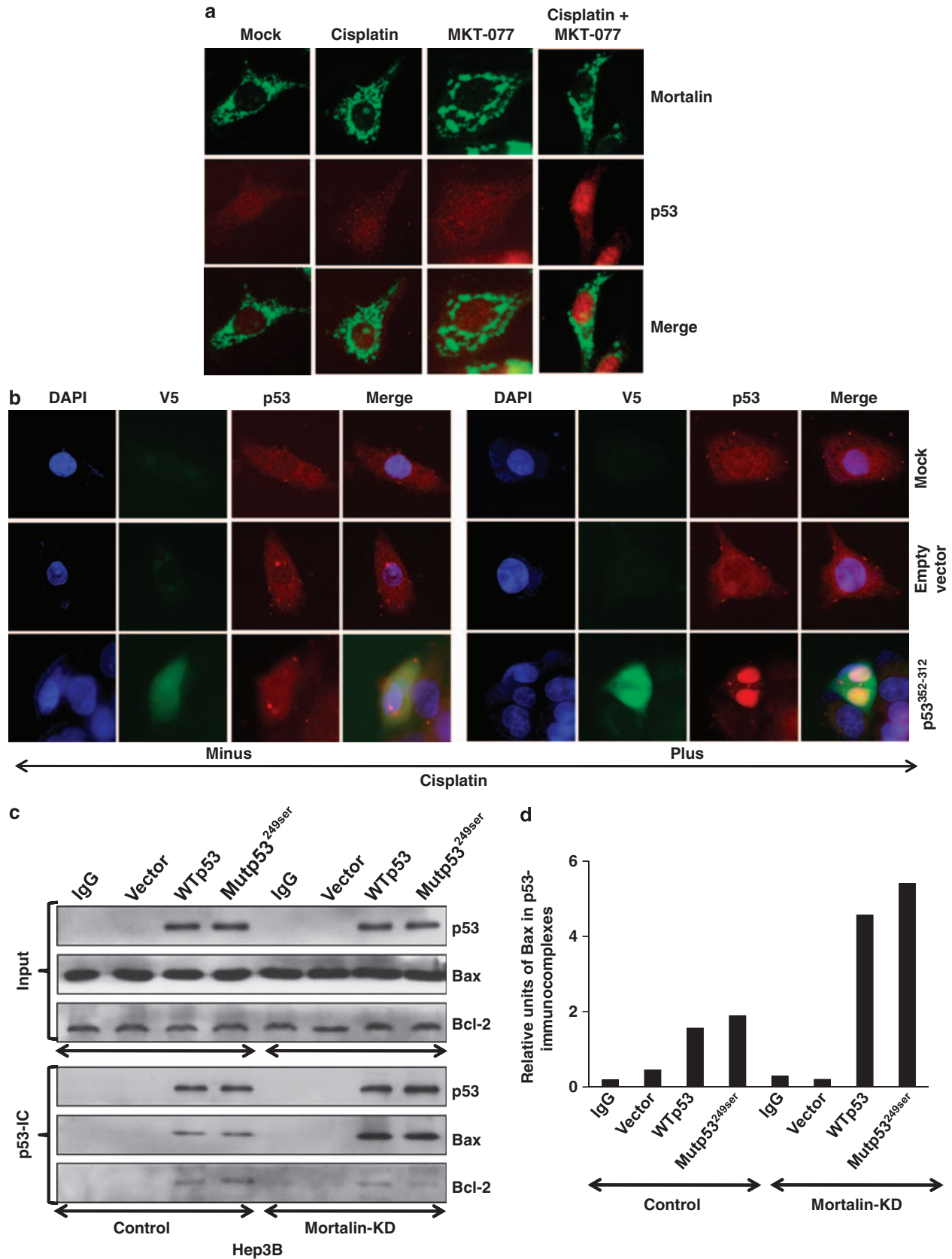


Figure 8 Nuclear accumulation of p53 in HepG2 cells. HepG2 cells treated with either MKT-077 (a) or peptides inhibitor (b) showed nuclear accumulation of p53, only when co-treated with cisplatin. (c) Mortalin knockdown cells (with wild-type or mutant p53) showed increased p53–Bax interaction; the quantitation of Bax present in p53 immunocomplexes in control and mortalin knockdown cells is shown in (d)

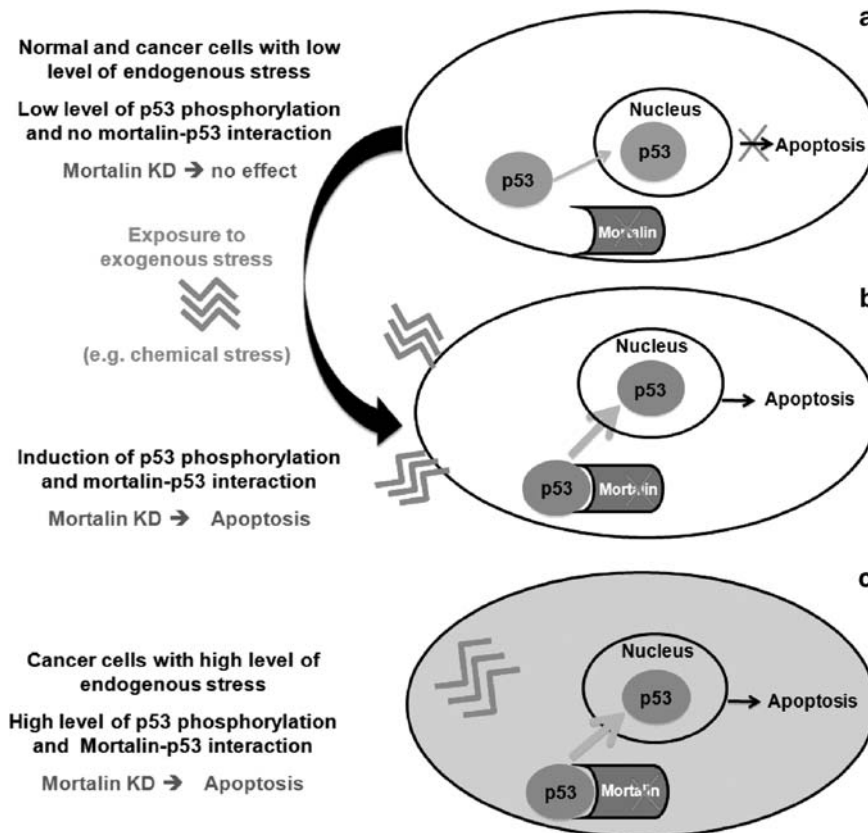


Figure 9 A schematic model showing the effect of mortalin knockdown and nuclear translocation of p53 in cells with different levels of stress. (a) In normal, immortalized and non-malignant cancer cells with low p53 phosphorylation, mortalin does not interact with p53, and hence p53 freely translocates from cytoplasm to nucleus. However, unphosphorylated p53 is inactive, unstable and insufficient for apoptotic activity. Mortalin knockdown has no effect in this category of cells. (b) Exposure of cells to stress (genotoxic or malignancy) causes p53 phosphorylation and accumulation that induces mortalin-p53 interaction. Mortalin silencing by shRNA in this scenario causes nuclear translocation of the phosphorylated p53 that is stable and active, resulting in apoptotic death of cells. (c) In malignant cancer cells with high stress, p53 is heavily phosphorylated and stable. In these cells, mortalin captures p53 in the cytoplasm and blocks its transcriptional activation, growth arrest and apoptotic functions. In this scenario, mortalin knockdown causes nuclear translocation of p53, leading to apoptotic death of cells. According to the proposed model, mortalin-p53 interaction is selective for stressed cancer cells, and hence could serve as a safe target for cancer therapeutics

Antibodies, drugs and plasmids. Antibodies bought were as follows: mortalin (H-155, sc-13967; Santa Cruz, CA, USA), p53 (DO-7; Dako, Carpinteria, CA, USA), phospho-p53 (9919; Cell Signaling, Danvers, MA, USA), actin (clone AC-74, Sigma-Aldrich, St. Louis, MO, USA), caspase-3 (9661 and 9662, Cell Signaling), V5-tag (R960-25, Invitrogen, USA), Hsp70 (4872, Cell Signaling), Bax (2772, Cell Signaling), Bcl-2 (2876, Cell Signaling), MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) (Invitrogen), Pifithrin- μ (PFT- μ , Merck, Whitehouse Station, NJ, USA),²⁶ cisplatin (Sigma-Aldrich), doxorubicin (Sigma-Aldrich), H₂O₂ (Sigma-Aldrich) and MKT-077.²³ The p53 shRNA plasmids were obtained from Addgene (Addgene Cambridge, MA 021399-1666, USA) plasmid depository. The Hsp60 and Hsp70 shRNA plasmids were constructed as described previously.³⁹

Mortalin shRNA plasmid construction and transfections. The following DNA template oligonucleotides corresponding to *HSPA9* gene (GenBank NM_004134) were synthesized: 5'-GCCAGAAGGACAACATATGTTCAGAGACA TATGTTGTCCCTTCTGGCTTTTTTGGAAA-3' (2166). These sequences were inserted into the *Bam*HI and *Hind*III sites of pSilencer2.1-U6 neo vector (Ambion, Austin, TX, USA) and were verified by DNA sequencing. For all the experiments, 3×10^5 cells were transfected with shRNA (2 μ g for single or 1 μ g for combinatorial transfection) using the Lipofectamine 2000 (Invitrogen).⁴⁰

Immunoblotting. Cell lysates were prepared in RIPA buffer, electrophoretically separated on 10% SDS-PAGE gels, electrotransferred to a nylon membrane and probed with the indicated antibody, followed by incubation

with horseradish peroxidase (HRP)-conjugated secondary antibody and ECL detection reagents (Amersham Pharmacia Biotech/GE Healthcare, Piscataway, NJ, USA).

Determination of cell viability. Cells were transfected with different vectors as indicated. Twelve hours post-transfection, each group was re-seeded with an equal density of 4000 cells per well in a 96-well plate. For viability assay, 10 μ l of MTT (5 mg/ml) was added, and plates were placed at 37°C for 2 h. In all, 100 μ l of dimethylsulfoxide (DMSO) was added to each well for cell lysis. Absorbance was measured at 570 nm with the reference filter (655 nm). All the experiments were done in triplicates.

TUNEL assay. Cells were grown on coverslips and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.4). The terminal TUNEL assay was carried out following the manufacturer's instructions (11684817910, Roche Applied Science, Indianapolis, IN, USA). Hematoxylin was used as a counterstain.

Treatment of transfected cells with drugs. Cisplatin (1 and 2 μ g/ml), doxorubicin (10 ng/ml), hydrogen peroxide (10 μ M) and MKT-077 (5 μ g/ml) treatments were given for 48–72 h.

Co-immunoprecipitation assay. Cell lysates (500 μ g protein in 400 μ l RIPA lysis buffer) were pre-cleared with protein A/G PLUS-Agarose beads (sc-2003, Santa Cruz) and were incubated with anti-mortalin antibody at 4°C

overnight. Immunocomplexes were separated by incubation with protein A/G Agarose beads and were resolved on SDS-PAGE. Immunoblotting was performed with antibodies as indicated.

Immunofluorescence staining. Cells were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100 for 15 min, immunostained for mortalin and p53 as described¹⁹ and visualized using an Eclipse E600 image analysis system (Nikon, Tokyo, Japan).

Conflict of interest

The authors declare no conflict of interest.

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