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MK5 is degraded in response to doxorubicin and negatively regulates doxorubicin-induced apoptosis in hepatocellular carcinoma cells

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ABSTRACT

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide. The mechanisms by which hepatoma cells resist apoptosis induced by doxorubicin are largely unknown. MAPKAPK5 (MK5), also named as p38-regulated/activated protein kinase (PRAK), has been identified as a crucial mediator of skin tumorigenesis in mouse and colon cancerogenesis in human. Here, we describe a novel role of MK5 in doxorubicin-induced apoptosis in human hepatoma cells. Expression of MK5 was highly upregulated in hepatoma cell lines. Doxorubicin rather than other chemotherapeutic drugs reduced MK5 protein level in a time- and concentration-dependent manner in hepatoma cells (HepG2 and Hep3B). We further showed that MK5 degradation induced by doxorubicin was *via* the 26S proteasome. Remarkably, stable overexpression of MK5 led to decreased cleavage of caspase-3 and PARP and attenuated doxorubicin-induced apoptosis, while stable knockdown of endogenous MK5 sensitized hepatoma cells to doxorubicin, which was coupled with increased cleavage of caspase-3 and PARP. Taken together, our results firstly demonstrate that MK5 is degraded in response to doxorubicin and negatively regulates doxorubicin-induced apoptosis, providing novel insights into the molecular mechanism of doxorubicin resistance in hepatoma cells.

1. Introduction

Hepatocellular carcinoma (HCC) ranks as the third leading cause of cancer mortality worldwide, with more than 600,000 new cases diagnosed annually [1]. Over the past decade, curative surgery has become the mainstay of treatment for HCC and the first option for patients with early-stage tumors [2,3]. However, many patients have been diagnosed with unresectable HCC at the time of initial diagnosis due to lack of early detection methods [2].

Chemotherapy has proven to be an effective treatment modality especially for patients with inoperable HCC. Doxorubicin (DOX), an anthracycline-based anticancer drug, is widely used for HCC among various chemotherapeutic drugs [4]. DOX acts as a DNA topoisomerase II inhibitor and blocks DNA synthesis through intercalation into the DNA strands, triggering intracellular apoptotic pathways in cancer cells [4]. Apoptosis is a tightly regulated cell suicide process and plays a vital role in tumor regression. DOX-induced apoptosis has been characterized by activation of caspase-3 which subsequently leads to cleavage of PARP [4]. Recent studies have demonstrated that the response rate to DOX becomes lower and lower [5,6]. Therefore, it is of great significance to search for novel molecular targets for developing more effective treatment methods.

Mitogen-activated protein kinase (MAPK)-activated protein kinase 5 (MK5), also described as p38-regulated/activated protein kinase (PRAK), has been identified as a serine/threonine kinase involved in p38 MAPK signaling pathway [7,8]. Previous researches on MK5-deficiency mice indicated that MK5 was an essential factor in tumor suppression [9,10]. The MK5-deficient mice were susceptible to dimethylbenzanthracene (DMBA)-induced skin carcinoma [9]. Intriguingly, a recent study on the same model revealed that once the tumor was formed, MK5 functioned as a critical factor to promote the progression of skin cancer [11]. Activation of MK5 was induced by tumor-secreted proangiogenic factors, resulting in endothelial cell migration and tumor angiogenesis [11]. All these evidence suggest that MK5 plays a dual role in the development of cancer, depending on the tissue type and the stage of carcinogensis. Moreover, MK5 was reported to be down-regulated in colon carcinomas and the negative feedback loop formed by MK5 and Myc was disrupted during colorectal tumorigenesis [10]. Despite the clear roles of MK5 in skin carcinogenesis and colorectal tumorigenesis, there is no evidence indicating that MK5 is involved in hepatocarcinogenesis.

MK5 was originally identified as a downstream target of p38 MAPK [7,12,13]. The members of p38 MAPK group have been

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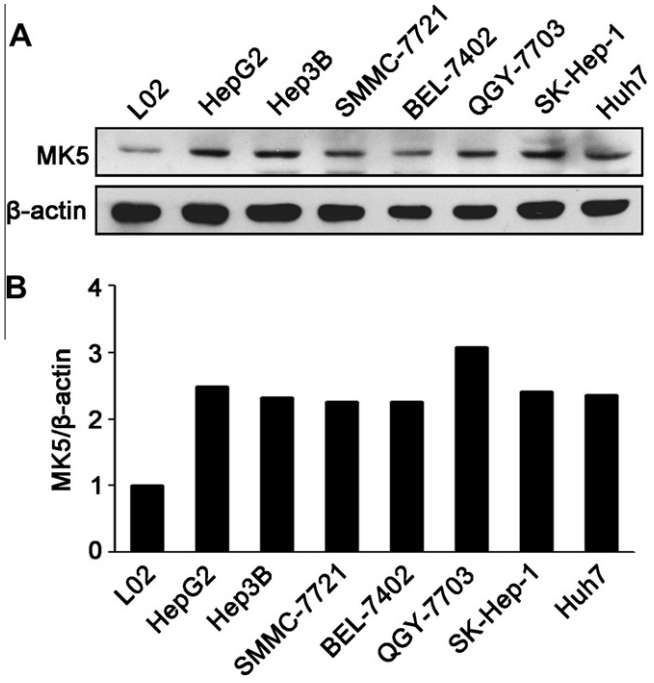

Fig. 1. Upregulation of MK5 expression in hepatoma cell lines. (A) Expression of MK5 protein was detected in seven hepatoma cell lines and the immortalized hepatic cell line L02 by Western blot. β-actin was used as a control for equal loading in all Western blot experiments. (B) Quantification of the immunoblots in panel A. Fold change of MK5 expression with respect to nontumorous specimens was normalized to β-actin.

shown to regulate apoptosis in response to various stimuli [14,15]. Previous findings indicated the involvement of p38 MAPK in both cisplatin-provoked apoptosis in epithelial renal tubule cells and TNF-α-induced apoptosis in differentiated PC12 cells [16,17]. On the basis of these results, we hypothesize that MK5 is likely to be associated with the stress-induced apoptosis.

We describe here the involvement of MK5 in DOX-triggered apoptosis. High expression of MK5 was detected in hepatoma cells. Proteasomal degradation of MK5 was specifically induced by DOX. Stable overexpression or knockdown of MK5 significantly attenuated or promoted, respectively, DOX-induced apoptosis in hepatoma cells.

2. Materials and methods

2.1. Cell lines and cell culture

The human hepatoma cell lines HepG2, Hep3B and SK-Hep-1 were purchased from the American Type Culture Collection. Other cell lines L02, SMMC-7721, BEL-7402, QGY-7703 and Huh7 were obtained from State Key Laboratory of Genetic Engineering, Fudan University (Shanghai, China). All these cell lines have been previously published [18,19]. Cells were maintained in DMEM with 10% FBS at 37 °C in an atmosphere of 5% CO_2.

2.2. Plasmid construction

The full-length *MK5* cDNA was cloned into the pcDNA 3.1/Myc-His vector for establishment of the stable cell lines overexpressing MK5. SuperSilencing shRNATM pGPU6/GFP/NeoU6 vector (purchased from Genepharma Shanghai, China) containing *MK5* target sequences was constructed for establishing MK5 knockdown cell lines. The target sequences are: shMK5-1: 5′-GCAAGCCAGCCAAG TAACA-3′. shMK5-2: 5′-GCAGGAGGCTTGGAAGTAT-3′. The sequence for negative control is: 5′-UUCUCCGAACGUGUCACGU-3′.

2.3. Transient transfection and selection of stable transfectants

For establishing stable cell lines with knockdown of MK5, shMK5-Mix (containing both shMK5-1 and shMK5-2 vectors) was transfected into HepG2 and Hep3B cells using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. At 24 h after transfection, the cells were portioned into new dishes and subject to selection with 500 μg/ml G418 (Invitrogen) for 14 days. Independent colonies were isolated and confirmed by Western blot. Control colonies stably transfected with shCtrl were also generated in parallel. Stable cell lines overexpressing MK5 were generated by the same method.

2.4. Western blot

Cell lysates were subjected to SDS–PAGE and proteins were transferred to nitrocellulose membranes (GE Healthcare). The membrane was blocked in TBS containing 5% non-fat milk and 0.1% Tween-20 for 1 h, and subsequently incubated with primary antibody for 2 h at room temperature, followed by incubation with secondary antibody at room temperature for 1 h. The proteins of interest were visualized using ECL chemiluminescence system (Santa Cruz).

2.5. RNA isolation and RT-PCR

Total RNAs were extracted from cells treated with indicated drugs using the Total RNA Extraction Reagent (Gene Solution, Shanghai, China) according to the manufacturer's protocol. cDNA was amplified using ReverTra Ace kit (Toyobo, Osaka, Japan). The primer sequences used for *MK5* and *GAPDH* are listed as follows: *MK5*, 5′-TCATGACAGGCAGTTTTGAGTT-3′ (forward) and 5′-CCGGTTTGACCTTCAGGA-3′ (reverse); *GAPDH*, 5-AGGGCTGCTTT-TAACTCTGGT-3′ (forward) and 5′-CCCCACTTGATTTTGGAGGGA-3′ (reverse). *GAPDH* was used as an internal control.

2.6. Flow cytometry analysis

For cell apoptosis analysis, doxorubicin (2 μM) was added 48 h before the cells were harvested. Cells were resuspended in PBS containing 0.1% Triton X-100 together with propidium iodide (50 μg/ml) and RNase (100 μg/ml) before being analyzed by FACSCalibur (BD Biosciences). At least 10,000 cells were acquired for each sample. Apoptosis was measured as the percentage of cells with sub-G1 DNA content.

2.7. Statistical analysis

The data in this study were expressed as the mean ± SD from at least three independent experiments. Statistical differences were assessed by Students unpaired *t*-test analysis. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. MK5 expression is upregulated in hepatoma cells

Previous studies have suggested the involvement of MK5 in skin carcinogenesis and colorectal tumorigenesis. To test whether MK5 plays a role in HCC, we firstly determined the expression of endogenous MK5 in hepatoma cell lines and an immortalized normal human liver cell line L02 by Western blot. As shown in Fig. 1A and B, MK5 protein levels were significantly higher in seven hepatoma cell lines than in L02 cells. These data indicate that expression of MK5 protein is frequently upregulated in hepatoma cells.

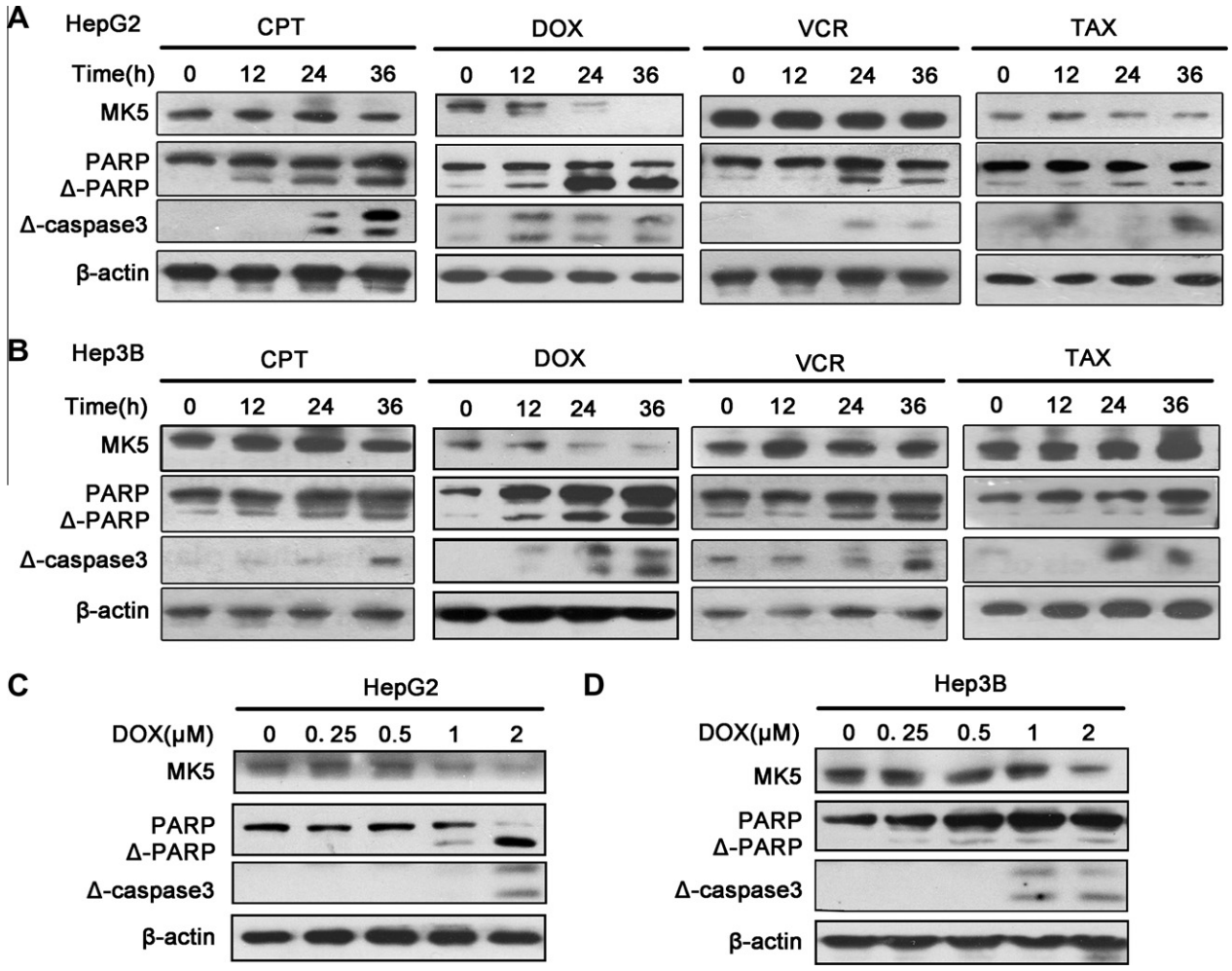

Fig. 2. MK5 is specifically downregulated by DOX in a time- and concentration-dependent manner. (A) Western blot analysis of MK5, cleaved-PARP (Δ-PARP) and cleaved-caspase3 (Δ-caspase3) in HepG2 cells exposed to CPT (2 μM), DOX (2 μM), VCR (2 μM) or TAX (2 μM) for indicated times. (B) Western blot analysis of MK5, cleaved-PARP (Δ-PARP) and cleaved-caspase3 (Δ-caspase3) in Hep3B cells exposed to CPT (2 μM), DOX (2 μM), VCR (2 μM) or TAX (2 μM) for indicated times. (C) Western blot analysis of MK5, cleaved-PARP (Δ-PARP) and cleaved-caspase3 (Δ-caspase3) in HepG2 cells exposed to different concentrations of DOX for 24 h. (D) Western blot analysis of MK5, cleaved-PARP (Δ-PARP) and cleaved-caspase3 (Δ-caspase3) in Hep3B cells exposed to different concentrations of DOX for 24 h.

3.2. MK5 expression is specifically downregulated by DOX in hepatoma cells

In order to examine whether MK5 is involved in hepatoma cell apoptosis triggered by chemotherapeutic drugs, expression of MK5 as well as two apoptotic marker proteins including cleaved caspase-3 and PARP in HepG2 and Hep3B cells treated with drugs were analyzed by Western blot. Two topoisomerase inhibitors (Camptothecin (CPT) and DOX) and two microtubule-targeting agents (Vincristine (VCR) and Paclitaxel (TAX)) were chosen for this test. HepG2 and Hep3B cells were treated with CPT, DOX, VCR or TAX, and harvested at indicated time points. All these drugs induced significant caspase-3 activation, followed by the cleavage of PARP in both HepG2 and Hep3B cells (Fig. 2A and B). Interestingly, MK5 expression was dramatically downregulated specifically in response to DOX in a time-dependent manner. We also monitored the expression of MK5 in response to DOX in another two hepatoma cell lines, SMMC-7721 and SK-Hep-1 cells. As shown in Fig. S1, DOX dramatically induced a decrease of MK5 expression in both hepatoma cells, suggesting that DOX-mediated downregulation of MK5 is probably a general mechanism in hepatoma cells. To determine whether downregulation of MK5 by DOX treatment is also in a concentration-dependent manner, we detected MK5 proteins levels in HepG2 and Hep3B cells exposed to 0, 0.25, 0.5, 1, and 2 μM DOX (Fig. 2C and D). Western blot analysis indicated that increasing concentrations of DOX resulted in decreasing protein levels of MK5, suggesting that DOX reduces MK5 protein level in a concentration-dependent manner. Based on these results, we conclude that MK5 is specifically downregulated by DOX in a time- and concentration-dependent manner.

3.3. DOX promotes MK5 degradation via the 26S proteasome pathway

We showed that MK5 protein level was decreased in response to DOX. On the contrary, the *MK5* mRNA levels in HepG2 and Hep3B cells treated with DOX were not significantly changed (Fig. 3A), indicating that DOX downregulates MK5 expression at the posttranscriptional level. We next investigated the pathway responsible for proteolytic degradation of MK5. Treatment with proteasome inhibitor MG132 increased MK5 protein levels in both hepatoma cell lines (Fig. 3B), suggesting that MK5 stability is regulated through the 26S proteasome pathway. To assess whether DOX regulates MK5 abundance at posttranslational level, we determined MK5 protein levels at the indicated intervals in HepG2 and Hep3B cells treated with or without DOX in the presence of cycloheximide (CHX), a protein synthesis inhibitor. As shown in (Fig. 3C–F), MK5 protein levels were more significantly reduced in DOX-treated cells than in untreated cells under CHX treatment,

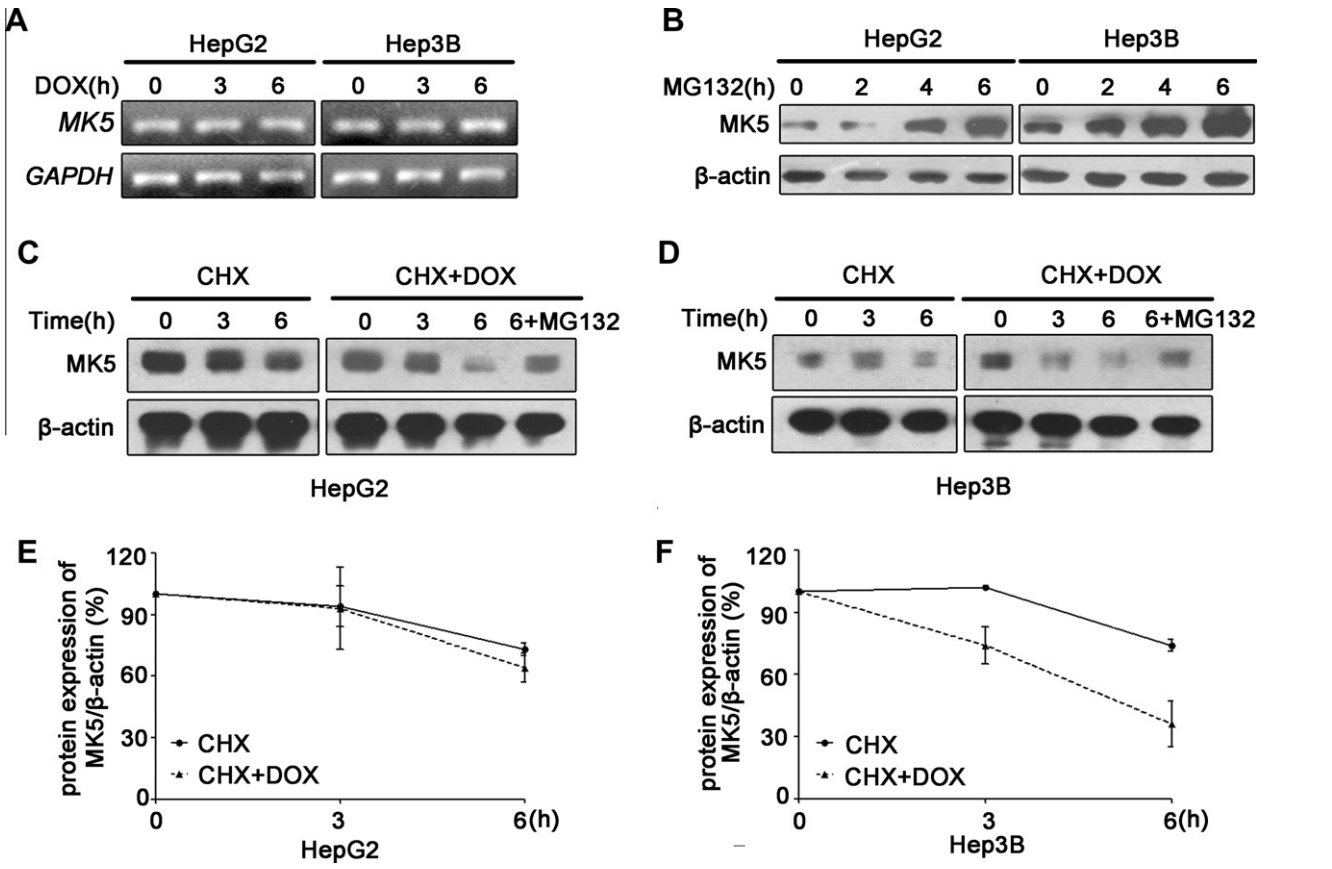

Fig. 3. DOX promotes degradation of MK5 *via* the 26S proteasome. (A) RT-PCR analysis of *MK5* mRNA levels in indicated hepatoma cell lines exposed to DOX (2 μM) for indicated times. *GAPDH* was used as an internal standard. (B) Western blot analysis of MK5 protein levels in indicated hepatoma cell lines exposed to MG132 (20 mM) for indicated times. (C and D) Western blot analysis of MK5 protein levels in HepG2 (C) and Hep3B (D) cells exposed to CHX (40 mM) in the presence or absence of DOX (2 μM) for the indicated times. (E and F) Relative quantification of MK5 protein levels shown in panel C and D. MK5 protein levels were normalized to β-actin. The data are expressed as the means ± SD obtained from three independent experiments.

implying a posttranslational regulation of MK5 stability by DOX. Interestingly, MG132 significantly blocked MK5 degradation at 6 h after DOX treatment (Fig. 3C and D), implying that DOX promotes MK5 degradation through the 26S proteasome pathway.

3.4. MK5 negatively regulates DOX-induced apoptosis in hepatoma cells

p38 MAPK, a direct upstream regulator of MK5, has been shown to mediate apoptosis in response to various stimuli. To identify the functional role of MK5 in mediating DOX-induced apoptosis, we generated stable cell lines with overexpression or knockdown of MK5 in HepG2 and Hep3B cells which were used for further functional analysis (Figs. 4A, B and S2). Hepatoma cells stably overexpressing MK5 and MK5 knockdown cells were treated with DOX and analyzed by FACSCalibur. Upon DOX treatment, significant decrease of apoptosis was observed in HepG2 and Hep3B cells overexpressing MK5 in comparison to the control cells (Fig. 4C). By contrast, knockdown of MK5 facilitated DOX-induced apoptosis in both cell lines (Fig. 4D). Meanwhile, the effects of MK5 on apoptotic resistance of hepatoma cells to CPT, VCR and TAX were not observed (Fig. S3). These physiological results suggest that MK5 is required for resistance to DOX-induced apoptosis in hepatoma cells. Meanwhile, protein levels of two important members in the apoptotic pathway, cleaved caspase-3 and PARP, were determined by Western blot. Compared with the control cells, decreased levels of the cleaved caspase-3 and PARP were observed in HepG2 and Hep3B cells overexpressing MK5 (Fig. 4E), while knockdown of MK5 increased the cleaved caspase-3 and PARP (Fig. 4F). Collectively, our results support the notion that MK5 plays a negative role in DOX- triggered apoptosis in hepatoma cells.

4. Discussion

In the present study, we have identified MK5 expression in hepatoma cells and the functional role of MK5 in DOX-induced hepatoma cell apoptosis. We have showed that MK5 protein is frequently highly expressed in hepatoma cells (Fig. 1). Previous studies have demonstrated that MK5 functions as an important regulator of skin carcinogenesis and colorectal tumorigenesis [10,11]. Our study firstly implies a potential novel function of MK5 in hepatocarcinogenesis. We further show that hepatoma cells stably overexpressing MK5 or MK5 knockdown cells are, respectively, specifically tolerant or sensitive to DOX rather than CPT, VCR and TAX (Figs. 4 and S2). These data suggest the involvement of MK5 in drug-induced apoptosis in a stimulus-depending manner. In addition, we demonstrate that MK5 is specifically responsive to DOX treatment. DOX downregulates the expression of MK5 in a time- and concentration-dependent manner (Fig. 2). These may explain the specific function of MK5 in DOX-triggered apoptosis.

Besides MK5, DOX treatment has been reported to downregulate a number of endogenous proteins [20–22]. However, the exact mechanism of this downregulation is currently unclear. Several studies have implicated that DOX activates ubiquitin–proteasome system and increases the expression of ubiquitin E3 ligases,

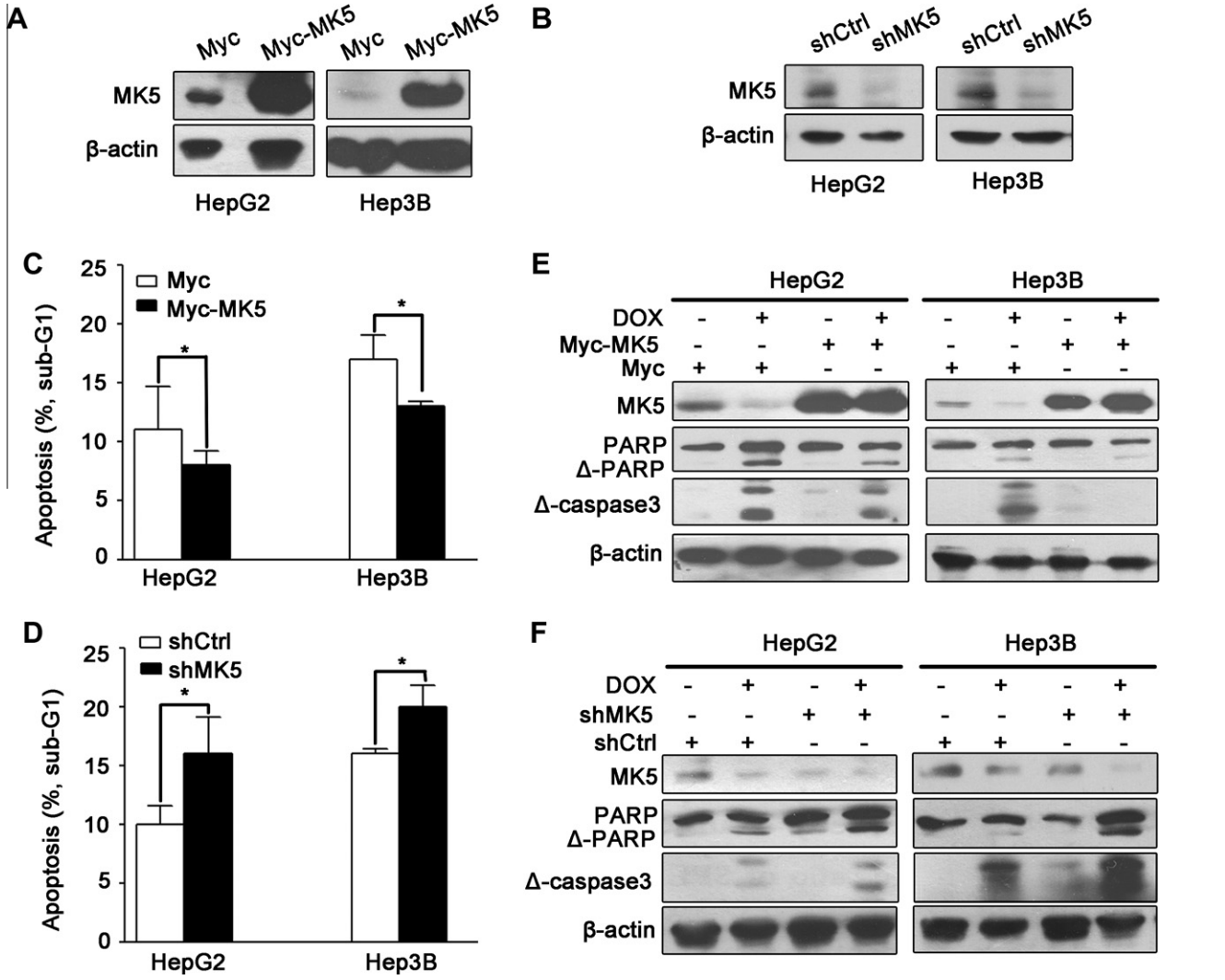

Fig. 4. MK5 is required for resistance to DOX-induced apoptosis in hepatoma cells. (A) Western blot analysis of MK5 expression in indicated hepatoma cell lines stably overexpressing MK5. HepG2 and Hep3B cells were transfected with pcDNA3.1-Myc-MK5 (Myc-MK5) or pcDNA3.1-Myc (Myc) vector and the positive clones were identified and analyzed by Western blot. (B) Western blot analysis of MK5 expression in indicated hepatoma cell lines with stable knockdown of MK5. HepG2 and Hep3B cells were transfected with shRNA vectors targeting *MK5* gene (shMK5) or a control vector (shCtrl) and the positive clones were identified and analyzed by Western blot. (C) Overexpression of MK5 attenuates doxorubicin-induced apoptosis. Indicated hepatoma cell lines overexpressing MK5 were exposed to DOX (2 μM) for 48 h and prepared as described in Section 2. Quantification of the percentage of cells at sub-G1 region was determined by FACSCalibur. (D) Knockdown of endogenous MK5 sensitized cells to doxorubicin. Indicated hepatoma cell lines with knockdown of MK5 were treated and analyzed as described in C. The data in panel C and D are expressed as the means + SD obtained from three independent experiments, $^{*}p < 0.05$ indicates statistical significance. (E) Overexpression of MK5 leads to decreased cleavage of caspase-3 and PARP. MK5-overexpressing cells and the control cells were treated with or without DOX (2 μM) for 24 h, and indicated proteins were analyzed by Western blot. (F) Knockdown of endogenous MK5 leads to increased cleavage of caspase-3 and PARP. MK5-knockdown cells and the control cells were treated with or without DOX (2 μM) for 24 h, and indicated proteins were analyzed by Western blot.

subsequently promoting the degradation of various substrates [23]. In this study, we demonstrate that DOX promotes the degradation of MK5 through the 26S proteasome pathway. However, the E3 ligase responsible for regulating MK5 stability remains to be identified. Recently, *in vitro* ubiquitination and binding assays indicated that MK5 was a potential substrate of Nedd4 [24]. Whether Nedd4 mediates DOX-induced MK5 degradation needs to be further explored.

Up to now, it has been well established that the biological function of MK5 is regulated through phosphorylation by upstream kinases [7,25]. p38α and p38β MAPKs as well as the atypical MAPK ERK3 and ERK4 are able to phosphorylate and activate MK5 [7,25–27]. This activation led to the translocation of MK5 from nuclei to the cytoplasm and subsequent activation of its downstream molecules, such as Hsp27 and F-actin [12,13,27–30]. However, our evidence provides a novel regulatory mechanism of MK5 through its proteasomal degradation. Treatment with proteasome inhibitor MG132 results in accumulation of MK5 protein in hepatoma cell lines (Fig. 3B) and DOX treatment induces MK5 degradation *via* the 26S proteosome (Fig. 3C and D). Poizat et al. (2005) reported that DOX treatment activated p38 MAPKs, which in turn induced the phosphorylation and degradation of p300, subsequently resulting in apoptosis [20]. Based on these results, we hypothesize that DOX-induced MK5 degradation is probably through p38-mediated phosporylation.

In conclusion, we present novel findings showing that MK5 is highly expressed in hepatoma cells. MK5 degradation is specifically induced by DOX through the 26S proteasome pathway. Importantly, MK5 is required for resistance to DOX-induced apoptosis in hepatoma cells.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.09.101.

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