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Knockdown of HURP inhibits the proliferation of hepacellular carcinoma cells via downregulation of gankyrin and accumulation of p53

Tzu-Ching Kuo [a,b], Pu-Yuan Chang [a], Shiu-Feng Huang [c], Chen-Kung Chou [b,d], Chuck C.-K. Chao [a,b,*]

[a] Department of Biochemistry and Molecular Biology, Chang Gung University, Taoyuan 333, Taiwan, ROC
[b] Graduate Institute of Biomedical Sciences, Chang Gung University, Taoyuan 333, Taiwan, ROC
[c] Division of Molecular and Genomic Medicine, National Health Research Institutes, Miaoli 350, Taiwan, ROC
[d] Department of Biomedical Sciences, Chang Gung University, Taoyuan 333, Taiwan, ROC

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ABSTRACT

We determined earlier that the hepatoma upregulated protein (HURP) is overexpressed in hepatocellular carcinoma (HCC), but the role of this protein during cancer development and progression remains unknown. Here, we observed that the overexpression of HURP in HEK293 cells promoted the ubiquitination of p53 and its degradation by the proteasome. In contrast, HURP knockdown using short-hairpin RNA reversed these effects. Knockdown of HURP promoted the accumulation of p53 in SK-Hep-1 cells (p53+/−), and these cells showed reduced proliferation, while the p53-mutant Mahlavu cells were not affected. HURP knockdown did not affect the proliferation of H1299 lung carcinoma cells and Hep3B HCC cells which lack p53. Knockdown of HURP also sensitized SK-Hep-1 cells to cisplatin. On the other hand, the expression of exogenous p53 in H1299 and Hep3B cells was decreased following overexpression of HURP, and these cells showed decreased sensitivity to cisplatin-induced apoptosis. Importantly, overexpression of HURP promoted the proliferation of HEK293 cells in an anchorage-independent manner, and inoculation of SK-Hep-1 cancer cells that expressed short-hairpin RNA to knockdown HURP resulted in smaller tumors in nude mice. Gankyrin, a positive regulator of the E3 ubiquitin ligase MDM2, was found to be upregulated following HURP expression, and gankyrin knockdown decreased the HURP-mediated downregulation of p53. Notably, we detected a positive correlation between elevated HURP and gankyrin protein levels in HCC patients ($r^2 = 0.778$; $N = 9$). Taken together, these results indicate that HURP represents an oncogene that may play a role in HCC progression and chemoresistance.

1. Introduction

Hepatocellular carcinoma (HCC) is associated with the deregulation of several oncogenes and tumor suppressor genes that usually protect the cell from forming cancer outgrowth. We observed earlier that the hepatoma upregulated protein (HURP) is elevated in HCC [1]. HURP represents a spindle-associated protein involved in the formation of spindle bipolarity and the stabilization of microtubule growth toward chromosomes during mitosis [2–4]. HURP also represents a target of aurora-A, a mitotic serine/threonine kinase with oncogenic properties [5]. In this context, phosphorylation of HURP by aurora-A provides a regulatory mechanism for the control of spindle assembly and functions [6]. In addition, HURP undergoes proteolysis via the Fbx7-associated SCF complex which acts as an E3 ubiquitin ligase [7]. However, the role of HURP in HCC and how this protein regulates cell growth and proliferation remain elusive.

The tumor suppression properties of p53 are attributed to its ability to exert suppression of cell growth and proliferation by inducing cell cycle arrest and apoptosis [8,9]. Five to 10% of all human tumors show overexpression of *mdm2*, an E3 ubiquitin ligase that mediates the ubiquitination and proteasomal degradation of p53 [10], a phenomenon which accounts for the development of several cancers, even in the presence of functional *p53* genes [11]. Gankyrin, an ankyrin-repeat oncoprotein [12], is also commonly overexpressed in HCC. Earlier studies have shown that gankyrin binds to the product of the retinoblastoma (Rb) gene and the S6 ATPase subunit of the 26S proteasome subunit, thereby increasing the degradation of Rb [12,13]. It was also found that gankyrin can control the E3 ubiquitin ligase activity of MDM2, thereby increasing p53 ubiquitination [14]. Recently, new evidence showed that the Rb protein may antagonize gankyrin and inhibit the MDM2-mediated p53 ubiquitination in cancer cells [15]. In this study, we show that HURP can act as an oncogene by

* Corresponding author at: Department of Biochemistry and Molecular Biology, Chang Gung University, Taoyuan 333, Taiwan, ROC. Tel.: +886 3 2118136; fax: +886 3 2118700.
E-mail address: cckchao@mail.cgu.edu.tw (Chuck C.-K. Chao).

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reducing the protein levels of the tumor suppressor p53 in both normal and cancer cells. These observations suggest that HURP might play a critical role in the progression of HCC.

2. Materials and methods

2.1. Cell lines and reagents

Human embryonic kidney cells (HEK293) and tumorigenic cell lines, including the lung cancer cells H1299 and the hepatocellular carcinoma cells Hep3B, Sk-Hep-1 and Mahlavu were maintained in Dulbecco's modified Eagle's medium (Gibco, Gaithersburg, MD, USA) supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (100 U/ml; Gibco) and streptomycin (100 μg/ml; Gibco). All cells were incubated at 37 °C in a humidified atmosphere of 5% (v/v) CO_2 in air. Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) or Cell Signaling Technology (Danvers, MA, USA). Chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless indicated otherwise.

2.2. Plasmids and transfection

HURP cDNA sequence (NCBI, NM_014750) was amplified by PCR using the following primers: forward primer (with additional HindIII sequence at the 5′-end), 5′-GAAGCTTATGTCTTCATCACAT-3′; reverse primer (with additional BamHI sequence at the 5′-end), 5′-GGGATCCCAAAATTCTCCTGG-3′. The amplified product was cloned in the pGEM-T easy vector (Promega, Madison, WI, USA). To construct CMV expression vectors, HURP open-reading frame (ORF) sequence was removed from pGEM-T easy vector and inserted in the pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA) using the restriction enzymes HindIII and BamHI. GFP expression plasmids were constructed in a similar manner using specific primers to isolate their ORF (GFP: forward primer, 5′-GGATATCC-TACCGGTCGCCACCATG-3′; reverse primer, 5′-CCTCGAGCTTGTA-CAGCTCGTCCATGCC-3′). Other expression plasmids driven by the CMV promoter included FLAG-HURP [1], HA-MDM2 [14], Myc-ubiquitin [1,7], histidine-tagged ubiquitin (provided by Dr. R.-H. Chen, Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan), pcDNA3.1-p53 [16]), and β-Gal [17]. Cells were transfected with plasmid cDNA and incubated for 48 h as previously described [18].

2.3. Quantitative real-time reverse transcription-PCR (qRT-PCR)

qRT-PCR, or in short qPCR, was performed as previously described [19]. The primers used were the following: p53, forward, 5′-TCAACAAGATGTTTTGCCAACTG-3′; reverse, 5′-ATGTGCTGTGA CTGCTTGTAGATG-3′; gankyrin, forward, 5′-GGAAGAAGCAAAACT GCTGG-3′; reverse, 5′-ACACTGGGGACAACAACACA-3′; and GAPDH, forward, 5′-TCCTGCACCACCAACTGCTT-3′; reverse, 5′-GAGGGGGC CATCCACGTCTT-3′. All unknown samples and controls were done in triplicate. Relative quantification was calculated by the ΔΔCt method and normalized based on GAPDH. Namely, the ΔCt for each candidate was calculated as ΔCt (candidate) = [Ct (candidate) − Ct (GAPDH)]. The relative abundance of the candidate gene X was shown as $2^{\Delta Ct(X)-\Delta Ct(GAPDH)}$.

2.4. Western blot analysis

Whole cell protein extracts were prepared as previously described [19]. Protein concentration was determined using the Bradford assay [19] and the BioRad dye reagent (BioRad, Hercules, CA, USA). Proteins (50 μg) from each sample were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [20], transferred onto PVDF membrane, and incubated with the antibodies according to the instructions of the manufacturer. The signal on the membrane was revealed using enhanced chemiluminescence according to the specifications of the supplier (Pierce, Rockford, IL, USA).

2.5. Knockdown of selected genes by shRNA

pLKO.1 plasmids expressing shRNA were purchased from the National RNAi Core Facility (Academia Sinica, Taipei, Taiwan). Luciferase shRNA (TRCN0000072244) was used as a negative control. Transient transfection was done by adding 2 μg/well (unless indicated otherwise) of shRNA plasmids along with 5 μl/well of Lipofectamine (Invitrogen) into cells suspensions kept in six-well plates (1.5×10^4 cells/well). The plasmids used included *HURP* (TRCN0000062232) and *gankyrin* (TRCN0000058077). The stable clones selected which expressed the shRNA plasmids via lentivirus as vector were established in normal or tumorigenic cells.

2.6. Ubiquitination assay

HEK293 cells were co-transfected for 24 h with 2 μg of expression vectors encoding either HURP or β-gal along with a vector coding for histidine-tagged ubiquitin. HEK293 cells were similarly co-transfected with expression vectors encoding either shHURP or shLuc along with the vector encoding histidine-tagged ubiquitin for 72 h. Cells were treated with MG132 (10 μM) for 6 h before harvesting. Cells were harvested in PBS and then lyzed in lysis buffer A (6 M guanidine–HCl, 10 mM imidazole, 0.1 M Na_2HPO_4/NaH_2PO_4, pH 8.0), and sonicated. Equal amounts of total protein lysates (1000 μg) were incubated with 20 μl of Ni-NTA resin (50% (v/v) histidine-bound resin, Novagen, Merck KGaA, Darmstadt, Germany) for 4 h at 4 °C. Next, the resin was washed twice with buffer A/TI (1 vol buffer A:3 vol buffer TI) and three times with buffer TI (25 mM Tris–HCl, pH 6.8, 20 mM imidazole). The ubiquitinated p53 was analyzed by Western blot using anti-p53 (DO-1) antibodies. HEK293 cells were also co-transfected with 2 μg of expression vectors encoding the indicated genes along with Myc-tagged ubiquitin for 48 h. Cells were treated with MG132 (10 μM) for 6 h before harvesting. Equal amounts of total protein lysates were subjected to IP with anti-HA antibody (F-7, Santa Cruz Biotechnology). The immunoprecipitation products were analyzed by Western blot using anti-myc (9E10, Santa Cruz Biotechnology) or the indicated antibodies.

2.7. Analysis of cell proliferation

We examined the effect of HURP knockdown on cell growth in HCC cells (Sk-Hep-1 and Mahlavu) and HURP expression in lung cancer cells (H1299) as described previously [21].

2.8. Anchorage-independent growth (AIG) assay

HEK293 cells stably expressing HURP or β-gal as well as shGankyrin or shLuc (1×10^4 cells/60 mm dish) were mixed with 3 ml of 0.4% agarose in DMEM containing 5% FBS and overlaid onto a solid layer of 0.4% agarose in DMEM. The cultures were maintained for 3 weeks, and cells were stained with 0.005% crystal violet for colonies counting and were photographed by microscopy. Colonies larger than 0.1 mm (small) or >0.5 mm (large) were counted.

2.9. In vivo studies of HCC tumor xenografts in nude mice

Female nude mice of six weeks of age were purchased from the National Laboratory Animal Center (NLAC, Taiwan). Tumors were

produced by subcutaneous injection of 1×10^7 Sk-Hep-1-shLuc cells and 1×10^7 Sk-Hep-1-shHURP cells in six nude mice each. Tumor size was measured as described before [22]. For these experiments, animal handling and experimental procedures were approved by the Animal Experimental Ethics Committee of Chang Gung University.

2.10. Statistical analysis

The data were reported as mean values ± standard deviation (SD). Three independent experiments were performed unless indicated otherwise. Statistical significance (p value) was calculated with a two-tailed Student's t test for single comparison.

3. Results

3.1. HURP overexpression decreases the level of the tumor suppressor p53

To determine whether the HURP protein affects p53, we expressed a HURP construct in HEK293 cells, and monitored the level of the p53 protein by Western blot. We observed that HURP overexpression increased the level of the anti-apoptotic protein Bcl-2 compared to GFP control while the level of the pro-apoptotic Bax remained unchanged (Fig. 1a). The overexpression of HURP in these experiments also led to a decrease in the accumulation of p53 when compared to the negative control GFP. When mRNA levels were determined by semi-quantitative RT-PCR, we found no difference in the amounts of p53 mRNA following overexpression of HURP or GFP control (Fig. 1b). These results show that HURP overexpression decreases the level of p53 protein and increases the level of the anti-apoptotic protein Bcl-2.

To confirm that HURP affects the stability of the p53 protein, we monitored the stability of p53 after treatment with cycloheximide (CHX), which prevents new protein synthesis. In these conditions, p53 protein levels in cells overexpressing HURP still decreased in a dose-dependent manner (Fig. 1c), showing that HURP affected the stability of p53 and had no effect on the synthesis of this protein. Although p53 slightly decreased in cells overexpressing the control GFP construct (Fig. 1c), quantification of protein levels by densitometry showed that the slope of p53 decrease in HURP-overexpressing cells (slope = −1.502) was 1.5-fold more pronounced than in control cells (slope = −1.033) (Fig. 1d). To confirm these observations, we ectopically overexpressed HURP and p53 in H1299 cells (p53-null) in the presence of CHX. Again, the level of p53 was quickly reduced in cells that overexpressed HURP compared to cells that overexpressed the controls GFP and β-gal (Fig. 1e). Quantification of the protein bands by densitometry produced slopes that were more pronounced for the HURP treatment (slope = −0.643) compared to control (slope = −0.264). 0.264). p53 level decreased 2.4 times faster in HURP-overexpressing H1299 cells compared to control (Fig. 1f). These observations indicate that HURP reduces the stability of the p53 protein in both normal and tumorigenic cells.

3.2. HURP overexpression enhances MDM2-dependent ubiquitination and degradation of p53

The stability of the p53 protein is regulated by its ubiquitination and subsequent degradation by the proteasome. MDM2 is one of the most potent E3 ubiquitin ligase that mediates p53 ubiquitination. To assess the possibility that HURP alters the stability of p53 via MDM2, we monitored the level of MDM2 protein in HEK293 cells. MDM2 protein levels appeared unchanged following overexpression of either HURP or β-gal control (Fig. 2a). While the level of p53 was reduced by HURP overexpression as noted earlier, this effect was prevented by treating the cells with Nutlin-3, which blocks the interaction between MDM2 and p53 (Fig. 2b). Densitometry data revealed significant differences in the level of p53 following treatments with Nutlin-3 in HURP-overexpressing cells (Fig. 2c). In order to exclude any non-specific effects of the inhibitor, we used MDM2 antisense DNA to inhibit protein expression of MDM2. In this condition, we found that the HURP-mediated p53 protein degradation could be reversed by MDM2 knockdown (Fig. 2d). Densitometry data also revealed significant difference in the level of p53 following treatment with MDM2 antisense DNA in HURP-overexpressing cells (Fig. 2e). To verify these results, we monitored the ubiquitination of p53 in the presence of the proteasome inhibitor MG132 (Fig. 2f). To facilitate the detection of ubiquitinated products, an ubiquitin protein tagged with histidine residues was also expressed in these experiments. Notably, the extent of p53 ubiquitination was significantly increased in cells that overexpressed HURP when compared to β-gal control, as revealed by Western blot experiments performed with antibodies reacting against p53 (Fig. 2f). These results suggest that HURP overexpression decreases the stability of p53 via increased MDM2-mediated ubiquitination and proteasome degradation.

3.3. Stabilization of gankyrin and the MDM2/p53/gankyrin complex is involved in HURP-induced p53 degradation

A previous study showed that gankyrin can enhance the MDM2-mediated ubiquitination of p53 and that this phenomenon can decrease the stability of p53 in HCC cells [14]. To elucidate how the E3 ubiquitin ligase activity of MDM2 is enhanced following expression of HURP, we assessed the level of gankyrin in cells that overexpress HURP. As expected, overexpression of HURP caused considerable accumulation of gankyrin in HEK293 cells compared to the β-gal control (Fig. 3a). Kinetic studies revealed that the mRNA levels of gankyrin did not vary in these cells (Fig. 3b). The decrease of HURP protein synthesis following cycloheximide treatment also produced a time-dependent decrease of gankyrin when assessed by Western blot (Fig. 3c). In this case, densitometry analysis of the protein bands showed that gankyrin degradation in HURP-overexpressing cells was about 1.77-fold slower than in control cells (Fig. 3d, slope −0.618 vs. −1.092). Gankyrin knockdown using stable expression of short-hairpin RNA (shRNA) reversed HURP-induced p53 degradation (Fig. 3e, compare lanes 3 and 6). This result was illustrated clearly by monitoring the density of the protein bands (Fig. 3f). These observations show that HURP affects the stability of p53 in a gankyrin-dependent manner.

3.4. HURP inhibits the MDM2-mediated ubiquitination and degradation of gankyrin

We have shown above that HURP caused gankyrin protein accumulation and enhanced the MDM2-mediated ubiquitination and degradation of the p53 protein. Next, we examined the manner in which HURP stabilizes the gankyrin protein. We hypothesized that MDM2 may act as an E3 ligase for gankyrin following p53 degradation. To assess this possibility, we overexpressed MDM2 for 48 h, and monitored the protein level of gankyrin in HEK293 cells. We observed reduced gankyrin protein levels in MDM2-overexpressing cells, and this effect could be reversed by overexpressing HURP (Fig. 4a). Furthermore, when we treated the cells with MG132 to block proteasome-mediated protein degradation, we found that ubiquitinated gankyrin increased, and that this ubiquitination could be decreased by overexpressing HURP (Fig. 4b). In this immunoprecipitation (IP)-Western blot assay, we did not observe any interaction between HURP and gankyrin or MDM2. We found that HURP overexpression did not affect the interaction between MDM2 and gankyrin. These data suggest that

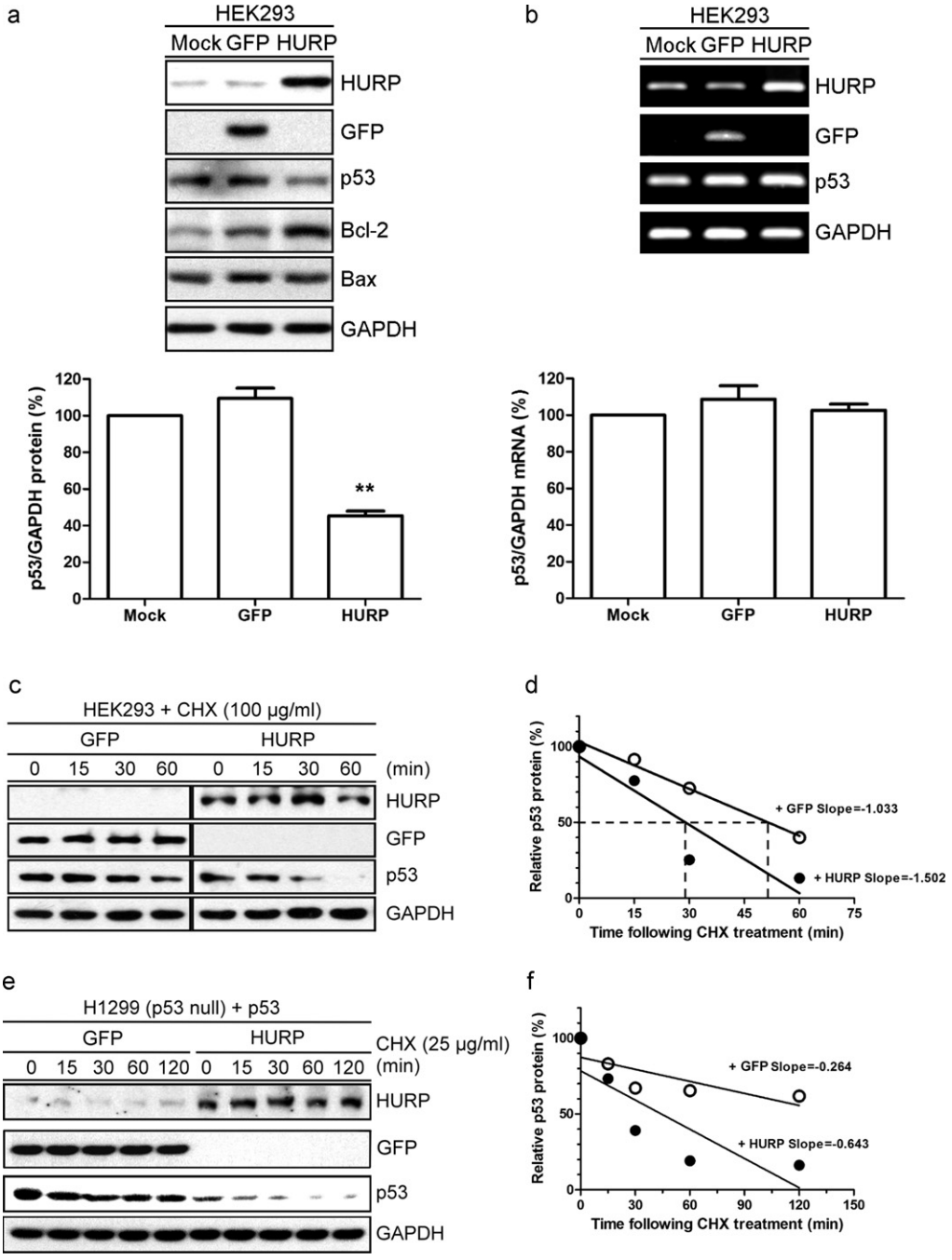

Fig. 1. Overexpression of HURP decreases the protein level of the tumor suppressor p53. (a) Western blots showing Bcl-2 and Bax protein levels after transfection of HURP plasmid (2 μg). Densitometry quantification of the protein bands was presented at bottom. (b) mRNA levels of HEK293 cells transfected with GFP or GFP-HURP were analyzed by semi-quantitative RT-PCR. Quantification of the mRNA bands was presented at bottom. The results represent means ± SD of experiments performed in triplicate. Statistical significance was expressed as $^{**}p < 0.01$ when compared to GFP-transfected cells. (c) HURP promotes the degradation of p53 protein. Plasmids coding for GFP or HURP were transfected in HEK293 cells for 24 h, followed by treatment with cycloheximide (CHX) for the time indicated. The degradation of p53 protein was examined by Western blot. (d) The amounts of p53 protein shown in (a) were quantified using densitometry and the results were plotted as shown. (e) β-gal, HURP, MDM2, p53, and/or GFP were transfected in H1299 lung cancer cells for 24 h, followed by treatment with cycloheximide for the time indicated. GFP expression was indicated for transfection efficiency and β-gal was used as a control. The degradation of p53 protein was examined by Western blot. (f) Amounts of p53 protein observed in (e) were quantitated using densitometry analysis.

HURP decreases the MDM2-induced ubiquitination of gankyrin, but that this process does not occur through disruption of the interaction between these two proteins. In order to confirm the existence of the proposed E3 ligase complex, we blocked proteasome-mediated protein degradation by treating cells with MG132. Afterwards, p53 was immunoprecipitated with anti-p53 antibodies (DO-1). In this p53 complex, we observed that more MDM2 and gankyrin was bound to p53 in HURP-overexpressing cells than in control cells. However, IP assays of p53 brought down MDM2 and gankyrin, but not HURP (Fig. 4c). These results suggest that HURP blocks the MDM2-mediated protein degradation of gankyrin. In turn, the accumulation of gankyrin subsequently enhances the MDM2-mediated ubiquitination and degradation of p53.

3.5. Reduced accumulation of p53 following HURP knockdown

Next, we assessed the effect of HURP knockdown on the level of p53 in HEK293 cells. We observed that p53 was slightly increased

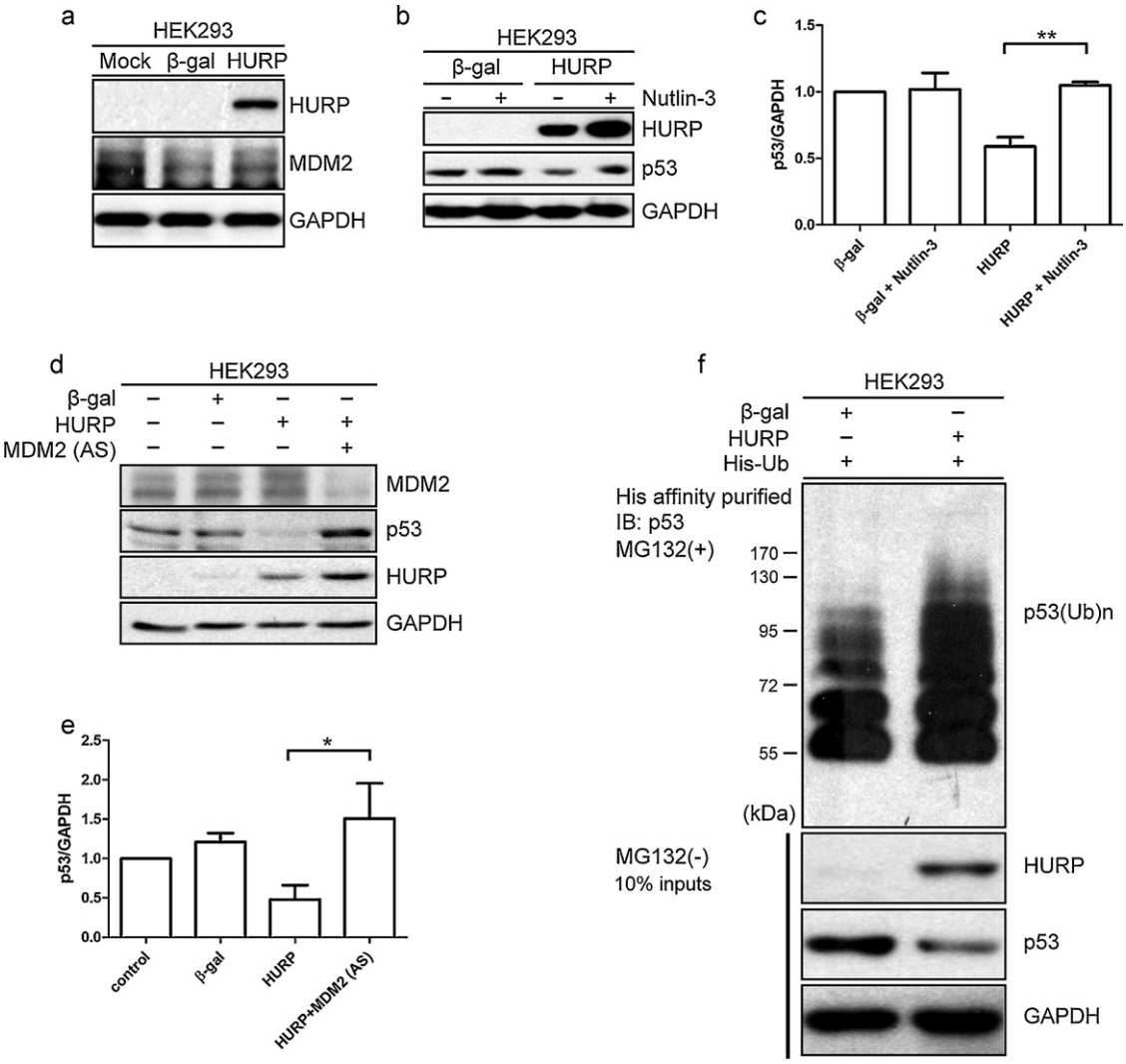

Fig. 2. HURP-induced degradation of p53 is dependent on the E3 ubiquitin ligase MDM2. (a) Representative Western blots showing the protein levels of MDM2 from HEK293 cells transfected with β-gal or HURP. Overexpression of HURP did not affect the protein level of MDM2. (b) β-gal or HURP were transfected in HEK293 cells for 24 h, followed by treatment with the MDM2 inhibitor Nutlin-3 (20 μM) for 12 h. p53 protein levels were examined by Western blot. (c) Amounts of p53 protein from (b) were quantified using densitometry and were normalized to GAPDH. The values shown represent means ± SD with statistical significance being expressed as $^{**}p < 0.01$ versus control β-gal-transfected cells. (d) β-gal, HURP and/or MDM2 antisense were transfected in HEK293 cells for 48 h, and protein levels of p53, MDM2 and HURP were presented by Western blots, representatively. (e) Amounts of p53 protein from (d) were quantified using densitometry and were normalized to GAPDH. The values shown represent means ± SD with statistical significance being expressed as $^{*}p < 0.05$ versus MDM2 antisense untransfected cells. (f) β-gal, HURP and/or His-ubiquitin were transfected in HEK293 cells for 24 h, followed by treatment with the proteasome inhibitor MG132 (10 μM) for 6 h. Ubiquitinated p53 was collected by His affinity purification and the level of p53ubiquitination was examined by immunoblotting (IB).

after HURP knockdown compared to control shLuc (Fig. 5a). Quantification of Western blot results confirmed this observation and showed that p53 level was 1.5-fold higher following knockdown of HURP (Fig. 5b). To examine whether ubiquitination was altered following the knockdown of HURP, we prepared HEK293 cells expressing either shLuc or shHURP, along with myc-ubiquitin, treated them with MG132, and used them for IP experiments. After p53 was immunoprecipitated, the ubiquitination of p53 was examined by Western blot with antibodies directed against either ubiquitin or p53. We noticed a low ubiquitination of p53 following knockdown of HURP compared to shLuc control (Fig. 5c). Consistent with our previous results, these experiments suggest that silencing of HURP using shRNA decreases the ubiquitination and degradation of p53.

3.6. Knockdown of HURP reduces cell proliferation by reducing gankyrin and p53

p53 is known to inhibit cell growth under conditions of cellular stress. To determine the effect of HURP on cell proliferation, we performed HURP knockdown in the HCC cell lines SK-Hep-1 and Mahlavu, which endogenously express wild-type and mutant p53, respectively. HURP knockdown in SK-Hep-1 cells which express low levels of HURP slightly decreased gankyrin and increased p53. On the other hand, HURP knockdown in Mahlavu cells, which express HURP at high levels, also decreased gankyrin but appeared to slightly decrease p53 (Fig. 6a). Densitometry analysis of Western blots experiments confirmed these observations and showed that p53 did not accumulate in Mahlavu cells after knockdown of HURP (Fig. 6a, bottom panel). Next, the proliferation of these cells was monitored using the MTT assay. The proliferation of SK-Hep-1 cells expressing shHURP was retarded compared to that of shLuc control (Fig. 6b). In contrast, this effect was not found in Mahlavu cells (Fig. 6c). These observations are consistent with the fact that SK-Hep-1 cells have one wild-type allele of the *p53* gene whereas Mahlavu cells have mutations that inactivate both *p53* alleles. We repeated these experiments in Hep3B cells which are p53-null and which express low levels of HURP. We observed that gankyrin accumulated following HURP overexpression in Hep3B cells

(Fig. 6d, compare lanes 1 and 2). Overexpression of p53, however, was reduced in Hep3B cells that co-expressed HURP (Fig. 6d, compare lanes 3 and 4). The proliferation rate of Hep3B cells expressing either HURP or GFP was similar (Fig. 6e). Interestingly, HepG2 cells which expressed p53 showed a higher proliferation rate in the presence of HURP compared to GFP control cells (Fig. 6f). We further repeated these experiments in H1299 cells which are p53-null and which express low levels of HURP. We also observed the positive regulation of HURP overexpression on gankyrin and proliferation rate in H1299 cells which overexpressed p53 (Fig. S1). These results support the notion that HURP regulates cell proliferation in a p53-dependent manner.

Supplementary material related to this article found, in the online version, at doi:10.1016/j.bcp.2011.12.034.

3.7. HURP promotes the transformation of HEK293 cells and enhances HCC tumor growth in vivo

To determine whether HURP represents an oncogene involved in cancer transformation and progression via accumulation of gankyrin, we overexpressed HURP and performed a knockdown of gankyrin in non-tumorigenic HEK293 cells, before monitoring the formation of colonies in soft agarose. HURP overexpression dramatically increased the formation of colonies compared to β-gal control (Fig. 7a, compare panel (i) and panel (ii)). The transformation effect of HURP on HEK293 cells could be reduced by knockdown of gankyrin (Fig. 7a, compare panels (iii) and (iv)). These effects were illustrated clearly by determining the number of colonies for each treatment (Fig. 7b). Next, we tested the effects of HURP on the progression of tumors produced by inoculation of SK-Hep-1 cancer cells in nude mice. We observed that the tumors that formed in mice injected with HURP-knockdown cancer cells were much smaller than those of control mice inoculated with cells expressing shLuc (Fig. 7c, compare the right panels with the left ones; representative tumors were encircled for clarity). The average size of the tumors found in six mice corresponding to each group was determined, and this analysis confirmed that tumors were considerably smaller in the mice that were inoculated with HURP-knockdown cells (Fig. 7d). Finally, we assessed whether HURP could play a role in preventing cisplatin-induced cell death in cancer cells. Cisplatin treatments of SK-Hep-1 cells decreased cell proliferation in a dose-dependent manner as revealed by the MTT assay (Fig. 7e). Interestingly, cisplatin-induced cell death was accentuated when HURP was down-regulated using shRNA (Fig. 7e). This effect could be reversed by treating the cells with the p53 inhibitor PFT-α. Furthermore, we observed that the co-knockdown of HURP and p53 produced higher cell viability in response to cisplatin treatment compared to

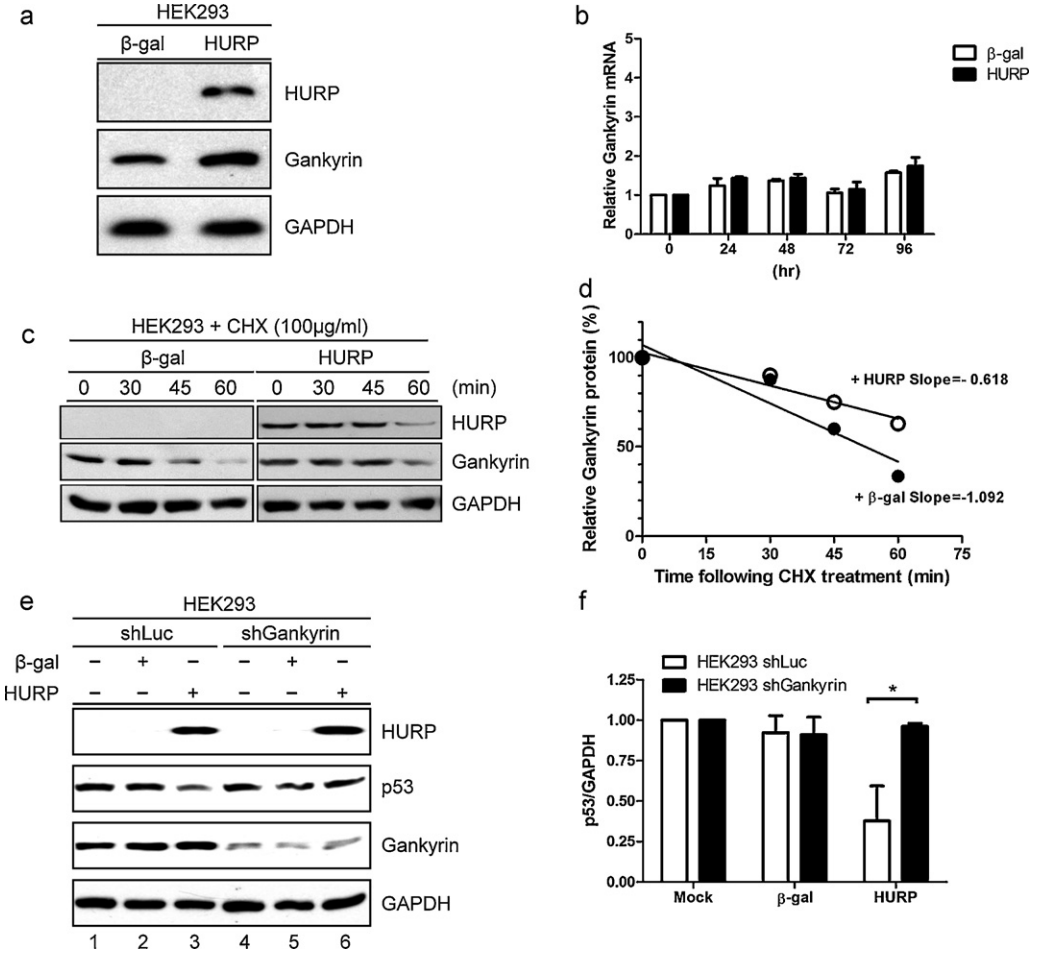

Fig. 3. HURP promotes the degradation of p53 by stabilizing gankyrin and by enhancing MDM2/p53/gankyrin complex formation. (a) Western blots of HEK293 cells transfected with β-gal or HURP showing the protein level of gankyrin. Overexpression of HURP led to accumulation of gankyrin. (b) Relative gankyrin mRNA levels of β-gal- or HURP-transfected HEK293 cells were determined by quantitative RT-PCR. Overexpression of HURP did not affect the mRNA level of gankyrin. (c) Plasmids coding for β-gal or HURP were transfected in HEK293 cells for 48 h, followed by treatment with cycloheximide (CHX) for the indicated. The degradation of gankyrin protein was examined by Western blot. (d) Levels of gankyrin protein observed in (c) were quantitated using densitometry. (e) HEK293 cells stably expressing shLuc or shGankyrin were transfected with β-gal or HURP for 48 h and the expression of p53 and gankyrin was examined by Western blot. (f) Protein levels of p53 were quantified using densitometry and normalized to GAPDH. The experiments were performed in triplicate ($^*p < 0.05$).

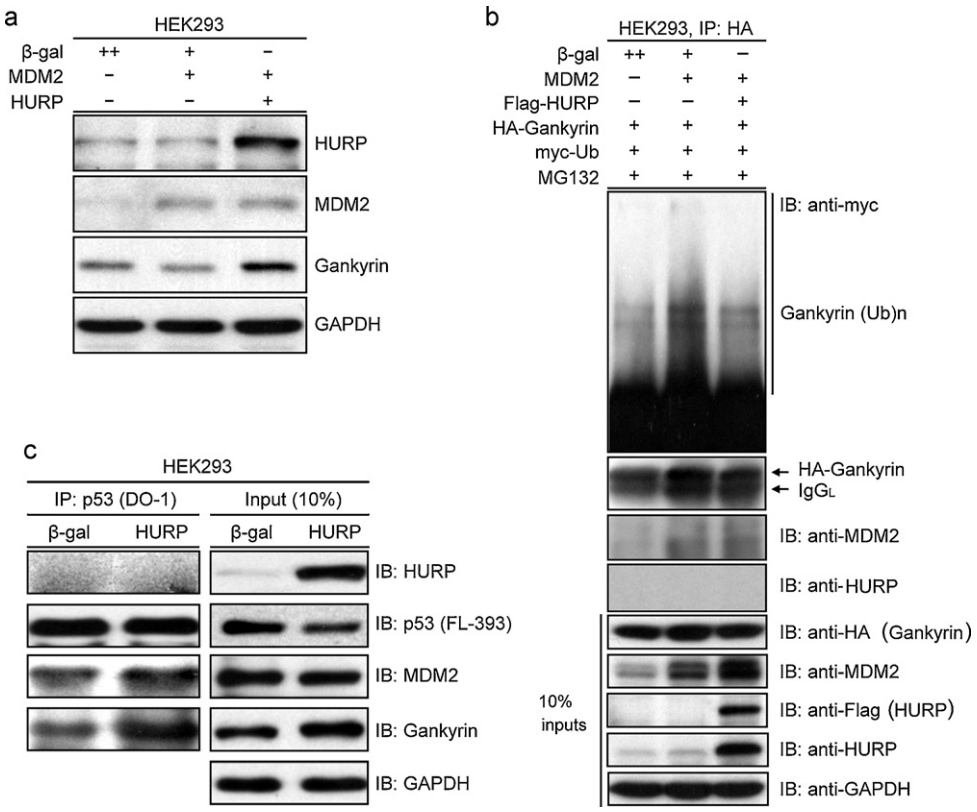

Fig. 4. HURP blocks MDM2-mediated ubiquitination and degradation of gankyrin. (a) Plasmids coding for MDM2 or HURP were transfected in HEK293 cells for 48 h. The gankyrin protein level was examined by Western blot. (b) HEK293 cells were transfected with HA-gankyrin, myc-Ub, MDM2, β-gal or HURP for 48 h, followed by treatment with MG132 (10 μM) for 6 h. After HA-gankyrin was immunoprecipitated (IP) with anti-HA antibodies (F-7), the level of its ubiquitination was examined by immunoblotting (IB). Proteins associated with the immunoprecipitated complexes were assessed by Western blot using the indicated antibodies. (c) HEK293 cells were transfected with β-gal or HURP for 24 h, followed by treatment with MG132 (10 μM) for 6 h. After p53 was immunoprecipitated with anti-p53 antibodies (DO-1). Proteins associated with the immunoprecipitated complexes were assessed by Western blot using the indicated antibodies.

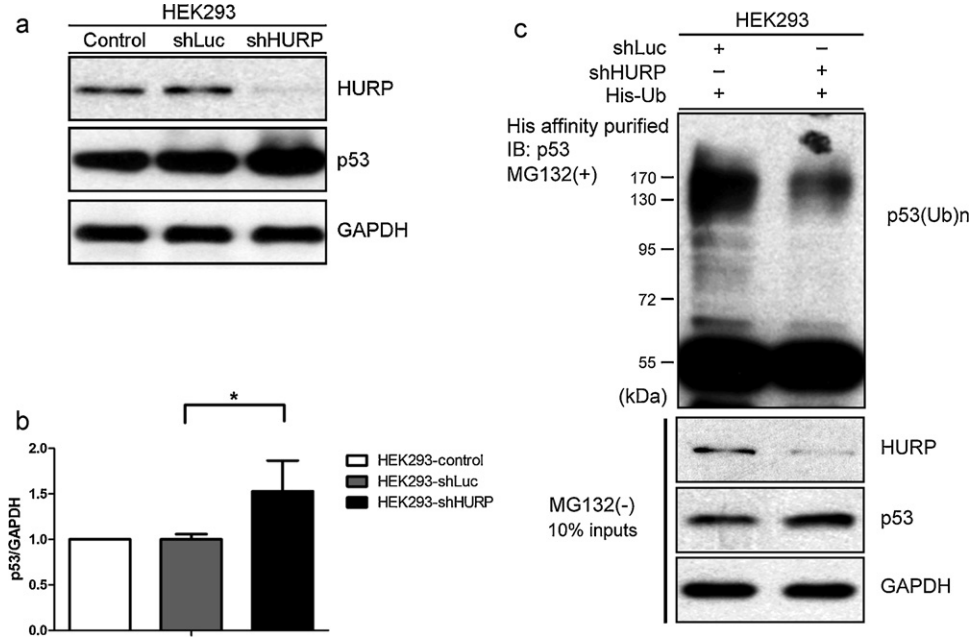

Fig. 5. Knockdown of HURP promotes the accumulation of p53. (a) Western blots of HEK293 cells stably expressing Luc or HURP shRNA were processed to determine the levels of p53. (b) Densitometry quantification of the p53 bands shown in (a) and normalized to GAPDH. The values shown represent mean ± SD of experiments performed in triplicate (*$p < 0.05$ versus shLuc cell line). (c) Luc shRNA, HURP shRNA, and/or histidine-ubiquitin were transfected in HEK293 cells for 72 h as indicated, followed by treatment with MG132 (10 μM) for 6 h. After His affinity purification, p53 ubiquitination was examined by Western blot.

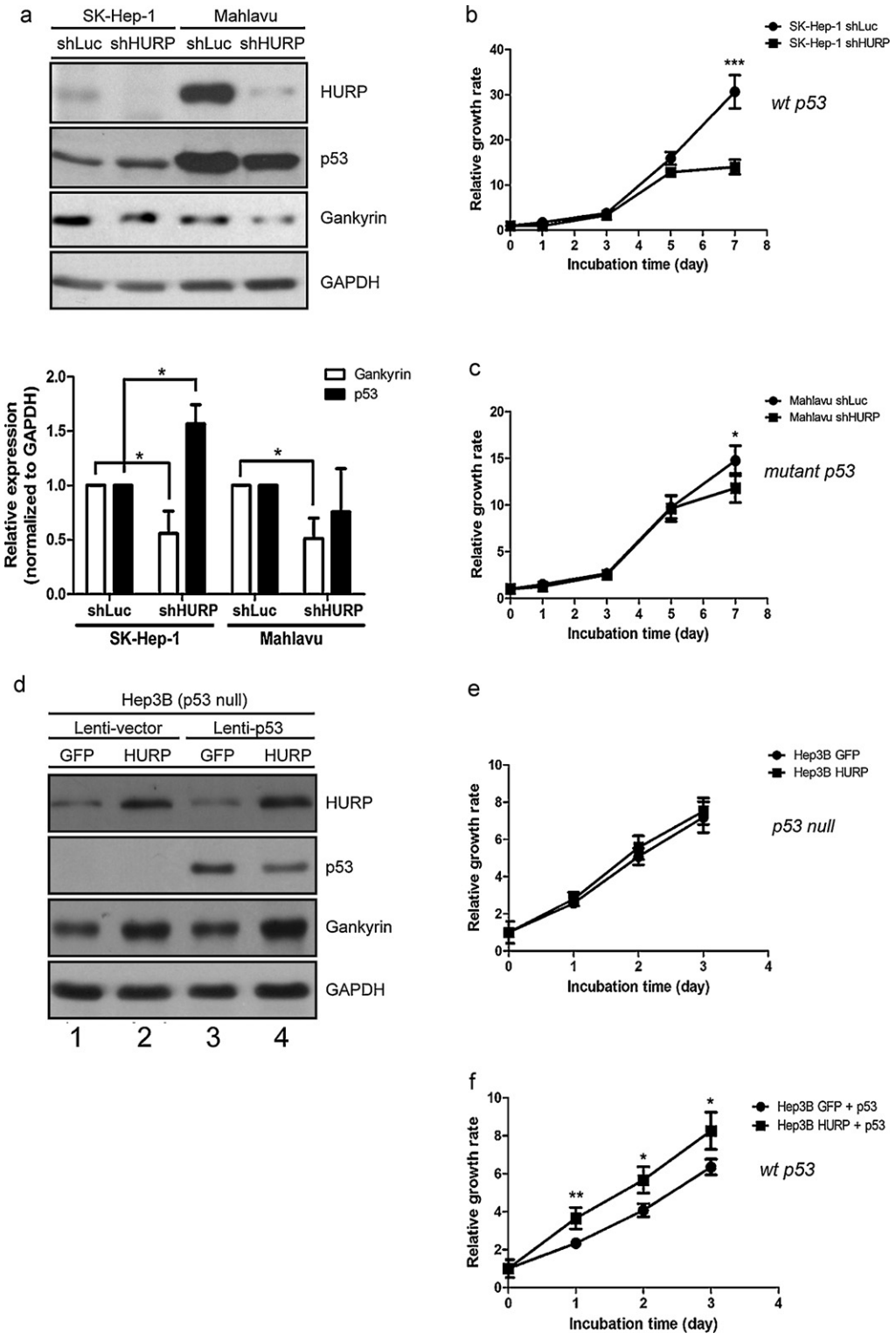

Fig. 6. HURP controls the expression of gankyrin and the proliferation of hepatoma and lung carcinoma cells in a p53-dependent manner. (a) Western blots experiments of hepatoma cell lines stably expressing shLuc or shHURP. Protein levels of gankyrin were quantitated using densitometry and were normalized to GAPDH (bottom panel). MTT cell proliferation assays of the hepatoma cell lines SK-Hep-1 (b) and Mahlavu (c) expressing the indicated shRNA. The status of the p53 gene for each cell line was indicated as a reference. (d) Representative Western blots of Hep3B hepatoma cells overexpressing either GFP or HURP as well as the empty lentivirus vector or the lentivirus vector coding for p53. (e) MTT cell proliferation assay of Hep3B cells expressing either GFP or HURP. (f) MTT assay of Hep3B cells co-expressing either GFP or HURP as well as p53. The MTT assays represent means ± SEM of experiments performed in quadruplicate ($^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ versus shLuc control or β-gal-transfected cells).

HURP knockdown alone (Fig. 7f). Taken together, these results indicate that HURP promotes anchorage-independent cell growth and stimulates tumor progression in vivo. In addition, HURP appears to be involved in blocking cisplatin-induced cell death.

3.8. Correlation between HURP and gankyrin proteins in HCC patients

We also wanted to determine whether gankyrin accumulates in liver tumors that overexpress HURP. Western blots of HCC patient

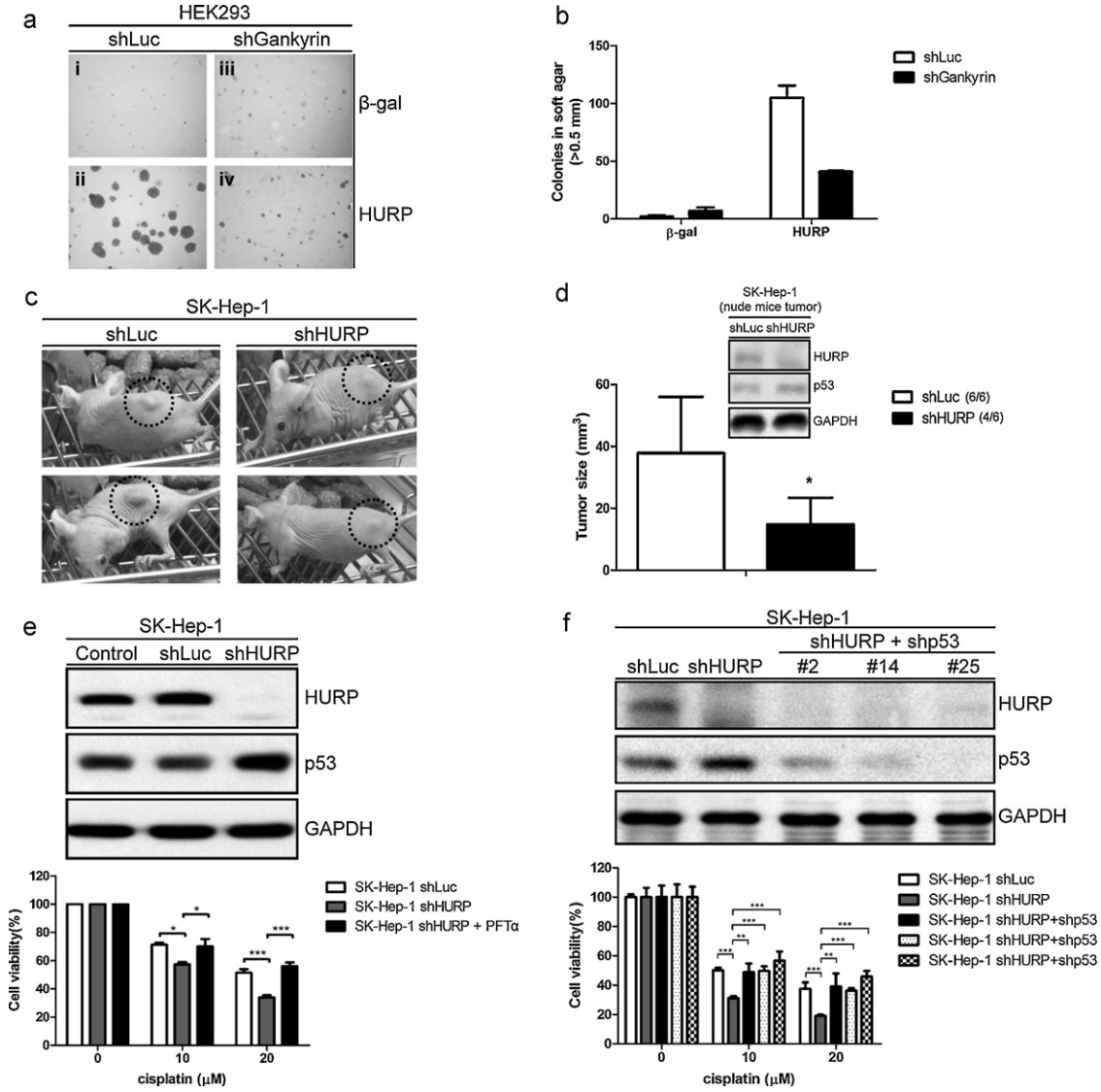

Fig. 7. HURP regulates malignant transformation, tumor growth, and apoptosis in vitro and in vivo. (a) Formation of colonies for the cells indicated was assessed in soft agarose for three weeks as described in Section 2. (b) The number of colonies was counted for each treatment ($^{**}p < 0.01$). (c) Representative photographs of two mice in each group were taken 17 days after inoculation. (d) Statistic analysis of tumor sizes of the nude mice of (c) (means ± SEM; $n = 6$, $^{*}p < 0.05$ versus shLuc group). Representative Western blots were also shown. (e) Cell viability of the indicated cells was measured using the MTT assay following treatment with cisplatin (10 or 20 μM). The p53 inhibitor pifithrin-α (PFT-α) was also used in these experiments at a concentration of 10 μM. The results represent means ± SD of experiments performed in quadruplicate ($^{*}p < 0.05$, $^{***}p < 0.001$ versus shLuc group or without PFT-α treatment group). (f) Cell viability of HURP and p53 double-knockdown cells was measured using the MTT assay. The results represent mean ± SD of experiments performed in quadruplicate ($^{*}p < 0.05$, $^{***}p < 0.001$ versus shLuc group or without p53 shRNA treatment group).

samples provided by the TLCN were presented (Fig. 8a). The nine HBV-associated HCC samples were scored for expression of HURP and gankyrin. The relative density of immunostaining was normalized by considering the first lane (T4) as 100%. Accordingly, we detected co-overexpression of gankyrin and HURP proteins in nine hepatitis B virus (HBV)-associated liver tumors (Fig. 8b, $r^2 = 0.778$). Although the correlation between the two proteins was less important in hepatitis C virus (HCV)-associated liver tumors, a high level of HURP was still detected in these tumors (data not shown). Since we described earlier that the HBV-encoded oncoprotein HBx can upregulate HURP [23], it is possible that viral proteins in HCV-associated liver tumors may induce HURP expression, a possibility which remains to be explored. Nonetheless, these observations performed on clinical samples further support the notion that HURP positively regulates gankyrin in liver tumors. Interestingly, most of the p53 protein was not detected in these patients that intrinsically expressed high levels of HURP. However, the p53 protein was also not detected in one HBV patient (T25) in which HURP and Gankyrin were not detected. Furthermore, p53 was detected in two HBV patients (T6 and T9) who also displayed intrinsically high levels of HURP.

3.9. Proposed model to illustrate the biological roles of HURP in human cancer

Based on the results presented here, HURP appears to play an oncogenic role in the cancer process seen in HCC. In summary, overexpression of HURP was shown to stabilize gankyrin by blocking MDM2-mediated ubiquitination, which in turn enhanced the formation of the MDM2/p53 complex and the E3 ubiquitin ligase activity of MDM2 (ref. [14]; Fig. 8c). This complex promoted the ubiquitination of p53 via the E3 ubiquitin ligase activity of MDM2, culminating in the degradation of p53 by the proteasome. Finally, alteration and degradation of p53 is associated with various anti-apoptotic effects that promote cell transformation and proliferation.

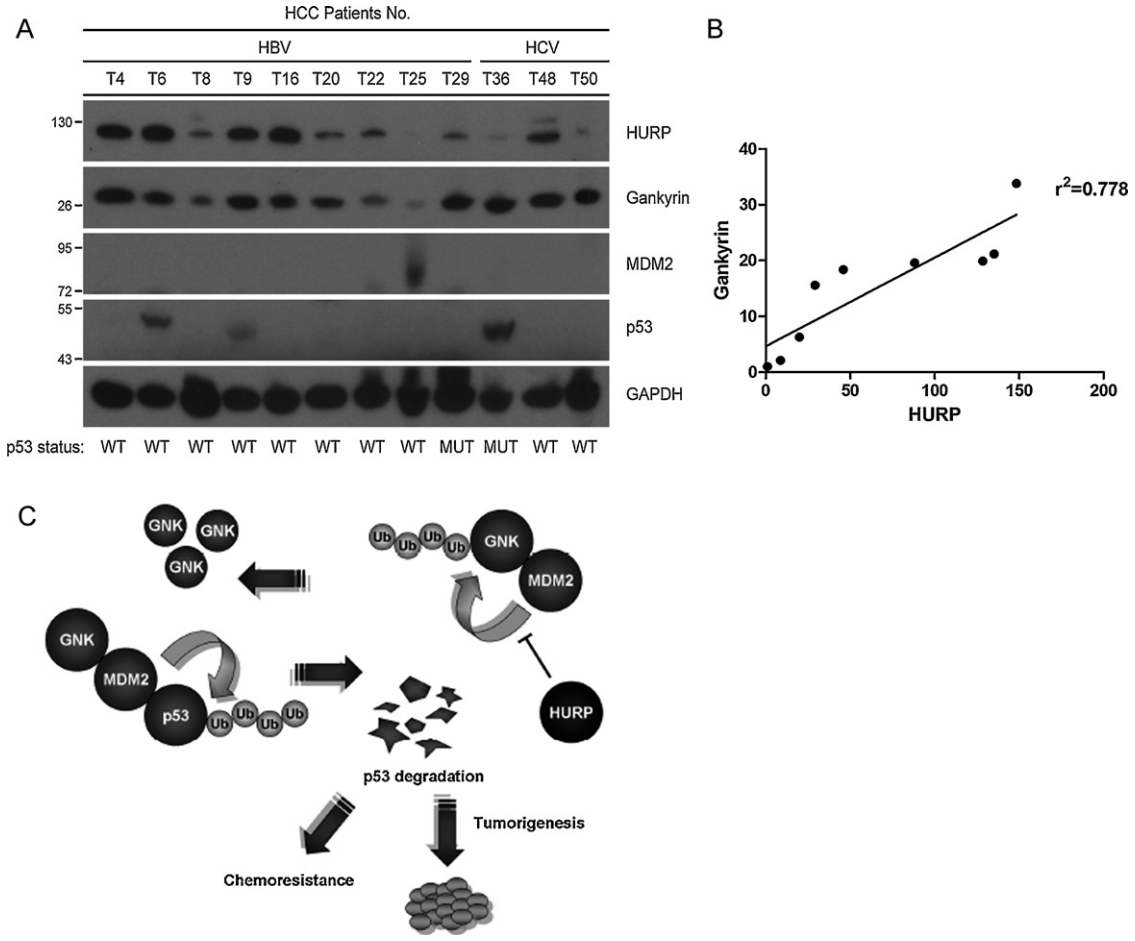

Fig. 8. Correlation between HURP and gankyrin proteins in HCC patients. (a) Representative Western blots of HCC patient samples provided by the TLCN. (b) Statistic correlation between HURP and gankyrin as shown in (a). The nine HBV-associated HCC samples were scored for expression of HURP and gankyrin. The relative density of immunostaining was normalized by considering the first lane (T4) as 100%. *r*, correlation coefficient between two proteins, according to their density of immunostaining. (c) Model to illustrate the oncogenic properties of HURP in human liver cancer. In this cycle of gankyrin/MDM2-enhanced p53 degradation, HURP weakens MDM2-mediated ubiquitination of gankyrin and leads to accumulation of gankyrin in normal cells and tumorigenic cells. The downstream effects of HURP appear to include malignant cell transformation and prevention of apoptosis induced by chemotherapeutic drug which in turn established chemoresistant phenotype.

4. Discussion

In this study, we examined whether HURP represents an oncogene involved in the development of HCC. We found that expression of HURP increased the levels of gankyrin, which in turn enhanced the E3 ligase activity of MDM2, culminating in the degradation of p53 in both non-tumorigenic and tumorigenic cells. The involvement of MDM2 in this process was confirmed by the observation that Nutlin-3 or MDM2 antisense DNA prevented the HURP-induced degradation of p53. Bcl-2 also accumulated following expression of HURP in HEK293 cells (see Fig. 1a), consistent with the notion that HURP has anti-apoptotic effects in this context. Accordingly, earlier studies found that p53 can directly repress *Bcl-2* gene expression [24,25]. We also observed that expression of HURP in HEK293 led to the formation of colonies in soft agarose. In addition, HURP knockdown decreased tumor sizes produced by inoculation of SK-Hep-1 cells in nude mice. These observations indicate that HURP has oncogenic properties which might play a role in the development of HCC.

Interestingly, Mahlavu cells, which harbor intrinsically high levels of HURP, also express very high levels of mutated p53. Mutated Mahlavu p53 did not accumulate after the shRNA-mediated knockdown of HURP but it was even reduced following this treatment (Fig. 6a). These observations are opposite to the results seen in cells that contain at least one wild type copy of p53. The p53 protein interacts with MDM2 via p53's N-terminal region and core domain. Yet, the p53 C-terminal region contains lysine residues that are ubiquitinated by MDM2 and that can bear post-translational modifications that prevent the p53-MDM2 interaction. However, there is only a G → T point mutation at codon 249 on Mahlavu p53 which is more stable than wild type p53 [26]. Surprisingly, complexes of p53 and MDM2 were detected at low levels in Mahlavu cell extracts using the IP/IB assay (data not shown), suggesting that the G → T point mutation at codon 249 (within the DNA-binding domain) on Mahlavu p53 may affect its conformation and its interaction with MDM2. Alternatively, MDM2 mutations which have not been characterized in Mahlavu cells may interfere with ubiquitination without interfering with the interaction with p53, as seen for the RING mutant MDM2 which is fully capable of binding to p53 without leading to ubiquitination [27]. In vivo knockin experiments have demonstrated that the major modification sites on p53 apparently are not essential for mouse p53 stabilization upon stress [28–30], suggesting that the mechanism of stress-induced p53 stabilization is complicate. These findings may partly explain the slight accumulation of wild type p53 in two patients with intrinsically high levels of HURP (Fig. 8).

A proposed mechanism illustrating how gankyrin may accumulate in cells that overexpress HURP is shown in Fig. 8c. We have shown that HURP stabilizes gankyrin by inhibiting the MDM2-

mediated ubiquitination of gankyrin but that this process is not due to the disruption of interactions between MDM2 and gankyrin. Alternatively, HURP might regulate the activity of yet-to-be defined de-ubiquitination enzymes by potentiating the enzyme binding to the gankyrin/MDM2 complex (not shown in Fig. 8c), which in turn might inhibit the MDM2-mediated ubiquitination and degradation of gankyrin. Further studies are required to examine whether HURP can affect the de-ubiquitination enzymes that interact with the MDM2/gankyrin complex.

Although MDM2 plays a key role in down-regulating p53 in various cellular conditions, increasing evidence challenges the view that MDM2 is an essential factor that regulates p53 turnover [31]. For example, degradation of p53 can also be mediated by the E3 ubiquitin ligase synoviolin which resides in the endoplasmic reticulum (ER) [32]. We observed earlier that cisplatin-induced p53 accumulation correlates with reduced expression of synoviolin [33]. Further studies are required to examine whether other E3 ubiquitin ligases or other factors like Rb or the S6 ATPase proteasome subunit are also involved in the destabilization of p53 by HURP.

The regulation of p53 target genes usually depends on the extent of DNA damage. Cell survival or apoptotic fates are largely orchestrated through the differential activation of distinct subsets of p53 target genes (reviewed in ref. [34]). For example, binding of CAS to p53 on chromatin favors the expression of pro-apoptotic genes and facilitates apoptosis [35]. In contrast, association of the zinc-finger protein Hzf with p53 favors its binding to promoters of growth-arrest genes and promotes cell survival [35,36]. The tumor suppression properties of p53 are attributed to its ability to exert suppression of cell growth and proliferation by inducing cell cycle arrest and apoptosis [8,9]. The HURP-mediated p53 degradation may thus be of relevance in HCC. Here, our findings reveal a new oncogenic pathway of HURP-induced malignant transformation that involves degradation of p53 and accumulation of gankyrin. In conclusion, the oncogenic properties of HURP reported here may offer new treatment strategies to defeat liver cancer in humans.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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