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Rock2 promotes the invasion and metastasis of hepatocellular carcinoma by modifying MMP2 ubiquitination and degradation

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ABSTRACT

Rho-associated coiled-coil-containing protein kinase 2 (Rock2) is a downstream effector of Rho that plays an important role in the tumorigenesis and progression of hepatocellular carcinoma (HCC). Matrix metalloproteinase 2 (MMP2) is a master regulator of tumor metastasis. In this study, we investigated the collections of Rock2 and MMP2 in HCCs and determined the potential role and molecular mechanism of Rock2 in MMP2-mediated invasiveness and metastasis. We found that Rock2 and MMP2 were markedly overexpressed in HCCs compared with the corresponding adjacent tissues, where a positive correlation in their expression was found. The knockdown of Rock2 significantly decreased MMP2 expression and inhibited the invasion and metastasis of HCC *in vitro* and *in vivo*. Additionally, the upregulation of MMP2 rescued the decreased migration and invasion induced by the knockdown of Rock2, whereas the knockdown of MMP2 decreased Rock2-enhanced HCC migration and invasion. Mechanistically, Rock2 stabilized MMP2 by preventing its ubiquitination and degradation. Together, our results link two drivers of invasion and metastasis in HCC and identify a novel pathway for MMP2 control.

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1. Introduction

Invasion and metastasis is the main cause of death in patients with hepatocellular carcinoma (HCC) [1]. The studies have reported that Rho-associated coiled-coil-containing protein kinase 2 (Rock2) plays an important role in HCC invasion and metastasis [2,3]. Rock2 belongs to a family of serine/threonine kinases, which are activated via interaction with Rho GTPases [4]. There have been an increasing number of studies on Rock2 in malignant tumors. These reports have shown that increased Rock2 expression is correlated with poor prognosis in many human cancers, including HCC, breast cancer and lung cancer [2,5,6]. Recently, emerging evidence links the biological function of Rock2 to tumor metastasis. For example, the overexpression of Rock2 in HCC is significantly associated with the presence of tumor microsatellite formation, and the stable knockdown of Rock2 markedly reduced HCC migration and invasion *in vitro* and *in vivo* [2]. Our previous study also showed that the knockdown of Rock2 can inhibit HCC cell growth and aggressiveness [4]. These studies have suggested that Rock2 may play a crucial role in the invasion and metastasis of HCC. However, its specific mechanism of action remains unclear.

Matrix metalloproteinase-2 (MMP2) is a family of zinc-containing proteolytic enzymes and participates in the breakdown of collagen type IV [7,8]. MMP2 expression can be regulated by a transcriptional mechanism, by ubiquitination modification and by the inhibition of enzymatic activity [9,10]. The reports have shown that MMP2 is overexpressed in HCC and is critical for HCC metastasis and poor prognosis [11–13]. A recent study found that the knockdown of BTB/POZ domain-containing protein 7 can repress Rock2 and MMP2 activation and inhibit HCC invasion and metastasis, and the study also found that a Rock2-specific inhibitor can significantly suppress the activity of MMP2 [14]. In addition, Vishnubhotla et al. also reported that Rock2 can regulate MMP2 activity in colon cancer [15]. However, it remains unclear whether

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Rock2 promotes HCC metastasis by regulating MMP2 expression, and its regulation mechanism has not been clarified.

In the present study, we first demonstrated that Rock2 and MMP2 are both commonly upregulated in HCC tissues compared with non-tumor liver tissues, and the expression analyses revealed a significantly positive correlation. In addition, the functional studies provided the first evidence that Rock2 facilitates HCC invasion *in vitro* and metastasis *in vivo* by upregulating MMP2 expression. Further investigations indicated that Rock2 can stabilize MMP2 expression by modulating the ubiquitination and degradation of MMP2.

2. Materials and methods

2.1. Samples

Human HCC specimens were collected from 77 patients who underwent HCC resection at the Second Affiliated Hospital of Nanchang University between January 2011 and June 2014. Informed consent was obtained from each patient, and the study protocol was approved by the Ethics Committee of the Second Affiliated Hospital of Nanchang University.

2.2. Cell culture

The human HCC cell lines HepG2, SMMC7721, Huh-7, HCCLM3 and MHCC97H were purchased from the Shanghai Institute of Cell Biology of China. The immortalized liver cell line HL-7702 was purchased from Shanghai Fu Xiang Biotechnology Co., Ltd. (China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with fetal bovine serum (Hyclone, Logan, Utah, USA) to a final concentration of 10% and were exposed to antibiotics at 37 °C with 5% CO_2.

2.3. Plasmids and reagents

Based on the Rock2 (NM_004850.3) and MMP2 (NM_004530.4) sequences, four short hairpin RNAs (shRNAs) were designed using the siRNA Target Finder (InvivoGen). The target sites of shRNA are detailed in Supplementary Table S1. The interference effects were confirmed by real-time quantitative polymerase chain reaction (qRT-PCR) and Western blotting (Supplementary Fig. S1).

The shRock2 and shMMP2 vectors that produced the most significant knockdown effect were used to transduce HCC cells. In the stable transfection, the shRock2 of HCC cells was selected based on resistance to hygromycin (600 μg/ml) (Invitrogen, Carlsbad, CA, USA), and HCC cells transfected with a negative control vector (shNC) were included as a control. The pcDNA3.1(+)-Rock2-expressing HCC cells were selected using G418 (700 μg/ml) (Invitrogen, Carlsbad, CA, USA), and an empty vector was used as the negative control. After four weeks of selection, individual colonies were isolated and expanded. pcDNA3.1(+)-Rock2, pcDNA3.1(+)-MMP2, pcDNA3.1(+)-Flag-Rock2, and pcDNA3.1(+)-HA-MMP2 were constructed in our laboratory as previously described. All of the primers are shown in Supplementary Table S1.

The following antibodies and reagents were used: Rock2, MMP2 and ubiquitin (Ub), (Proteintech, Chicago, IL, USA); protein A/G PLUS-agarose (Santa Cruz, CA, USA); Lipofectamine LTX (Invitrogen, Carlsbad, CA, USA); a total protein extraction kit (Applygen, Beijing, China); a BCA protein quantitation kit (Beyotime, Jiangsu, China); and MG132 and cyclohexamide (CHX) (Sigma–Aldrich, St. Louis, MO, USA).

2.4. qRT-PCR, Western blotting analysis, H&E analysis and Co-immunoprecipitation (Co-IP)

qRT-PCR, Western blotting, H&E analyses and Co-IP were performed as previously described [16]. The specific primers used for PCR amplification are shown in Supplementary Table S1.

2.5. Wound-healing assays

The cells were grown to 80–90% confluence in 60-mm dishes. Artificial wounds were generated by scraping a pipette tip across the cell surface. After the removal of the detached cells by gentle washing with PBS, the cells were fed fresh complete medium and incubated over time to allow the cells to migrate into the open area. The cell movement during wound closure was measured by phase-contrast photography at 37 °C during incubation for 0, 24, and 36 h, and three randomly selected wound areas were analyzed.

2.6. Cell migration and invasion assay

For the migration assay, 5×10^4 cells were resuspended in serum-free medium and placed in the upper chambers, and for the invasion assays, 1×10^5 cells were seeded in a Matrigel-coated chamber (BD Biosciences, Bedford, MA, USA). After 24 h (to examine migration) or 48 h (to examine invasion) of incubation, the non-migrated cells on the upper surface of the membrane were removed, and the cells on the lower surface were fixed and stained with 0.1% crystal violet. The cells in five random microscopic fields were counted and imaged using a light microscope with a DP70 CCD system (Olympus Corp., Tokyo, Japan).

2.7. Metastasis assays in vivo

For the *in vivo* metastasis assays, the mice were inoculated subcutaneously in the right dorsal part near the lower pole of the right kidney with 1×10^7 HCCLM3, which could induced a high incidence of lung metastases when implanted either subcutaneously or orthotopically in nude mice [17]. The mice were sacrificed six weeks after tumor implantation. The lungs were then processed and embedded in paraffin. The animal work was approved by the Ethics Committee for Animal Experiments of the Second Affiliated Hospital of Nanchang University and was performed in accordance with the "Guide for the Care and Use of Laboratory Animals" (revised 1985).

2.8. Immunofluorescence staining and confocal laser microscopy

The cells were seeded in two-chamber slides, fixed in 4% paraformaldehyde for 10 min, washed twice with PBS, permeabilized with 0.5% Triton X-100 for 15 min, and then blocked with 5% albumin from bovine serum (BSA) (Sigma–Aldrich, St. Louis, MO, USA). The cells were then incubated with primary antibodies (mouse anti-Rock2 and rabbit anti-MMP2; 1:200 dilution) overnight at 4 °C. After thorough washing, the cells were incubated with FITC-conjugated donkey anti-mouse IgG (1:300 dilution, Invitrogen, Carlsbad, CA, USA) or Texas Red-conjugated donkey anti-rabbit IgG (1:300 dilution, Invitrogen, Carlsbad, CA, USA) for 30 min. The nuclei were stained with ProLong Gold antifade reagent with DAPI (Invitrogen, Carlsbad, CA, USA). The localization of the proteins was observed using a confocal laser scanning microscope (SP-II; Leica Microsystems, Wetzlar, Germany).

2.9. Statistical analysis

All of the data were analyzed using SPSS 16.0 (SPSS, Inc., Chicago, IL, USA). The results are presented as the means ± SD from three independent experiments. The differences between the groups were analyzed by Student's *t* test when two groups were compared or by one-way ANOVA when more than two groups were compared. The test results were considered significant at $p < 0.05$.

3. Results

3.1. Rock2 and MMP2 expression are positively correlated in HCC tissues

To investigate the expression correlation between Rock2 and MMP2 in HCCs, we first determined the expression levels of Rock2 and MMP2 in 77 pairs of HCC and adjacent nontumor samples by qRT-PCR and Western blot analyses. The qRT-PCR results revealed that the average fold change of Rock2 and MMP2 mRNA expression in tumor tissues was significantly higher than that found in the paired nontumor tissues (Fig. 1A). The Western blot analysis results showed that Rock2 protein was overexpressed in 77.92% (60/77) of the HCC tissue samples, whereas MMP2 protein was overexpressed in 62.34% (48/77) of the HCC tissue samples (Fig. 1B). These results indicated that Rock2 and MMP2 were frequently overexpressed in HCCs. Moreover, the scatter plots showed that the Rock2 and MMP2 mRNA and protein expression levels were positively correlated in HCC tissues (Fig. 1C).

3.2. Downregulation of Rock2 reduces MMP2 expression and represses HCC invasion and metastasis in vitro and in vivo

To investigate whether Rock2 modulates the MMP2 expression levels in HCC cells, we first examined the levels of Rock2 and MMP2 in a variety of HCC cells by qRT-PCR and immunoblotting assays. The results determined that the Rock2 levels were positively correlated with the levels of MMP2 (Fig. 2A). Second, we manipulated the Rock2 levels by the stable transfection of Rock2 shRNA or Rock inhibitor (Y27632) and examined the expression of Rock2 and MMP2. The immunoblot analysis results showed that the downregulation of Rock2 could decrease the MMP2 expression levels in the HepG2 and HCCLM3 cells (Fig. 2B). Furthermore, we found that cells with the stable knockdown of Rock2 displayed a

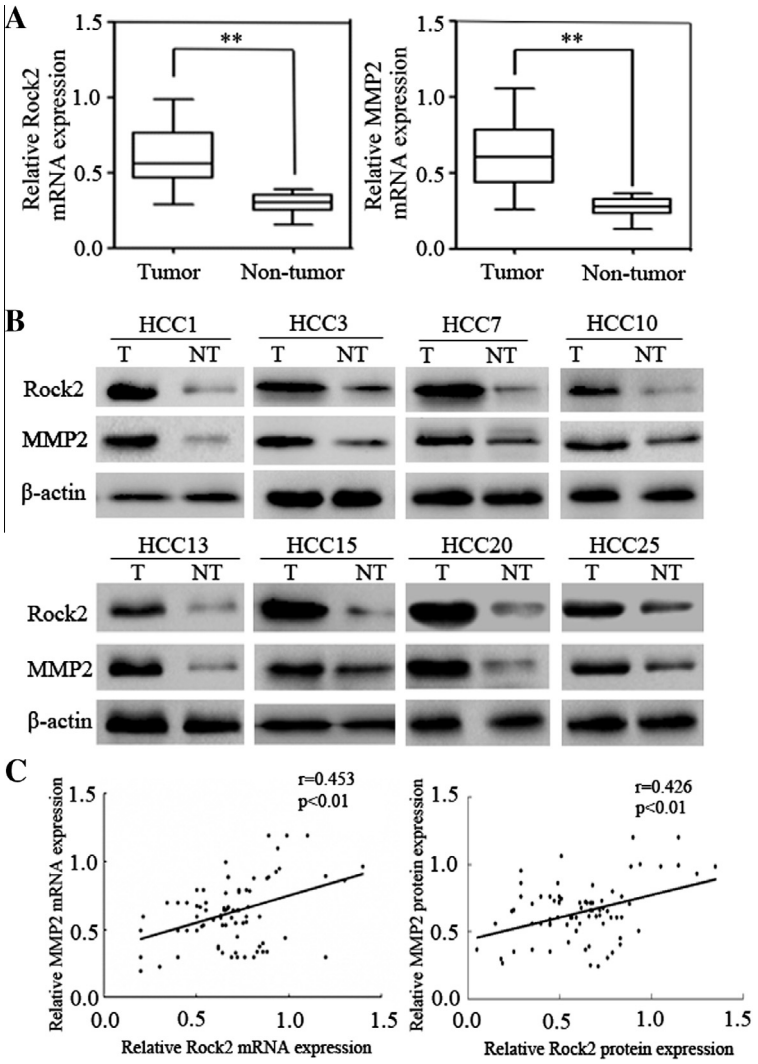

Fig. 1. Rock2 overexpression correlates with MMP2 expression in human HCCs. (A) qRT-PCR analysis of Rock2 and MMP2 mRNA expression in 77 HCC tumors and peritumoral liver tissues (**$p < 0.01$, paired Student's *t*-test). (B) Representative Western blotting analysis of Rock2 and MMP2 protein expression (T: tumor, NT: nontumorous tissues). (C) Scatter plots show a positive correlation between Rock2 and MMP2 at the mRNA and protein levels in 77 HCCs ($r = 0.453$, $p < 0.01$ and $r = 0.426$, $p < 0.01$).

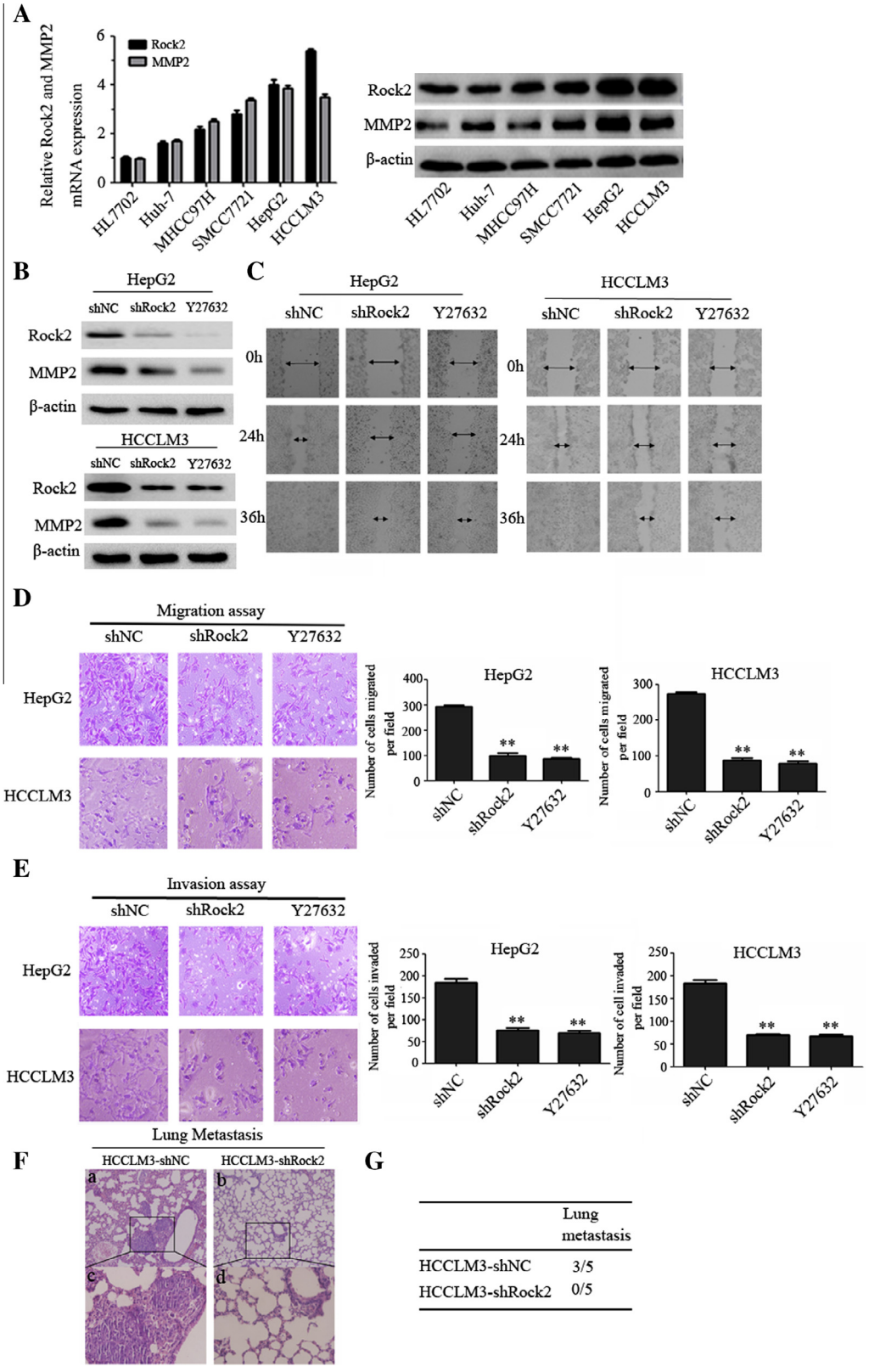

Fig. 2. Stable knockdown of Rock2 decreases MMP2 expression and suppresses HCC invasion and metastasis *in vitro* and *in vivo*. (A) qRT-PCR and Western blot analyses of Rock2 and MMP2 expression in human normal hepatocyte and HCC cell lines. (B) Western blotting was used to detect Rock2 and MMP2 expression in HepG2 and HCCLM3 cells that are stably transfected with the shNC or shRock2 plasmid or treated with a Rock inhibitor (Y27632). (C) Wound healing assay. Wound closure was delayed in stable Rock2-knockdown or Y27632-treated cells compared with the shNC control at both the 24- and 36-h time points. (D) Transwell migration assays of HepG2 and HCCLM3 cells with Rock2 expression stable inhibited (**$p < 0.01$). (E) Transwell invasion assays of HepG2 and HCCLM3 cells treated with Rock2-shRNA or Y27632. (F) H&E-stained sections of distal metastatic nodules in the lung formed by HCCLM3-shRock2 or shNC (left panel). Magnification: a and b, 100×; c and d, 400×. Summary of the pathological analysis of HCCLM3-shNC and shRock2-derived tumors in the lungs of nude mice (right panel). (G) Incidence of lung metastases in the HCCLM3-shNC and HCCLM3-shRock2 groups of nude mice.

significant decrease in cell migration ability compared with those transfected with the vector control. Similar findings were also observed in HCC cells after treatment with the Rock inhibitor Y27632 compared with the control cells ($p < 0.01$) (Fig. 2C and D). In addition, using a Matrigel-coated transwell chamber, we observed that the stable Rock2-knockdown HCC cells invaded through the matrix slower than the control cells (Fig. 2E).

We further examined the effects of Rock2 on HCC metastasis by establishing an HCC metastasis model in nude mice with stable Rock2-knockdown cell lines. The histological analysis results demonstrated that three cases developed lung metastasis in the shNC group. However, in the shRock2 group, there was no obvious lung metastases (Fig. 2F and G). These results indicate that the stable knockdown of Rock2 can reduce MMP2 expression and thus inhibit HCC invasion and metastasis.

3.3. MMP2 is critical for Rock2-mediated HCC cell migration and invasion

To further determine whether Rock2 facilitates HCC migration and invasion by regulating MMP2 expression, we first overexpressed MMP2 in Rock2-downregulated HCC cells and then determined the expression levels of the Rock2 and MMP2 proteins and the cell migration and invasion abilities by immunoblotting and transwell assay. The immunoblotting results showed that the knockdown of Rock2 decreased MMP2 expression, whereas the upregulation of MMP2 attenuated the loss of MMP2 expression in Rock2-knockdown HepG2 and HCCLM3 cells (Fig. 3A and Fig. S2A). We also found that the downregulation of Rock2 markedly decreased the migration and invasion abilities of HepG2 and HCCLM3, whereas the upregulation of MMP2 rescued the decreased migration and invasion abilities induced by Rock2 knockdown (Figs. 3B and S2B).

We then knocked down MMP2 expression in Rock2-overexpressing HCC cells and then detected the Rock2 and MMP2 protein expression and cell migration and invasion abilities. The immunoblotting results showed that the upregulation of Rock2 significantly enhanced MMP2 expression, whereas the downregulation of MMP2 could inhibit the increased MMP2 expression induced by Rock2 in Huh-7 cells (Fig. 3C). In addition, the downregulation of MMP2 significantly reduced the Rock2-enhanced cell

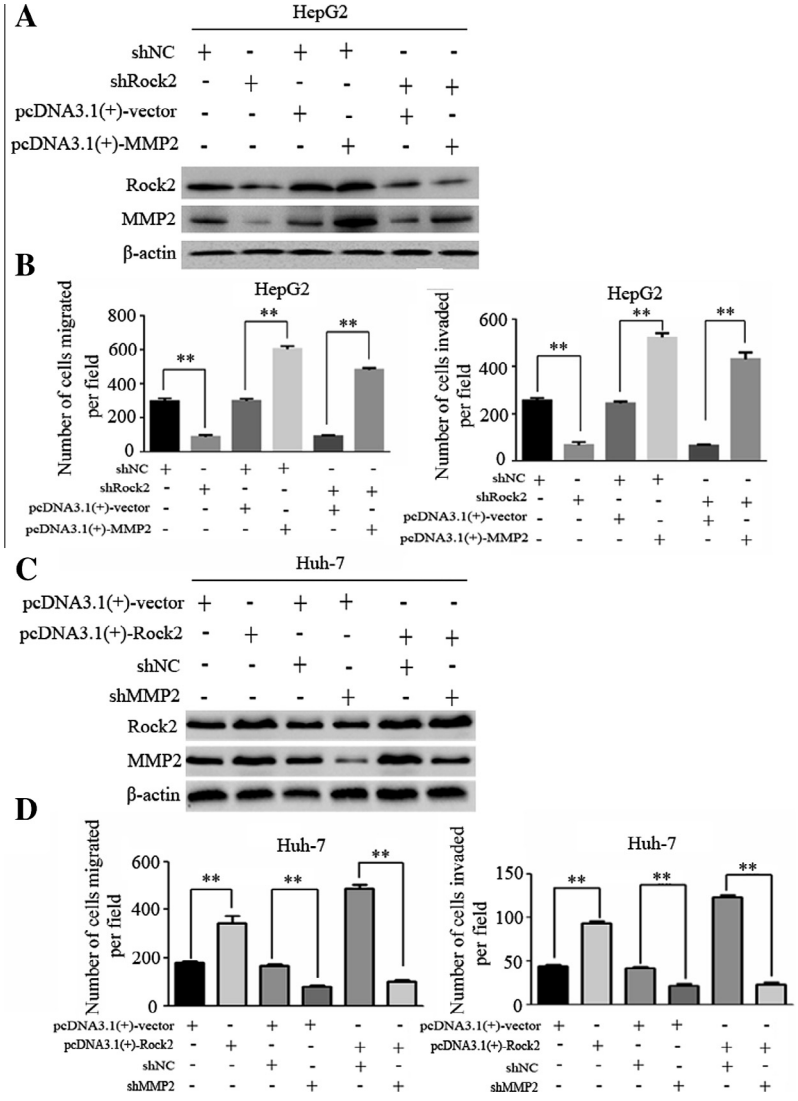

Fig. 3. MMP2 is essential for Rock2-mediated HCC migration and invasion. (A) Western blotting was used to detect the expression of Rock2 and MMP2. The upregulation of MMP2 attenuated the loss of MMP2 expression in HepG2-shRock2 cells. (B) Transwell assays showed that the upregulation of MMP2 significantly rescued the cell migration and invasion in HepG2-shRock2 cells ($^{**}p < 0.01$). (C) The protein levels of Rock2 and MMP2 were detected by Western blot analysis. The knockdown of MMP2 expression markedly inhibited the increase of MMP2 expression in Huh-7-Rock2 cells. (D) Transwell assays showed that MMP2 inhibition reduced the Rock2-enhanced cell migration and invasion ($^{**}p < 0.01$).

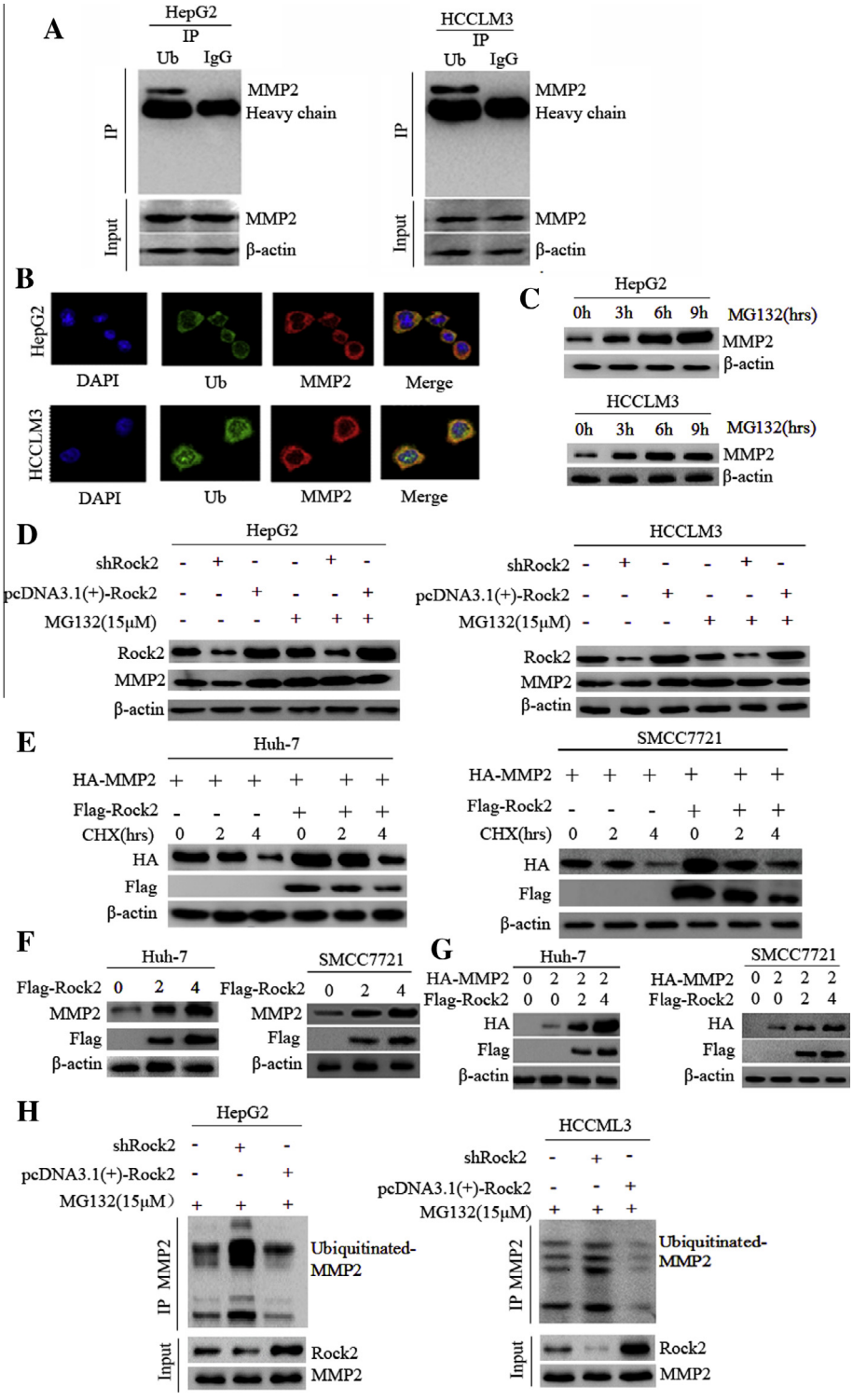

Fig. 4. Rock2 stabilizes MMP2 protein by preventing its ubiquitination and degradation in HCC cells. (A) Co-IP between endogenous MMP2 and ubiquitin in HepG2 and HCCLM3 cells. (B) Colocalization studies were performed on HepG2 and HCCLM3 cells using Ub antibody (1:200, green) and anti-MMP2 antibody (1:200, red), followed by DAPI nuclear counterstaining (blue). The merged images of Ub (green) and MMP2 (red) with DAPI (blue) are also shown. (C) HepG2 and HCCLM3 cells were treated with MG132 (Z-Leu-Leu-Leu-CHO, 15 μM) for the indicated time, and the levels of MMP2 were then detected. (D) Rock2 had no effect on MMP2 expression, as assessed by MG132 after transfection with shRock2 or pcDNA3.1(+)-Rock2 plasmid in HepG2 and HCCLM3 cells. (E) Huh-7 and SMCC7721 cells were transfected with the expression plasmid encoding HA-MMP2 with or without the Flag-Rock2 plasmid. The cells were then exposed to CHX (20 μM) for the indicated times, and the degradation of MMP2 was detected with anti-HA antibody. (F) Huh-7 and SMCC7721 cells were transfected with increasing amounts of Flag-Rock2 plasmid. The expression levels of the endogenous MMP2 were detected with anti-MMP2 antibody. (G) Huh-7 and SMCC7721 cells were left untreated or transfected with a single dose of expression plasmid encoding HA-MMP2 with or without being combined with increasing amounts of the Flag-Rock2 plasmid. The expression levels of MMP2 were detected with anti-HA antibody. (H) The knockdown or exogenous expression of Rock2 altered the ubiquitination of MMP2. The cells in each group were treated with the proteasomal inhibitor MG132 (15 μM). Cell lysates were prepared and subjected to immunoprecipitation with anti-MMP2 antibody. The level of ubiquitin-attached MMP2 was detected by Western blotting analysis with Ub antibody.

migration and invasion (Fig. 3D). Thus, these results confirm that MMP2 is essential for Rock2-mediated HCC metastasis.

3.4. Rock2 stabilizes MMP2 expression by modifying MMP2 degradation

We then further assessed the mechanisms through which Rock2 regulates MMP2. The study has reported that the MMP2 protein undergoes degradation via the ubiquitin proteasome system (UPS) in prostate cancer cells [10]. In addition, we previously demonstrated that Rock2 can regulate the ubiquitin degradation of Cdc25A [4]. Thus, we presumed that Rock2 may also regulate MMP2 by affecting MMP2 ubiquitination degradation. To test this hypothesis, we first determined whether the ubiquitination and degradation of MMP2 proteins also occurs in HCC cells. The IP results showed that the endogenous MMP2 and ubiquitin are directly bound in HepG2 and HCCLM3 cells (Fig. 4A). Colocalization assays using confocal microscopy was used to further confirm the interaction of ubiquitin and MMP2 in HepG2 and HCCLM3 cells (Fig. 4B). Furthermore, treatment with the proteasome inhibitor MG132 for the indicated time resulted in significant accumulation of the endogenous MMP2 protein in HepG2 and HCCLM3 cells (Fig. 4C). These results demonstrated that MMP2 is also degraded by the UPS in HCC cells.

Furthermore, to determine whether Rock2 is involved in regulating the degradation of MMP2 protein, we transfected the shRock2 and pcDNA3.1(+)-Rock2 vectors into HepG2 and HCCLM3 cells and examined the effects of variable Rock2 on MMP2 expression, either with or without MG132. The results showed that the down- or upregulation of the expression of Rock2 had no significant impact on MMP2 expression in HepG2 and HCCLM3 cells after treatment with MG132 (Fig. 4D). In addition, the degradation dynamics assay showed that the half-life of the ectopically expressed MMP2 was significantly increased in the Rock2-overexpressing Huh-7 and SMCC7721 cells (Fig. 4E). These results suggest that Rock2 is involved in the degradation of MMP2.

Moreover, we further assessed the role of Rock2 in the process of MMP2 degradation. The results showed that the ectopic dose-dependent effect of Rock2 upregulation caused a significant increase of endogenous MMP2 proteins in Huh-7 and SMCC7721 cells (Fig. 4F). Consistently, a single dose of HA-MMP2 and an increasing amount of Flag-Rock2 were co-transfected into Huh-7 and SMCC7721 cells, and a dose-dependent effect of Rock2 upregulation on MMP2 expression was found (Fig. 4G). These data suggest that Rock2 can stabilize the expression of MMP2.

Finally, to determine the mechanism through which Rock2 stabilizes MMP2 expression, we added MG132 to HepG2 and HCCLM3 cells that were transfected with the shRock2 and pcDNA3.1(+)-Rock2 vectors. The immunoprecipitation results showed that the inhibition of Rock2 can increase the levels of MMP2 ubiquitination, whereas Rock2 overexpression significantly decreased the levels of MMP2 ubiquitination (Fig. 4H). These results suggest that Rock2 stabilizes MMP2 expression by modifying MMP2 ubiquitination and degradation.

4. Discussion

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related deaths globally. The high mortality rate of HCC is related mainly to tumor invasion, metastasis, and tumor recurrence after surgical resection. Although some recent progress has been made with regard to the clinical detection of and treatment methods for HCC, the metastasis and recurrence rates after radical resection of HCC remain high [18,19]. Understanding the molecular mechanisms underlying HCC metastasis is of crucial significance to the development of therapeutic strategies for advanced HCC patients.

Rho-associated coiled-coil containing protein kinase 2 (Rock2) belongs to a family of serine/threonine kinases that are activated via interaction with Rho GTPases [4]. There is accumulating evidence that Rock2 is upregulated in a variety of malignancies, including HCC, glioblastoma, lung cancer, and colorectal cancer [2,5,6,20]. Our previous study also found that Rock2 is overexpressed in HCCs and that the knockdown of Rock2 inhibits HCC cell growth and promotes cell-cycle arrest at the G1/S phase [4]. In addition, Wong and colleagues reported that the downregulation of Rock2 can suppress the metastasis and progression of HCC [2]. The results of previous studies, when combined with our findings, suggest that Rock2 plays an important role in the processes of HCC tumorigenesis and metastasis. MMP2 is a type IV collagenase that contributes to the degradation and damage of the extracellular matrix and basement membrane [7]. The studies have indicated that MMP2 is overexpressed in HCC and is a master regulator of HCC invasion and metastasis [12,13]. Together, these studies suggest that Rock2 and MMP2 individually promote metastatic progression in HCC; however, the overexpression of both Rock2 and MMP2 in HCCs and the interactions between them in HCC invasion and metastasis remain unclear. In this study, our data first indicated that Rock2 and MMP2 overexpression exhibit a positive correlation in HCC tissues. Furthermore, we also found that Rock2 inhibition can reduce MMP2 expression and decrease the invasion and metastasis of HCC *in vitro* and *in vivo*. Moreover, the upregulation of MMP2 rescued the decreased migration and invasion induced by Rock2 knockdown, whereas MMP2 inhibition significantly decreased the Rock2-enhanced migration and invasiveness. These results confirm that MMP2 is essential for Rock2-mediated HCC invasion and metastasis.

Next, the mechanism through which Rock2 regulates MMP2 expression was investigated. Because the study reported that MMP2 is degraded by the UPS in prostate cancer cells [10], our previous study showed that Rock2 can affect Cdc25A degradation through the UPS in HCC cells [4]. Therefore, we observed whether Rock2 can regulate MMP2 ubiquitination and degradation in HCC cells. Interestingly, our results confirmed that Rock2 regulates MMP2 expression by controlling MMP2 protein stability through the UPS. First, we demonstrated that MMP2 is degraded by the UPS in HCC cells. Second, we found that Rock2 is involved in the degradation process of MMP2 and can function as a stabilizer for MMP2. Furthermore, Rock2 overexpression has the effect of suppressing MMP2 ubiquitination and degradation. These data demonstrated a novel mechanism through which MMP2 protein stability can be regulated by Rock2 through the UPS.

In summary, we demonstrated that Rock2 promotes HCC metastasis by regulating MMP2 expression. We also found that Rock2 is an important regulator of MMP2 expression through a mechanism in which Rock2 stabilizes MMP2 via the proteasomal pathway. Thus, we identified a novel mechanism for the regulation of MMP2 expression, which providing a greater insight into our understanding of the regulation and mechanism of metastasis in human HCC.

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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.2014.09.061.

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