

High-Mobility Group Box 1 Promotes Hepatocellular Carcinoma Progression through miR-21-Mediated Matrix Metalloproteinase Activity

Man Chen^{1,2}, Yao Liu^{1,3}, Patrick Varley¹, Ying Chang², Xing-xing He², Hai Huang¹, Daolin Tang¹, Michael T. Lotze¹, Jusheng Lin², and Allan Tsung¹

Abstract

Liver inflammation plays a critical role in hepatocellular carcinoma (HCC) etiology. Damage-associated molecular patterns (DAMP), such as high-mobility group box 1 (HMGB1), and dysregulated miRNAs involved in inflammatory disease states, such as miR-21, may participate in the link between inflammation and cancer. We sought to determine the role of HMGB1 signaling in HCC tumor progression. We first document the concordant expression increase of HMGB1 and miR-21 in HCC cell lines and primary HCC tumor samples and subsequently show that HMGB1 stimulation results in overexpression of miR-21. These changes were found to be dependent on the IL6/STAT3 signaling axis. Invasion and migration of HCC cells *in vitro* were inhibited by both STAT3 and miR-21 antagonists, suggesting a role for this pathway in HCC tumor progression. We verified that

HMGB1-induced expression of miR-21 in HCC provides a post-transcriptional repression of the matrix metalloproteinase (MMP) inhibitors RECK and TIMP3, which are known to impact HCC progression and metastases. Finally, we found that inhibition of miR-21 in murine HMGB1-overexpressing HCC xenografts led to reduced tumor MMP activity through released repression of the miR-21 targets RECK and TIMP3, which ultimately impeded tumor progression. The prototypical DAMP, HMGB1, is released during liver inflammation and provides a favorable environment for HCC growth. HMGB1 signaling increases miR-21 expression to mediate the enhanced activity of MMPs through RECK and TIMP3. These findings provide a novel mechanism for HMGB1-mediated HCC progression through the IL6/Stat3-miR-21 axis. *Cancer Res*; 75(8); 1645–56. ©2015 AACR.

Introduction

Hepatocellular carcinoma (HCC) is a common complication of chronic liver disease and the third leading cause of cancer deaths worldwide (1). Despite advances in diagnosis and treatment, the majority of patients with HCC have an extremely poor prognosis due to advanced disease at presentation and lack of effective treatment modalities (2). HCC has been closely linked to underlying chronic liver diseases such as hepatitis B virus (HBV), hepatitis C virus (HCV), alcoholic liver disease, and nonalcoholic steatohepatitis (NASH; ref. 1). Inflammation is a unifying feature of these disease processes and has also been implicated as a critical

factor in carcinogenesis and progression of HCC (3). Understanding the relationship between inflammatory stimuli and HCC tumor biology is therefore critical to the development of novel therapeutic strategies for HCC.

The role of proinflammatory transcription factors, such as NF- κ B, HIF1 α , and STAT3, in the development and progression of cancer is well established, and has prompted active investigation into the regulatory mechanisms of these pathways (4). Previous work from our group and others suggest that high-mobility group box-1 protein (HMGB1) is an important member of this inflammatory cascade (5, 6). HMGB1 is a highly conserved nuclear protein that acts as a chromatin-binding factor by bending DNA and promoting access to transcriptional protein assemblies on specific DNA targets (7). More importantly, the role of HMGB1 as a damage-associated molecular pattern (DAMP) has led to its implication in a variety of disease states mediated by inflammatory stimuli, including sepsis, arthritis, ischemia-reperfusion injury, and cancer (8, 9). After passive release from necrotic cells or active release from inflammatory cells, HMGB1 binds pattern recognition receptors (PRR), including the receptor for advanced glycation end products (RAGE), Toll-like receptors (TLR)-2, TLR-4, and TLR-9, and, as a negative signaling molecule, CD24. These interactions subsequently mediate the host response to infection and injury (7, 10, 11), as well as exert an influence on the oncogenesis, progression, and metastases of various malignancies (5, 6).

miRNAs are a class of evolutionarily conserved, single-stranded, small (approximately 19–23 nucleotides), endogenously

¹Department of Surgery, University of Pittsburgh, Pittsburgh, Pennsylvania. ²Institute of Liver Diseases, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China. ³Department of Gastroenterology, The First Affiliated Hospital of Gannan Medical College, China.

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

Corresponding Authors: Allan Tsung, University of Pittsburgh, 3d459 Fifth Avenue, UPMC Montefiore, 7 South, Pittsburgh, PA 15213-2582. Phone: 412-692-2001; Fax: 412-692-2002; E-mail: tsunga@upmc.edu; and Jusheng Lin, Institute of Liver Diseases, Tongji Hospital, 1095# Jie-Fang Avenue, Wuhan 430030, China. Phone: 86-27-83663661; Fax: 86-27-83663661; E-mail: jslin@tjh.tjmu.edu.cn

doi: 10.1158/0008-5472.CAN-14-2147

©2015 American Association for Cancer Research.

Chen et al.

expressed, and nonprotein-coding RNAs that act as posttranscriptional regulators of gene expression in a broad range of animals, plants, and viruses (12, 13). The alteration in miRNA expression profiles is a common characteristic of all human tumors (14, 15), suggesting their importance in the progression and prognosis of cancer (16). miR-21 is a well-described oncomir, overexpressed in a number of malignancies including neuroblastoma, glioblastoma, leukemia, lung, breast, pancreas, and colorectal cancers, HCC, and lymphoma (17–20). Like HMGB1, miR-21 has also been associated with multiple inflammatory pathways, including those regulated by TLRs. Specifically, knockout of TLR-4 has been linked to a reduction in IL6 and miR-21, which then protects mice from radiation-induced lymphoma (21). Given the importance of both miR-21 and HMGB1 in the inflammatory processes linked to cancer, we hypothesized that they may both be part of a common pathway.

In this study, we show that HMGB1 and miR-21 are overexpressed in human HCC tumors. We describe the role of IL6 and the transcription factor STAT3 in HMGB1-mediated induction of miR-21 expression. We also show that this pathway influences tumor progression through its targets RECK and TIMP3, inhibitors of matrix metalloproteinases (MMP), and demonstrate that the inhibition of miR-21 reduces HMGB1-induced tumor progression in a murine model of HCC. Taken together, our body of work defines a significant relationship between inflammation, miRNA, and HCC, which may provide novel targets for future therapeutic strategies.

Materials and Methods

Cell lines, patient samples, and reagents

Following an approved Institutional Review Board protocol and after informed consent of the tissue donors, samples of HCC tissue and nonadjacent liver tissue were collected from 20 patients with HCC undergoing curative surgery at the Liver Cancer Center of the University of Pittsburgh Medical Center (Pittsburgh, PA). Hepatocyte isolation was performed as previously described (22). Cell lines and reagents are described in the Supplementary Materials and Methods.

RNA and protein extraction

Total RNA and cellular protein were extracted as described previously (23).

Antibodies and immunoblot analysis

Rabbit anti-Stat3, anti-phospho-STAT3 (P-STAT3) (tyr705), anti-RECK, and anti-TIMP3 were purchased from Cell Signaling Technology. Whole-cell protein was extracted with cell lysis buffer (Sigma-Aldrich), and anti-HMGB1 (Abcam) along with anti-actin antibody (Sigma) were used for Western blot analysis (22).

miRNA-related reagents, siRNAs, and transfection

miRNA precursor, inhibitor, negative control, and HMGB1 interfering RNA (siRNA) were purchased from Ambion. Lipofectamine 2000 (Invitrogen) was used to transfect 50 nmol/L miRNA inhibitors, 100 nmol/L siRNAs, and 100 nmol/L miRNA precursor according to manufacturer instructions.

Reverse transcription and qRT-PCR

Reverse transcription and TaqMan real-time PCR for miRNAs were performed as described previously (23). Real-time PCR for

mRNA detection was performed using SYBR Green PCR Master Mix (Ambion). Primers are listed in Supplementary Table S1.

Luciferase assay

To verify predicted targets of miR-21, the pMIR-REPORT system (Ambion) was used. Briefly, 55-mer reversion-inducing cysteine-rich protein with Kazal motifs precursor (*RECK*) and 57-mer metalloproteinase inhibitor 3 precursor (*TIMP3*) fragments of the wild-type 3' untranslated region (UTR) containing the putative binding site (Supplementary Table S2) were synthesized and ligated between the SpeI and HindIII restriction sites of the pMIR-REPORT Luciferase vector. Mutant 3'UTRs lacking the miR-21-binding site were also synthesized. The wild-type and mutant 3'UTRs were ligated between the SpeI and HindIII restriction sites of the pMIR-REPORT Luciferase vector to create the pLUC-targets and pLUC-mutTargets constructs. Huh7 cells were transfected with different reporter vectors (p-Luc-Empty, p-Luc-targets, or p-Luc-mutTargets) and cotransfected with negative control or miR-21 inhibitor. Huh7 cells were transfected with these reporter vectors in addition to a miR-21 inhibitor or negative control. pMIR-REPORT β -gal was also transfected for use in transfection normalization. At 36 hours after transfection, luciferase and β -galactosidase activities were measured using the Dual-Light System (Promega).

ELISA

IL6 and HMGB1 levels in the supernatants from HCC cell lines or primary human hepatocytes were detected by ELISA (IBL), according to the manufacturer's instructions.

Cell proliferation assay

Cells were plated in 96-well plates at a density of 3,000 per well and incubated at 37°C. Cell proliferation was assessed at 24, 48, and 72 hours after transfection using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay Kit (Promega).

Cell migration and invasion assays

The migration and invasion assays were performed by using Transwell insert chambers (6.5 mm diameter, 8 mm pore size, Corning) as previously described (24).

Establishment of stable HMGB1-expressing cells

A total of 2×10^5 Huh7 cells were seeded onto 6-well plates and allowed to recover for 24 hours. Transfection was accomplished using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction, and cells were then cultured in DMEM plus 700 μ g/mL G418 (Sigma-Aldrich) to select stable transfectants. After 4 weeks, clones with G418 resistance were selected and expanded.

Animals

Athymic nude mice (NU/J), male, 4–6 weeks old, and 16–20 g) were purchased from Jackson ImmunoResearch Laboratories and bred in pathogen-free conditions. Animal protocols were approved by the Animal Care and Use Committee of the University of Pittsburgh and the experiments were performed in adherence to the NIH Guidelines for the Use of Laboratory Animals. To establish HCC xenograft model, 2×10^6 Huh7-HMGB1 or Huh7-vector control were suspended in 100 μ L serum free-DMEM and subcutaneously inoculated into the flanks of nude mice. Eight days after inoculation, a subgroup of these mice received either intratumoral injection (1×10^8 copies in 50 μ L PBS) of a

lentiviral-based expression vector for the miR-21 inhibitor (anti-miR-21) or negative control (Lenti-GFP-NC). Every 4 days after inoculation, tumor length (L) and width (W) were measured with Vernier calipers, with volume (V) calculated by the formula $V = (L \times W^2) \times 0.5$.

In vivo imaging of MMP activity

Three days after lentiviral injection, 150 μ L of MMPsense 680 (PerkinElmer Inc.) was given to the mice intravenously. At 24 hours, the mice were imaged using IVIS Lumina. Relative intensities were determined using ImageJ software (NIH).

Statistical analysis

Quantitative data were presented as mean \pm SD as analyzed by the Student t test or ANOVA, with $P < 0.05$ considered as

statistically significant. Qualitative data were representative of three independent experiments.

Results

HMGB1 and miR-21 are upregulated in HCC

Using qRT-PCR, HMGB1 and miR-21 mRNA expression were measured in three human HCC cell lines (Huh7, HepG2, Hep3B) and compared with primary human hepatocyte controls. HMGB1 and miR-21 mRNA levels were significantly increased in all three HCC cell lines (Fig. 1A and C). This observation was corroborated *in vivo* by comparing HMGB1 and miR-21 mRNA levels in HCC tumors and corresponding background liver tissue (Fig. 1B and D). Because the role of HMGB1 as a proinflammatory DAMP depends on its presence in the extracellular environment, we also confirmed

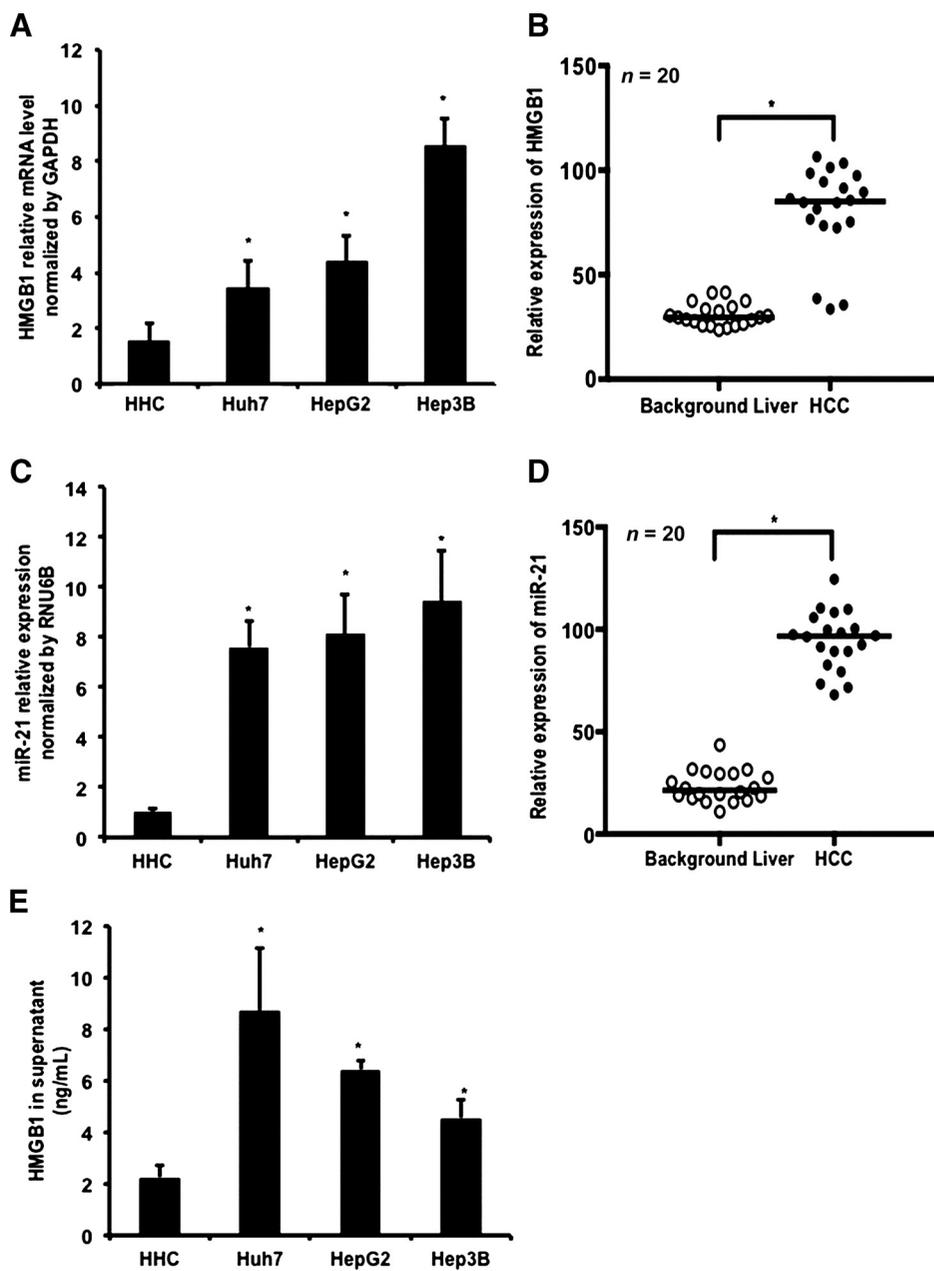


Figure 1.

HMGB1 and miR-21 are upregulated in HCC cell lines and HCC tumor samples. miR-21 and HMGB1 levels were measured in HCC cell lines (Huh7, HepG2, and Hep3B) and human HCC tumor samples using qRT-PCR. Comparison was made to primary human hepatocytes (HHC) or background liver tissue from the same patient. Levels of HMGB1 (A and B) and miR-21 (C and D) were significantly increased in both HCC cell lines and HCC tumor samples. E, ELISA was used to measure HMGB1 levels in the cell culture media of HCC cell lines and primary human hepatocytes. Data are presented as mean \pm SE and are representative of three independent experiments. *, $P < 0.05$.

the presence of elevated HMGB1 levels in the supernatant of HCC cell lines compared with primary hepatocytes (Fig. 1E).

HMGB1 induces the expression of miR-21 in HCC cell lines

To evaluate whether HMGB1 can regulate miR-21 expression, Huh7 and HepG2 HCC cells were treated with varying concentrations of recombinant human HMGB1 (rhHMGB1). HMGB1 stimulation resulted in a dose-dependent increase in miR-21 levels (Fig. 2A). HMGB1 was also found to amplify miR-21 expression in a time-dependent manner (Fig. 2B). We next established that the blockade of HMGB1 signaling with HMGB1-neutralizing antibody (anti-HMGB1) in both Huh7 and HepG2 cells prevented the expression of miR-21 (Fig. 2C). To further confirm the finding that HMGB1 mediates miR-21 expression, Huh7, HepG2, and Hep3B cells were each treated with siRNA to HMGB1 (si-HMGB1). HMGB1 knockdown was confirmed with decreased HMGB1 protein expression and release from all three cell lines (Fig. 2D). More importantly, knockdown of HMGB1 resulted in decreased miR-21 expression, suggesting that tumor-derived HMGB1 functions in a paracrine fashion to regulate miRNA expression in nearby cells (Fig. 2E).

MiR-21 expression is stimulated by HMGB1 in an IL6/Stat3-dependent manner

IL6 and Stat3 have recently been described as mediators of miR-21 expression in lymphoma and glioma, respectively (17, 25). Taken with our initial observations, we hypothesized that the IL6/Stat3 signaling pathway serves as the bridge between HMGB1 and miR-21 expression. To first determine whether IL6 is upregulated in HCC, we stimulated Huh7 cells with rhHMGB1 and measured IL6 levels in the cell culture media. Media from cells treated with rhHMGB1 showed a significant increase in IL6 levels, but this effect was attenuated by addition of anti-HMGB1 antibodies (Fig. 3A). Similar results were found with HepG2 cells under the same experimental conditions (Supplementary Fig. S1A).

Stat3 not only is an important downstream target of IL6, but also has been implicated in the relationship between inflammation and HCC (26). Accordingly, Western blot analysis of HCC tumor samples revealed a significant increase in the active/total STAT3 ratio in tumor tissues compared with normal background tissue (Fig. 3B). In addition, treatment of Huh7 cells with HMGB1 induced Stat3 activation *in vitro* (Fig. 3C). This effect was abrogated with anti-HMGB1 treatment. HepG2 cells were used in the same experiments with similar results (Supplementary Fig. S1B). The importance of IL6 in the HMGB1-Stat3 pathway was further confirmed by treating Huh7 cells with anti-IL6, anti-IL6 receptor (anti-IL6R) antibodies, and WP1066 (an inhibitor of Stat3 phosphorylation), all of which inhibited Stat3 activation by HMGB1 (Fig. 3D).

Next, to determine whether HMGB1 induces miR-21 expression in HCC through the IL6/Stat3 signaling pathway, Huh7 and HepG2 cells were treated with HMGB1 in the presence of IL6 and Stat3 inhibitors. As anticipated, HMGB1 treatment alone resulted in a significant increase in miR-21 levels, while with inhibition of HMGB1, IL6, or Stat3 each limited miR-21 expression (Fig. 3E and Supplementary Fig. S1C). The effects of HMGB1 on the expression of pre-miR-21 (precursor mRNA for miR-21) were similar to that of the effects of HMGB1 on mature miR-21 (Supplementary Fig. S1C), which confirmed that our findings were related to alteration in miR-21 transcription and not merely generalized changes in miRNA processing.

RECK and TIMP3 are downstream targets of miR-21

To better evaluate the importance of the HMGB1-miR-21 signaling pathway in HCC, we sought to identify biologically relevant and measurable targets of miR-21. From the predicted targets of miR-21 determined by the TargetScan (27) algorithm, we chose to focus on the MMP inhibitors RECK and TIMP3 (Fig. 4A). These two proteins were first chosen because analysis of the HCC samples from our tissue bank show that not only are RECK and TIMP3 repressed in tumor tissue compared with matched background liver controls but, as expected, the abundance of miR-21 and HMGB1 is inversely correlated with levels of these proteins (Fig. 4B). RECK and TIMP3 were also selected because their expression has been linked to HCC prognosis (28), and they participate in regulating MMPs, which mediate invasion and metastases through degradation of the extracellular matrix (ECM; ref. 29). In addition to being predicted targets of miR-21, we also observed that the expression of RECK and TIMP3 is sensitive to HMGB1 signaling. HMGB1 stimulation represses these proteins while, in contrast, their expression is increased with siRNA knockdown of HMGB1 (Fig. 4C).

To confirm the validity of the predicted targets, the 3'UTRs containing the putative miR-21-binding sites of both *RECK* or *TIMP3* were cloned into pLUC expression vectors (pLUC-target). After transfection of the expression vectors, Huh7 cells were treated with anti-miR-21 or an anti-miRNA negative control (anti-miR-NC), and luciferase expression was measured. Cells treated with the pLUC-target vector and anti-miR-NC showed basal repression of the luciferase activity due to the presence of endogenous miR-21, while addition of anti-miR-21 then reversed this repression (Fig. 4D, left). Luciferase activity was not suppressed when the vectors containing mutated target sequences (pLuc-mutTarget) were treated with pre-miR-21 but was inhibited as expected when vectors with the normal target sequence were treated with pre-miR-21 (Fig. 4D, right). These data confirm that translational repression is mediated by the specific binding of miR-21 to the identified target sequences within the 3'UTR of both *RECK* and *TIMP3*. These observations demonstrate that endogenous levels of miR-21 can repress expression of RECK or TIMP3 and that the repression is subsequently released with inhibition of miR-21.

The effect of miR-21 on RECK and TIMP3 expression was measured *in vitro* by transfection of normal hepatocytes with pre-miR-21. As expected, pre-miR-21 treatment resulted in a significant reduction of RECK and TIMP3 protein (Fig. 4E). Interestingly, RECK and TIMP3 mRNA were also downregulated by addition of pre-miR-21 in hepatocytes (Fig. 4F), suggesting that post-transcriptional regulation by miR-21 occurs through both mRNA destabilization and transcriptional blockade. Because miR-21 is overexpressed in HCC, we also investigated the effects of miR-21 knockdown in Huh7 cells. As expected, anti-miR-21 treatment resulted in significant increases in both RECK and TIMP3 protein (Fig. 4E) and mRNA levels (Fig. 4F). Together, these data show that the overexpression of miR-21 is associated with oncogenesis and provides a mechanism for modulation of RECK and TIMP3.

miR-21 is essential for HMGB1-mediated HCC invasion, migration, and proliferation

To evaluate the importance of the HMGB1-miR-21-RECK/TIMP3 pathway in HCC progression, we utilized the Transwell invasion/migration assay. Invasion of both Huh7 (Fig. 5A) and HepG2 (Supplementary Fig. S2A) cells were significantly increased after HMGB1 treatment, while anti-miR-21 treatment

resulted in reduced invasive potential. Inhibition of Stat3 with WP1066 was also capable of abrogating HMGB1-mediated invasion. Similar effects on migration were observed in Huh7 (Fig. 5B)

and HepG2 cells (Supplementary Fig. S2B) after either HMGB1 or anti-miR-21 treatment. In contrast with miR-21 inhibition, treatment of Huh7 cells with pre-miR-21 promoted invasion/

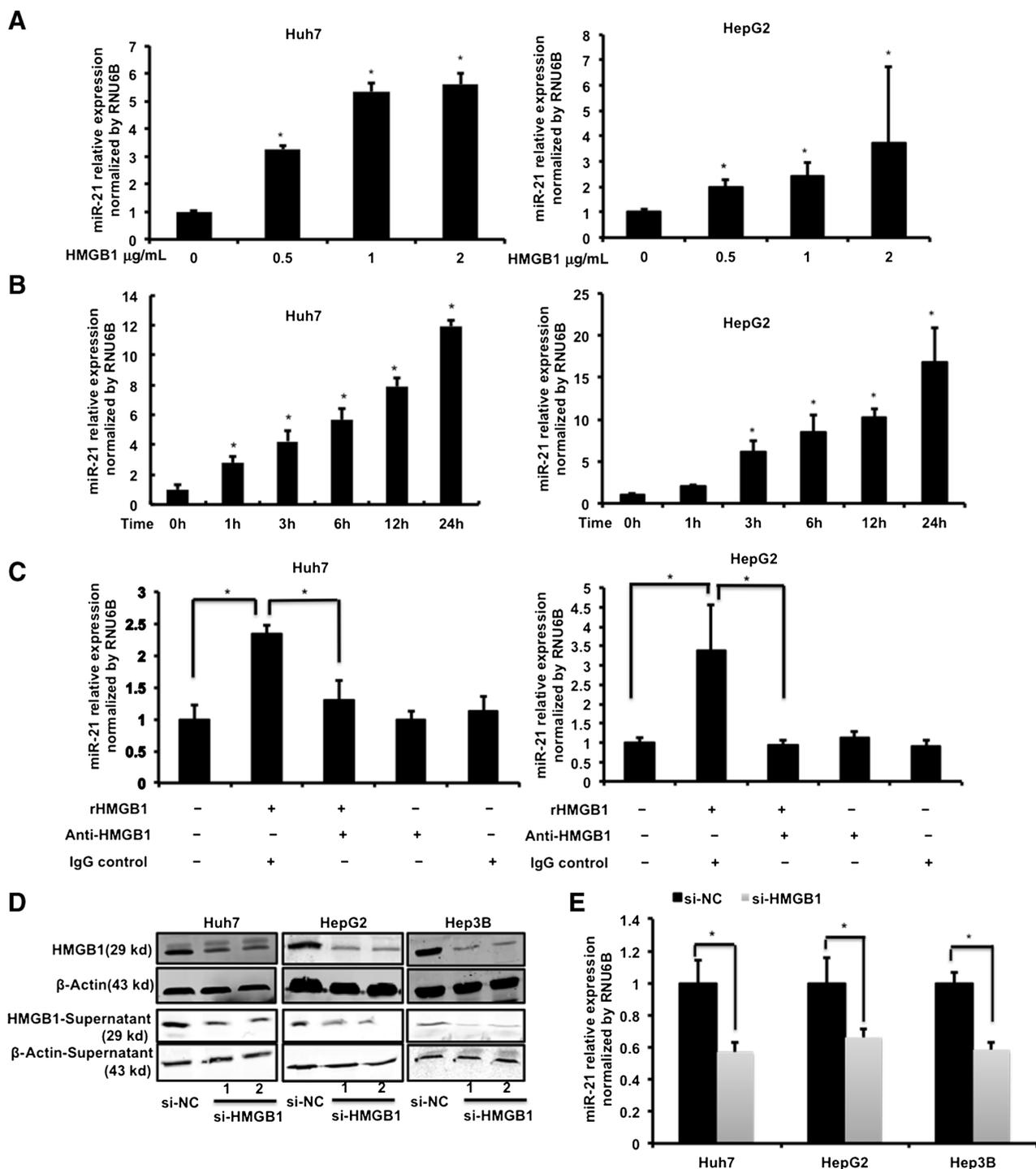
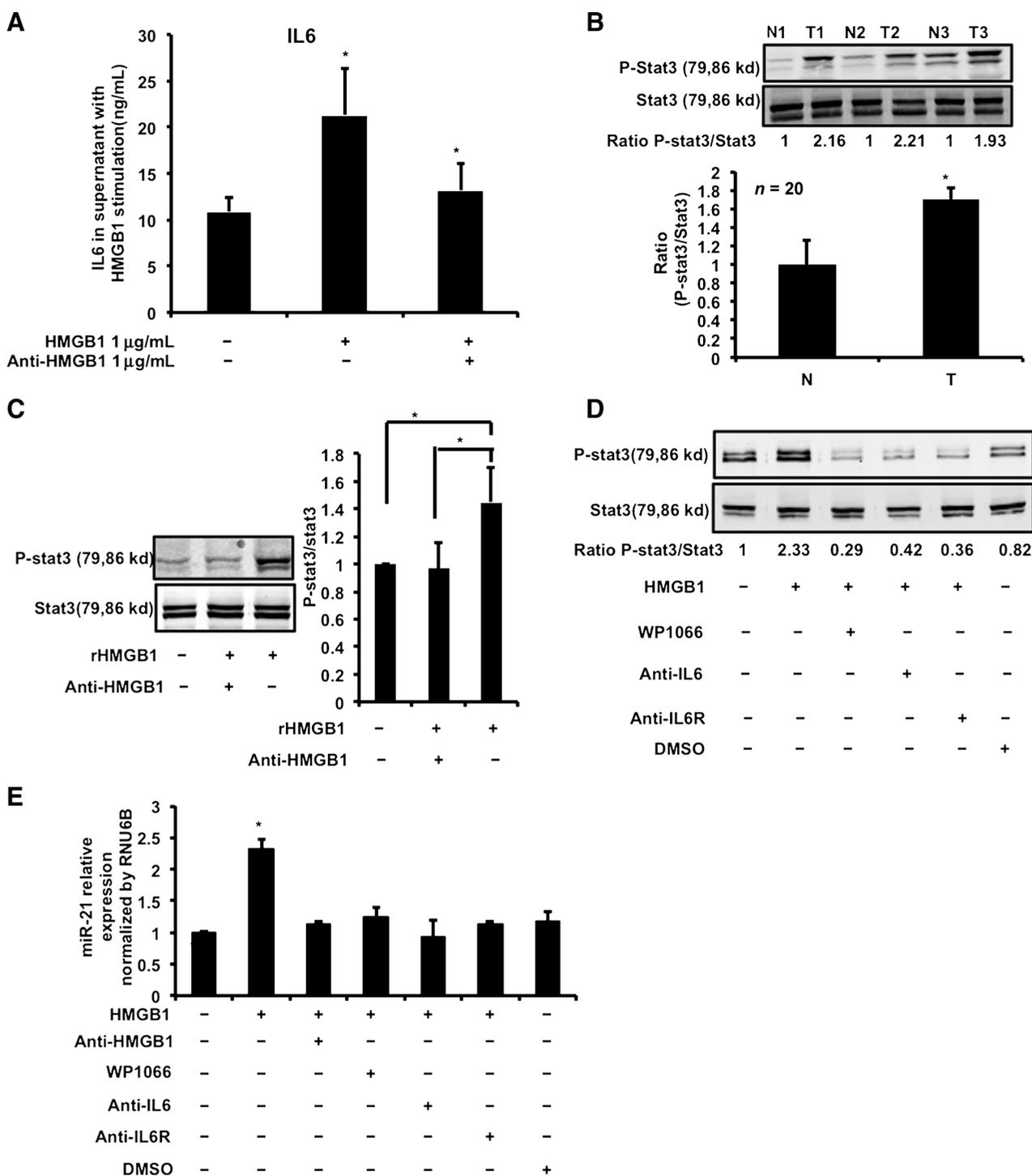


Figure 2.

HMGB1 stimulates miR-21 expression in a dose- and time-dependent manner. A, miR-21 expression in Huh7 and HepG2 cells was measured after treatment with 0, 0.5, 1, and 2 $\mu\text{g}/\text{mL}$ of rHMGB1. B, miR-21 expression in Huh7 and HepG2 cells was measured at 0, 1, 3, 6, 12, and 24 hours after treatment with 1 $\mu\text{g}/\text{mL}$ of rHMGB1. C, Huh7 and HepG2 cells were cotreated with rHMGB1 (1 $\mu\text{g}/\text{mL}$) and an HMGB1-neutralizing antibody (anti-HMGB1, 1 $\mu\text{g}/\text{mL}$) or IgG antibody control and miR-21 expression was measured. D, Huh7, HepG2, and Hep3B cells were treated with an HMGB1 siRNA (si-HMGB1) or negative control siRNA (si-NC). Western blot analysis characterized HMGB1 content in the whole-cell lysates and cell culture media. E, miR-21 expression was measured by qRT-PCR. Data are presented as mean \pm SE and are representative of three independent experiments. *, $P < 0.05$.

Chen et al.

**Figure 3.**

HMGB1-mediated miR-21 expression is dependent upon the IL6/Stat3 pathway. A, Huh7 cells were treated with rHMGB1 (1 µg/mL) alone or in combination with HMGB1-neutralizing antibody (anti-HMGB1). ELISA was used to measure IL6 levels in the cell culture media. B, Stat3 and P-stat3 (tyr705) levels were measured in paired samples of human HCC tumors (T) and background liver (N) using Western blot analysis with densitometric analysis, selected samples are representative of 20 unique paired samples, $n = 20$. C, Huh7 cells were treated with rHMGB1 (1 µg/mL) alone and in combination with anti-HMGB1 (1 µg/mL). Stat3 and P-stat3 levels were measured by Western blot analysis with densitometric analysis. Stat3 activation was characterized by calculating the ratio of P-stat3 to Stat3. D and E, Huh7 cells were treated with rHMGB1 alone (1 µg/mL) or in combination with IL6-neutralizing antibody (anti-IL6), IL6 receptor antibody (Anti-IL6R), or a known Stat3 inhibitor (WP1066). DMSO was used as a vehicle control. D, Stat3 activation was measured by calculation of the P-stat3 to Stat3 ratio after Western blot analysis with densitometric analysis. E, miR-21 expression was measured. Data are presented as mean \pm SE and are representative of three independent experiments. *, $P < 0.05$.

A

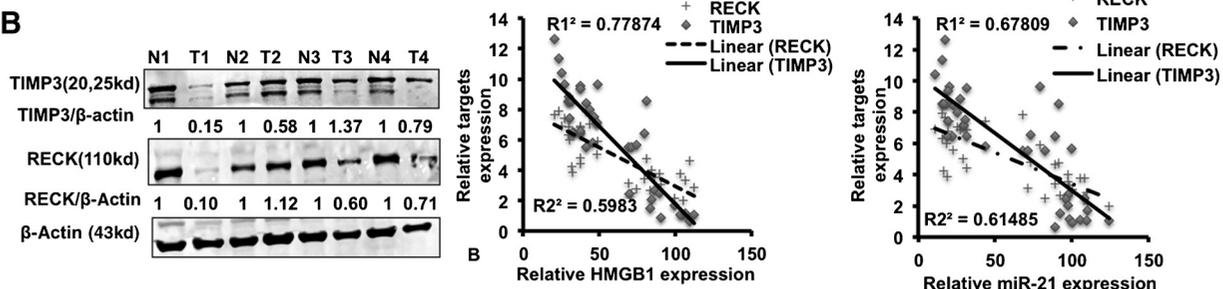
TIMP3: Metalloproteinase inhibitor 3 precursor (TIMP3)

	Predicted consequential pairing of target region(top) and miRNA(bottom)
Position 1032-1039 of TIMP3 3' UTR	5' ...UACCCACAUGGGGACAUAAGCUA... 3' AGUUGUAGUCAGACUAUUCGAU
Position 2385-2392 of TIMP3 3' UTR	5' ...GGUUUUGAGCUUUCUAUAAGCUA... 3' AGUUGUAGUCAGACUAUUCGAU

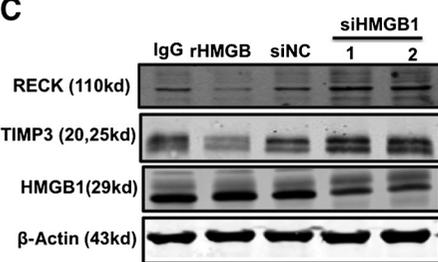
RECK: Reversion-inducing cysteine-rich protein with Kazal motifs precursor (hRECK)

	Predicted consequential pairing of target region(top) and miRNA(bottom)
Position 1138-1145 of RECK 3' UTR	5' ...GUUUCACAGUUUGAAAUAAGCUA... 3' AGUUGUAGUCAGAC -- UAUUCGAU

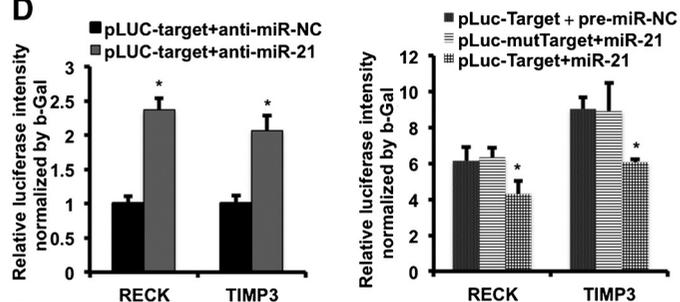
B



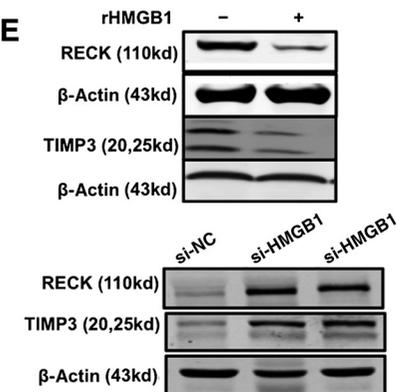
C



D



E



F

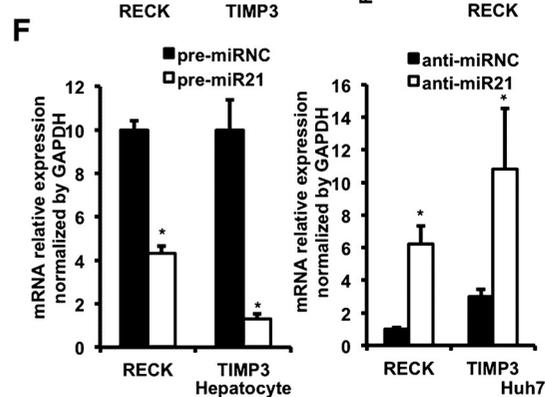
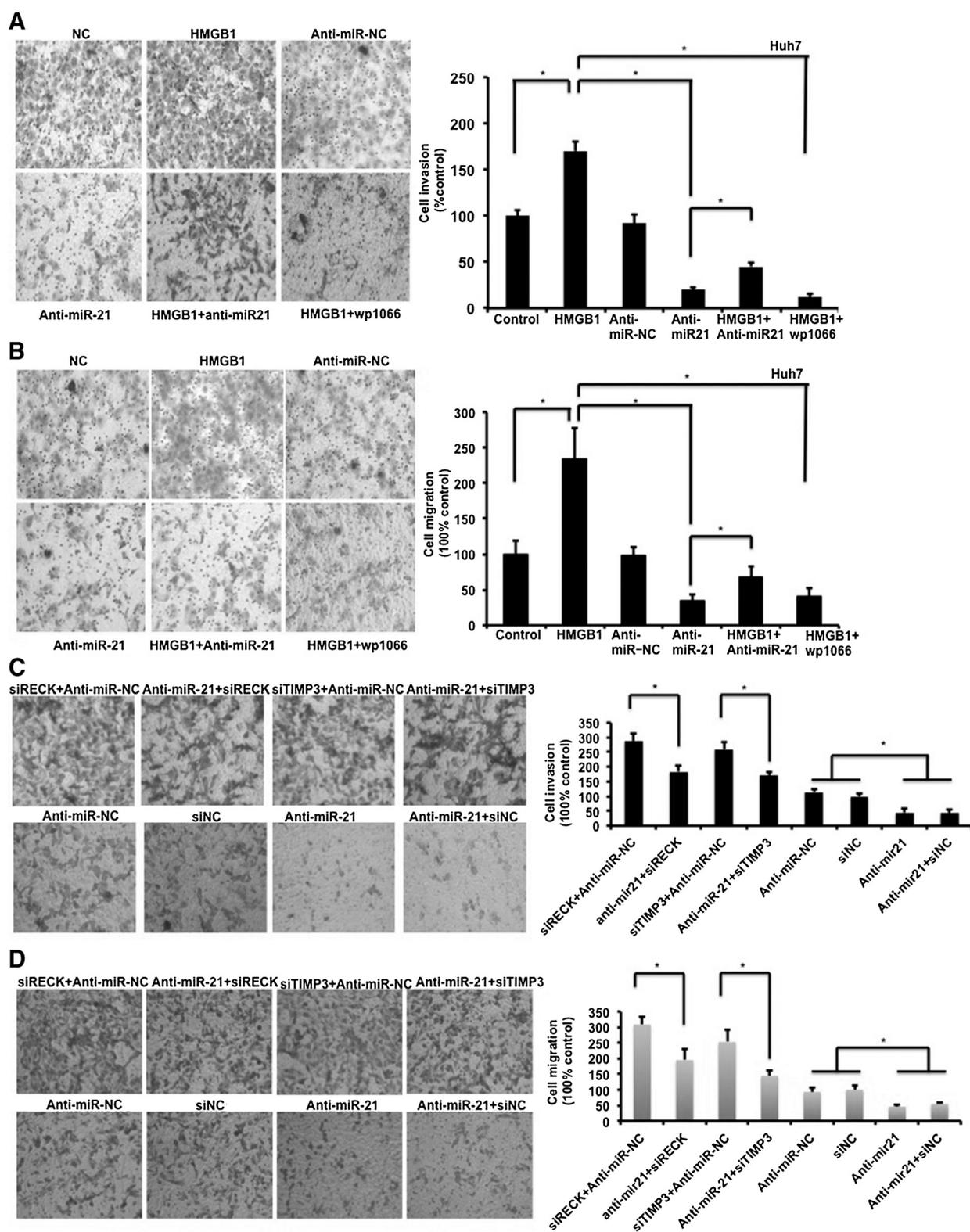


Figure 4.

RECK and TIMP3 are repressed by miR-21 in HCC. A, TargetScan alignment of miR-21 and the 3'UTRs of RECK and TIMP3. B, paired tumor (T) and background liver (N) samples were analyzed for RECK and TIMP3 expression by Western blot analysis. Results were standardized to β -actin by densitometric analysis. Linear regression was used to identify the correlation between HMGB1 or miR-21 expression and RECK/TIMP3. Displayed results are representative of 20 unique paired samples. C, Huh7 cells were treated with HMGB1 (1 μ g/mL) for 24 hours and HMGB1 expression in Huh7 cells was inhibited by transfection of HMGB1 siRNA (si-HMGB1). IgG and siNC serve as the negative controls for rHMGB1 and siHMGB1, respectively. Results utilizing two unique HMGB1 siRNAs are shown. RECK and TIMP3 expression was measured by Western blot analysis. D, fragments of the wild-type RECK (55-mer) and TIMP3 (57-mer) 3-UTR containing the predicted miR-21-binding sites were cloned into the pLUC expression vector (pLUC-target). Mutated targets were cloned into the same vector as a negative control (p-Luc-mutTarget). Vectors were cotransfected with anti-miR-21/anti-miR-NC in Huh7 cells or pre-miR-21/pre-miR-NC in primary hepatocytes. Luciferase activity was measured 36 hours after transfection and normalized to β -Gal. E, primary human hepatocytes were transfected with miR-21 precursor (pre-miR-21) or precursor miRNA negative control (pre-miR-NC). Huh7 cells were treated with anti-miR-21 or anti-miR-NC. RECK and TIMP3 expression was measured by Western blot analysis 48 hours after transfection. F, hepatocytes were transfected with pre-miR-21 or pre-miR-NC and Huh7 cells were transfected with anti-miR-21 or anti-miR-NC. RECK and TIMP3 mRNA levels were measured with qRT-PCR 48 hours after transfection. Data are presented as mean \pm SE and are representative of three independent experiments. *, $P < 0.05$.

Chen et al.

**Figure 5.**

Blockade of the miR-21 prevents HCC invasion and migration. Huh7 cells were pretreated with rHMGB1 alone or in combination with anti-miR-21 or the Stat3 inhibitor WP1066. Cells were then seeded in Transwell chambers and counted by light microscopy after 48 hours. Representative micrographs and cell counts for invasion (A) and migration (B) are shown. Phenotype rescue experiments were completed by transfecting TIMP3 and RECK siRNA into HMGB1-overexpressing Huh7 cells with anti-miR-21 or anti-miR control. Representative micrographs and cell counts for invasion (C) and migration (D) are shown. Data are presented as mean \pm SE and are representative of three independent experiments. *, $P < 0.05$.

migration (Supplementary Fig. S2C). In addition to its role in HCC invasion and migration, we also noted that miR-21 has a significant impact on HCC cell proliferation as measured by the MTT assay (Supplementary Fig. S2D).

To further confirm that the observed changes in invasion and migration were related to the differential expression of RECK and TIMP3, we performed phenotype rescue experiments. RECK and TIMP3 siRNAs were cotransfected into HMGB1-overexpressing Huh7 cells with anti-miR-21 or anti-miR-NC, and invasion/migration were measured using Transwell assays. In these cells, elevated miR-21 levels resulted in repression of RECK and TIMP3 and enhanced invasion and migration, while treatment with anti-miR-21 reduced their invasive potential. However, cells subjected to knockdown of RECK and TIMP3 after miR-21 inhibition exhibit restoration of their invasive potential (Fig. 5C and D). This finding is consistent with the conclusion that the regulation of RECK and TIMP3 is a critical element of miR-21's influence on the invasive potential of HCC.

HMGB1-miR-21 signaling inhibits RECK/TIMP3 and promotes tumor growth

The relevance of our *in vitro* findings was next confirmed *in vivo* using an HCC xenograft model. First, Huh7 cells with stable HMGB1 overexpression (Huh7-HMGB1) were generated through transfection and G418 selection. These cells were then injected into the flanks of nude mice. Eight days after tumor engraftment, one group of mice was treated with an intratumoral injection of a lentiviral-based miR-21 inhibitor (lenti-anti-miR-21) or a negative control (lenti-GFP-NC; Fig. 6A). Infection efficiency of lenti-anti-miR-21 and HMGB1 overexpression was confirmed by qRT-PCR and Western blot analysis, respectively (Supplementary Fig. S3D). MMPsense 680 and IVS lumina imaging were then used to analyze MMPs activity within the tumors. Consistent with our expectations, HMGB1-mediated MMP activity was suppressed by treatment with lenti-anti-miR21 (Fig. 6B). Two mice without any treatment were used as negative controls for the imaging agent (Supplementary Fig. S3C). After 6 weeks, Huh7-HMGB1 tumors were significantly larger than control, whereas lenti-anti-miR-21 mitigated the effect of HMGB1 overexpression on tumor growth (Fig. 6C and D and Supplementary Fig. S3A and S3B). To ensure that *in vivo* treatment with anti-miR-21 affects RECK and TIMP3 expression, we performed Western blot analysis of the explanted xenografts. As expected, anti-miR-21 treatment resulted in derepression of RECK and TIMP3 (Fig. 6E). Taken together, these findings suggest that HMGB1-miR-21 signaling leads to MMP activity through downregulation of RECK and TIMP3 and subsequently promotes HCC tumor progression *in vivo*.

Discussion

Over the past decade, our understanding of the complex molecular mechanisms responsible for HCC tumorigenesis and progression has improved dramatically, implicating a number of classical cellular signaling pathways (30). However, this knowledge has not translated into improved chemotherapeutic strategies for advanced and recurrent disease, allowing HCC to remain the third most common cause of cancer deaths worldwide (31). In this study, we find that HMGB1 stimulates miR-21 overexpression through the IL6/Stat3 axis in HCC. Furthermore, we show that the

HMGB1-miR-21 signaling cascade promotes tumor progression by repression of the MMP inhibitors, RECK and TIMP3, in a murine xenograft model.

The importance of inflammation in the initiation and progression of cancer is well established (4). With respect to HCC, over 90% of HCC cases occur in the setting of chronic liver inflammation related to underlying liver diseases such as viral hepatitis, NASH, and alcoholic liver disease. Thus, it is no surprise that HCC tumor biology is strongly influenced by mediators of the inflammatory response (32). Our previous work has focused on characterizing the role of DAMPs, including histones and HMGB1, in liver inflammation (8, 33). Importantly, we have shown that release of HMGB1 not only mediates the acute phase of liver injury associated with ischemia-reperfusion (25), but also promotes HCC invasion and metastasis by activating caspase-1 (6). Though not classified as a DAMP, miR-21 shares similarities with these molecules in that it bridges pathways linking inflammation to cancer (34). In breast and ovarian adenocarcinomas, hypoxia induces miR-21 expression through Akt-2, and confers resistance to apoptosis by targeting programmed cell death 4 (*PDCD4*) and *Sprouty1* (35). With regard to HCC, miR-21 overexpression is associated with advanced tumor stage and poor prognosis (36), and this effect has previously been attributed to modulation of the tumor suppressors *PTEN* and *PDCD4* (37, 38). Similar to recent findings that HMGB1 can stimulate miRNA expression in cardiac regeneration (39) and papillary thyroid cancer (40), our study demonstrates that HMGB1 and miR-21 participate in a common pathway linking inflammation to HCC progression.

The effects of HMGB1 release can be correlated with its ability to signal through the PRRs TLR2, TLR4 and the receptor for RAGE (41). Ligand of these cellular receptors activate the NF- κ B, ERK, and p38 signaling cascades, ultimately resulting in the production of the prosurvival cytokines IL6, TNF, and IFN γ (42). Consistent with these previous observations, our work demonstrates that IL6 expression is induced by HMGB1 treatment of HCC cell lines. Furthermore, IL6 signaling is known to play an important role in the tumor microenvironment by promoting growth and inhibiting apoptosis (43, 44). This effect is dependent upon activation of the proinflammatory transcription factor STAT by Janus kinase (JAK), but the targets of STAT are less well characterized. Our observation that activation of the IL6/Stat3 axis by HMGB1 is not only sufficient, but also necessary to stimulate miR-21 expression therefore adds significant insight into the mechanism for inflammation-mediated growth of HCC.

As a classic gene family, MMPs are primarily involved in cancer development and progression via ECM degradation and/or the activation of pre-progrowth factors (45). Previous work from other groups has also shown that MMP activity can be used as a marker of disease progression in animal tumor models (46, 47). We corroborate these observations in HCC by showing that increased MMP activity correlates with the growth of tumor xenografts. Results from analysis of breast cancer cell lines have previously identified RECK and TIMP3 as targets of miR-21 in gastric (48) and breast (49) cancer, respectively. Our analysis of human HCC samples shows that RECK and TIMP3 expressions are significantly repressed when compared with background liver tissue, and we further confirm that posttranscriptional regulation by miR-21 contributes to this repression. Thus, we conclude that modulation of RECK and TIMP3 through the HMGB1-miR-21 axis plays a significant role in HCC tumor progression. It is important to note that the role of miR-21 in HCC is not limited

Chen et al.

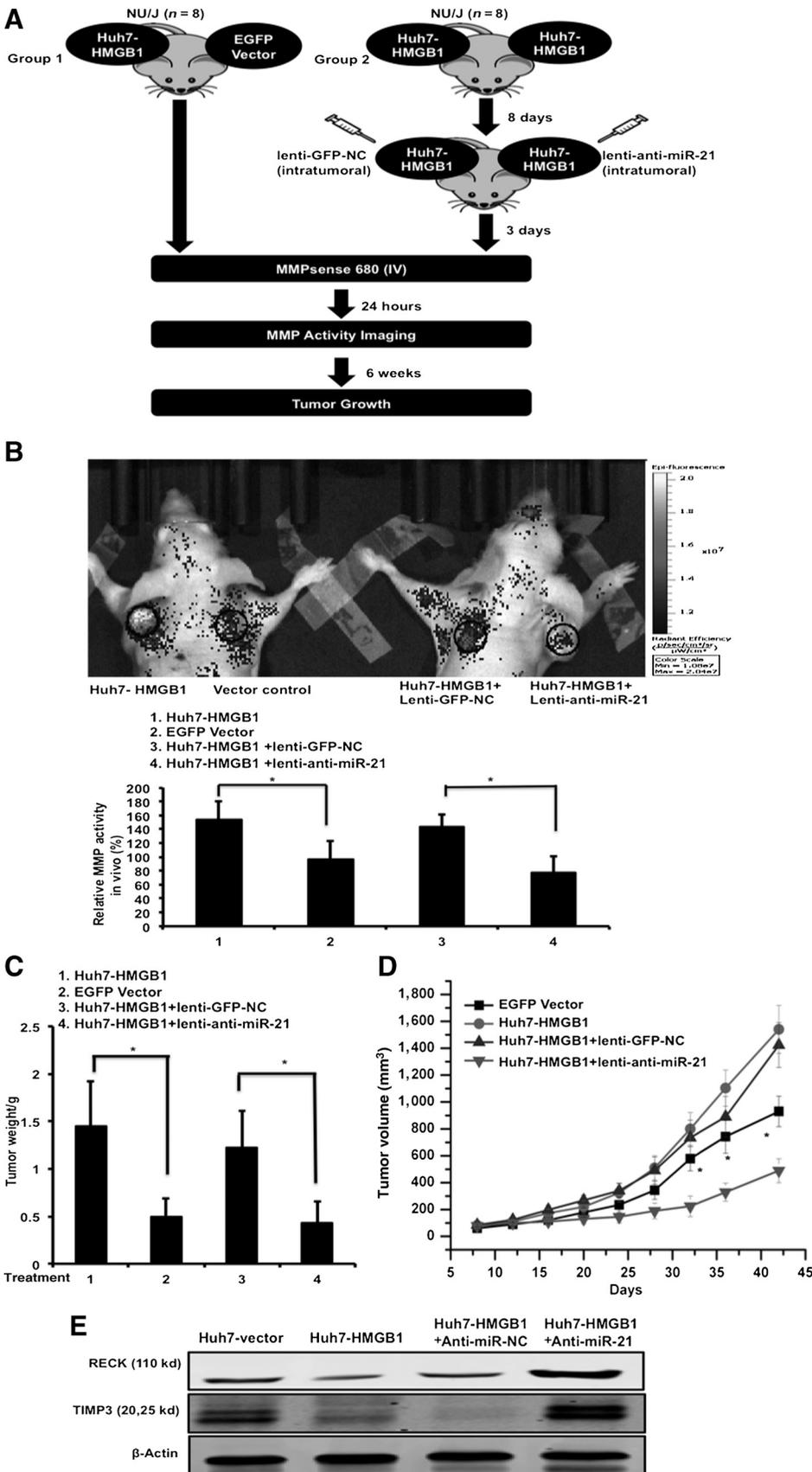


Figure 6. miR-21 blockade inhibits HMGB1-mediated progression of murine HCC xenografts. A, Huh7 cells with stable HMGB1 overexpression (Huh7-HMGB1) were generated through transfection and selection. Nude (NU/J) mice then received bilateral flank injection of tumor cells. Group 1 received Huh7-HMGB1 and Huh7-vector control, while group 2 received Huh7-HMGB1 in both flanks. Eight days after engraftment, group 2 mice received intratumoral injection of lentiviral vectors containing a miR-21 expression cassette (right) or negative control cassette (left). B, tumor MMP activity was measured by IVIS Lumina imaging after injection of MMPsense 680 injection. Quantification of the relative fluorescent intensities was performed with ImageJ (NIH). $N = 4$, with two representative mice shown. C, final tumor weight was documented after 6 weeks. D, tumor dimensions were documented with Vernier calipers every 4 days after tumor engraftment. Volume was calculated by the formula $V = (L \times W^2) \times 0.5$. E, Western blot analysis was used to analyze RECK and TIMP3 levels in explanted xenografts. Data, mean \pm SE. *, $P < 0.05$.

to its effects on these two targets. The focus of our work, however, was not the identification of all downstream targets of miR-21 but rather the upstream pathway that links HMGB1 and miR-21 expression. RECK and TIMP3 therefore provided validated, biologically relevant, and experimentally measurable targets to assess the importance of miR-21 regulation by HMGB1.

In summary, we present a novel IL6/Stat3-dependent mechanism for miR-21 expression stimulated by HMGB1 in HCC, and further describe the importance of miR-21 in tumor growth and progression through its regulation of MMP activity. These observations provide insight into the pathways responsible for miRNA regulation, and further define the complex link between inflammation and cancer. Our results lend credibility to HMGB1 and miR-21 as potential targets for the development of novel therapeutic agents for HCC; and, future endeavors should work to translate these findings from animal models to the clinical setting.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: M. Chen, Y. Chang, X.-X. He, M.T. Lotze, J. Lin, A. Tsung
Development of methodology: M. Chen, Y. Chang, X.-X. He, M.T. Lotze, A. Tsung

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Chen, H. Huang, A. Tsung

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Chen, Y. Liu, P. Varley, Y. Chang, D. Tang, A. Tsung
Writing, review, and/or revision of the manuscript: M. Chen, P. Varley, D. Tang, M.T. Lotze, A. Tsung

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Chen, M.T. Lotze, A. Tsung

Study supervision: M. Chen, H. Huang, M.T. Lotze, J. Lin, A. Tsung

Other (developed strategies for miR analysis at the UICSHS): M.T. Lotze

Acknowledgments

The authors thank Xinghua Liao, Kimberly Ferrero, and Nicole Martik-Hays for their expert technical assistance.

Grant Support

This work was supported by Howard Hughes Medical Institute Physician-Scientist Award (A. Tsung), R01-GM95566 (A. Tsung), The National Natural Science Foundation of China (No. 81101824), and by the Outstanding Youth Science Foundation of Tongji Hospital (No. YXQN005).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received July 23, 2014; revised December 11, 2014; accepted December 24, 2014; published OnlineFirst February 26, 2015.

References

- El-Serag HB, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. *Gastroenterology* 2007;132:2557-76.
- Llovet JM, Di Bisceglie AM, Bruix J, Kramer BS, Lencioni R, Zhu AX, et al. Design and endpoints of clinical trials in hepatocellular carcinoma. *J Natl Cancer Inst* 2008;100:698-711.
- Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. *Cell* 2010;140:883-99.
- Multhoff G, Molls M, Radons J. Chronic inflammation in cancer development. *Front Immunol* 2011;2:98.
- Tang D, Kang R, Zeh HJ, Lotze MT. High-mobility group box 1 and cancer. *Biochim Biophys Acta* 2011;1799:131-40.
- Yan W, Chang Y, Liang X, Cardinal JS, Huang H, Thorne SH, et al. High-mobility group box 1 activates caspase-1 and promotes hepatocellular carcinoma invasiveness and metastases. *Hepatology* 2012;55:1863-75.
- Lotze MT, Tracey KJ. High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. *Nat Rev Immunol* 2005;5:331-42.
- Tsung A, Klune JR, Zhang X, Jeyabalan G, Cao Z, Peng X, et al. HMGB1 release induced by liver ischemia involves Toll-like receptor 4 dependent reactive oxygen species production and calcium-mediated signaling. *J Exp Med* 2007;204:2913-23.
- Kang R, Zhang Q, Zeh HJ, Lotze MT, Tang D. HMGB1 in cancer: good, bad, or both? *Clin Cancer Res* 2013;19:4046-57.
- Scaffidi P, Misteli T, Bianchi ME. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature* 2002;418:191-5.
- Tian J, Avalos AM, Mao S-Y, Chen B, Senthil K, Wu H, et al. Toll-like receptor 9-dependent activation by DNA-containing immune complexes is mediated by HMGB1 and RAGE. *Nat Immunol* 2007;8:487-96.
- O'Hara SP, Mott JL, Splinter PL, Gores GJ, LaRusso NF. MicroRNAs: key modulators of posttranscriptional gene expression. *Gastroenterology* 2009;136:17-25.
- Dong H, Lei J, Ding L, Wen Y, Ju H, Zhang X. MicroRNA: Function, Detection, and Bioanalysis. *Chem Rev* 2013;113:6207-33.
- Di Leva G, Croce CM. Roles of small RNAs in tumor formation. *Trends Mol Med* 2010;16:257-67.
- Lee YS, Dutta A. MicroRNAs in cancer. *Annu Rev Pathol* 2009;4:199-227.
- Ryan BM, Robles AI, Harris CC. Genetic variation in microRNA networks: the implications for cancer research. *Nat Rev Cancer* 2010;10:389-402.
- Chan JA, Krichevsky AM, Kosik KS. MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells. *Cancer Res* 2005;65:6029-33.
- Iorio M V, Ferracin M, Liu C-G, Veronese A, Spizzo R, Sabbioni S, et al. MicroRNA gene expression deregulation in human breast cancer. *Cancer Res* 2005;65:7065-70.
- Schetter AJ, Leung SY, Sohn JJ, Zanetti KA, Bowman ED, Yanaihara N, et al. MicroRNA expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma. *JAMA* 2008;299:425-36.
- Liu C, Yu J, Yu S, Lavker RM, Cai L, Liu W, et al. MicroRNA-21 acts as an oncomir through multiple targets in human hepatocellular carcinoma. *J Hepatol* 2010;53:98-107.
- Liu C, Gao F, Li B, Mitchell REJ, Liu X, Lin J, et al. TLR4 knockout protects mice from radiation-induced thymic lymphoma by downregulation of IL6 and miR-21. *Leukemia* 2011;25:1516-9.
- Du Q, Park KS, Guo Z, He P, Nagashima M, Shao L, et al. Regulation of human nitric oxide synthase 2 expression by Wnt beta-catenin signaling. *Cancer Res* 2006;66:7024-31.
- Chang Y, Yan W, He X, Zhang L, Li C, Huang H, et al. miR-375 inhibits autophagy and reduces viability of hepatocellular carcinoma cells under hypoxic conditions. *Gastroenterology* 2012;143:177-87.e8.
- He X-X, Chang Y, Meng F-Y, Wang M-Y, Xie Q-H, Tang F, et al. MicroRNA-375 targets AEG-1 in hepatocellular carcinoma and suppresses liver cancer cell growth *in vitro* and *in vivo*. *Oncogene* 2012;31:3357-69.
- Tsung A, Sahai R, Tanaka H, Nakao A, Fink MP, Lotze MT, et al. The nuclear factor HMGB1 mediates hepatic injury after murine liver ischemia-reperfusion. *J Exp Med* 2005;201:1135-43.
- He G, Karin M. NF- κ B and STAT3—key players in liver inflammation and cancer. *Cell Res* 2011;21:159-68.
- Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 2005;120:15-20.
- Furumoto K, Arai S, Mori A, Furuyama H, Gorriñ Rivas MJ, Nakao T, et al. RECK gene expression in hepatocellular carcinoma: correlation with invasion-related clinicopathological factors and its clinical significance. Reverse-inducing-cysteine-rich protein with Kazal motifs. *Hepatology* 2001;33:189-95.
- Chen J-S, Wang Q, Fu X-H, Huang X-H, Chen X-L, Cao L-Q, et al. Involvement of PI3K/PTEN/AKT/mTOR pathway in invasion and metastasis in hepatocellular carcinoma: Association with MMP-9. *Hepatology* 2009;39:177-86.
- Aravalli RN, Steer CJ, Cressman ENK. Molecular mechanisms of hepatocellular carcinoma. *Hepatology* 2008;48:2047-63.

Chen et al.

31. El-Serag HB. Hepatocellular carcinoma. *N Engl J Med* 2011;365:1118–27.
32. Berasain C, Castillo J, Perugorria MJ, Latasa MU, Prieto J, Avila M a. Inflammation and liver cancer: new molecular links. *Ann N Y Acad Sci* 2009;1155:206–21.
33. Huang H, Evankovich J, Yan W, Nace G, Zhang L, Ross M, et al. Endogenous histones function as alarmins in sterile inflammatory liver injury through Toll-like receptor 9 in mice. *Hepatology* 2011;54:999–1008.
34. Tili E, Michaille J-J, Croce CM. MicroRNAs play a central role in molecular dysfunctions linking inflammation with cancer. *Immunol Rev* 2013;253:167–84.
35. Polytaichou C, Iliopoulos D, Hatziaepostolou M, Kottakis F, Maroulakou I, Struhl K, et al. Akt2 regulates all Akt isoforms and promotes resistance to hypoxia through induction of miR-21 upon oxygen deprivation. *Cancer Res* 2011;71:4720–31.
36. Karakatsanis A, Papaconstantinou I, Gazouli M, Lyberopoulou A, Polymeneas G, Voros D. Expression of microRNAs, miR-21, miR-31, miR-122, miR-145, miR-146a, miR-200c, miR-221, miR-222, and miR-223 in patients with hepatocellular carcinoma or intrahepatic cholangiocarcinoma and its prognostic significance. *Mol Carcinog* 2013;52:297–303.
37. Meng F, Henson R, Wehbe-Janek H, Ghoshal K, Jacob ST, Patel T. MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. *Gastroenterology* 2007;133:647–58.
38. Qiu X, Dong S, Qiao F, Lu S, Song Y, Lao Y, et al. HBx-mediated miR-21 upregulation represses tumor-suppressor function of PDCD4 in hepatocellular carcinoma. *Oncogene* 2013;32:3296–305.
39. Limana F, Esposito G, D'Arcangelo D, Di Carlo A, Romani S, Melillo G, et al. HMGB1 attenuates cardiac remodeling in the failing heart via enhanced cardiac regeneration and miR-206-mediated inhibition of TIMP-3. *PLoS ONE* 2011;6:e19845.
40. Mardente S, Mari E, Consorti F, Di Gioia C, Negri R, Etna M, et al. HMGB1 induces the overexpression of miR-222 and miR-221 and increases growth and motility in papillary thyroid cancer cells. *Oncol Rep* 2012;28:2285–9.
41. Park JS, Svetkauskaite D, He Q, Kim J-Y, Strassheim D, Ishizaka A, et al. Involvement of Toll-like receptors 2 and 4 in cellular activation by high mobility group box 1 protein. *J Biol Chem* 2004;279:7370–7.
42. Park JS, Arcaroli J, Yum H-K, Yang H, Wang H, Yang K-Y, et al. Activation of gene expression in human neutrophils by high mobility group box 1 protein. *Am J Physiol Cell Physiol* 2003;284:C870–9.
43. Liu Y, Fuchs J, Li C, Lin J. IL-6, a risk factor for hepatocellular carcinoma: FLLL32 inhibits IL-6-Induced STAT3 phosphorylation in human hepatocellular cancer cells. *Cell Cycle* 2010;9:3423–7.
44. Lepiller Q, Abbas W, Kumar A, Tripathy MK, Herbein G. HCMV activates the IL-6-JAK-STAT3 axis in HepG2 cells and primary human hepatocytes. *PLoS ONE* 2013;8:e59591.
45. Li L, Li H. Role of microRNA-mediated MMP regulation in the treatment and diagnosis of malignant tumors. *Cancer Biol Ther* 2013;14:796–805.
46. Gabrieli G, Wurdinger T, Kesari S, Esau CC, Burchard J, Linsley PS, et al. MicroRNA 21 promotes glioma invasion by targeting matrix metalloproteinase regulators. *Mol Cell Biol* 2008;28:5369–80.
47. Yan X-L, Jia Y-L, Chen L, Zeng Q, Zhou J-N, Fu C-J, et al. Hepatocellular carcinoma-associated mesenchymal stem cells promote hepatocarcinoma progression: role of the S100A4-miR155-SOCS1-MMP9 axis. *Hepatology* 2013;57:2274–86.
48. Zhang Z, Li Z, Gao C, Chen P, Chen J, Liu W, et al. miR-21 plays a pivotal role in gastric cancer pathogenesis and progression. *Lab Invest* 2008;88:1358–66.
49. Song B, Wang C, Liu J, Wang X, Lv L, Wei L, et al. MicroRNA-21 regulates breast cancer invasion partly by targeting tissue inhibitor of metalloproteinase 3 expression. *J Exp Clin Cancer Res* 2010;29:29.

Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

High-Mobility Group Box 1 Promotes Hepatocellular Carcinoma Progression through miR-21–Mediated Matrix Metalloproteinase Activity

Man Chen, Yao Liu, Patrick Varley, et al.

Cancer Res 2015;75:1645-1656. Published OnlineFirst February 26, 2015.

Updated version	Access the most recent version of this article at: doi: 10.1158/0008-5472.CAN-14-2147
Supplementary Material	Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2015/02/27/0008-5472.CAN-14-2147.DC1

Cited articles	This article cites 49 articles, 11 of which you can access for free at: http://cancerres.aacrjournals.org/content/75/8/1645.full.html#ref-list-1
-----------------------	--

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org .
Permissions	To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org .