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RN181 Suppresses Hepatocellular Carcinoma Growth by Inhibition of the ERK/MAPK Pathway

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The activation of oncogenes and the inactivation of tumor suppressor genes by mutations or chronic hepatitis virus infections play key roles in the pathogenesis of hepatocellular carcinoma (HCC). Here we report that RN181, a really interesting new gene finger domain-containing protein, was down-regulated in highly malignant cell lines and in tumor cells of 139 HCC clinical samples in comparison with adjacent normal liver tissues. The expression of RN181 was strongly associated with the pathological grade of HCC. Alterations of the expression of RN181 by retrovirus-transduced up-regulation and short hairpin RNA–mediated down-regulation demonstrated the function of RN181 as a tumor suppressor because it decreased the proliferation and colony formation of HCC cells *in vitro* and inhibited tumor growth *in vivo* by suppressing cell proliferation and enhancing cell apoptosis in xenografted tumors. Proteomic analyses showed that RN181 regulates the expression of many proteins that are important in many cellular processes. Statistical analyses identified 33 proteins with consistent changes ($\geq$2-fold) in RN181-transformed cells. Ten of these proteins were up-regulated by RN181, and 23 were down-regulated. Representative proteins were validated by western blotting. Interaction network investigations revealed that 20 RN181-regulated proteins could integrate several key biological processes such as survival, metabolism, and mitogen-activated protein kinase (MAPK) pathways. Remarkably, 11 of the 33 proteins are associated with MAPK signaling in one or more ways. RN181 suppressed the tyrosine phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) in cell lines and in tumor cells of xenografts and HCC clinical samples, and removing the suppression increased tumor growth. *Conclusion:* We have shown that RN181 suppresses the tumorigenesis of HCC through the inhibition of ERK/MAPK signaling in the liver. Our results provide new insights into the pathogenesis of HCC and may help with the development of novel therapeutic strategies. (HEPATOLOGY 2011;53:1932-1942)

epatocellular carcinoma (HCC) is one of the most common malignant tumors and the third-leading cause of death from cancer worldwide with 600,000 deaths per year.[1] HCC has characteristics of rapid growth, early vascular invasion, and high resistance to standard chemotherapy. For early or localized disease, surgical resection or liver transplantation is a curative treatment. However, approximately 80% of HCC patients present with advanced disease that is not amenable to surgical

resection or transplantation and thus have a poor prognosis.[2] Recently, in a randomized phase III trial, sorafenib, a multikinase inhibitor with potent activity against v-raf-1 murine leukemia viral oncogene homolog 1 (RAF1), v-raf murine sarcoma viral oncogene homolog B1 (BRAF), vascular endothelial growth factor receptor 2, vascular endothelial growth factor receptor 3, c-kit, and platelet-derived growth factor receptor α (among others),[3] was shown to prolong the median survival time of HCC patients from 7.9 to 10.7 months.[4] Significantly, this was the first time that a systemic therapy produced clinical benefits in patients with advanced HCC, and the results will facilitate the identification of novel targets for the development of targeted therapies.

HCC has a complicated molecular tumorigenesis in which two mechanisms may predominate.[5] One is cirrhosis associated with hepatic regeneration after tissue damage caused by a chronic hepatitis B or C virus infection, chronic alcohol consumption, toxins, or metabolic influences. The other includes the activation of oncogenes and/or inactivation of tumor suppressor genes by mutations or chronic hepatitis infections.[6] In HCC, frequently mutated genes are those encoding p53, cyclin-dependent kinase inhibitor 2A, and β-catenin, but deregulated genes include those encoding axis 1, axis 2, v-Ha-ras Harvey rat sarcoma viral oncogene homolog (HRAS), v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS), neuroblastoma RAS viral (v-ras) oncogene homolog, phosphatase and tensin homolog, hepatocyte nuclear factor 1A, interleukin-6 signal transducer, retinoblastoma 1, SMAD2, SMAD3, SMAD4, phosphoinositide-3-kinase catalytic alpha polypeptide (PIK3CA), insulin-like growth factor 2 receptor, hepatocyte growth factor, hepatocyte growth factor receptor (MET), colony stimulating factor 1 receptor, serine/threonine kinase 11, E-cadherin, and cyclooxygenase 2.[6,7] Both pathogenic mechanisms are associated with abnormalities in numerous critical cellular signaling pathways that could perpetuate the tumorigenic process of HCC.[5] One of the major pathways is mitogen-activated protein kinase (MAPK) signaling. The mammalian MAPK family consists of extracellular signal-regulated kinase (ERK), p38, and c-Jun N-terminal kinase [JNK; also known as stress-activated protein kinase (SAPK)]. Each of these enzymes has several isoforms: ERK1 to ERK8; p38α (MAPK14), p38β (MAPK11), p38γ (MAPK12), and p38δ (MAPK13); and JNK1 (MAPK8), JNK2 (MAPK9), and JNK3 (MAPK10). Each MAPK signaling cascade consists of at least three layers: an MAPK kinase kinase [also known as MAP3K, serine/threonine-specific protein kinase (RAF), and MEKK], an MAPK kinase [also known as MAP2K, dual-specificity mitogen-activated protein kinase kinase (MEK), and MKK], and an MAPK. Activated MAPKs phosphorylate numerous substrates such as cyclin D, c-Myc, p53, activator protein 1, and c-Jun. Activation of the ERK/MAPK pathway (also known as the RAF/MEK/ERK pathway), which can result from the activation of mutations in RAS, BRAF, and MEK1, the loss of the tumor suppressor neurofibromin 1, or the mutation-mediated upstream activation, amplification, or ligand-mediated activation of cell surface receptors,[8] regulates many important cellular processes such as proliferation, differentiation, angiogenesis, survival, and cell adhesion.[9] Importantly, the ERK/MAPK pathway is constitutively activated in HCC (including RAF proto-oncogene serine/threonine-protein kinase (CRAF), MEK1/2, and ERK).[5]

RN181 (also known as HSPC238) is a member of the family of really interesting new gene finger proteins, which have been recognized mainly in protein-protein interactions and protein dimerization.[10] RN181 is known to be able to interact with the cytoplasmic regulatory domain of integrin αIIbβ3 in platelets and contains E3 ubiquitin ligase activity for auto-ubiquitination.[11] However, the physiological substrates and biological function of RN181 in cancer biology are unknown. Here we report that RN181 was downregulated in tumor cells of clinical HCC samples. The expression of RN181 was strongly associated with the

pathological grade. Alterations of RN181 expression demonstrated the function of RN181 as a tumor suppressor controlling tumor growth *in vitro* and *in vivo*. Proteomic analyses showed that RN181 regulates the expression of many proteins involved in many important cellular processes. A large number of the proteins are associated with MAPK signaling. We demonstrate here that ERK/MAPK signaling is the main pathway by which RN181 suppresses the tumorigenesis of HCC.

Materials and Methods

Cell Culture. Human HCC cell lines (SMMC-7721, HepG2, and Chang) were purchased from the Shanghai Institutes for Biological Sciences (China). All cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin (complete medium) and were maintained at 37°C with 5% carbon dioxide.

Patients and Tissue Samples. One hundred thirty-nine clinical tissue samples were collected from HCC patients who underwent hepatic resection at the Sun Yat-Sen University Cancer Center from December 2007 to February 2009 and were used in our clinicopathological investigation. Patients' consent and approval by the local ethics committee were obtained for the use of the clinical materials in research. They included 128 males and 11 females with a median age of 50 years (range = 21-76 years). Formalin-fixed, paraffin-embedded HCC specimens paired with adjacent nontumor liver tissues were used for the immunohistochemical study.

Tumor Xenografts. BALB/c nude mice that were 4 to 5 weeks old were purchased from the Experimental Animal Center of the Guangzhou University of Traditional Chinese Medicine (China) and were maintained under standard pathogen-free conditions. Tumor cells (5×10^6) in 0.2 mL of serum-free Dulbecco's modified Eagle's medium were subcutaneously implanted into the flanks of each mouse. The left flank was implanted with control tumor cells, whereas the right side was injected with the tested tumor cells. Each group consisted of six mice. Tumor growth was monitored by the measurement of the length and width of the tumor with a caliper. Tumor sizes were calculated with the following formula:

$$\text{Size} = \text{Length} \times \text{Width}^2 \times (\pi/6)$$

After the mice were sacrificed, xenografted tumors were collected, photographed, and then fixed in paraffin blocks.

Experimental Assays. All experimental assays, including quantitative real-time polymerase chain reaction, western blotting, DNA transfection, cell proliferation and colony formation assays, immunohistochemistry, proteomic analyses, and statistical analyses, are described in the supporting information.

Results

Production and Characterization of the Anti-RN181 Antibody. We made a recombinant RN181 protein and used it to immunize mice for the production of a specific antibody. As shown in Fig. 1AB, the immune serum specifically recognized endogenous RN181 either by western blotting or by immunochemical staining of nontumor liver tissue. Pre-absorption of the serum with an excess amount of recombinant RN181 eliminated the antibody-antigen reaction. RN181 was highly expressed in the Chang cell line, moderately expressed in the HepG2 cell line, and weakly expressed in the SMMC-7721 cell line (Fig. 1C). This suggests that highly malignant tumor cells may have a lower expression level of RN181.

Expression of RN181 in Clinical HCC Tissues. We analyzed 139 pairs of HCC and adjacent normal tissues by immunohistochemistry with the antibody developed in house. Representative scores of immunohistochemical staining of RN181 for tumor and stroma cells are shown in Fig. 2A. As shown in Table 1, RN181 was strongly expressed in normal tissues adjacent to HCC tissues; all 139 samples of adjacent normal tissues had a score $\geq 2+$. However, in comparison with adjacent normal tissues, RN181 expression in tumor tissues was significantly decreased ($Z = -10.241$, $P < 0.001$). As shown in Fig. 2B, within a tumor, RN181 was highly expressed in normal stoma cells but was weakly expressed in malignant tumor cells (representative cases 1 and 2). Interestingly, the expression of RN181 was strongly associated with the pathological grade of HCC ($Z = 59.138$, $P < 0.001$; Table 1); well-differentiated tumor cells showed a high level of expression (cases 1 and 2), whereas poorly differentiated tumor cells exhibited a low level of expression (case 3). The expression of RN181 was significantly decreased in grade III HCC tumors versus grade I/II tumors (see Table 1). However, RN181 expression did not seem to be associated with the sex, age, or clinical stage of the HCC patients. Thus, the results suggest that decreased expression of RN181 may be associated with the tumorigenesis of HCC.

Effects of RN181 on the Proliferation and Colony Formation of HCC Cells **In Vitro.** To investigate the role of RN181 in the tumorigenesis of HCC *in vitro*, we up-regulated RN181 in tumor cells by retrovirus-mediated transduction. RN181 was significantly up-

Fig. 1. Characterization of the mouse anti-RN181 antibody. (A) Western blotting showed the specificity of the anti-RN181 antibody. Recombinant RN181 was used to immunize the mice (left panel). The recognition of endogenous RN181 in the lysate of Chang cells by the antibody (middle panel) was fully removed by pre-absorption of the lysate with excess recombinant RN181 (right panel). (B) Immunochemical staining of normal liver tissues with the anti-RN181 antibody. The recognition of endogenous RN181 in liver cells by the antibody (left panel) was fully absorbed with excess recombinant RN181. (C) Western blotting with the anti-RN181 antibody showed RN181 expression in three liver cancer cell lines. RN181 was expressed highly in Chang cells, moderately in HepG2 cells, and weakly in SMMC-7721 cells. Abbreviation: GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
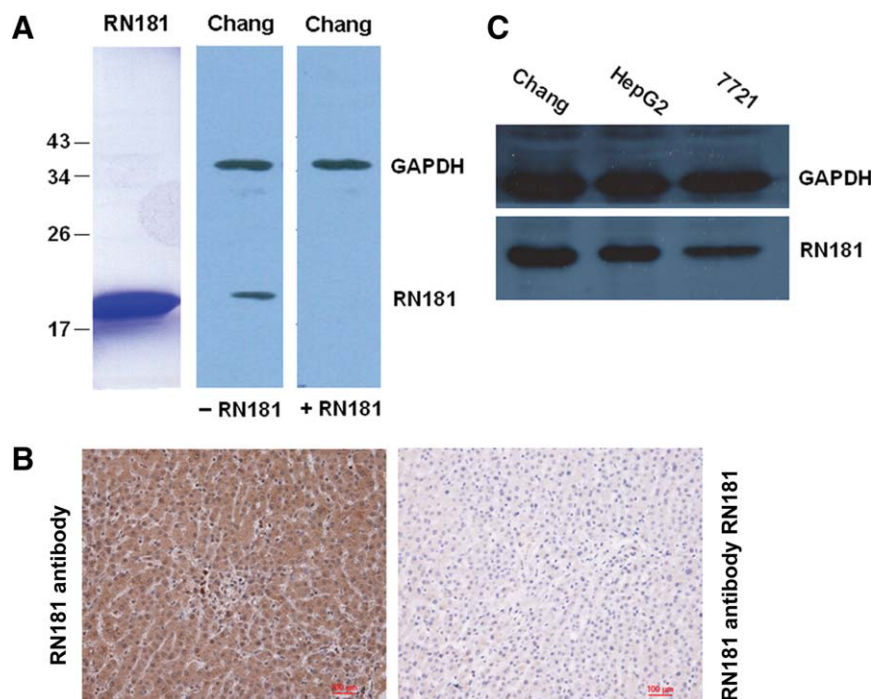
regulated at both messenger RNA and protein levels in SMMC-7721/RN181 cells in comparison with control SMMC-7721/empty cells (Fig. 3A and Supporting Fig. 1A). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) proliferation assays showed that up-regulation of RN181 strongly inhibited the growth of SMMC-7721/RN181 cells in comparison with control cells ($P < 0.01$; Fig. 3B). Colony formation assays demonstrated that up-regulation of RN181 significantly reduced the colony formation of SMMC-7721/RN181 cells in comparison with control cells ($P < 0.01$; Fig. 3C). To verify these data, we down-regulated the expression of endogenous RN181 in SMMC-7721 cells by the transfection of a short hairpin RNA (shRNA)–containing vector. RN181 was significantly knocked down at either the messenger RNA level or the protein level (Fig. 3D and Supporting Fig. 1B). In agreement with the up-regulation results, the down-regulation of RN181 significantly increased the proliferation (Fig. 3E) and colony formation (Fig. 3F) of SMMC-7721/knockdown (KD) cells in comparison with control SMMC-7721/negative control (NC) cells ($P < 0.01$). Thus, the results suggest that RN181 functions as a tumor suppressor and thus controls the growth of HCC cells *in vitro*.

Effects of RN181 on the Growth of HCC Cells In Vivo. To determine whether RN181 regulates the growth of HCC tumors *in vivo*, we engrafted SMMC-7721 cells into nude mice (Fig. 4A). Up-regulation of RN181 (Supporting Fig. 1E) significantly decreased

the tumor growth of SMMC-7721/RN181 in comparison with the SMMC-7221/empty control (top panels in Fig. 4A). Consistently, down-regulation of RN181 (Supporting Fig. 1F) strongly increased the tumor growth of SMMC-7721/KD in comparison with the SMMC-7721/NC control (bottom panels in Fig. 4A). Similar results were observed from xenografted tumors of Chang cells in which RN181 was knocked down (Supporting Fig. 2AB).

The immunohistochemistry of tumor sections confirmed the high expression of RN181 at the protein level in SMMC-7721/RN181 tumors (Fig. 4B). The cell proliferation of SMMC-7721/RN181 *in vivo* was significantly decreased ($P < 0.01$), as demonstrated by anti–proliferating cell nuclear antigen (anti-PCNA) staining (middle panels), whereas the cell apoptosis of SMMC-7721/RN181 was significantly increased in comparison with the cell apoptosis of the control tumor ($P < 0.01$), as revealed by anti–activated caspase-3 staining (bottom panels). Consistently, down-regulation of RN181 increased tumor cell proliferation and decreased tumor cell apoptosis in SMMC-7721/KD tumors versus SMMC-7721/NC tumors (Supporting Fig. 3). Taken together, these data suggest that RN181 acts as a tumor suppressor and thus inhibits the growth of HCC tumors *in vivo*.

Differential Proteomic Expressions Regulated by RN181. To explore molecular mechanisms of RN181-mediated transformation, we performed proteomic analyses by comparing expression profiles of SMMC-
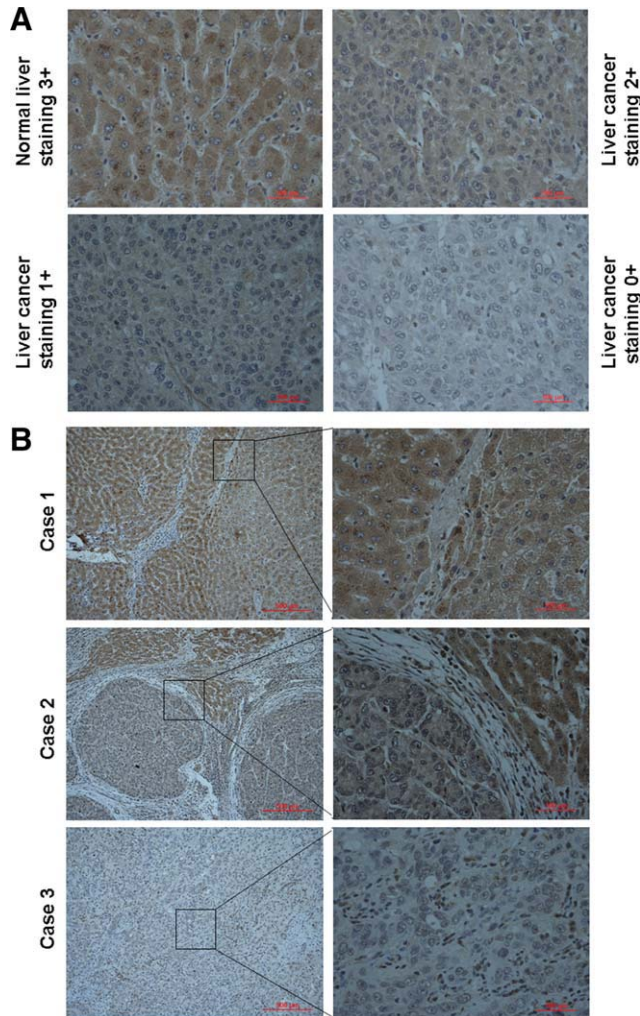
Fig. 2. Immunohistochemical analyses of normal liver and HCC tissues in humans. (A) Representative immunostaining scores of RN181 expressed in tumor and stroma cells. Normal liver tissues showed high expression of RN181 (score = 3+), whereas HCC tissues showed moderate (score = 2+), low (score 1+), or very week/undetectable expression (score 0+) of RN181. (B) Representative immunostaining of tumor and stroma cells in HCC tissues with different pathological grades. RN181 was expressed highly in normal liver cells but weakly in tumor cells (cases 1 and 2). The expression of RN181 was much higher in well-differentiated HCC cells (case 1) versus poorly differentiated HCC cells (case 3). The original magnifications were ×10 (left panels) and ×40 (right panels).

7721/RN181 cells and SMMC-7721/empty cells. Representative two-dimensional gel images are shown in Fig. 5A. More than 1200 spots were detected in each gel. A number of proteins were regulated by RN181. According to potential cellular functions, these proteins could be grouped into five general categories: actin remodeling and cell migration, cell proliferation and invasion, apoptosis, metabolism, and signal transduction. Statistical analyses of liquid chromatography/tandem mass spectrometry data sets revealed 33 proteins from more than 1200 spots showing changes in

abundance (≥2-fold) with the ratio of SMMC-7721/RN181 cells to SMMC-7721/empty cells (Supporting Table 1). Ten of these proteins were significantly up-regulated in SMMC-7721/RN181 cells, whereas 23 were significantly down-regulated. The differential expression changes were reproduced in independent experiments. Thus, the results suggest that up-regulation of RN181 in tumor cells regulates numerous biological processes that could suppress the tumorigenesis of HCC.

To confirm the proteomic results, we validated the expression of two up-regulated proteins and four down-regulated proteins by western blotting. As shown in Fig. 5B, the expression levels of enolase 1 (ENO1) and glutathione S-transferase π1 (GSTP1) were significantly increased in SMMC-7721/RN181 cells versus SMMC-7721/empty cells, whereas the expression levels of tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein epsilon polypeptide (YWHAE), lectin galactoside-binding soluble 1 (LGALS1), S100 calcium binding protein A6 (S100A6), and stathmin 1 (STMN1) were decreased in SMMC-7721/RN181 cells versus SMMC-7721/empty cells.

Regulation of the ERK/MAPK Pathway by RN181. We investigated interaction networks of 33 proteins obtained from proteomic profiling with Osprey and IntAct. Twenty proteins regulated by RN181 were shown to integrate several key biological processes such as survival, metabolism, and MAPK pathways (Fig. 6A). Remarkably, one-third of the identified proteins (11/33) are associated with MAPK signaling in one or more ways [i.e., YWHAE, LGALS1, STMN1, S100A6, ENO1, GSTP1, nucleophosmin (NPM1), chaperonin containing TCP1 subunit 3 (CCT3), phosphoglycerate kinase 1 (PGK1), adenosylhomocysteinase (AHCY), and nonmetastatic cells 1 (NME1)]; this suggests that MAPK signaling might be the main pathway by which RN181 suppresses the tumorigenesis of HCC.

To investigate whether RN181 affects ERK/MAPK signaling, we performed western blotting with the antibody against tyrosine-phosphorylated extracellular signal-regulated kinase 1/2 (pERK1/2). As shown in Fig. 6B, up-regulation of RN181 (Supporting Fig. 1A) decreased the pERK1/2 level in SMMC-7721/RN181 cells versus SMMC-7721/empty cells, whereas down-regulation of RN181 (Supporting Fig. 1B-D) strongly increased pERK1/2 levels in the SMMC-7721, Chang, and HepG2 cell lines. However, neither up-regulation nor down-regulation of RN181 seemed to significantly affect the total ERK1/2 level.

To determine whether the inhibition of the ERK/MAPK pathway by RN181 contributes to the

Table 1. Correlation of the Expression of RN181 With Clinicopathological Features of 139 Patients With HCC

Feature	n	RN181 Expression				Mean Rank	Z Value	P Value
		0+	1+	2+	3+			
Sex								
Male	128	19	70	39		70.63	−0.693	0.488
Female	11	3	5	3		60.73		
Age (years)								
<50	69	12	37	20		68.52	−0.477	0.633
≥50	70	10	38	22		71.46		
Clinical stage								
1	17	5	3	9		76.71	0.744	0.689
2	101	14	59	28		69.50		
3	21	3	13	5		67.00		
Differentiation								
1	25	0	4	21		109.14	59.138	<0.001
2	78	5	52	21		72.64		
3	36	17	19	0		37.10		
Location								
Tumor tissue	139	22	75	42	0		−10.241	<0.001
Adjacent tissue	139	0	0	20	119			

suppression of tumor growth by RN181, we treated SMMC-7721 cells with 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene (U0126), a highly selective inhibitor of both MEK1 and MEK2.[12] U0126 effectively inhibited the tyrosine phosphoryla-

tion of ERK1/2 (Supporting Fig. 4A) and totally abolished the increases in proliferation (Fig. 6C) and colony formation (Fig. 6D and Supporting Fig. 4B) caused by down-regulation of RN181. Thus, the results suggest that RN181 suppresses the tumor
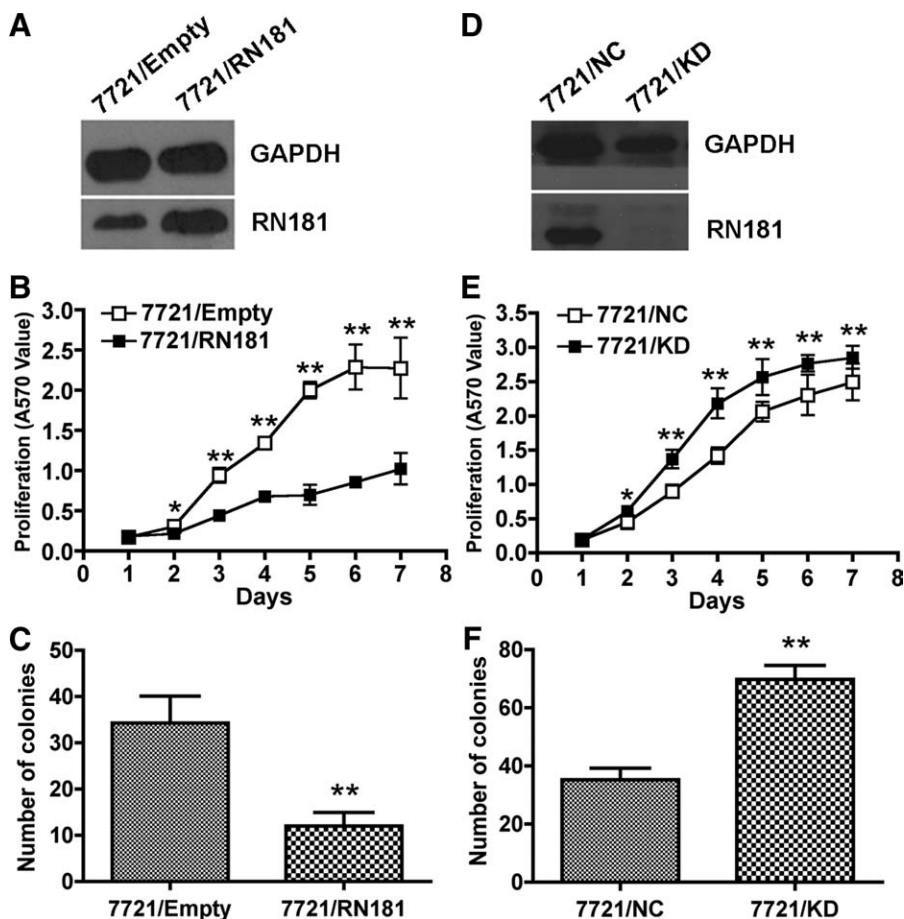
Fig. 3. Alternations of RN181 expression modulated the growth and colony formation of SMMC-7721 cells *in vitro*. (A) Western blotting revealed that retrovirus-mediated transduction up-regulated RN181 in SMMC-7721 cells at the protein level. (B) MTT proliferation assays showed that up-regulation of RN181 inhibited the growth of SMMC-7721 cells *in vitro*. (C) Colony formation assays showed that up-regulation of RN181 inhibited the colony formation of SMMC-7721 cells *in vitro*. (D) Western blotting revealed that transfection of the shRNA-containing vector down-regulated RN181 in SMMC-7721 cells at the protein level. (E) MTT proliferation assays showed that down-regulation of RN181 increased the growth of SMMC-7721 cells *in vitro*. (F) Colony formation assays showed that down-regulation of RN181 increased the colony formation of SMMC-7721 cells *in vitro*. Means and standard errors are presented (n = 4). *$P < 0.05$ and **$P < 0.01$. Abbreviations: A570, absorbance at 570 nm; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Fig. 4. RN181 suppressed tumor growth by decreasing tumor cell proliferation and increasing tumor cell apoptosis *in vivo*. (A) Up-regulation of RN181 in SMMC-7721 cells d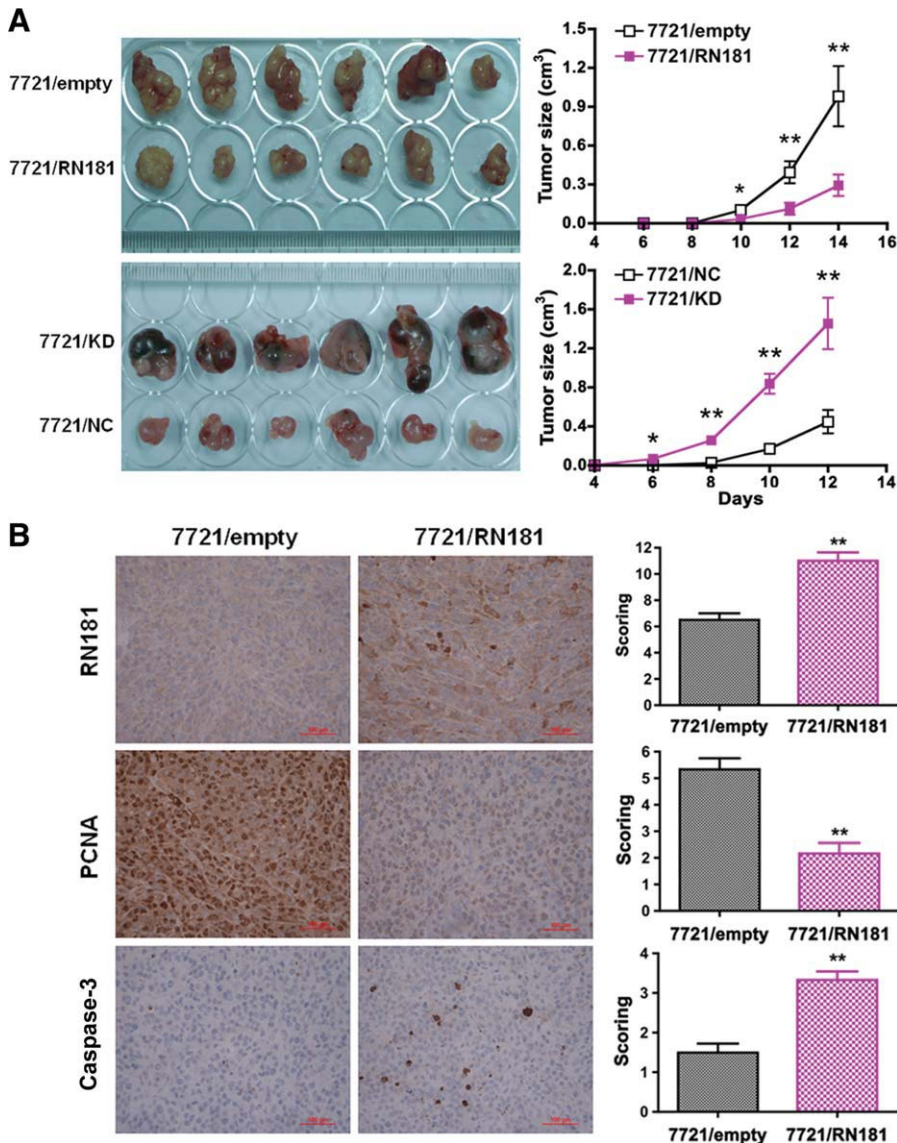ecreased tumor growth in nude mice, whereas down-regulation of RN181 in SMMC-7721 cells increased tumor growth in nude mice (paired comparisons). (B) Up-regulation of RN181 in SMMC-7721 cells (top panels) decreased tumor cell proliferation (anti-PCNA staining in the middle panels) but increased tumor cell apoptosis (anti–activated caspase-3 staining in the bottom panels) in xenografted tumors. Scale bars represent 100 μm; means and standard errors are presented (n = 6). *$P < 0.05$ and **$P < 0.01$.

growth of HCC through the inhibition of ERK/MAPK signaling in tumor cells.

Suppression of ERK/MAPK Signaling by RN181 in Xenografts and HCC Tumors. To investigate whether the suppressor action of RN181 occurs *in vivo*, we analyzed the correlation of RN181 expression with the ERK/MAPK activity in HCC tumors. As shown in Fig. 7A, immunohistochemical staining revealed that up-regulation of RN181 dramatically reduced the pERK1/2 level in SMMC-7721/RN181 xenografted tumors versus the SMMC-7721/empty control (top panels). Consistently, down-regulation of RN181 significantly increased the pERK1/2 level in SMMC-7721/KD xenografts versus the SMMC-7721/NC control (bottom panels). Similar results were obtained from xenografted tumors of Chang cells in which RN181 was knocked down (Supporting Fig. 2C).

We further evaluated the expression of RN181 and the ERK/MAPK activity in 40 pairs of HCC clinical samples. As shown in Fig. 7B, those tumor tissues with moderate expression of RN181 had a lower level of pERK1/2, whereas those tumor tissues with low expression of RN181 displayed a higher level of pERK1/2 (right panels). In contrast, adjacent normal liver tissues exhibited high expression of RN181 but very low or undetectable levels of pERK1/2 (left panels). Pearson correlation analyses showed that RN181 expression was reversely associated with the pERK1/2 levels in HCC tumors ($r = -0.432$, $P < 0.05$).

Discussion

The major etiologies of HCC and the risk factors for its development are well defined, but the
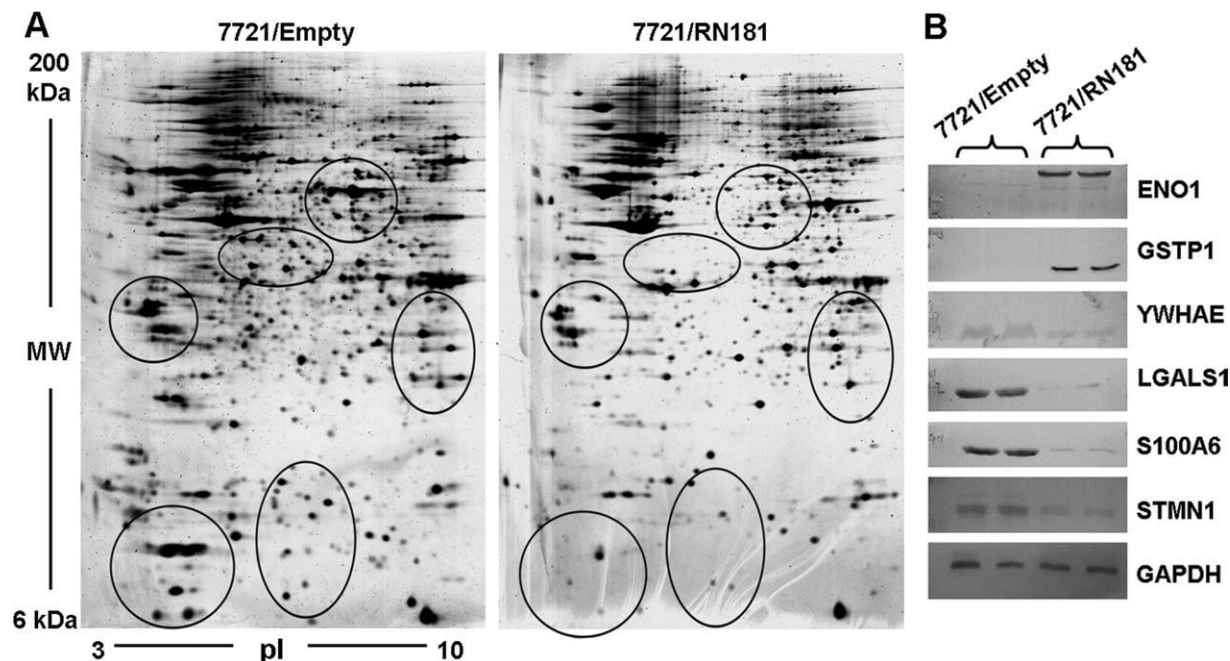
Fig. 5. Proteomic analyses of RN181-regulated proteins. (A) An overview of two-dimensional gel images of SMMC-7721 cells. Highlighted in circles are the areas in which significant and consistent differences in protein expression levels were found between SMMC-7721/RN181 cells and SMMC-7721/empty cells. (B) Validation of representative proteins obtained from the proteomic analyses by western blotting. ENO1 and GSTP1 were up-regulated by RN181, whereas YWHAE, LGALS1, S100A6, and STMN1 were down-regulated by RN181. Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MW, molecular weight.

mechanisms of hepatocellular carcinogenesis are still unclear.[6,13] The activation of oncogenes and the loss of tumor suppressor genes are believed to play key roles in the pathogenesis of HCC.[6] Here we report that RN181 was down-regulated in highly malignant cell lines (SMMC-7721 < HepG2 < Chang) and in tumor cells from 139 HCC clinical samples in comparison with adjacent normal liver tissues and stroma cells within tumors by immunohistochemical staining. Interestingly, the expression of RN181 was strongly associated with the pathological grade. These results suggest that RN181 may control hepatocellular differentiation to suppress the tumorigenesis of HCC.

To investigate the possibility that RN181 could suppress the tumorigenesis of HCC, we altered the expression of RN181 in tumor cells by up-regulation and down-regulation. We found that up-regulation of RN181 significantly inhibited the growth and colony formation of SMMC-7721/RN181 cells *in vitro*; consistently, down-regulation of RN181 enhanced the growth and colony formation of SMMC-7721/KD cells *in vitro*. More importantly, up-regulation of RN181 decreased the tumor growth of SMMC-7721/RN181 *in vivo*, whereas down-regulation of RN181 promoted the tumor growth of both SMMC-7721/KD and Chang/KD cells by the manipulation of tumor cell proliferation and apoptosis in xenografted

tumors. Taken all these together, we have concluded that RN181 acts as a tumor suppressor controlling the tumorigenesis of HCC.

To investigate underlying suppression mechanisms, we used a high-throughput proteomic approach to screen global differential expression profiles of SMMC-7721/RN181 and SMMC-7721/empty cells. We found that RN181 regulated the expression of many proteins that are important in many cellular processes. Thirty-three proteins were identified from more than 1200 spots showing changes (≥2-fold) in RN181-transformed cells versus control cells. Twenty-three of these proteins were significantly down-regulated by RN181, whereas 10 were significantly up-regulated. Interestingly, among the 23 down-regulated proteins, at least 10 molecules [PGK1,[14] guanosine 5′-monophosphate synthetase,[15] protein arginine methyltransferase 1,[16] tropomyosin 3 (TPM3),[17] STMN1,[18] LGALS1,[19] YWHAE,[20] S100A6, S100A10, and S100A11[21]] have been reported to promote tumorigenesis or tumor progression. On the other hand, 4 of the 10 up-regulated proteins [AHCY, NME1, histidine triad nucleotide binding protein 1 (HINT1), and GSTP] are known to be involved in suppressing tumorigenesis.[22-25] Together, these data may help to explain why RN181 can suppress the tumorigenesis of HCC.
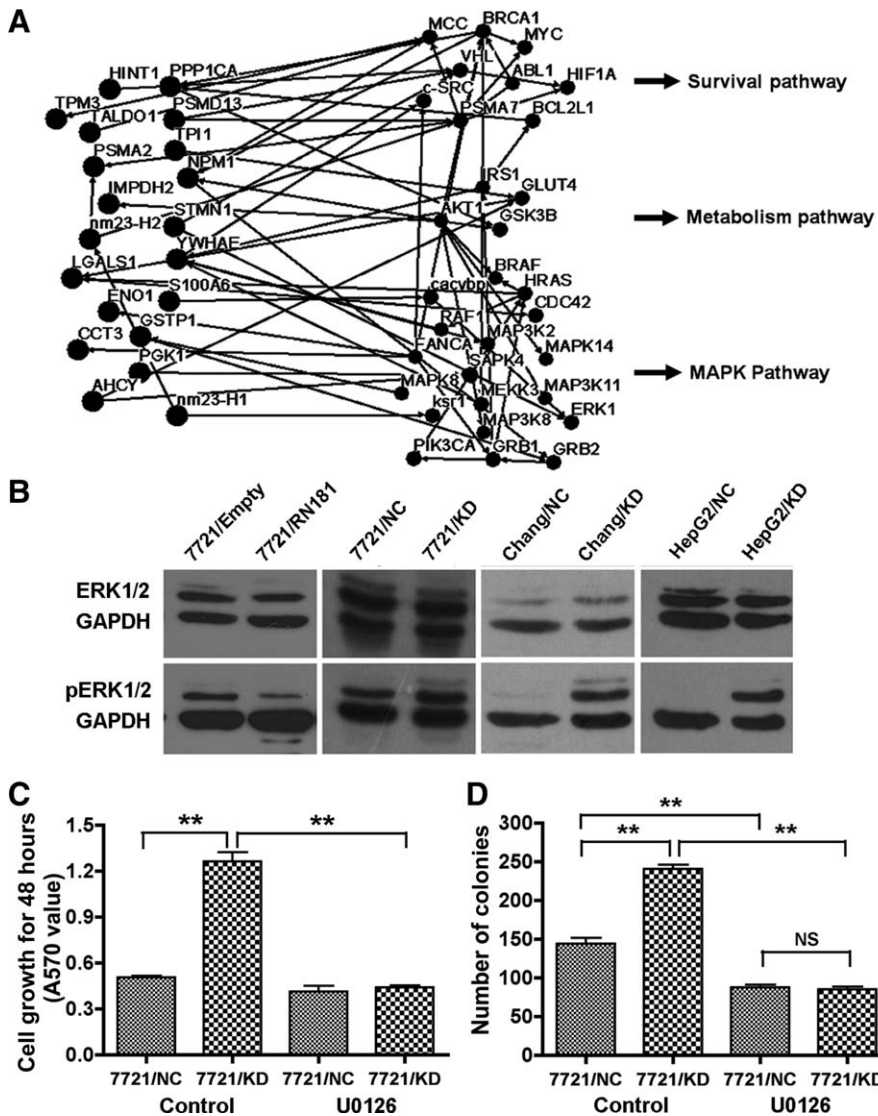
Fig. 6. Regulation of the MAPK pathway by RN181. (A) Analyses of interaction networks of RN181-regulated proteins by Osprey and IntAct revealed interactions of RN181-regulated proteins with many biological processes. Twenty of 33 proteins that were identified as targets of RN181 by proteomic analyses (left side) were shown to integrate survival, metabolism, and MAPK pathways (right side). (B) Western blot analyses of pERK1/2. Up-regulation of RN181 decreased pERK1/2 levels, whereas down-regulation of RN181 increased pERK1/2 levels. Alterations of RN181 did not affect the total ERK1/2 level. GAPDH served as a loading control. (C) Increased cell growth by down-regulation of RN181 in SMMC-7721 cells was eliminated by the addition of 20 μM U0126 to the cells cultured in vitro. Means and standard errors are presented (n = 4). **$P < 0.01$. (D) Increased colony formation by down-regulation of RN181 in SMMC-7721 cells was eliminated by the addition of 20 μM U0126 to the cells cultured in vitro. Means and standard errors are presented (n = 4). **$P < 0.01$. Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NS, not significant.

To better understand the mechanisms, we investigated the interaction networks of the 33 proteins identified from the proteomic analyses. We found that 20 proteins regulated by RN181 could integrate several key biological processes such as survival, metabolism, and MAPK pathways. Surprisingly, one-third of the identified proteins (11/33) are associated with MAPK signaling in one or more ways (i.e., YWHAE, LGALS1, S100A6, STMN1, ENO1, GSTP1, NPM1, CCT3, PGK1, AHCY, and NME1). YWHAE, a multifunction protein of the 14-3-3 family, can activate Ras-mediated signaling and inhibit cell migration induced by MEK5.[26] LGALS1 is known to activate ERK, JNK, and activator protein 1. S100A6 can activate JNK.[27] STMN1, a substrate of all four isoforms of p38,[28] is highly expressed in HCC and has been associated with local invasion, early recurrence, and a poor prognosis for HCC patients.[29] ENO1, up-regulated by RN181, has been shown to bind to the c-Myc promoter and act

as a transcriptional repressor.[30] ENO1 can inhibit prostate cancer cell growth by regulating the MEK5-mediated signaling pathway and suppressing nuclear factor kappa B.[31] GSTP1 is capable of protecting cells from toxins (drugs, pesticides, and carcinogens) and oxidative stress[32] and inhibits tumor necrosis factor receptor-associated factor 2 (TRAF2)-induced activation of both JNK and p38 but not nuclear factor kappa B.[33] NPM1, up-regulated by RN181, can regulate KRAS plasma membrane interactions and activate ERK/MAPK signaling.[34] NME1 can phosphorylate kinase suppressor of Ras (KSR), a scaffold protein for the MAPK cascade, and reduce levels of phosphorylated MAPK.[35] Considering all these, we propose that MAPK signaling may be the major pathway that RN181 regulates in the liver to suppress the tumorigenesis of HCC.

To verify this hypothesis, we investigated tyrosine phosphorylation of ERK1/2 because tyrosine phosphorylation of ERK1/2 is required to activate the ERK/
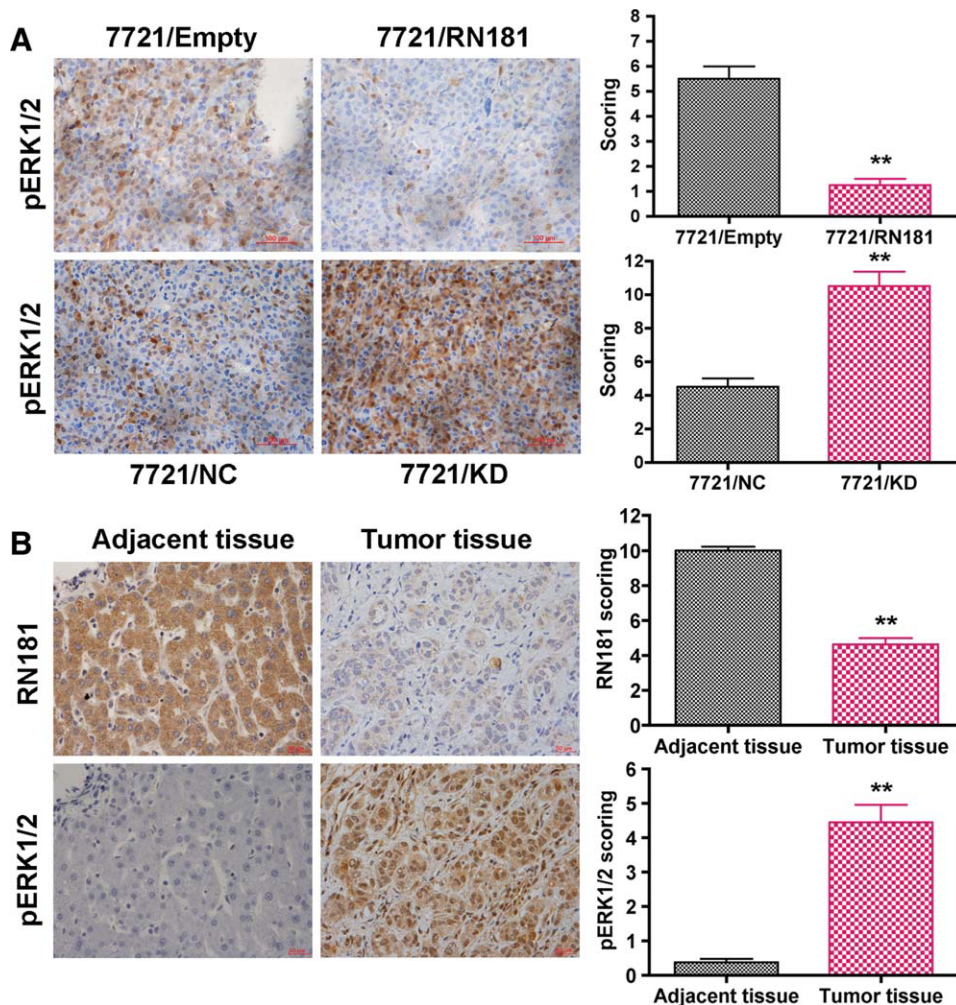
Fig. 7. Suppression of ERK/MAPK signaling by RN181 in xenografts and HCC tumors. (A) Up-regulation of RN181 resulted in a decrease in the pERK1/2 level (top panels), whereas down-regulation of RN181 led to an increase in the pERK1/2 level (bottom panels) in SMMC-7721 xenografted tumors. Means and standard errors are presented (n = 6). **$P < 0.01$. (B) The expression of RN181 was inversely correlated with the pERK1/2 levels in HCC tissues. High expression of RN181 resulted in low pERK1/2 levels in adjacent normal tissues, whereas low expression of RN181 led to high pERK1/2 levels in tumor tissues. Means and standard errors are presented (n = 40). **$P < 0.01$.

MAPK pathway. Indeed, we revealed that the level of pERK1/2 was decreased in RN181–up-regulated cells (SMMC-7721) and increased in RN181–down-regulated cell lines (SMMC-7721, HepG2, and Chang). Inhibition of ERK/MAPK signaling by U0126 eliminated the increases in proliferation and colony formation observed in RN181–down-regulated cells and thus highlighted the role of ERK/MAPK signaling in RN181 suppressor action for the tumorigenesis of HCC. Furthermore, we demonstrated that the expression of RN181 was reversely correlated with the pERK1/2 level in xenografted tumors and in HCC clinical samples and thus confirmed the relevance of the ERK/MAPK pathway in the suppression of tumorigenesis by RN181 in vivo. It has been noted that the ERK/MAPK pathway is constitutively activated in HCC (including CRAF, MEK1/2, and ERK).[5] ERK is highly expressed in tumor tissues and has been significantly correlated with the HCC tumor size.[36] ERK1/2 activation has been associated with aggressive tumor behavior and can serve as an independent prognostic marker for decreased overall survival.[37] Taken all together, we have demonstrated that RN181 suppresses the tumorigenesis of HCC through the inhibition of the ERK/MAPK pathway in the liver. Clearly, the ways in which RN181 regulates those proteins integrating the ERK/MAPK pathway and the downstream substrates of ERK/MAPK signaling that are affected by RN181 in the liver need to be further investigated in the future.

In summary, we have demonstrated that RN181 is down-regulated in HCC tumor cells and that the expression of RN181 is strongly associated with the pathological grade. In the liver, RN181 functions as a tumor suppressor to control the tumorigenesis of HCC because it decreases tumor growth in vitro and in vivo by suppressing cell proliferation and promoting cell apoptosis of HCC. Proteomic analyses revealed that RN181 regulates many proteins involved in several important cellular processes such as the MAPK pathway. RN181 has been shown to suppress ERK/MAPK signaling, and disrupting this suppression will increase HCC growth. We conclude that RN181 suppresses the tumorigenesis of HCC mainly through the inhibition of the ERK/MAPK pathway in the liver.

Our results provide new insights into the pathogenesis of HCC and may help with the development of novel therapeutic strategies.

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