

RPB5-mediating Protein Is Required for the Proliferation of Hepatocellular Carcinoma Cells*

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RPB5-mediating protein (RMP) is associated with the RNA polymerase II subunit RPB5. RMP functionally counteracts the transcriptional activation of hepatitis B virus X protein that has been shown to play a role in the development of hepatocellular carcinoma (HCC). However, the effect of RMP on the growth of HCC remains unclear. In this study, we characterized the potential role of RMP in the proliferation of human HCC cells using two cell lines, SMMC-7721 and HepG2. We found that RMP expression increased when HCC cells were treated with ⁶⁰Co γ -irradiation. Cell growth and colony formation assays suggest that RMP plays an antiapoptotic role in the proliferation and growth of HCC cells. We also show that RMP depletion induced the G₂ arrest of HCC cells characterized by the decreased expression of Cdk1 and Cyclin B. Tumor formation assays further confirmed the *in vivo* requirement of RMP during HCC growth. In conclusion, our results demonstrate that RMP is a radiation-sensitive factor, and it may play essential roles in HCC growth by affecting the proliferation and apoptosis of HCC cells.

Eukaryotic RNA polymerase II transcribes mRNA and microRNA genes (1, 2). RNA polymerase II consists of 12 subunits, including RPB5, and functions by interacting with other transcription factors (3, 4). RPB5 is also one of the targets of transcriptional regulation in signal transduction (5, 6). Previous studies have suggested that RPB5 binds specific partners of RNA polymerase II, such as Taf1 and the RAP30 subunit of TFIIE, to regulate transcription and downstream gene expression (7). Another protein that interacts directly with RPB5 is hepatitis B virus X protein (HBx),⁴ a protein known to be a

causative agent in the formation of hepatocellular carcinoma (HCC) (8–10).

RMP is a RPB5-associated protein. The RMP gene was first isolated and cloned from a human HepG2 cDNA library more than a decade ago (11). Since then, multiple RMP variants or homologs have been reported in humans and other species. These RMP variants play multiple functions during development. The unconventional prefoldin RPB5 interactor (URI), an alternative form of RMP, was shown to participate in a nutrient-related signaling pathway that is required for gene expression (12). URI-1 is the RMP homolog in *Caenorhabditis elegans*. It has been demonstrated that URI-1 is required to maintain genome stability by playing an important function in controlling cell cycle (13). Depletion of URI-1 increases DNA breaks and induces G₂/M cell cycle arrest and apoptosis (13). Uri, the RMP homolog in *Drosophila*, has also been shown to be required for normal development by playing essential roles in transcriptional regulation and genome integrity maintenance (14). In humans, RMP/URI is a component of the multiprotein PAF1 complex that is involved in histone methylation, gene expression, and cell cycle regulation. URI interacts with Paf1 and parafibromin, a tumor suppressor. Dysfunction of parafibromin and Paf1 promotes cell progression into S phase, suggesting that RMP/URI is involved in cell cycle regulation (15).

As a major regulator of transcription and the cell cycle, RMP may also play a role in tumor formation. It has been reported that NNX3, a variant of RMP that lacks the first 50 N-terminal amino acids, is a potential marker for Reed-Sternberg cells in Hodgkin lymphoma (16).

We have shown previously that RMP associates with RPB5, suppressing transcriptional activation via HBx. In addition, we have shown that the overexpression of HBx releases the inhibitory effect of RMP on transcriptional activation (11, 17). Given the multiple functions of RMP, we surmised that RMP might be involved in the growth of HCC by affecting proliferation, transformation, or apoptosis. To investigate the biological functions of RMP, we generated the human HCC cell lines SMMC-721 and HepG2, whereby RMP was either overexpressed or depleted. The potential role of RMP in tumor cell proliferation was examined in these HCC cell lines. Our results showed that RMP strongly promoted the proliferation of HCC cells, whereas the depletion of RMP resulted in growth inhibition and apoptosis.

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⁴ The abbreviations used are: HBx, hepatitis B virus X protein; AFP, α -fetoprotein; Gy, gray; HCC, hepatocellular carcinoma; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI, propidium iodide; RMP, RPB5-mediating protein; RMPi, RMP-depleting cells; RMPo, RMP-overexpressing cells; SCR, scrambled; URI, unconventional prefoldin RPB5 interactor.

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Moreover, our tumor formation assays further demonstrated that RMP is required for HCC growth *in vivo*.

EXPERIMENTAL PROCEDURES

Plasmids, Cell Lines, and Reagents—The plasmid pFLAGCMV4 was a gift from Dr. Danny Reinberg (University of Medicine and Dentistry of New Jersey). NKCFLAG-RMP was obtained from Dr. Seishi Murakami (Kanazawa University, Japan). pGPU6-Neo was purchased from Jima Co. (Shanghai, China). SMMC-7721 and HepG2 human HCC cell lines were obtained from the laboratory of the Department of Cellular and Molecular Biology at Soochow University. Cells were maintained in minimal essential medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Sino-American Biotechnology Co, Shanghai, China). The Annexin V-FITC/propidium iodide (PI) apoptosis detection kit was purchased from BD Biosciences (Shanghai, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma.

Antibodies against Bax, Bcl-2, c-Myc, α -fetal protein (AFP), and Cyclin B1 were purchased from Boster Company (Wuhan, China). Antibodies for p53, p21, Cdk1, and β -actin were purchased from Biosynthesis Biotechnology Company (Beijing, China). The RMP antibody was a gift from Dr. Seishi Murakami.

Constructs for RMP Interference and Overexpression—For depletion of RMP, three small interfering RNAs (siRNAs) targeting different regions of human RMP were designed and commercially synthesized (Jima Co., Shanghai, China). The sequences of the sense strand of three siRNAs are as follows: RMPi, 5'-GGA UUU GCU AGC UGA UAA ATT-3'; RMPi1, 5'-GGU AGA UAA UGA CUA UAA UTT-3'; and RMPi2, 5'-AGA GGG UUU CAA AGU UUA ATT-3'. To generate double-stranded siRNAs, equal molar amounts of complementary sense and antisense strands were mixed, annealed, and cooled slowly to 10 °C in 50 μ l of reaction buffer (100 mM NaCl and 50 mM HEPES, pH 7.4). Because RMPi showed the most efficient depletion of RMP, its complementary sense strand DNA was synthesized (5'-CAC CGG ATT TGC TAG CTG ATA AAT TCA AGA GAT TTA TCA GCT AGC AA A TCC TTT TTT GGA TCC-3'). As a control, a scrambled sequence (SCR) of RMPi was also synthesized. The forward sequence of SCR was 5'-CAC CGT TCT CCG AAC GTG TCA CGT CAA GAG ATT ACG TGA CAC GTT CGG AGA ATT TTT TG-3'. The annealed, double-stranded oligomers were digested and inserted into the BamHI and BbsI sites of pGPU6-Neo. For the overexpression construct, RMP was amplified by PCR with the following primers: 5'-TCA AGC TTA TGA GGC TAG GAA ATG-3' and 5'-ATG GAT CCC TAG TCT TTC TGT TGC-3'. The RMP fragment was digested and inserted into the BamHI and HindIII sites of plasmid pFLAGCMV4.

Cell Culture, Transfection, and Western Blot Analysis—A total of 2×10^5 SMMC-7721 or HepG2 cells were seeded into each well of a 24-well plate. After incubating for 24 h, these cells were transfected with LipofectamineTM2000 (Invitrogen) according to the manufacturer's protocol. Cells were transfected with plasmids pGPU6/Neo-RMPi for RMP depletion and pFLAGCMV4-RMP for RMP overexpression. Both pGPU6/Neo-SCR and vector alone (pFLAGCMV4) were used

as negative controls. Selection of stably transfected cells and Western blot analysis were conducted as described previously (18). For Western blot analysis of xenograft tumors, tissue samples were first homogenized and lysed in Nonidet P-40 lysis buffer (150 mM NaCl, 1.0% Nonidet P-40, 50 mM Tris, pH 8.0, 1 mM phenylmethylsulfonyl fluoride) at 4 °C for 1 h. Then, crude lysates were collected and centrifuged at 15,000 rpm for 15 min at 4 °C. The supernatants were then subjected to Western blot analysis.

Cell Viability Determination—MTT assays were carried out as described previously (18). Briefly, after SMMC-7721 and HepG2 cells were cultured for 24 h, 30 μ l of MTT (5 mg/ml) was added to each well. Crystals were formed and then dissolved by adding 300 μ l of isopropyl alcohol acidified with HCl (0.04 N) containing 10% Triton X-100. Then, 200 μ l of the blue formazan mixtures was transferred to 96-well plates. The plates were read at 570 nm using a Microplate Reader (model 550; Bio-Rad).

Colony Formation Assays—A total of 1×10^5 SMMC-7721 cells were seeded into each well of a 24-well plate and incubated at 37 °C with 5% CO₂. After incubation, the cultures were fixed in 100% ethanol and stained with 0.005% crystal violet (Sigma). Cell colonies were visualized by microscopy. Colony-forming efficiency was performed by counting the number of colonies that had at least 50 cells. Experiments were performed in triplicate, and colony numbers are an average of these three experiments.

Analysis of Apoptosis and Cell Cycle by Flow Cytometry—SMMC-7721 cells were exposed to ionizing radiation using a ⁶⁰Co γ -irradiator at a dose of 2 or 6 Gy. Cells were then stained with Annexin V-FITC and PI and analyzed for apoptosis according to the manufacturer's instructions. Briefly, after two treatments with washing buffer, cells were resuspended in 400 μ l of Dulbecco's phosphate-buffered saline (PBS). Then, 100 μ l of this cell suspension was incubated with 10 μ l of PI (50 μ g/ml) and 5 μ l of Annexin V-FITC for 15 min at room temperature in the dark. Cells were then analyzed by flow cytometry. Cells that stained positive for only Annexin V-FITC were in the early stage of apoptosis, whereas cells that stained positive for both Annexin V-FITC and PI were in the stage of late apoptosis or primary necrosis. Hoechst 33258 staining was used to investigate the changes in the nucleus of apoptotic cells. Apoptotic bodies containing nuclear fragments were found in apoptotic cells by fluorescence microscopy. Cell cycle analysis by flow cytometry was carried out as described (18).

Tumor Formation Assay in Nude Mice—For the *in vivo* tumor formation assay, 5×10^6 SMMC-7721 cells in 0.1 ml of PBS were injected subcutaneously into the right flank of 15–20-g female nude mice (Animal Center of Soochow University). Each group consisted of five mice. Tumor size and volume were measured every 2 days. To examine the effect of RMP on the growth of established tumors, mice with established xenograft tumors were subjected to RMP treatment 2 weeks later. Briefly, 100 μ g of various RMP expression vectors was injected into tumors every other day for 2 weeks. The tumor size and volume were also measured every 2 days. At the end of the 4th week, tumors from mice with or without RMP treatment were dissociated and evaluated. The animal operations and proce-

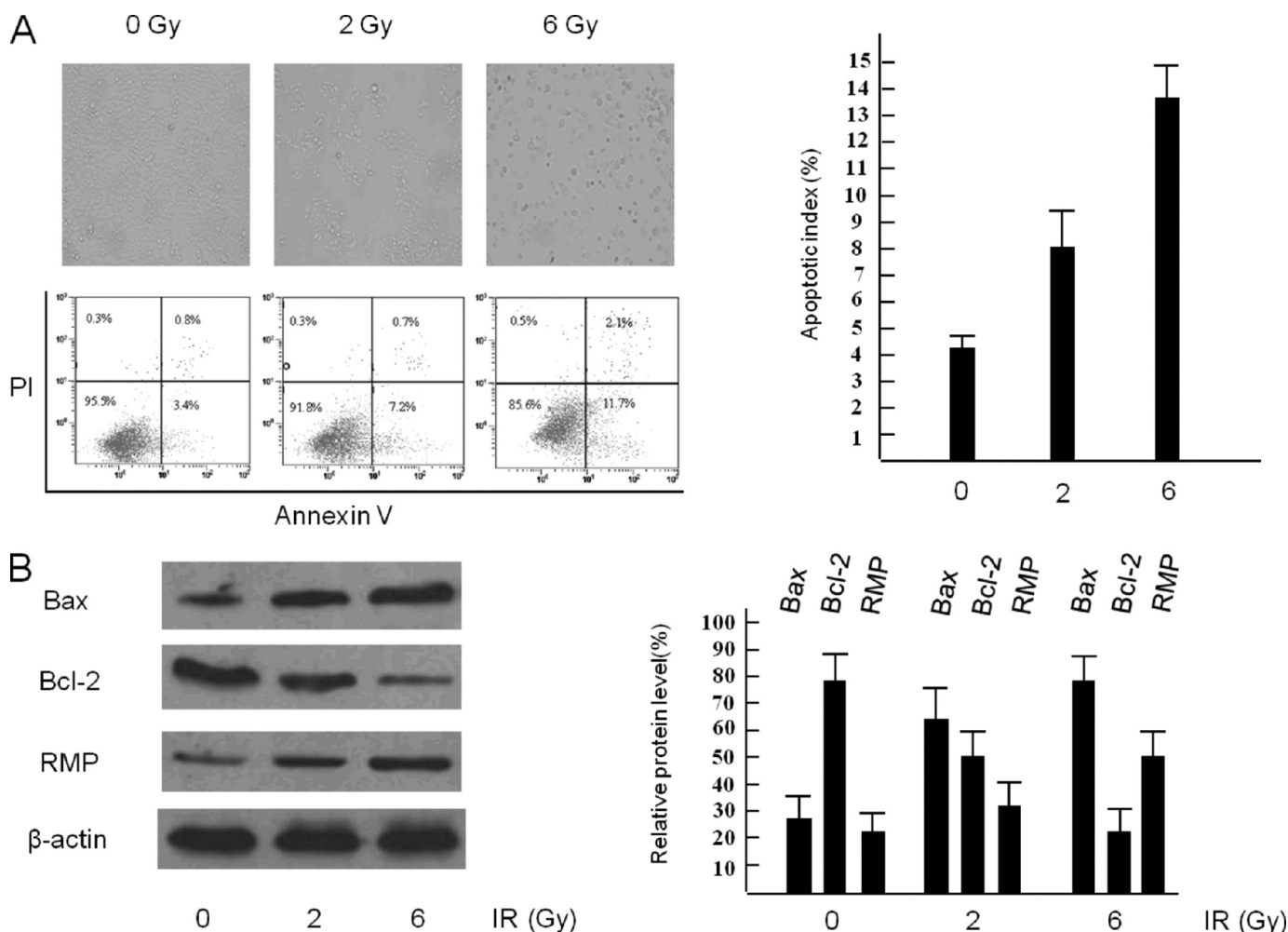


FIGURE 1. **RMP is a radiation-sensitive factor in HCC cells.** A, HCC SMMC-7721 cells were exposed to 0, 2, or 6 Gy of ^{60}Co γ -irradiation. The morphology of the treated cells was observed under a microscope (left upper panel), and cell apoptosis was analyzed by flow cytometry (left lower panel) 24 h after irradiation. Quantitation of the percentage of apoptotic cells is presented graphically on the right. B, Western blot analysis of Bax, Bcl-2, and RMP expression levels in irradiated cells. The relative levels of protein expression were normalized against β -actin and are depicted graphically on the right. Results are reported as the mean \pm S.D. of three independent experiments. IR, ionizing radiation.

dures were approved by the Committee on the Use of Live Animals in Teaching and Research of Soochow University.

Reverse Transcription-PCR—Total RNA was extracted using the RNeasy Mini kit (Qiagen) and was reverse-transcribed using the Thermoscript RT system (Invitrogen), according to the manufacturer's protocol. PCR was performed using 2 μl of complementary DNA and 0.2 unit of Hot start TaqDNA polymerase in a 20- μl reaction volume. The reaction was performed for 30 cycles with 94 $^{\circ}\text{C}$ denaturation for 30 s, 56 $^{\circ}\text{C}$ annealing for 30 s, and 72 $^{\circ}\text{C}$ elongation for 45 s. Primers for RMP were 5'-TCA AGC TTA TGA GGC TAG GAA ATG-3' and 5'-ATG GAT CCC TAG TCT TTC TGT TGC-3'. Primers for GAPDH were 5'-TGA TGA CAT CAA GAA GGT GGT GAA-3' and 5'-TCC TTG GAG GCC ATG TGG GCC AT-3'.

Immunohistochemistry Detection—Slides for both cultured cells and xenograft tumors were prepared for subsequent immunohistochemistry assays. Specifically, SMMC-7721 cells cultured on slides for 48 h were washed with PBST (0.2% Triton-X) and then fixed for 4 min in 1% formaldehyde in PBST before being freeze-cracked. The slides were transferred to -20°C cold methanol for 6 min and washed in PBS three times

for 5 min each. Meanwhile, xenograft tumors dissociated from nude mice were subjected to formalin fixation, paraffin embedding, and sectioning. The slides were blocked for 30 min in PBST containing 3% BSA (PBST-BSA) at 37 $^{\circ}\text{C}$ and incubated overnight at 4 $^{\circ}\text{C}$ with mouse monoclonal antibodies (1:500 in PBST-BSA). Slides were then washed three times in PBST (10 min at room temperature) and incubated for 2 h with the secondary antibody (anti-mouse HRP, 1:200) in PBST at room temperature. After incubation, slides were washed three times (10 min each) in PBST and mounted with 3 μl of Vectashield for further analysis.

Mitotic Index Analysis—SMMC-7721 cells, either exposed to 6 Gy of irradiation or not, were cultured for 72 h in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin. Cells were arrested in mitosis by a 30-min treatment of colcemid (0.12 mg/ml; Invitrogen) before being harvested and incubated in 75 mM KCl for 15 min at 37 $^{\circ}\text{C}$. Cells were centrifuged, and 10^7 cells were incubated in 5 ml of freshly prepared fixative (acetic acid/methanol at a 1:3 v/v ratio). The fixation step was repeated twice, and cells were dropped on ethanol-cleaned microscope slides. Slides were aged for a min-

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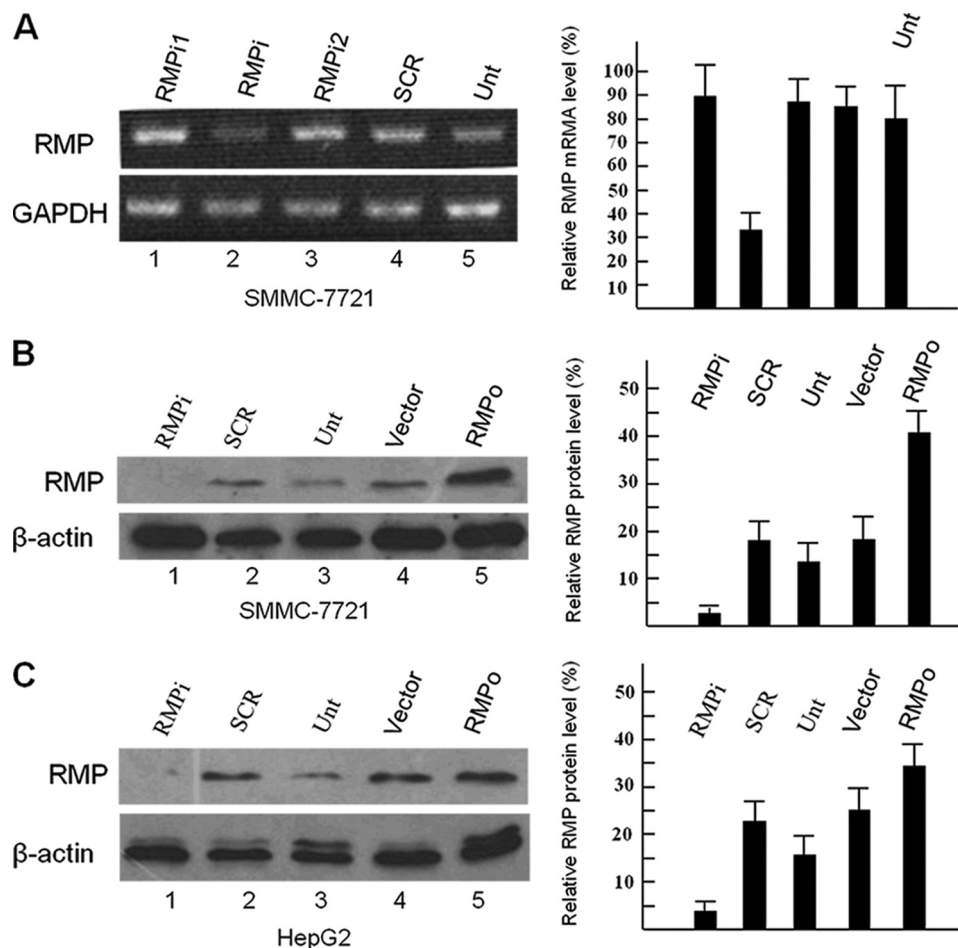


FIGURE 2. RMP expression in transfected HCC cells. *A*, expression levels of RMP were examined by RT-PCR in SMMC-7721 cells transfected with siRNAs (*left panel*). The cells were transiently transfected with double-stranded siRNA of RMPi, RMPi1, RMPi2, or scrambled RMPi (SCR). *B*, expression levels of RMP were examined by Western blot analysis in SMMC-7721 cells. SMMC-7721 cells were stably transfected with plasmids pGPU6-Neo-RMPi (RMPi), pGPU6-Neo-SCR (SCR), pFLAGCMV4-RMP (RMPo), or vector alone pFLAGCMV4 (Vector). *C*, same experiment as in *B* was repeated in the transiently transfected HepG2 cells (*left panel*). Relative levels of RMP were normalized against GAPDH (*A*) or β -actin (*B* and *C*) and plotted in the *right panels*. The untransfected SMMC-7721 cells (Unt) were applied as controls in (*A–C*). Results are reported as means \pm S.D. of three independent experiments.

imum of 1 week in ambient air at 20 °C and then sealed in plastic bags and stored at -20 °C. Slides were stained by Giemsa and observed under a microscope. The mitotic index was calculated as the percentage of dividing cells of the total HCC cells counted.

Statistical Analysis—Values are expressed as the means \pm S.E. Student's *t* test was used to determine the significance of the difference between compared groups. $p < 0.05$ indicated significant differences.

RESULTS

RMP Is a Radiation-sensitive Factor in HCC Cells—URI-1, the homolog of human RMP in *C. elegans*, is associated with the maintenance of genome stability (13). We investigated whether human RMP responded to DNA damage in a similar way.

DNA damage was induced in SMMC-7721 cells by ^{60}Co γ -irradiation, after which RMP expression was examined. As shown in Fig. 1A, SMMC-7721 cells that were γ -irradiated at 2 and 6 Gy appeared shrunken and displayed fewer intercellular connections compared with untreated cells (*left upper panel*). Flow cytometry analyses showed that increasing amounts of

γ -irradiation resulted in more apoptotic cells in SMMC-7721 cultures (Fig. 1A, *left lower* and *right panels*).

The expression level of the proapoptotic factor Bax and the antiapoptotic factor Bcl-2 was examined by Western blotting. The results demonstrated that Bax expression was increased and Bcl-2 expression was decreased sharply after increasing amounts of irradiation (Fig. 1B). Notably, the expression of RMP in SMMC-7721 cells significantly increased upon exposure to γ -irradiation (Fig. 1B), suggesting that RMP is a radiation-sensitive gene and may play a role in the apoptosis of HCC cells.

RMP Is Required for the Proliferation of HCC Cells—To investigate the biological function of RMP, three double-stranded siRNAs (RMPi, RMPi1, and RMPi2) were synthesized and examined for their ability to knock down RMP expression in SMMC-7721 cells. Among the three interfering sequences, RMPi showed the highest efficiency for the depletion of RMP (Fig. 2A). Therefore, we established a stable cell line that constantly expresses RMPi, effectively knocking down RMP expression. As seen in Fig. 2B, RMPi resulted in a dramatic inhibition of RMP expression compared with a SCR interfer-

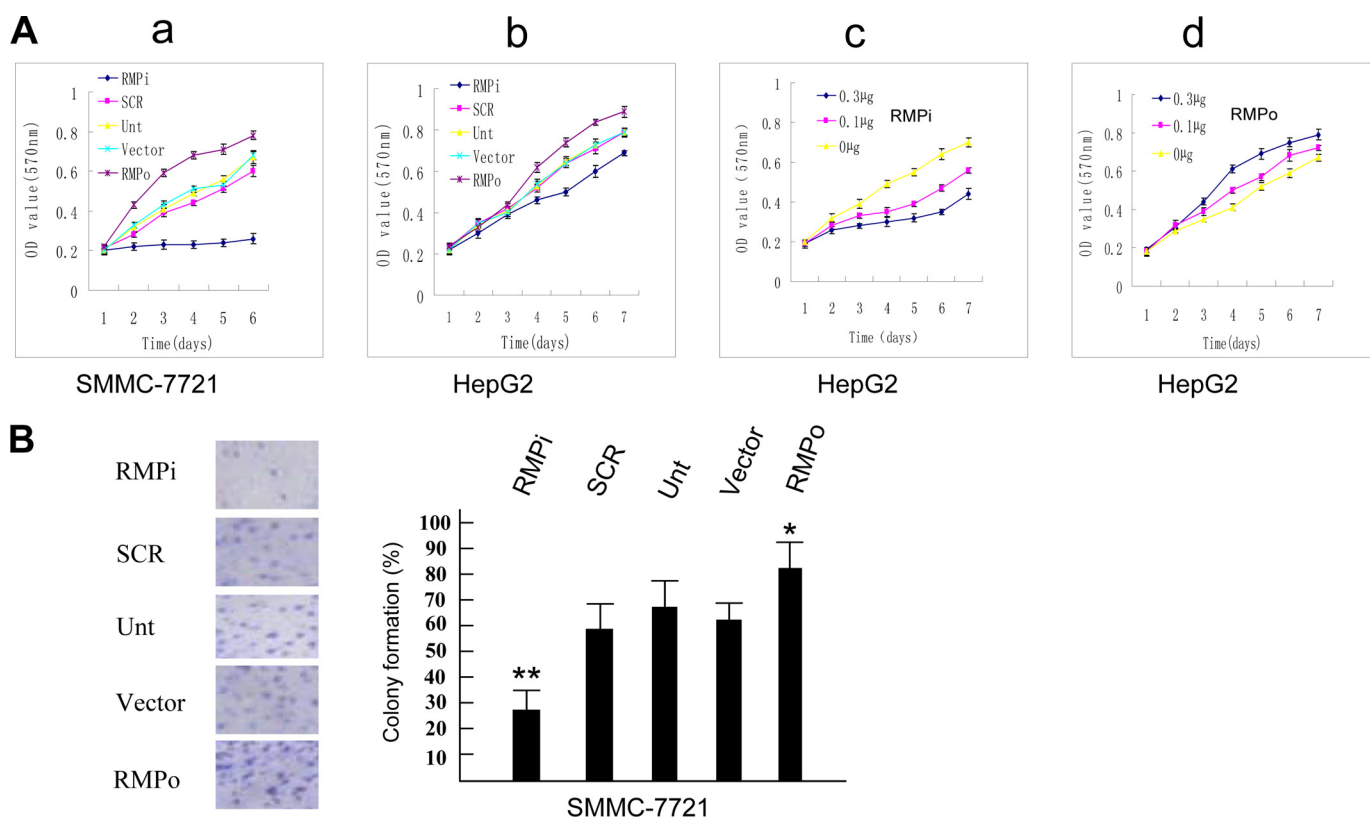


FIGURE 3. RMP is required for the proliferation of HCC cells. *a*, MTT assay. *a*, SMMC-7721 cells stably transfected with plasmids as indicated were subjected to the MTT proliferation assay. *b*, MTT assay was carried out with HepG2 cells transiently transfected with plasmids as indicated in *a*. Similar results were observed when compared with stably transfected cells as in *a*. *c* and *d*, HepG2 cells were transiently transfected with 0, 0.1, and 0.3 μg of pGPU6-Neo-RMPi (*c*) or pFLAGCMV4-RMP (*d*). MTT assay was performed on the days indicated. Each measurement was made in triplicate. *B*, colony formation assay. SMMC-7721 cells (1×10^5) were stably transfected with the indicated plasmids and cultured in plates. Cells were stained with 0.005% crystal violet (left panel) after culture for 4 weeks. Colony numbers were scored and are depicted graphically on the right. Student's *t* test, *, $p < 0.05$ and **, $p < 0.01$, relative to controls.

ence sequence. We also generated a stable SMMC-7721 cell line that overexpressed RMP (RMPo) compared with control cells transfected with vector alone or untransfected cells (Fig. 2*B*). Notably, when HepG2 cells were transiently transfected with the RMPi or RMPo expression plasmids, RMP depletion or expression was also observed (Fig. 2*C*).

Using SMMC-7721 stable cell lines, we examined the effects of RMP on the proliferation of HCC cells. As shown in Fig. 3*Aa*, MTT assays revealed that RMP depletion resulted in decreased cell proliferation, whereas control cells transfected with SCR or vector alone were unaffected compared with the untransfected SMMC-7721 cells. SMMC-7721 cells overexpressing RMP showed the highest growth rate of all stable cell lines examined (Fig. 3*Aa*).

HepG2 cells were also transfected with the RMP-depleting vector (RMPi). However, we failed to establish a HepG2 cell line with a stable depletion of RMP. All transfected cells died within 3 weeks of selection, suggesting that HepG2 cells are more sensitive to RMP depletion than are the SMMC-7721 cells.

To examine the effect of RMP on the growth of HepG2 cells, we performed MTT assays with transiently transfected HepG2 cells. As shown in Fig. 3*Ab*, HepG2 cells transfected with RMPi demonstrated delayed growth, whereas cells overexpressing RMP grew faster than control cells either transfected with vector alone or untransfected.

A dose-dependent effect of RMP on HepG2 cell growth was also observed. Increasing amounts of RMP-depleting vector

(RMPi) resulted in stronger growth inhibition, as shown in Fig. 3*Ac*. Additionally, increasing amounts of the RMP-expressing vector (RMPo) promoted proliferation of HepG2 cells, as shown in Fig. 3*Ad*.

To confirm the finding that RMP is required for the proliferation of HCC cells, we performed colony formation assays. The results showed that the colony-forming capacity of RMP-depleted transfectants (RMPi) was significantly lower than that of controls (Fig. 3*B*). However, SMMC-7721 cells overexpressing RMP (RMPo) demonstrated a significantly higher colony formation capacity than controls (Fig. 3*B*).

RMP Promotes *in Vivo* Growth of Xenograft Tumors—To investigate the biological impact of RMP on HCC growth *in vivo*, xenograft tumors were generated in nude mice. SMMC-7721 cells stably transfected with RMPi, RMPo, SCR, or vector alone were injected into 6-week-old female nude mice. As expected, mice injected with cells overexpressing RMP developed much larger solid tumors compared with mice injected with SCR or vector only. However, no tumors developed in any of the mice injected with SMMC-7721 cells depleted of RMP (RMPi cells) (Fig. 4*A*, upper and lower left panels). There was no change in the mean body weight of mice treated with RMPo and RMPi compared with mice treated with control cells (Fig. 4*A*, lower right panel).

We also examined the effects of RMP on the growth of established tumors in nude mice. Xenograft tumors formed in nude mice 2 weeks after injection. Then, we locally administered

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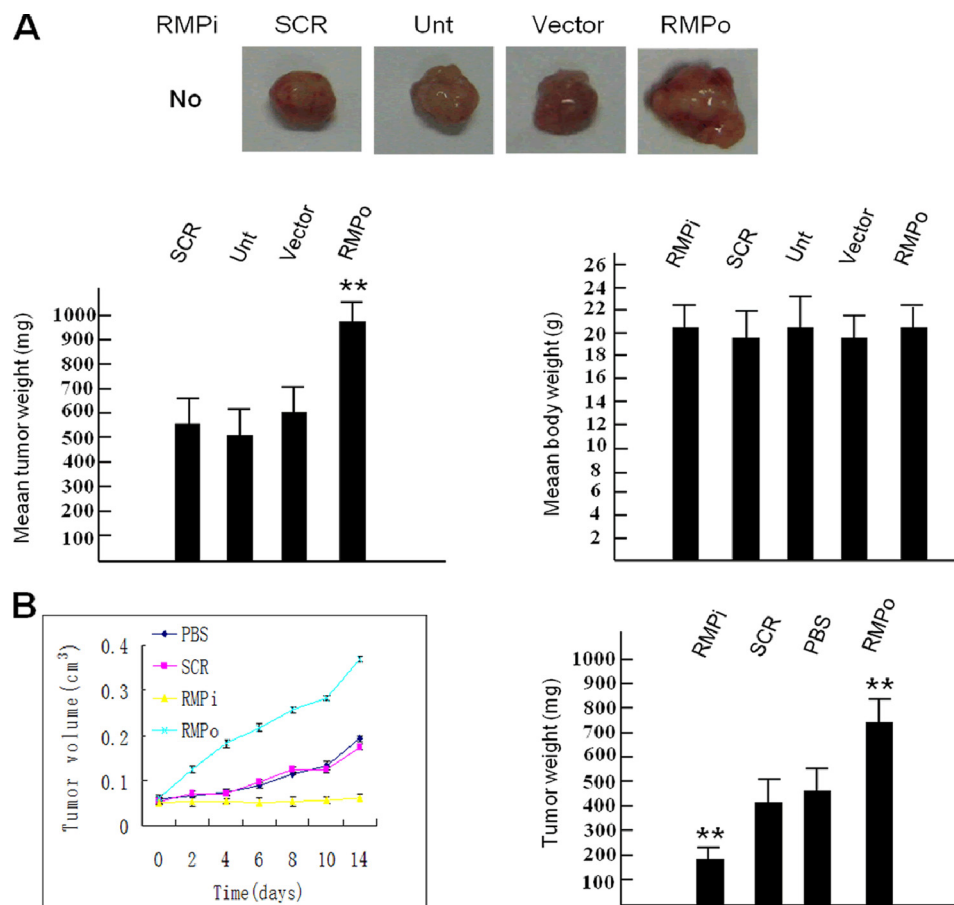


FIGURE 4. RMP promotes the growth of xenograft tumors in mice. *A*, tumors from xenograft mice were observed. SMMC-7721 cells (5×10^6) stably transfected with the indicated plasmids were inoculated subcutaneously into the dorsal flanks of nude mice (five mice in each group). Tumors were dissected at the end of 4 weeks. Photographs of a representative tumor from each group are shown (*top panels*). Quantitation of mean tumor weight and mean total body weight of mice is shown at the *bottom*. *B*, RMP promotes the growth of established tumors. Two weeks after inoculation, the nude mice were treated every other day by an intratumor injection of PBS or the indicated plasmids at $100 \mu\text{g}/\text{animal}$. Tumor size was expressed as the mean volume of tumors from five mice. Tumors were dissected 4 weeks after inoculation. Quantitation of tumor weight is shown in the *right panel*. Student's *t* test, **, $p < 0.01$, relative to the controls.

either the RMP-depleting vector (RMPi) or the RMP-expressing vector (RMPo) by intratumor injection. Our results demonstrated that the growth of tumors treated with RMPi was significantly inhibited compared with that of the controls treated with SCR vector or PBS (Fig. 4*B*). In contrast, tumors injected with RMPo grew at the fastest rate of all groups examined (Fig. 4*B*). These results demonstrated that RMP is a critical modulator in the growth of xenograft tumors, which is consistent with the *in vitro* results as shown in Fig. 3.

RMP Is an Antiapoptotic Factor in HCC Cells—As the results above suggested that RMP was required for the proliferation of HCC cells, we tested its effect on apoptosis with stable SMMC-7721 cell lines expressing RMPi, RMPo, or SCR. Cells were exposed to ^{60}Co γ -irradiation (6 Gy) to induce DNA damage and apoptosis. Then, the apoptosis level was analyzed by flow cytometry at 0, 24, and 48 h after irradiation. Although both early and late apoptotic cells were increased in all groups after irradiation, many more apoptotic cells were observed when RMP was down-regulated compared with SCR control cells. In contrast, the smallest increase in the apoptotic rate was seen in cells overexpressing RMP (RMPo) compared with cells transfected with RMPi and SCR (Fig. 5, *A* and *B*).

To confirm the effect of RMP knockdown on apoptosis, SMMC-7721 cells were also stained with Hoechst. These studies demonstrated that cells with RMP depletion showed significant DNA condensation and more fragmented nuclei compared with control cells with or without SCR transfection (Fig. 5*C*). These observations indicated that RMP depletion could induce apoptosis in SMMC-7721 cells.

RMP Depletion Induces G_2 Arrest—To investigate the mechanism of RMP on HCC proliferation, SMMC-7721 cells depleted of RMP were compared with the SCR control cells and untransfected SMMC-7721 cells. As shown in Fig. 6, *A* and *B*, more cells with RMP depletion were arrested in the G_2/M phase than control cells with or without SCR transfection. These results indicate that RMP plays a critical role in regulating the G_2/M phase transition during the cell cycle.

To confirm further the effect of RMP depletion on G_2/M arrest, SMMC-7721 cells were exposed to ^{60}Co γ -irradiation because G_2 is a radiosensitive phase of the cell cycle (19–21). Cell cycle analysis was carried out by flow cytometry at 24, 48, and 72 h after irradiation. As shown in Fig. 6, *A* and *B*, increasing numbers of cells were arrested in the G_2/M phase after

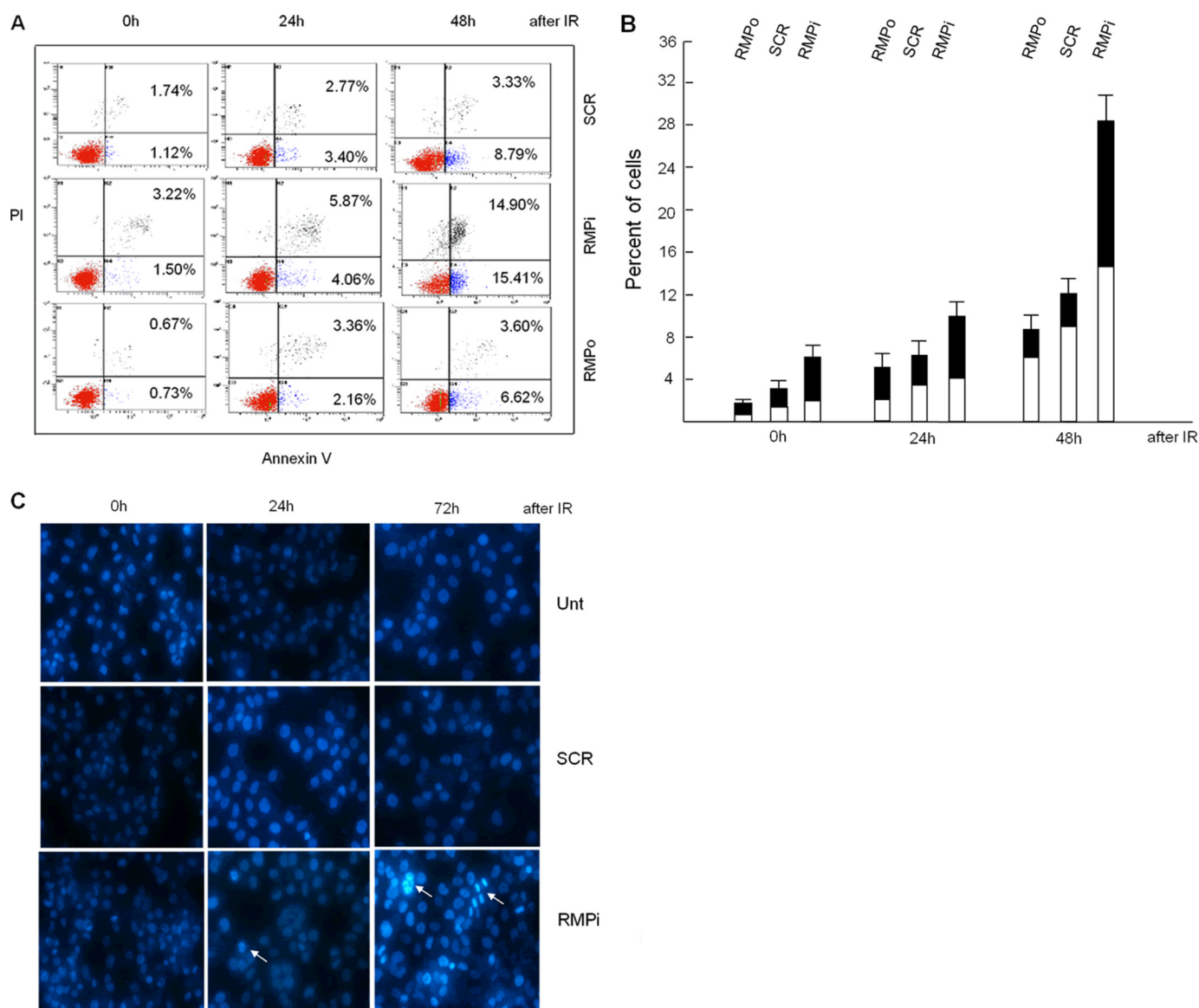


FIGURE 5. RMP is an antiapoptotic factor in HCC cells. *A*, stably transfected SMMC-7721 cells were irradiated at 6 Gy. After irradiation, these cells were cultured for an additional 0, 24, or 48 h and then subjected to apoptosis analysis by flow cytometry. *B*, percentages of early (white bar) and late (black bar) apoptotic cells are depicted. *C*, SMMC-7721 cells were irradiated at 6 Gy. These cells were cultured for an additional 0, 24, or 72 h and then stained with Hoechst 33258. Arrows indicate condensed DNA and apoptotic cells. Unt, untransfected.

irradiation. However, there were more RMP-depleted cells that were arrested in the G₂/M phase compared with control cells (untransfected and SCR) (Fig. 6, *A* and *B*). To test whether this arrest occurred in the G₂ or M phase, the cells with RMP depletion were subjected to mitotic chromosome analysis (22). The results showed that the mitotic index was 1.2% before irradiation and 1.8% at 72 h after irradiation (Fig. 6*C*). There was no significant difference between these mitotic indexes, indicating that cells were arrested in the G₂ phase rather than in M phase.

RMP Regulates the Genes Related to Apoptosis and Cell Cycle—To elucidate the mechanism of RMP in apoptosis, we examined the expression of apoptotic genes in xenograft tumors by immunohistochemistry. As expected, the expression of RMP and Bcl-2 was elevated in tumors overexpressing RMP, whereas Bax expression was decreased in the RMPo tumors

compared with the vector only control (Fig. 7*A*). Notably, we also observed an increased expression of AFP in tumors overexpressing RMP (Fig. 7*A*).

Western blot analysis was also performed to confirm the expression of apoptotic genes in xenograft tumors. As shown in Fig. 7*B*, RMP expression increased in the tumor that was transfected with the RMP expression plasmid (RMPo), whereas the expression level of Bax was lower in the RMPo tumor compared with the vector control (Fig. 7*B*). In contrast, the expression of the antiapoptotic factor Bcl-2 in RMPo tumors was higher than that in the tumors treated with vector alone.

Because no tumors developed in nude mice injected with RMPi cells, we examined the expression of apoptotic genes in the cultured RMPi cells by immunostaining. We found that the expression of both Bax and p53 was increased, but Bcl-2 expression was decreased in cells depleted of RMP compared with the

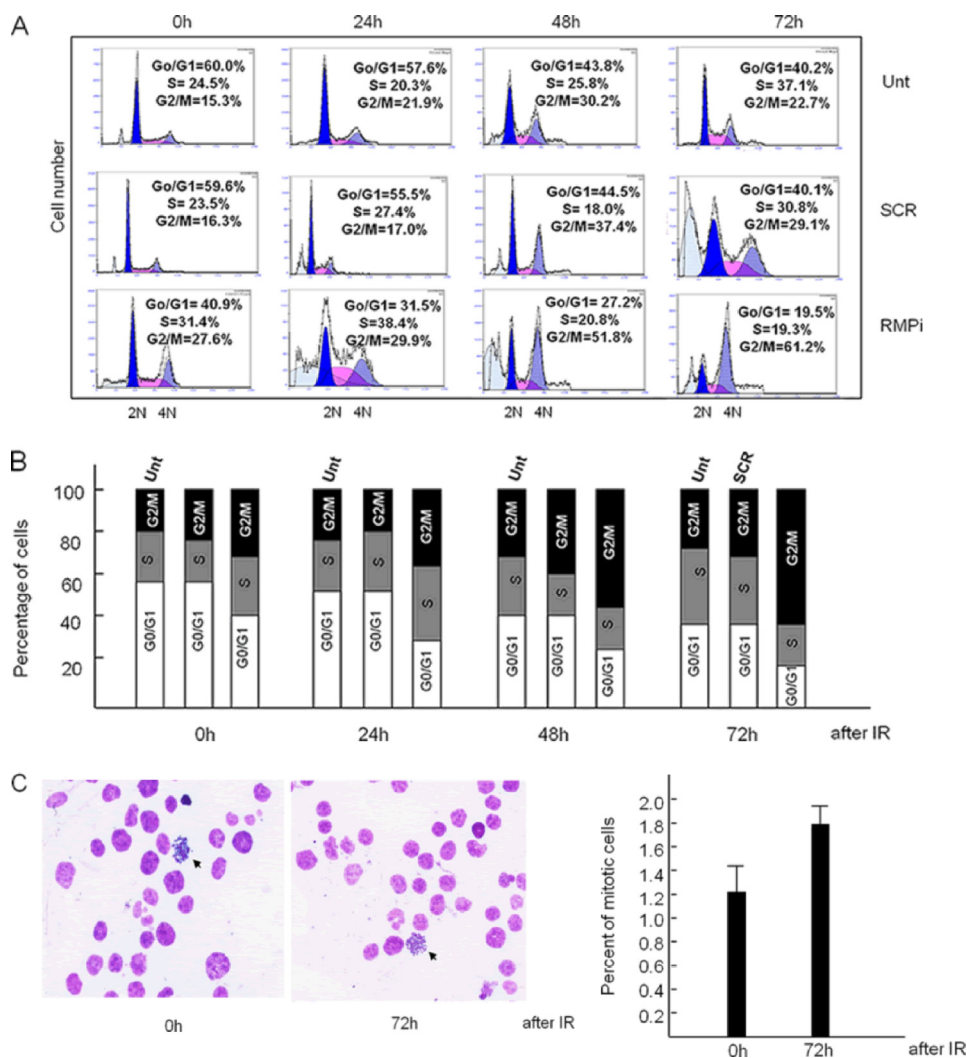


FIGURE 6. **RMP depletion induces G₂ arrest in human HCC cells.** *A*, SMMC-7721 cells stably transfected with plasmids as indicated were irradiated at 6 Gy. These cells were then cultured for an additional 0, 24, 48, or 72 h and subjected to cell cycle analysis by flow cytometry. *B*, percentages of cells in G₀/G₁ (white bars), S (gray bars), and G₂/M (black bars) were scored and are depicted graphically. *C*, chromosomes were prepared from RMPi-transfected SMMC-7721 cells both before and 72 h after irradiation. Arrows at the top indicate chromosome spreads. Quantitation of mitotic index is depicted graphically at the bottom.

SCR control (Fig. 7C). RMP depletion also reduced the expression of AFP (Fig. 7C).

We further investigated the expression of apoptotic factors in the SMMC-7721 cells by Western blotting. As shown in Fig. 7D, the expression of the proapoptotic factor Bax was elevated in the RMP-depleted cells (RMPi) compared with SCR or untransfected cells. The expression of the proapoptotic factor c-Myc was also increased by RMP depletion, in a manner similar to Bax. In contrast, the expression of the antiapoptotic factor Bcl-2 was decreased in RMPi cells (Fig. 7D). Additionally, Western blot analysis also showed the down-regulation of AFP in RMPi cells, consistent with the immunostaining studies shown in Fig. 7C.

We also examined the expression of G₂ relevant checkpoint regulators. The Cdk1/Cyclin B complex is the most critical regulator for the G₂ checkpoint (19–21). We observed a significantly decreased expression of both Cdk1 and Cyclin B1 in the RMP-depleted cells compared with the control cells (Fig. 7D). However, the expression of p53 and p21, the upstream regula-

tors of the Cdk1/Cyclin B complex, was up-regulated by RMP depletion (Fig. 7D). Overall, these observations suggest that RMP may play an important role in the proliferation and apoptosis of HCC cells.

DISCUSSION

Given the regenerative characteristics of the liver and the hypothesis that RMP might be a cell cycle regulator, we investigated the underlying molecular mechanisms regulating HCC cell cycle, apoptosis and proliferation. RMP appears to compete with HBx for RPB5 binding and antagonize the coactivation of HBx. Here, we provide evidence that RMP is an antiapoptotic factor in HCC. As demonstrated in the HCC cells SMMC-7721 and HepG2, RMP promoted cell proliferation. RMP depletion suppressed HCC cell growth, whereas overexpression of RMP promoted the proliferation of HCC cells, which was confirmed by both HCC cell proliferation and colony formation assays. Overexpression of RMP inhibited the apoptosis of HCC cells, and the depletion of RMP induced apoptosis. Enhanced apo-

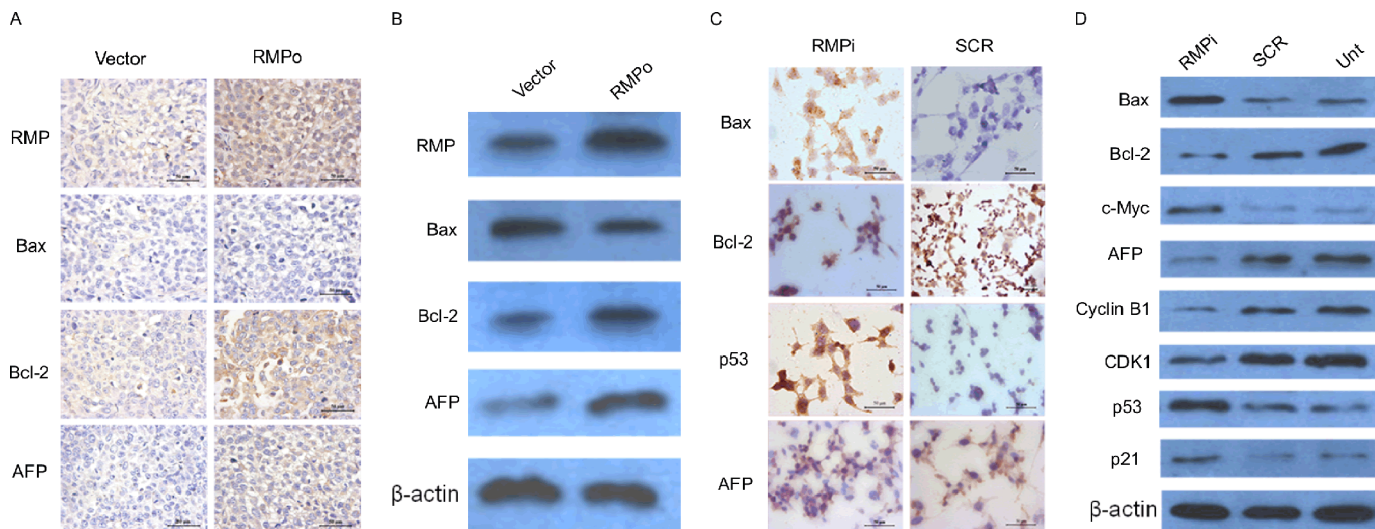


FIGURE 7. **RMP regulates genes related to apoptosis and cell cycle.** *A*, immunohistochemical examination of RMP, Bax, Bcl-2, and AFP in tumors from mice injected with SMMC-7721 cells stably transfected with RMPo or vector alone. *B*, Western blot analysis shows expression of RMP, Bax, Bcl-2, and AFP in tumor tissues from xenograft mice. *C*, expression of Bax, Bcl-2, p53, and AFP was examined in SMMC-7721 cells stably transfected with RMPi and SCR. *D*, Western blot analysis in the same cells as in *C*.

ptosis by RMP depletion was also observed when HCC cells were treated by irradiation, whereas the overexpression of RMP suppressed apoptosis. This antiapoptotic property of RMP could also be attributed, at least in part, to its role in the regulation of the G_2 cell cycle checkpoint.

Bax and Bcl-2 are key factors that regulate apoptosis. Bax homodimers are known to promote apoptosis, whereas Bcl-2 homodimers block apoptosis. The relative expression levels of Bax and Bcl-2 play a critical role in the apoptotic response. Higher levels of Bax promote apoptosis, but higher levels of Bcl-2 block apoptosis to support cell survival. In our study, the depletion of RMP in SMMC-7721 cells up-regulated Bax expression, whereas Bcl-2 expression was down-regulated. This result explains the observed apoptosis in these RMP-depleted cells and the increased apoptosis induced by irradiation. However, overexpression of RMP inhibited apoptosis and favored cell survival, which supports the antiapoptotic role of RMP in HCC cells.

SMMC-7721 cells contain wild-type p53 (23), which activates transcription of the Bax gene and inhibits transcription of the Bcl-2 gene. We found that the depletion of RMP down-regulated the expression of Bcl-2, but up-regulated Bax, p53 and p21. These results suggest that RMP regulation of Bax and Bcl-2 in SMMC-7721 cells might be through a p53-dependent pathway.

The c-Myc oncogene plays dual functions both in apoptosis and in cell proliferation (24). Our data show that RMP knock-down decreases Bcl-2 expression and at the same time increases the expression of Bax and c-Myc. This result suggests that RMP may promote HCC cell survival by increasing the expression of Bcl-2 while decreasing the expression of Bax and c-Myc. c-Myc might play a proapoptotic rather than a proliferative role in HCC cells under the regulation of RMP. These data also imply that RMP-induced proliferation of HCC cells might work through a c-Myc-independent mechanism.

Cell cycle progression is regulated primarily at the G_1/S and G_2/M phase transitions by a series of checkpoint regulators,

including Cdk1 and Cyclin B. Cdk1 (*cdc2*) is responsible for controlling the transition of G_1/S and G_2/M (25, 26). The kinase activity of Cdk1 is controlled during the cell cycle by association with Cyclin B, another cell cycle regulator overexpressed in multiple tumors (25–29). In our studies, we found that RMP contributes to the regulation of the G_2 checkpoint in response to radiation-induced DNA damage. A possible mechanism of RMP at this checkpoint is through regulating the expression of the Cyclin B/Cdk1 complex because depletion of RMP in HCC cells significantly decreased both Cdk1 and Cyclin B1 levels, whereas the overexpression of RMP up-regulated Cdk1 and Cyclin B1.

The transcriptional functions of RMP might be distinct from its roles in hepatocarcinogenesis. RMP was previously shown to counteract transcriptional activation by HBx (11). However, both RMP and HBx might possess oncogenic properties, although cooperative or overlapping oncogenic effects could exist between RMP and HBx.

Although further investigation is required, our novel discovery that RMP affects the proliferation of HCC cells suggests that RMP plays important roles in the pathways controlling apoptosis and cell cycle. This function makes RMP a candidate molecule that is essential for the elucidation of hepatocarcinogenesis and might also facilitate the identification of novel therapeutic targets of HCC.

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