

MAF1 Suppresses AKT-mTOR Signaling and Liver Cancer Through Activation of PTEN Transcription

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The phosphatidylinositol 3-kinase/phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase/protein kinase B/mammalian target of rapamycin (PI3K-PTEN-AKT-mTOR) pathway is a central controller of cell growth and a key driver for human cancer. MAF1 is an mTOR downstream effector and transcriptional repressor of ribosomal and transfer RNA genes. MAF1 expression is markedly reduced in hepatocellular carcinomas, which is correlated with disease progression and poor prognosis. Consistently, MAF1 displays tumor-suppressor activity toward *in vitro* and *in vivo* cancer models. Surprisingly, blocking the synthesis of ribosomal and transfer RNAs is insufficient to account for MAF1's tumor-suppressor function. Instead, MAF1 down-regulation paradoxically leads to activation of AKT-mTOR signaling, which is mediated by decreased PTEN expression. MAF1 binds to the PTEN promoter, enhancing PTEN promoter acetylation and activity. **Conclusion:** In contrast to its canonical function as a transcriptional repressor, MAF1 can also act as a transcriptional activator for PTEN, which is important for MAF1's tumor-suppressor function. These results have implications in disease staging, prognostic prediction, and AKT-mTOR-targeted therapy in liver cancer. (HEPATOLOGY 2016;63:1928-1942)

Upon stimulation by growth factors and nutrients, mammalian cells rapidly relay mitogen signals to regulate diverse cellular processes. A pivotal mitogenic pathway is the phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K-AKT-mTOR) pathway.⁽¹⁾ PI3K activates AKT, a key kinase that promotes growth, metabolism, survival, and motility.⁽²⁾ Another important component of this pathway is mTOR kinase that controls protein translation, ribosome biogenesis, and metabolism.⁽³⁻⁵⁾ Phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase (PTEN), a phosphatidylinositol 3-phosphate (PI3P) phosphatase and a major tumor suppressor, antagonizes PI3P's mitogenic signal.⁽⁶⁾ Because of its pivotal role in growth, metabolism, and survival, the PI3K-AKT-mTOR pathway is a

Abbreviations: ACC1, acetyl-coenzyme A carboxylase 1; AKT, protein kinase B; ANOVA, analysis of variance; bp, base pair; ChIP, chromatin immunoprecipitation; CoA, coenzyme A; EdU, 5-ethynyl-2'-deoxyuridine; FASN, fatty acid synthase; FOXO, forkhead box O; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IGF1, insulin-like growth factor 1; IHC, immunohistochemistry; mRNA, messenger RNA; mTOR, mammalian target of rapamycin; P-AKT, phosphorylated AKT; PCR, polymerase chain reaction; PI3K, phosphatidylinositol 3-kinase; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha; PI3P, phosphatidylinositol 3-phosphate; Pol, polymerase; PTEN, phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase; rRNA, ribosomal RNA; shRNA, small hairpin RNA; siRNA, small interfering RNA; SNK, Student-Newman-Keuls; TBP, TATA box-binding protein; tRNA, transfer RNA.

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major oncogenic driver that is frequently activated during carcinogenesis.⁽¹⁾ Therefore, understanding the molecular details of this pathway has profound implications in the etiology, diagnosis, and therapy of human malignancies. mTOR controls protein synthesis in response to growth factors and nutrients,^(7,8) which is mediated by cap-dependent translational regulation through 4E-BP1 phosphorylation,⁽⁹⁾ and biogenesis of ribosomal RNA (rRNA) and transfer RNA (tRNA).⁽¹⁰⁾ mTOR regulates transcription of rRNA and tRNA genes by RNA polymerases I and III (Pol I and Pol III),⁽¹¹⁻¹⁸⁾ which is, in part, mediated by MAF1, a general transcriptional repressor and mTOR downstream effector.⁽¹⁹⁾ MAF1 was originally identified in yeast as a transcriptional repressor of Pol III.⁽²⁰⁾ MAF1 binds to and prevents Pol III's transcriptional initiation and elongation.⁽²¹⁻²³⁾ In mammals, MAF1 also represses Pol II target genes, such as TATA box-binding protein (TBP), and lipogenic genes,⁽²⁴⁻²⁷⁾ and 45S rRNA gene indirectly through TBP.⁽²⁵⁾ Upon mitogen stimulation, mTOR binds to and phosphorylates MAF1, relieving its transcriptional repression and promoting the synthesis of ribosomal and transfer RNAs.^(17,28,29) More recently, PTEN was shown to regulate MAF1 through a forkhead box O (FOXO)-dependent, yet unknown posttranslational mechanism, which is important for suppressing hepatocarcinogenesis.⁽²⁷⁾

Liver cancer is a leading cause of cancer deaths worldwide.⁽³⁰⁾ The PI3K-AKT-mTOR pathway is a major driver for liver carcinogenesis and a therapeutic target.⁽³¹⁾ For example, chronic activation of AKT and mTOR attributed to liver-specific PTEN deletion in mice leads to hepatocellular carcinoma (HCC), the

predominant form of liver cancer.⁽³²⁾ AKT-mTOR pathway activation in many malignancies is predominantly attributed to activating mutation of phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA) or inactivating mutations of PTEN.⁽³³⁾ Although hyperactive AKT-mTOR signaling is commonly observed in HCC, PIK3CA, and PTEN mutation is relatively rare,⁽³¹⁾ at approximately 2%-6% according to cBioPortal. How the AKT-mTOR pathway is activated remains not fully understood. Because MAF1 is a major effector of AKT-mTOR signaling, we investigated its potential function in liver cancer. Our study provides new insights into the transcriptional and tumor-suppressive functions of MAF1.

Materials and Methods

XENOGRAFT TUMORS

Xenograft tumors were generated as described.^(34,35) Briefly, female athymic nu/nu mice age 5-6 weeks were injected with 5×10^6 of tumor cells in 0.2 mL of serum-free Dulbecco's modified Eagle's medium subcutaneously into the flank of each mouse. The left flank was implanted with control tumor cells whereas the right side was injected with testing tumor cells. Intra-gastric administration was performed each day with 200 ug/400 uL of doxycycline per mouse. Tumor growth was monitored by measuring length and width of tumor using a caliper. Tumor sizes were calculated from the formula: $\text{Size} = \text{Length} \times \text{Width}^2 \times (\pi/6)$. After mice were sacrificed, xenograft tumors were

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collected, photographed, and then fixed in paraffin. Animal procedures were approved by the Sun Yat-Sen University Animal Care and Use Committee (Guangzhou, China).

PRIMARY HUMAN HCC TUMORS

Fifty-six clinical tissue samples of HCC were collected from HCC patients who underwent hepatic resection at the Sun Yat-Sen University Cancer Center from December 2007 to February 2013. The HCC tissue array (HLiv-HCC180Sur-01), containing 90 HCC tumors with clinicopathological information, was purchased from Shanghai Outdo Biotech (<http://www.superchip.com.cn/>). Altogether, 146 formalin-fixed, paraffin-embedded specimens of deidentified HCCs paired with adjacent nontumour liver tissues were used for the immunohistochemistry (IHC) study. Patients' consent and approval from the Sun Yat-sen University Cancer Center Ethics Committee were obtained for research purposes in use of the clinical materials. There are 123 males and 23 females with a median age of 51.2 years (range, 26-73).

CHROMATIN IMMUNOPRECIPITATION ASSAY

Chromatin immunoprecipitation (ChIP) assay was performed based on the Upstate EZ ChIP Kit instruction manual as described.⁽³⁶⁾ The identified MAF1 regulating PTEN was confirmed using independent ChIP-PCR (polymerase chain reaction) assays with immunoprecipitated DNA fragments pulled down by anti-MAF1 antibodies (ab95971; Abcam, Cambridge, MA), anti-histone H3ac (pan-acetyl; no.: 39139), or pooled immunoglobulin G from rabbit (Santa Cruz Biotechnology, Santa Cruz, CA) as a negative control. DNA was amplified by quantitative PCR using Terra qPCR Direct SYBR Premix (Clontech Laboratories Inc., Mountain View, MA) on the 7500 System (Applied Biosystems, Foster City, CA). PTEN primer sequences are: -1364 to -1022 upstream forward primer, 5'-ATGTGGGTGCTTGTGTAACC-3', reverse primer, 5'-AAAGTACGGAACGGTAGGAAGCT-3'. Primers for pre-tRNA^{Leu} are forward, 5'-GTCAGGATGGCCGAGTGGTCTAAG-3', and reverse, 5'-CCACGCCT CCATACGGAGAACCA-GAAGACCC-3') as described.⁽³⁷⁾

STATISTICAL ANALYSIS

All statistical analyses were performed by using SPSS software (version 11.0; SPSS Inc., Chicago, IL). Differences among categorical variables were analyzed by one-way analysis of variance (ANOVA)/Student-Newman-Keuls (SNK) test or independent-sample Student *t* test. Xenograft tumor size and immunostaining scores of MAF1 in paired clinical samples were performed using a sample-paired *t* test. Kaplan-Meier's analysis was used to analyze overall survival data. Univariate and multivariate survival analyses were conducted using a Cox's proportional hazards regression model. The correlation of MAF1 expression and the PTEN/P-AKT (phosphorylated AKT) score was analyzed using Pearson's correlation test. Immunostaining scores of MAF1 were performed using the nonparametric Mann-Whitney's U test. Statistical significance was considered significant when $P < 0.05$ (indicated in corresponding figures with one asterisk for $P < 0.05$ and two asterisks for $P < 0.01$).

For additional materials and methods, see the [Supporting Information](#).

Results

MAF1 OVEREXPRESSION SUPPRESSES LIVER CANCER GROWTH, PROLIFERATION, AND INVASION

To investigate the role of MAF1 in liver cancer, we transiently expressed MAF1 in liver cancer cell lines Hep3B, QGY7703, SMMC7721, and QGY7701 (Fig. 1A,B). Proliferation of liver cancer cells moderately expressing ectopic MAF1 (1- to 2-fold increase over endogenous MAF1) was notably reduced. MAF1 also displayed an antiproliferation effect as measured by the 5-ethynyl-2'-deoxyuridine (EdU) assay (Fig. 1C and [Supporting Fig. S1A](#)), colony formation (Fig. 1D and [Supporting Fig. S1B](#)), invasiveness (Fig. 1E and [Supporting Fig. S1C](#)), and xenograft tumor growth assays. MAF1 significantly decreases xenograft tumor burden (Fig. 2A,B and [Supporting Fig. S2A, B](#)). IHC staining of tumor sections showed markedly reduced proliferation as judged by Ki67 staining (Fig. 2C,D). Our results are consistent with a recent observation that MAF1 ectopic expression increases the doubling time of Huh7 cells and reduces the growth rate of Huh7-derived xenograft tumors.⁽²⁷⁾ Of note,

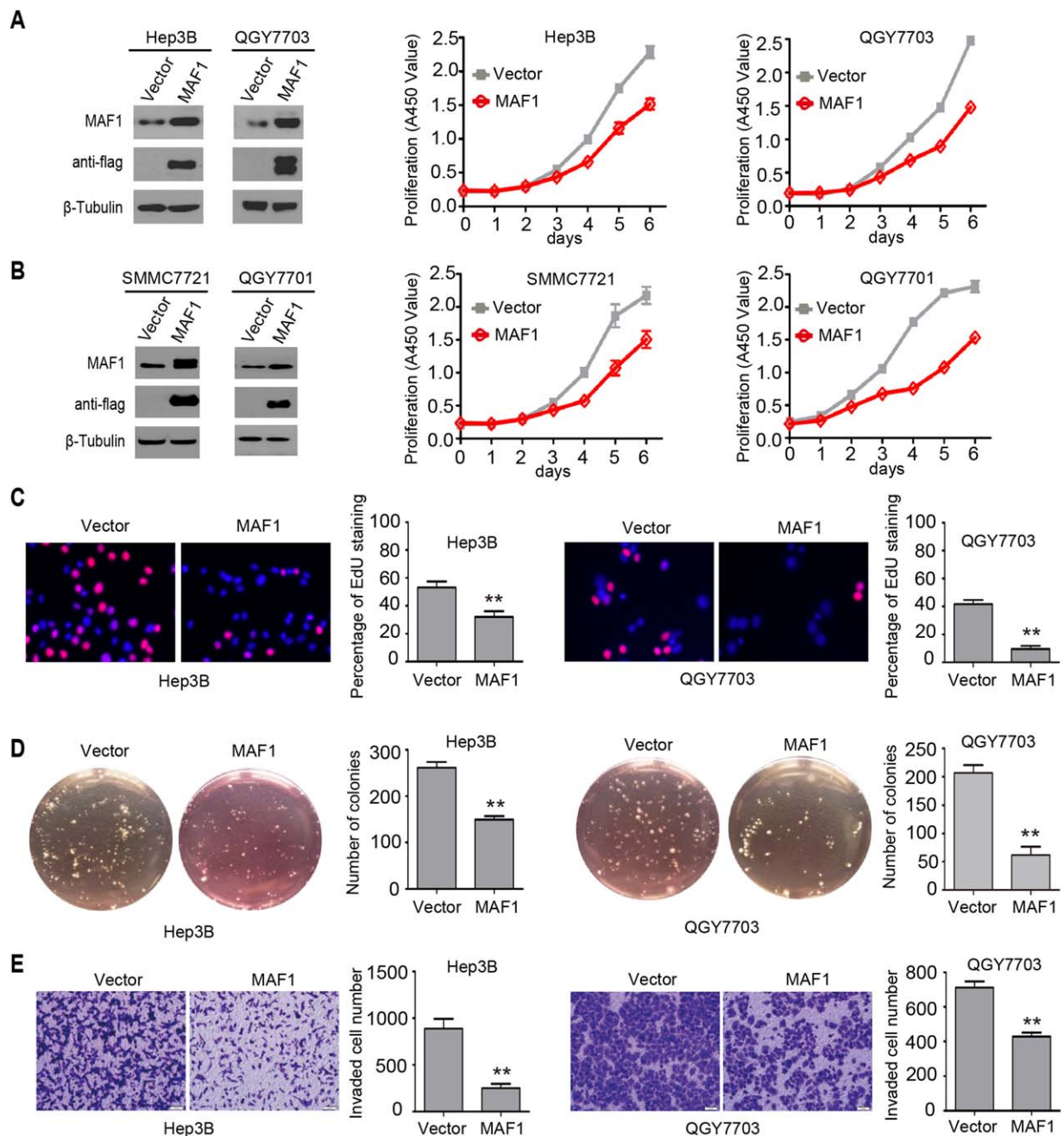


FIG. 1. MAF1 overexpression suppresses proliferation and invasion of HCC. (A) Ectopic expression of MAF1 blocks proliferation of Hep3B and QGY7703 cells. MAF1 is transiently expressed in Hep3B and QGY7703 cells. Left panel: immunoblotting of ectopic MAF1-Flag protein. Middle and right panels: MAF1 overexpression inhibits proliferation of Hep3B and QGY7703 cells, respectively, as measured by the Cell Counting Kit-8 assay. Data represent mean \pm standard deviation (SD; $n = 3$ in quadruplets). (B) Ectopic expression of MAF1 blocks proliferation of SMMC7721 and QGY7701 cells. Same as (A), except that SMMC7721 and QGY7701 cells were used. (C) MAF1 overexpression inhibits growth of Hep3B and QGY7703 cells *in vitro*, as measured by EdU proliferation assay. Data (mean \pm SD; $n = 6$) were analyzed by Student *t* test; ** $P < 0.01$. (D) Soft agar growth assay shows that MAF1 overexpression inhibits anchorage-independent growth of Hep3B and QGY7703 cells *in vitro*. Data (mean \pm SD; $n = 6$ in quadruplets) were analyzed by Student *t* test; ** $P < 0.01$. (E) Transwell assay shows that MAF1 overexpression suppresses invasion of Hep3B and QGY7703 cells *in vitro*. Data (mean \pm SD; $n = 6$ in triplets) were analyzed by Student *t* test; ** $P < 0.01$.

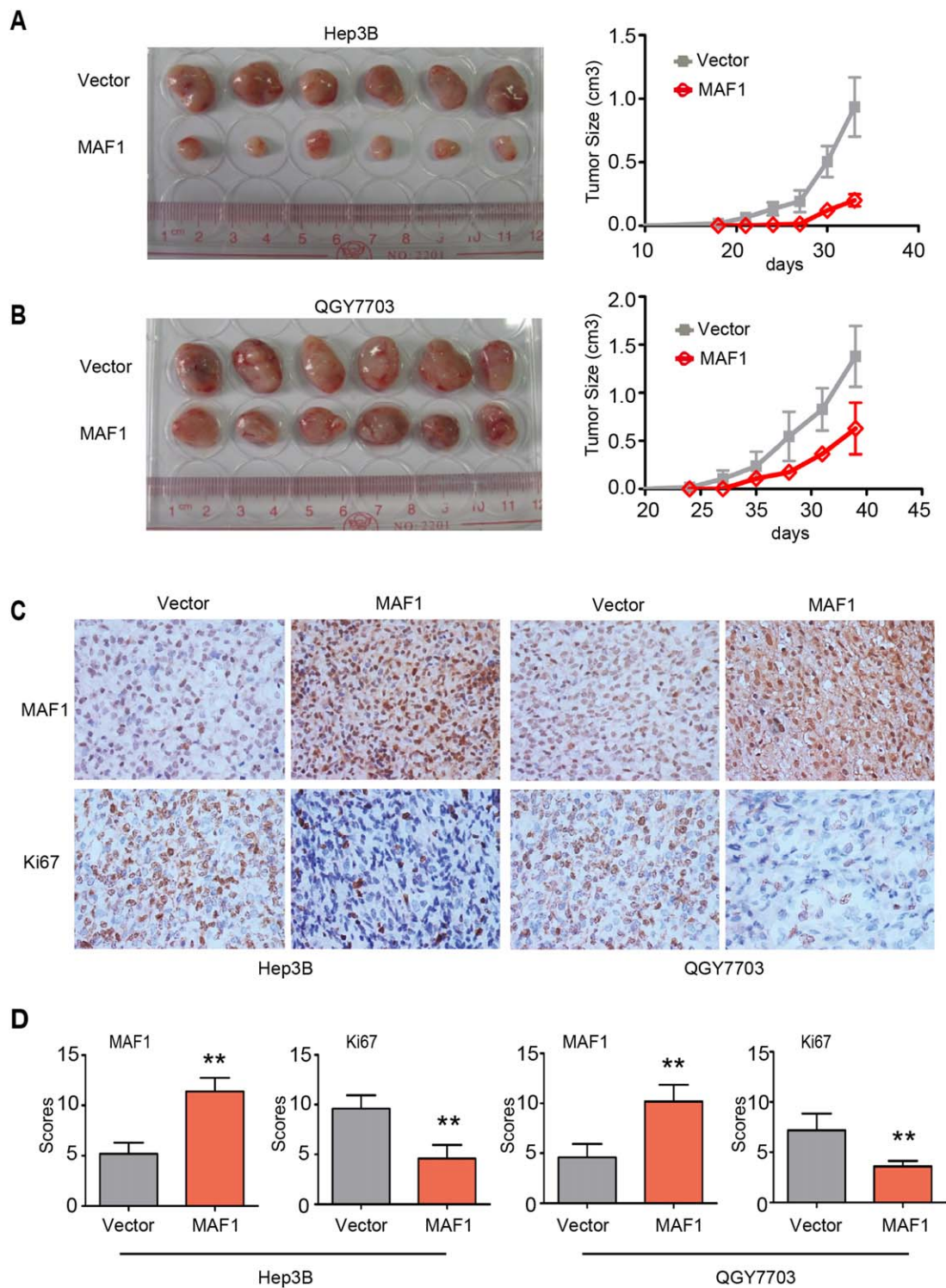


FIG. 2. MAF1 suppresses HCC growth *in vivo*. (A) MAF1 overexpression suppresses growth of Hep3B xenograft tumors. Growth of xenograft tumors derived from Hep3B cells overexpressing MAF1 or carrying a control vector was measured by tumor volume. Data (mean \pm standard deviation [SD]; n = 6) were analyzed by sample-paired *t* test; $**P < 0.01$. (B) MAF1 overexpression suppresses growth of QGY7703 xenograft tumors. Growth of QGY7703 xenograft tumors was measured by tumor volume. Data (mean \pm SD; n = 6) were analyzed by sample-paired *t* test; $**P < 0.01$. (C) Representative images of IHC staining show that MAF1 overexpression decreases liver cancer cell proliferation (Ki67) in xenograft tumors (20 \times magnification). (D) Quantification of IHC scores for MAF1 and Ki-67 staining in Hep3B and QGY7703 xenograft tumors. Data (mean \pm SD; n = 6) were analyzed by Student *t* test; $**P < 0.01$.

MAF1 inhibits liver cancer growth more potently in xenograft tumor than *in vitro* cultured cells, suggesting that MAF1 is a stronger tumor suppressor under the *in vivo* tumor environment.

DOWN-REGULATION OF MAF1 PROMOTES LIVER CANCER CELL GROWTH, PROLIFERATION, AND INVASION

To further evaluate the function of MAF1 in liver cancer, we knocked down MAF1 by lentiviral small hairpin RNA (shRNA). Among four MAF1 shRNAs tested, shRNA1-1 and 1-4 generated the most consistent knockdown results across Hep3B, QGY7703, and QGY7701 cell lines and were thus chosen for subsequent studies. Both shRNAs reduced MAF1 messenger RNA (mRNA) and protein level by 50%-60% in different cell lines (Fig. 3A,B and Supporting Fig. S3A). In contrast to MAF1 overexpression, down-regulation of MAF1 led to enhanced growth and proliferation (Fig. 3C,3D and Supporting Fig. S3B,C) and invasiveness of HCC (Fig. 3E and Supporting Fig. S3D). These results are consistent with the MAF1 overexpression data and show that MAF1 suppresses growth, proliferation, and invasiveness of liver cancer cells.

MAF1 EXPRESSION IS FREQUENTLY DOWN-REGULATED IN PRIMARY HUMAN HCC, WHICH IS CORRELATED WITH DISEASE PROGRESSION AND POOR PROGNOSIS

To evaluate the clinical significance of MAF1 in liver cancer, we investigated MAF1 expression by IHC staining of 146 pairs of hepatitis B virus (HBV)-positive human HCCs and matching adjacent nontumor liver tissues. MAF1 expression is significantly decreased in tumors as indicated by lower IHC staining scores compared with the adjacent hepatocytes ($Z = -8.864$; $P < 0.001$; Fig. 4A-C). Of the 146 HCC samples, 14 cases (9.6%) displayed high MAF1 staining (score +++), 77 (52.7%) showed moderate staining (score ++), and 55 (37.7%) had weak or undetectable staining (score + or -). Clinicopathological analyses showed no correlation between MAF1 expression and sex, age, or tumor size (Table 1). MAF1 staining is inversely

associated with tumor differentiation ($\chi^2 = 6.318$; $P < 0.042$) and clinical stage ($\chi^2 = 29.863$; $P < 0.001$; Table 1). Weak MAF1 staining is correlated with poor differentiation and late clinical stages, whereas higher MAF1 expression is correlated with well-differentiated tumors (Table 1). Kaplan-Meier's survival analysis indicates that low MAF1 staining is strongly associated with poor overall survival ($P < 0.001$), with a median survival time of 22 months in the low-MAF1-expression group, compared to 65 months for the high-MAF1-staining group (Fig. 4D). To strengthen independent prognostic significance, we analyzed overall survival using Cox's regression analysis. Multivariate analysis shows that whereas age, sex, clinical stage, and histological differentiation did not have prognostic significance, tumor size and MAF1 staining were independent prognostic factors for overall survival of HCC patients (Supporting Table S1). Consistent with the role of MAF1 in suppressing HCC invasiveness, lower MAF1 expression is correlated with much higher metastasis rate in HCC (Supporting Table S2). During the course of this work, another study found reduced MAF1 expression in three HBV/hepatitis C virus (HCV)-negative HCC tumors.⁽²⁷⁾ Thus, MAF1 down-regulation is a common phenomenon in both HBV-positive and -negative HCCs.

REPRESSION OF RRNA AND TRNA GENES IS NOT SUFFICIENT TO ACCOUNT FOR MAF1'S TUMOR-SUPPRESSOR FUNCTION

Consistent with MAF1's tumor-suppressive function, MAF1 blocks insulin-like growth factor 1 (IGF1), insulin- and serum-induced proliferation, and invasion of liver cancer cells (Fig. 5A,B). Interestingly, MAF1's inhibitory effect under the stimulated conditions is markedly stronger than the steady-state culture condition (Figs. 1 and 2), indicating that MAF1 has a more prominent role in mitogen-stimulated liver cancer cell proliferation and motility. Although mitogen stimulation up-regulates 45S pre-rRNA and pre-tRNA^{Met} genes, surprisingly, MAF1 overexpression does not block growth-factor-induced increase in 45S pre-rRNA and pre-tRNA^{Met} (Fig. 5C). This is in contrast to marked inhibition of 45S pre-rRNA and pre-tRNA^{Met} by MAF1 under serum-starved or steady-state culture conditions (Fig. 5C and Supporting Fig. S4A). Thus, blocking expression of rRNA and tRNA genes is not correlated with inhibition of cell growth by MAF1. Indeed, pharmacological

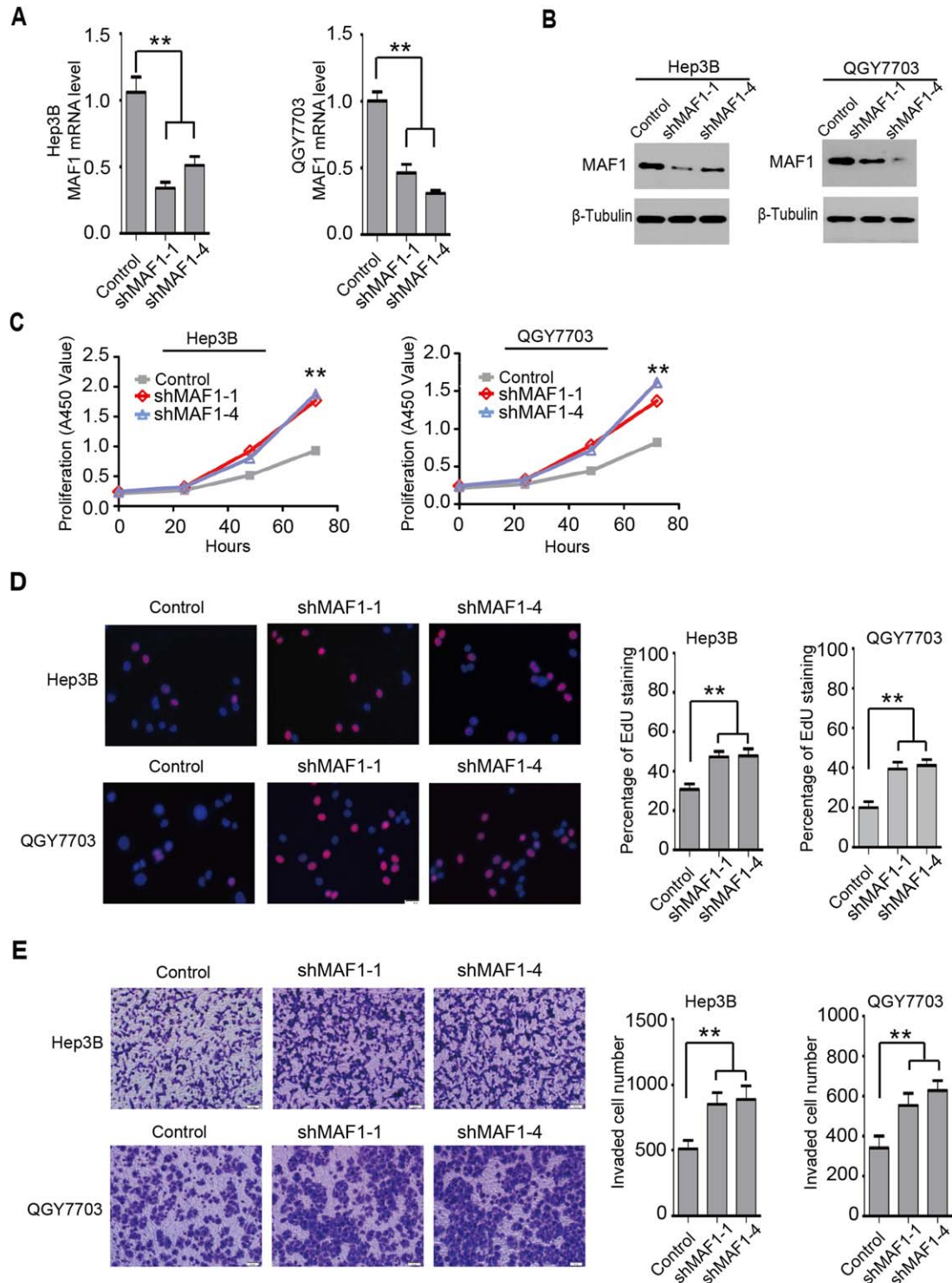


FIG. 3. MAF1 knockdown accelerates proliferation and invasiveness of HCC cells. (A) Hep3B and QGY7703 cells were infected with lentiviral MAF1 shRNA1-1 and shRNA1-4, or a control shRNA. MAF1 mRNA level was measured by reverse-transcription PCR. Data represent mean \pm standard deviation (SD; $n = 3$). (B) MAF1 immunoblotting in Hep3B and QGY7703 cells infected with lentiviral MAF1 shRNA1-1 and shRNA1-4, or a control shRNA. (C) MAF1 knockdown enhances HCC cell proliferation. Proliferation of Hep3B and QGY7703 cells infected with lentiviral MAF1 shRNA1-1 and shRNA1-4, or a control shRNA, was measured by the Cell Counting Kit-8 assay. Data (mean \pm SD; $n = 6$) were analyzed by Student t test; $**P < 0.01$. (D) Same as (C), except EdU assay was used. Data (mean \pm SD; $n = 6$) were analyzed by Student t test; $**P < 0.01$. (E) MAF1 knockdown enhances invasiveness of HCC cells. Hep3B and QGY7703 cells infected with lentiviral MAF1 shRNA1-1 and shRNA1-4, or a control shRNA, were measured for invasiveness by transwell assay. Data (mean \pm SD; $n = 6$) were analyzed by Student t test; $**P < 0.01$.

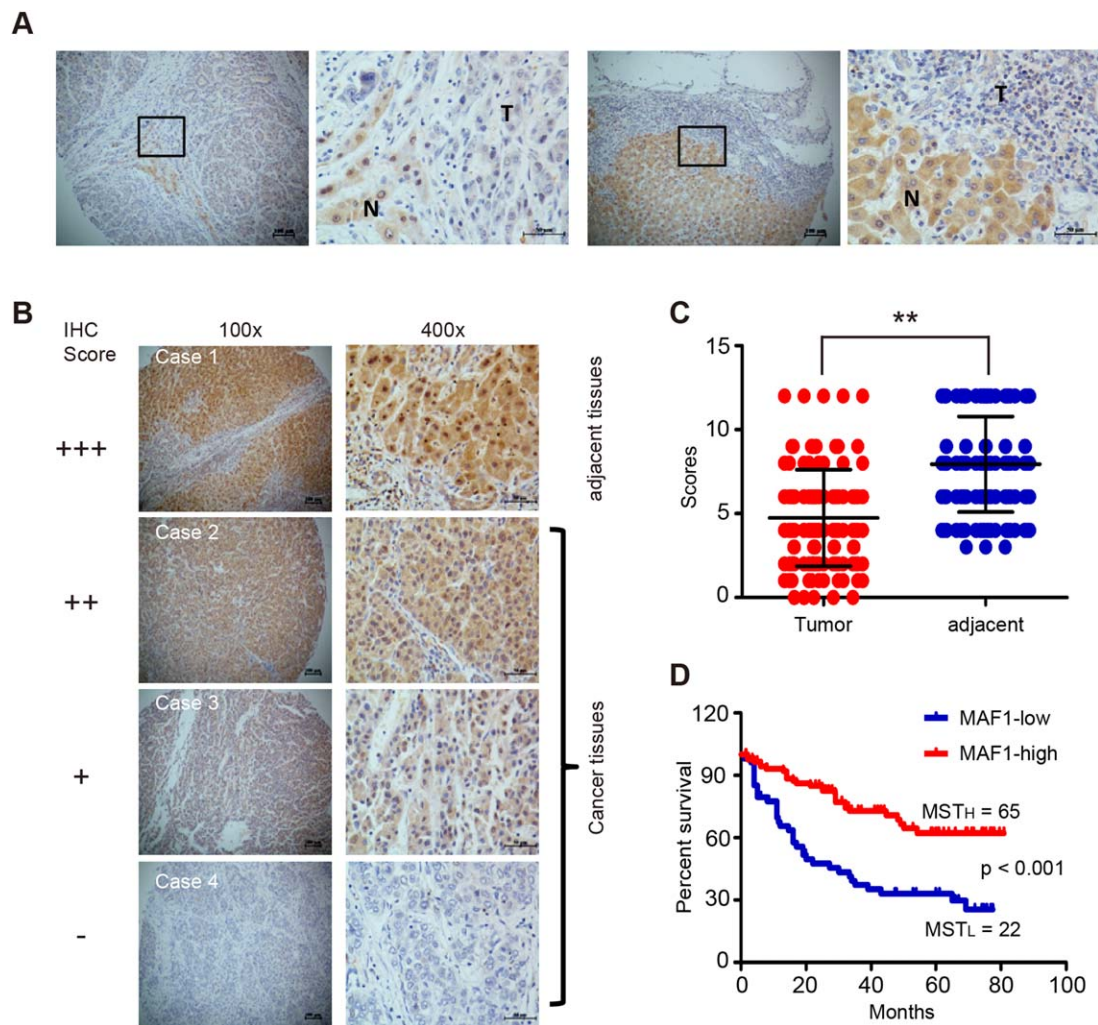


FIG. 4. MAF1 expression is reduced in HCC, which is associated with disease progression and poor survival. (A) Representative IHC staining of MAF1 in human primary HCC and adjacent nontumor liver tissue (magnifications 100 \times and boxed area 400 \times). (B) Scoring of MAF1 staining in human primary HCC tissues with different pathological grade (range: +++, high; ++, moderate; +, weak; -, negative). MAF1 level is higher in well-differentiated (e.g., case 2) than poorly differentiated HCC tissues (e.g., cases 3 and 4). Magnifications: 100 \times and 400 \times . (C) Dot distribution graph of MAF1 IHC staining scores in HCC and adjacent nontumor tissue. Data (mean \pm standard error; n = 146) were analyzed by sample-paired *t* test. (D) Kaplan-Meier's survival analysis of HCC with high (Score \geq ++) and low (Score \leq +) MAF1 IHC staining. Median survival time of high-expression group (MST_H = 65 month) versus median survival time of low-expression group (MST_L = 22 month): n = 146; *P* < 0.0001.

inhibition of Pol I (CX546, Pol I-i) or Pol III (CAS577784-91-9, Pol III-i) completely abrogates expression of rRNA and tRNA, but is insufficient to prevent the increased proliferation that resulted from MAF1 knockdown (Fig. 5D,E and Supporting Fig. S4B). Essentially the same results are obtained with QGY7703, QGY7701, and SMMC7721 cell lines (Supporting Fig. S4C). These observations indicate that MAF1's tumor-suppressive activity is not attributed to derepression of rRNA and tRNA genes.

MAF1 INHIBITS AKT-MTOR SIGNALING IN HCC

MAF1 is a substrate of mTOR, and phosphorylation by mTOR inhibits MAF1's transcriptional repressor activity.⁽¹⁹⁾ Unexpectedly, MAF1 overexpression reduces phosphorylation of AKT and S6 in HCC cells (Fig. 6A and Supporting Fig. S5A-D), indicative of inhibition of AKT-mTOR signaling. MAF1 also inhibits phosphorylation of AKT and S6 in xenograft tumors (Fig. 6B and Supporting Fig. S5E).

TABLE 1. Clinical Characteristics of 146 HCC Samples With Different MAF1 Level

Items	No. of Cases	MAF1 Expression			Mean Rank	Statistic	P Value
		Low	Moderate	High			
Sex						Z = -1.653	0.098
Male	123	43	67	13	75.74		
Female	23	12	10	1	61.54		
Age, years						Z = -1.434	0.152
≤55	90	38	44	8	70.18		
>55	56	17	33	6	78.84		
Differentiation grade						$\chi^2 = 6.318$	0.042
High	19	3	12	4	93.16		
Moderate	60	22	34	4	72.83		
Low	67	30	31	6	68.52		
Clinical stage						$\chi^2 = 29.863$	0.000
I	17	4	10	3	86.50		
II	62	10	43	9	89.96		
III	67	42	23	2	54.57		
Tumor size, diameter						$\chi^2 = 2.683$	0.261
≤2.5 cm	14	5	7	2	76.93		
2.5 < d ≤ 7 cm	78	25	45	8	77.51		
>7 cm	54	25	25	4	66.81		
Survival time, months						Z = -2.063	0.039
<60	107	43	59	5	69.60		
≥60	39	12	18	9	84.19		

Conversely, MAF1 knockdown increases AKT-mTOR signaling (Fig. 6A and [Supporting Fig. S5A-D](#)). Moreover, MAF1 abrogates mitogen-induced increase in AKT and S6 phosphorylation (Fig. 6C). In sharp contrast, pharmacological inhibition of Pol I and Pol III does not interfere with mitogen-stimulated AKT-mTOR signaling (Fig. 6D and [Supporting Fig. S5F](#)). Thus, blockage of AKT-mTOR signaling by MAF1 is not an indirect effect from repression of rRNA and tRNA genes. On the other hand, pharmacological inhibition of AKT (MK2206) and mTOR (rapamycin) only moderately inhibits HCC cell proliferation, but completely abrogates the increased proliferation resulting from MAF1 knockdown (Fig. 6E and [Supporting Fig. S5G](#)). Taken together, these data indicate that MAF1 suppresses HCC proliferation through blocking AKT-mTOR signaling rather than rRNA and tRNA synthesis.

MAF1 INHIBITS AKT-MTOR SIGNALING AND HCC PROLIFERATION THROUGH PTEN

Because MAF1 overexpression inhibits AKT-mTOR signaling, we investigated whether PTEN, a tumor suppressor and negative regulator of the AKT-mTOR pathway, is involved. MAF1 ectopic

expression leads to increased PTEN protein in Hep3B and QGY7703 cells (Fig. 7A). Conversely, MAF1 knockdown causes reduced PTEN protein and mRNA (Fig. 7A). PTEN expression is also enhanced in xenograft tumors expressing MAF1 ([Supporting Fig. S6](#)). Consistently, IHC staining of consecutive sections of 55 primary HCC tumors shows that MAF1 expression is negatively correlated with P-AKT level, but positively correlated with PTEN level ([Supporting Fig. S7](#)). To ask whether altered PTEN expression plays a role in MAF1 suppression of HCC proliferation and invasion, we used small interfering RNA (siRNA) to knock down PTEN in MAF1-overexpressing Hep3B and QGY7703 cells (Fig. 7B). PTEN knockdown restores IGF-induced AKT-mTOR signaling ([Supporting Fig. S8A](#)), as well as proliferation and invasion of MAF1-overexpressing HCC cells (Fig. 7B and [Supporting Figs. S9 and S10](#)). On the other hand, PTEN overexpression blunts the ability of MAF1 shRNA to promote phosphorylation of AKT, mTOR, and S6 ([Supporting Fig. S8B](#)) and HCC cell proliferation ([Supporting Fig. S9](#)). These observations indicate that MAF1 positively regulates PTEN expression, which is important for MAF1 to suppress growth and invasiveness of HCC cells. Of note, PTEN was recently reported to regulate MAF1 protein level through AKT-FOXO1 through an unidentified

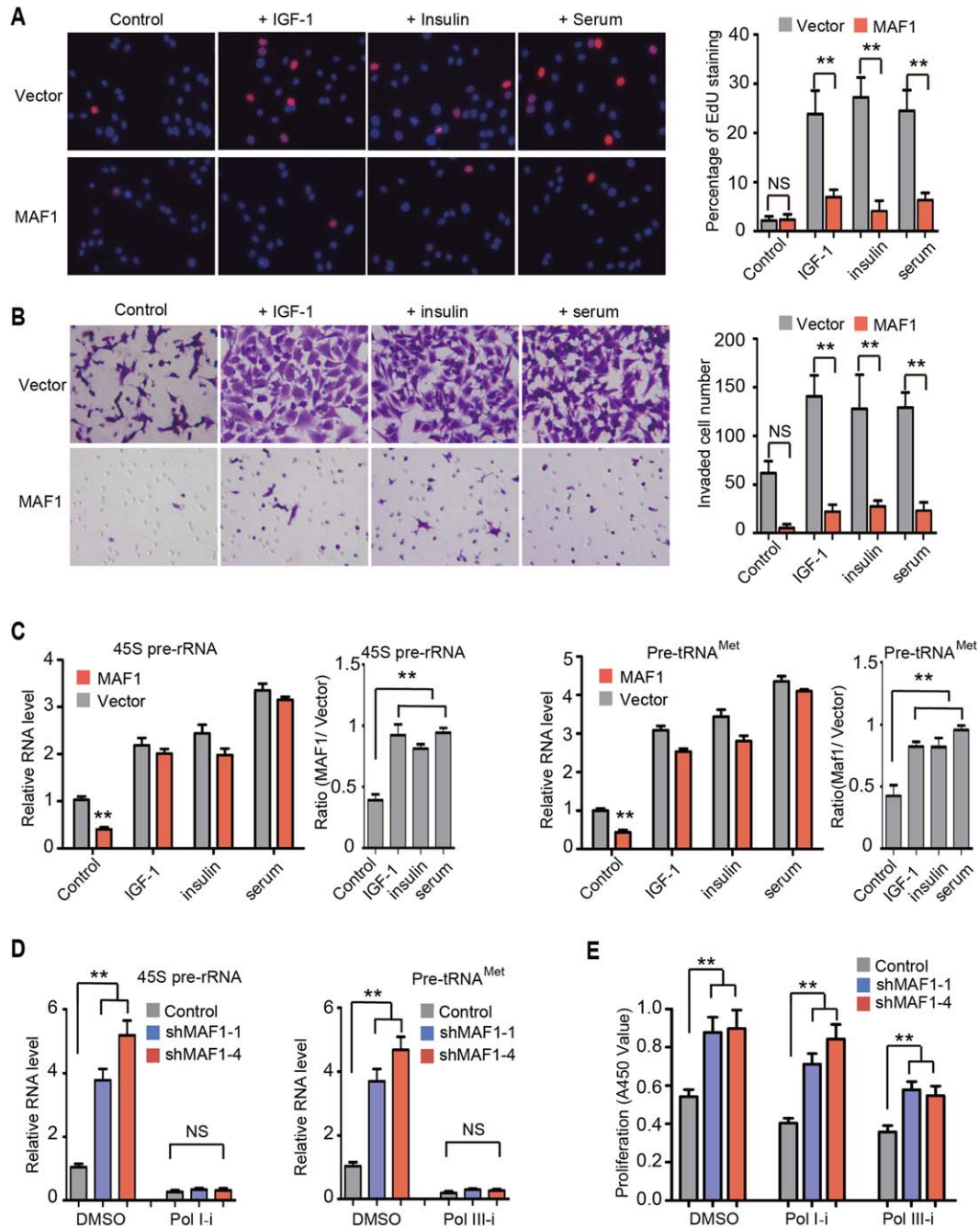


FIG. 5. Inhibition of Pol I and Pol III is insufficient to account for MAF1's tumor-suppressive activity in HCC. (A) MAF1 inhibits growth-factor-induced HCC proliferation. Hep3B cells with or without MAF1 overexpression were serum starved for 24 hours and then stimulated with IGF-1 (100 ng/mL), insulin (100 nM), or 20% serum for 4 hours. Cell proliferation was measured by EdU incorporation. Data (mean \pm SD; $n = 6$) were analyzed by Student t test; $**P < 0.01$. (B) MAF1 inhibits growth-factor-induced invasion of HCC cells. Hep3B cells with or without MAF1 overexpression same as (A), except invasion of HCC cells was measured by transwell assay. Data (mean \pm standard deviation [SD]; $n = 6$) were analyzed by Student t test; $**P < 0.01$. (C) MAF1 does not significantly inhibit growth-factor-induced transcriptional activity of Pol I and Pol III. Hep3B cells with or without MAF1 overexpression were serum starved for 24 hours and then stimulated with IGF-1 (100 ng/mL), insulin (100 nM), or 20% serum for 4 hours. Pre-45S rRNA and pre-tRNA^{Met} transcripts were measured by reverse-transcriptase PCR. The ratio of transcriptional induction is calculated for MAF1/Vector cells. Data represent mean \pm SD ($n = 3$). (D) MAF1 knockdown stimulates synthesis of pre-45S rRNA and pre-tRNA^{Met}. Hep3B cells expressing MAF1 shRNA or a control shRNA were treated with or without 100 nM of Pol I-i (CX546) or 15 μ M of Pol III-i (CAS577784-91-9). Transcripts of pre-45S rRNA (top panel) and pre-tRNA^{Met} (bottom panel) were measured by reverse-transcriptase PCR. The ratio of transcriptional induction calculated for MAF1/Vector cells. Ratios (mean \pm SD; $n = 3$) were analyzed by one-way ANOVA/SNK test; $**P < 0.01$. (E) Stimulation of HCC cell proliferation by MAF1 knockdown is largely unaffected by inhibition of Pol I and Pol III. Hep3B cells expressing MAF1 shRNA or a control shRNA were treated with or without 100 nM of Pol I-i (CX546) or 15 μ M of Pol III-i (CAS577784-91-9). Cell proliferation was measured by the Cell Counting Kit-8 assay. Data represent mean \pm SD ($n = 6$). $**P < 0.01$. Abbreviations: DMSO, dimethyl sulfoxide; NS, not significant.

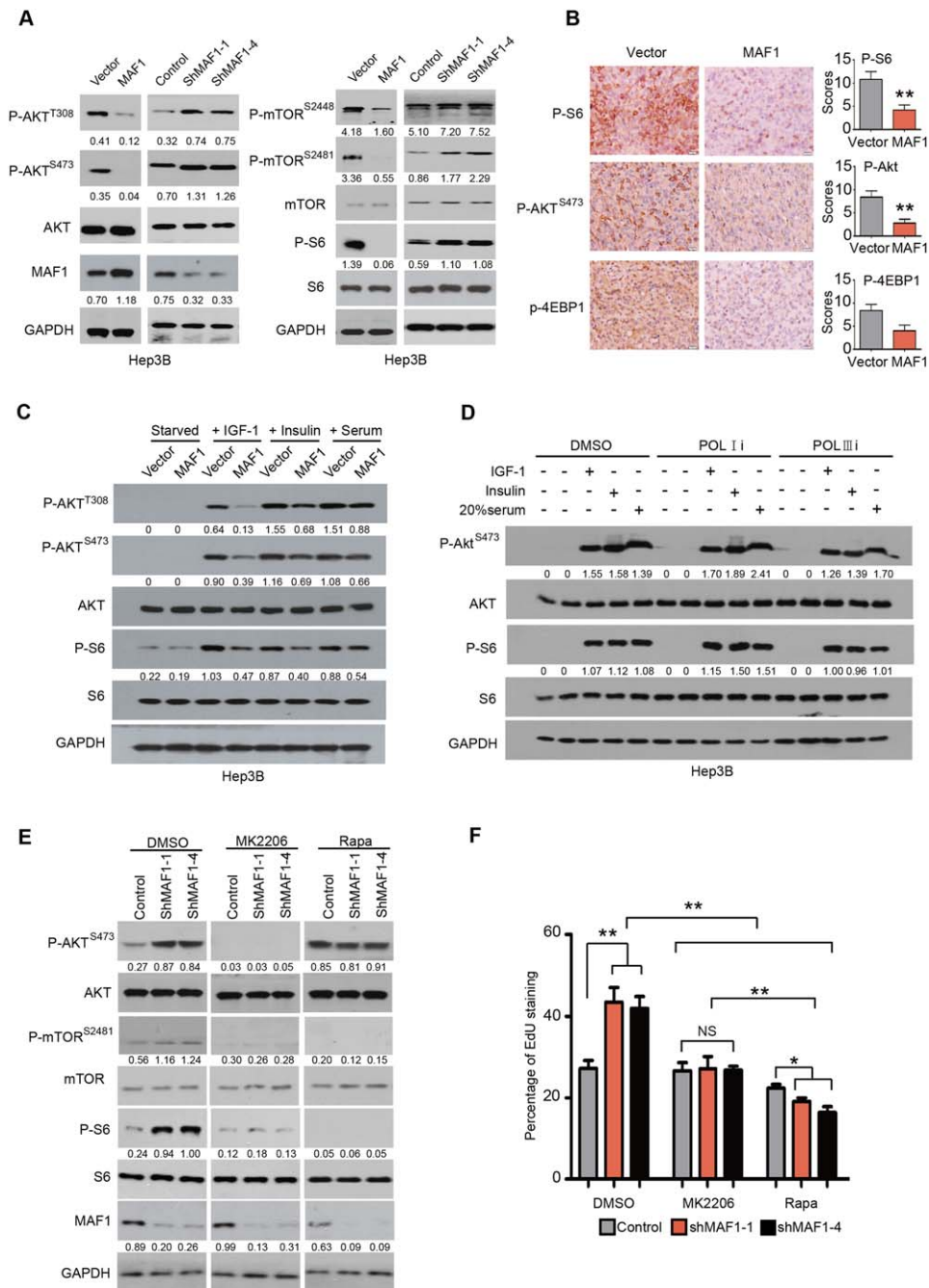
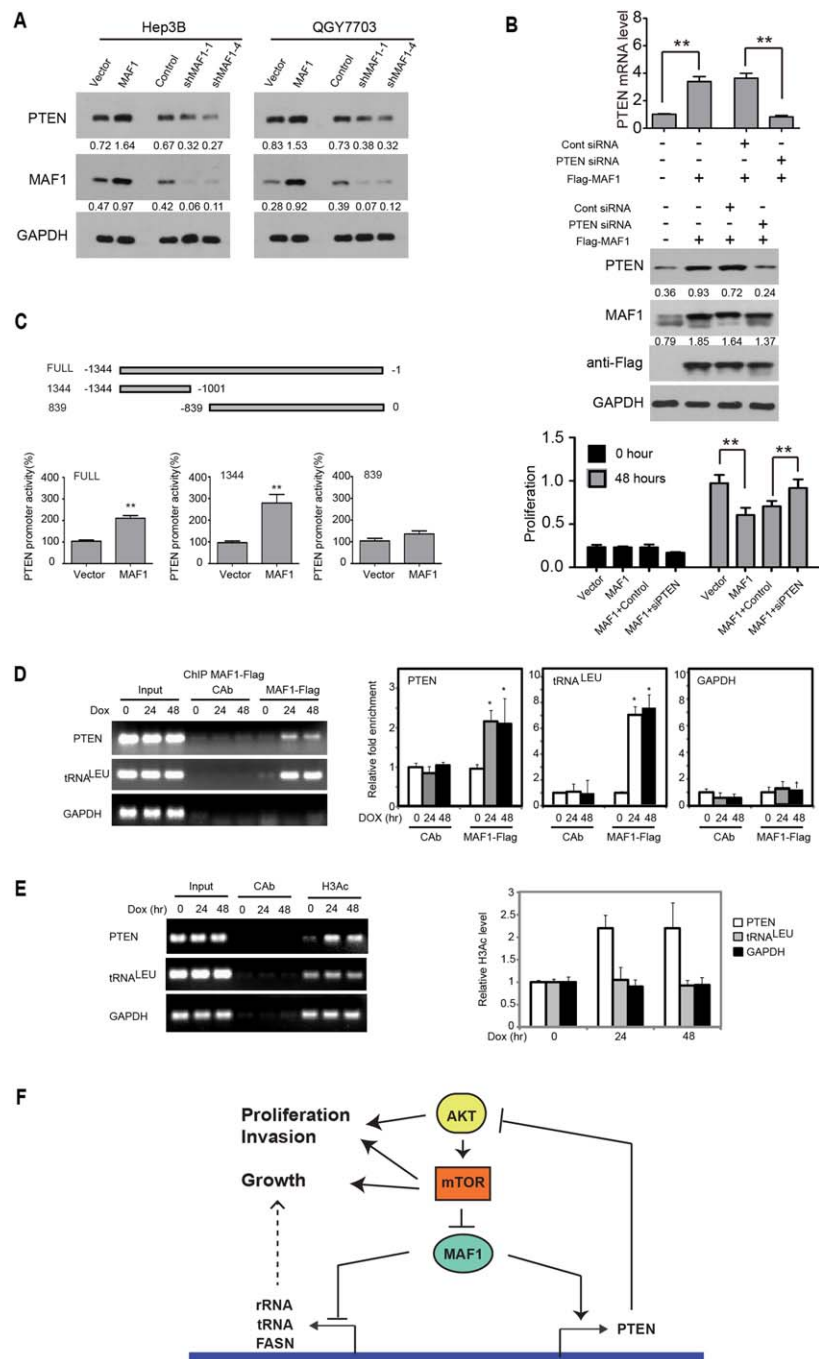


FIG. 6. MAF1 negatively regulates AKT-mTOR signaling in HCC cells. (A) MAF1 inhibits mTOR signaling in liver cancer cells. Hep3B cells expressing ectopic MAF1 or MAF1 shRNAs were analyzed for mTOR signaling by immunoblotting for P-AKT and P-S6. GAPDH was used as a loading control. Numbers show the ratio of phosphor-protein/total protein (arbitrary unit). Result is representative of at three independent experiments. (B) MAF1 inhibits AKT-mTOR signaling in liver xenograft tumors. Xenograft tumors derived from Hep3B cells expressing ectopic MAF1 or carrying a control vector were analyzed for the level of P-AKT and P-S6 by IHC. Left panels show quantification of the IHC staining results. Data represent mean \pm standard deviation (SD; $n = 3$); $**P < 0.01$. (C) MAF1 inhibits the ability of growth factors to stimulate AKT-mTOR signaling. Serum-starved Hep3B cells expressing ectopic MAF1 were stimulated by IGF-1 (100 ng/mL), insulin (100 nM), or 20% serum and analyzed for phosphorylation of AKT and S6. GAPDH was used as a loading control. (D) Inhibition of Pol I and Pol III does not affect growth-factor-induced AKT-mTOR signaling. Serum-starved Hep3B cells expressing ectopic MAF1 were stimulated by IGF-1 (100 ng/mL), insulin (100 nM), or 20% serum in the presence or absence of 100 nM of Pol I-i (CX546) or 15 μ M of Pol III-i (CAS577784-91-9) and analyzed for phosphorylation of AKT and S6. GAPDH was used as a loading control. (E) Pharmacological inhibition of PI3K-AKT-mTOR signaling abrogates MAF1 knockdown-induced activation of AKT-mTOR signaling. Hep3B cells were transfected with MAF1 shRNAs or a control shRNA and then treated with AKT and mTORC1 inhibitors MK2206 (1 μ M) and rapamycin (100 nM), respectively. Their effect on AKT-mTOR signaling was analyzed by immunoblotting. (F) Blockage of PI3K-AKT-mTOR signaling abrogates the growth-stimulatory effect of MAF1 knockdown. Hep3B cells were transfected with MAF1 shRNAs or a control shRNA and then treated with MK2206 (1 μ M) and rapamycin (100 nM). Proliferation of Hep3B cells was measured by the Edu assay. Data represent mean \pm SD ($n = 6$); $**P < 0.01$; NS, not significant. Abbreviations: DMSO, dimethyl sulfoxide; P-mTOR, phosphorylated mTOR; Rapa, rapamycin.

FIG. 7. MAF1 suppresses proliferation and invasiveness of liver cancer cells by stimulating PTEN expression. (A) MAF1 regulates PTEN expression in liver cancer cells. Hep3B and QGY7703 cells expressing ectopic MAF1, or MAF1 shRNAs, were analyzed for PTEN protein expression by immunoblotting. Numbers indicate quantification of relative protein amount. GAPDH served as a loading control. (B) MAF1 overexpression suppresses liver cancer growth in a PTEN-dependent manner. Hep3B cells without or with ectopic MAF1 expression were transfected with PTEN siRNA. Level of PTEN mRNA (upper panel) and protein (middle panel) was measured by reverse-transcriptase PCR and immunoblotting, respectively. Cell proliferation was measured by the Cell Counting Kit-8 assay (lower panel). Data represent mean \pm SD (n = 6); ***P* < 0.01. (C) MAF1 regulates PTEN promoter activity. PTEN luciferase reporter plasmid was cotransfected with a control Renilla plasmid into Hep3B cells carrying a doxycycline-inducible MAF1 or a vector control. Cells were treated with doxycycline for 24 hours and measured for luciferase activity. Upper panel shows luciferase reporter fused with different regions of the PTEN promoter. Lower panel shows the activity of different luciferase reporters in the absence or presence of MAF1 overexpression. Data represent mean \pm SD (n = 3); ***P* < 0.01. (D) MAF1 binds to the PTEN promoter. Expression of MAF1 was induced by doxycycline for different times (hours) in Hep3B cells. Binding of MAF1 to the promoter of PTEN, tRNA^{LEU}, and GAPDH was assayed by ChIP assay. Right panel shows the activity of PCR results. tRNA^{LEU} and GAPDH genes are used as positive and negative control, respectively. Data represent mean \pm SD (n = 3); ***P* < 0.01. (E) MAF1 induces histone acetylation of the PTEN promoter. MAF1 expression was induced by doxycycline for different times as indicated. Acetylation of PTEN, tRNA^{LEU}, and GAPDH promoters was assayed by ChIP with a pan-acetylated histone antibody. A control antibody (CAb) was used as a negative control. Data represent mean \pm SD (n = 3); ***P* < 0.01. (F) A model shows the mechanism of MAF1 in feed-forward regulation of the PI3K-PTEN-AKT-mTOR pathway in tumor suppression. Abbreviation: Cont, control.



posttranscriptional mechanism.⁽²⁷⁾ However, down-regulation of PTEN only slightly decreases the overall MAF1 protein level in this system (Fig. 7B), indicating that MAF1-dependent regulation of PTEN is a major event here.

MAF1 REGULATES PTEN TRANSCRIPTION BY ENHANCING ACETYLATION OF PTEN PROMOTER

To ask whether MAF1 regulates PTEN through transcription, we analyzed expression of luciferase under the control of the PTEN promoter, and found that ectopic MAF1 expression stimulates the activity

of the full length PTEN promoter (−1,344 to −1 base pair [bp]; Fig. 7C). Further analysis reveals that the MAF1-responsive region resides within −1,344 to −1,001 bp, but not −839 to 0 bp, of the PTEN promoter (Fig. 7C). MAF1 was found to be associated with the PETN promoter, as judged by ChIP assay (Fig. 7D). In the same experiment, MAF1 was also detected at the tRNA^{leu} promoter, but not the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) promoter (Fig. 7D), demonstrating the specificity of the ChIP assay. Histone acetylation is known to promote gene expression.⁽³⁸⁾ ChIP analysis, using a pan-histone H3 antibody, showed that ectopic MAF1 expression causes increased histone H3 acetylation at the PTEN promoter, but not at the tRNA^{leu} and GAPDH promoters (Fig. 7E). Together, our results show that, in contrast to its previously known function as a transcriptional repressor, MAF1 can act as a transcriptional activator to enhance acetylation and activity of the PTEN promoter.

Discussion

PI3K-AKT-mTOR is frequently activated, but mutations in PIK3CA and PTEN are rare in HCC.⁽³¹⁾ This study shows how MAF1 expression is frequently down-regulated in HBV-positive HCC, which is associated with disease progression, metastasis, and poor prognosis. Reduced MAF1 expression was also observed in 3 cases of HBV-/HCV-negative liver tumors.⁽²⁷⁾ Thus, MAF1 down-regulation appears to be a common event among different subtypes of HCC. Of note, in a panel of immortalized hepatocyte (LO2) and liver cancer cell lines (QGY7701, QGY7703, SMMC7721, and Hep3B), basal MAF1 expression level of SMMC7721 was similar to that of LO2 whereas MAF1 level was significantly lower in the other three HCC cell lines (Supporting Fig. S11). Interestingly, SMMC7721 shows much lower tumorigenicity than QGY7701, QGY7703, and Hep3B (Fig. 2A,B and Supporting Fig. S2). Consistently, MAF1 down-regulation activates AKT-mTOR signaling and enhances liver cancer cell growth, proliferation, and invasion *in vitro* and xenograft tumors, indicating that inactivation of MAF1 provides oncogenic advantage and promotes hepatocarcinogenesis.

Driven by Pol I and Pol III, ribosome biogenesis generates ribosomes to support cell growth and proliferation, a process using an estimated 90% total tran-

scriptional activity.⁽¹⁰⁾ Elevated synthesis of ribosomal and transfer RNAs is a hallmark of malignant growth.^(18,39) Thus, the antiproliferative activity of MAF1 has been attributed to its transcriptional suppression of ribosome biogenesis. Surprisingly, MAF1 suppresses mitogen-induced proliferation without significantly affecting serum-stimulated synthesis of ribosomal and transfer RNAs. Moreover, pharmacological inhibition of Pol I and Pol III only slightly affected the increase in HCC cell proliferation attributed to MAF1 knockdown. Together, our studies suggest that blockage of AKT-mTOR signaling is the main contributor to MAF1's tumor-suppressor activity (Fig. 7F).

MAF1 is well known as a general transcriptional repressor,⁽¹⁹⁾ with direct target genes including tRNA and 5S rRNA genes, gluconeogenic genes, TBP, and lipogenic genes (e.g., fatty acid synthase [FASN] and acetyl-CoA [coenzyme A] carboxylase 1 [ACC1]) and indirect target 45S rRNA through TBP. Here, we show that MAF1 can also act as a transcriptional activator, which is distinguished from its canonical repressor function. In this role, MAF1 enhances histone acetylation at the PTEN promoter, a chromatin modification that increases promoter activity. Thus, MAF1 can act as a versatile transcription factor to both positively and negatively regulate gene expression, depending on specific target genes and signaling inputs. It would be of considerable interest to further understand the mechanisms for transcriptional activation by MAF1.

PTEN regulates MAF1 by inhibiting mTOR kinase, preventing MAF1 phosphorylation. In addition, PTEN has also been shown to regulate MAF1 protein level through FOXO1 in an unknown post-transcriptional mechanism.⁽²⁷⁾ Down-regulation of PTEN only slightly decreases the overall MAF1 protein level in Hep3B cells (Fig. 7B and Supporting Fig. S12C), suggesting that posttranslational regulation of MAF1 by PTEN is cell-type dependent. PTEN knockdown enhances expression of lipogenic genes (FASN, ACC1, and stearoyl-CoA desaturase 1) in the presence or absence of MAF1 overexpression. Conversely, PTEN overexpression decreases lipogenic genes in the absence or presence of MAF1 knockdown (Supporting Fig. S11). Together, these results suggest that PTEN regulates expression of lipogenic genes through MAF1-dependent and -independent mechanisms. Thus, in addition to directly acting on lipogenic

genes, MAF1 also inhibits lipogenic genes, in part, through PTEN.

PI3K-AKT-mTOR is a major mitogen-signaling pathway that regulates diverse cellular processes related to growth, proliferation, survival, and motility, which is negatively regulated by PTEN, a commonly mutated tumor suppressor in human cancer. When phosphorylated by mTORC1, MAF1 is relieved from repressing ribosome biogenesis. Paradoxically, this transcriptional repressor function does not appear to play a major role in liver cancer suppression. Instead, MAF1 inhibits cancer proliferation mainly by suppressing AKT-mTOR signaling through activation of PTEN transcription. MAF1 and AKT-mTOR regulate each other, creating a feed-forward mechanism that magnifies both positive and negative signals (Fig. 7F). This enables robust mitogenic regulation of this key growth-regulatory pathway.

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