The Critical Role of Dysregulated FOXM1–PLAUR Signaling in Human Colon Cancer Progression and Metastasis

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Abstract

Purpose: The mammalian Forkhead Box (Fox) transcription factor FOXM1 is implicated in tumorigenesis including mouse intestinal cancer. However, the clinical significance of FOXM1 signaling in human colorectal cancer pathogenesis remains unknown.

Experimental Design: We investigated FOXM1 expression in 203 cases of primary colon cancer and matched normal colon tissue specimens and explored the underlying mechanisms of altered FOXM1 expression and the impact of this altered expression on colon cancer growth and metastasis using in vitro and animal models of colon cancer.

Results: We found weak expression of FOXM1 protein in the colon mucosa, whereas we observed strong FOXM1 expression in tumor-cell nuclei of colon cancer and lymph node metastases. A Cox proportional hazards model revealed that FOXM1 expression was an independent prognostic factor in multivariate analysis. Experimentally, overexpression of FOXM1 by gene transfer significantly promoted the growth and metastasis of colon cancer cells in orthotopic mouse models, whereas knockdown of FOXM1 expression by siRNA did the opposite. Promotion of colon tumorigenesis by FOXM1 directly and significantly correlated with activation of urokinase-type plasminogen activator receptor (PLAUR) expression and elevation of invasion and metastasis.

Conclusions: Given the importance of FOXM1 in regulation of the expression of genes key to cancer biology, dysregulated expression and activation of FOXM1 may play important roles in colon cancer progression and metastasis. Clin Cancer Res; 19(1); 62–72. ©2012 AACR.

Introduction

Colorectal cancer is one of the most common malignancies worldwide and a prevalent cause of morbidity and mortality (1). The age-adjusted incidence rate was 46.3 per 100,000 per year worldwide, with only 12% 5-year survival rate for advanced stage of colorectal cancer in the latest statistics (2). Approximately 20% of the patients have distant metastases at the time of presentation (2). Approximately 50% to 60% of patients diagnosed with colorectal cancer will develop colorectal metastases, and 80% to 90% of these have unresectable metastatic liver disease (3). Recently, KRAS and BRAF mutation status has been used to predict the response of treatment and prognosis of colorectal cancer (4). Also, DNA mismatch repair status is an independent prognostic biomarker for disease-free survival in patients with colon cancer receiving adjuvant chemotherapy (5). Identification of molecular markers associated with the progression of this disease and understanding the key pathogenic processes involved remain crucial for designing novel and effective therapeutic strategies.

Invasion and metastasis has been shown to be one of the most important hallmarks of cancer (6). Extracellular proteolysis is crucial for tumor cell invasion and metastasis, not only because of its ability to degrade the extracellular matrix (ECM) surrounding tumor cells, but also because of its impact on cell proliferation, cell adhesion and migration, and angiogenesis (7, 8). One important part of the pericellular network of interacting proteolytic systems is the urokinase-type plasminogen activator (PLAUI) system consisting of the serine protease PLAUI, its receptor PLAUR receptor (PLAUR), and its inhibitor PLAUI inhibitor (PAI-1). PLAUR...
FOXM1 Regulates Colon Cancer Progression and Metastasis

Translational Relevance

We have used colon cancer tissue microarray and molecular biology and animal models to evaluate the activation and function of FOXM1/PLAUR pathway in human colon cancer. Our clinical and mechanistic findings indicate that urokinase-type plasminogen activator receptor (PLAUR) is a direct transcriptional target of FOXM1 and that frequently dysregulated FOXM1 overexpression leads to aberrant PLAUR expression. Moreover, FOXM1 positively regulates colon cancer cell migration, invasion, and growth, suggesting a novel molecular basis for the critical role of FOXM1 activation in colon cancer development and progression and the deregulated FOXM1/PLAUR signaling could be a promising new molecular target for designing novel preventive/therapeutic strategies to control this malignancy. Therefore, our findings may have a significant effect on clinical management of patients with colorectal cancer.

(CD87) is a glycosylated, glycan lipid-anchored membrane protein that consists of 3 structurally homologous domains DI, DII, and DIII (9). PLAUR binds and activates PLAU, which is then able to convert plasminogen to active plasmin, which in turn is able to degrade components of the ECM, thus facilitating invasion and metastasis (10). In addition, PLAUR has important signaling properties (via interactions with membrane-bound integrins) that are capable of affecting malignant phenotypes such as cell migration and proliferation, including intracellular cascades such as MEK/ERK (extracellular signal-regulated protein kinase), PI3K/Akt (phosphoinositide 3-kinase-serine and threonine kinase), and FAK signaling pathways (11–13). Furthermore, PLAUR has been proven to be prognostic markers in several types of cancer and a potential role in therapeutic implications (14, 15). However, the underlying mechanism of transactivation of PLAUR remains to be elucidated in colorectal cancer.

We previously showed that the mammalian Forkhead Box (Fox) transcription factor FOXM1, play a critical role in the carcinogenesis and metastasis of several malignancies including gastric cancer, pancreatic cancer, and glioma (16–21). FOXM1 previously known as HFH-11B, Trident, WIN, or MPP2, is a member of the Fox transcription factor family that share homology within the winged-helix/Forkhead DNA-binding domain, which involved in the regulation of organism development, cell proliferation, and differentiation (22, 23). FOXM1 is considered to be a master regulator of both G1–S and G2–M phases of the cell-cycle and mitotic-spindle integrity (24, 25). It has been noted that FOXM1 is abundantly expressed in a wide range of human cancers, suggesting that targeting FOXM1 could be a therapeutic strategy against human malignancies (26–37). FOXM1 has been implicated in the DNA-damage response pathway, for example DNA repair genes, XRCC1 and BRCA2 were identified as direct transcriptional targets of FOXM1 (38). We also found that abnormal activation of FOXM1 in human glioma leads to overexpression of multiple angiogenic genes downstream of FOXM1, such as matrix metalloproteinase-2 (MMP-2; ref. 17). We discovered that FOXM1 directly bound to the promoter region of Cav-1 gene and positively transactivated its activity, promoting pancreatic cancer epithelial–mesenchymal transition (EMT), invasion, and metastasis (18). Therefore, abnormal activation of FOXM1 causes overexpression of multiple proinvasion and -metastasis molecules, which in turn render tumor cells highly aggressive.

However, the precise role and underlying signaling cascade of FOXM1 involved in colorectal cancer progression and metastasis remain unclear. In the present study, we sought to determine the role of FOXM1 expression in colon cancer pathogenesis and the underlying molecular mechanisms. We discovered a novel FOXM1–PLAUR signaling pathway, which is altered in colorectal cancer and critically regulates colon cancer progression and metastasis.

Materials and Methods

**Human tissue specimens and TMA construction**

The tissue microarray (TMA) construction and immunohistochemical analysis was conducted as described previously (39). Briefly, the primary colon cancer in these patients was diagnosed (and later confirmed by at least 2 pathologists) and the patients were accepted for colectomy at Affiliated First People’s Hospital from 2001 to 2003. The 203 formalin-fixed, paraffin-embedded specimens were selected to represent all of the stages and histologic types of colon cancer. Tumor staging for the specimens was carried out according to the American Joint Committee on Cancer staging criteria (40). The patients’ disease-free survival and overall survival (OS) durations were defined as the interval from initial surgery to clinically or radiologically proven recurrence or metastasis and from initial surgery to death, respectively. The follow-up period for this analysis concluded on June 29, 2008. The use of human specimens was approved by proper Institutional Review Boards.

**Immunohistochemical analysis**

Sections (4–5 μm) of paraffin-embedded colon cancer specimens were prepared and standard immunohistochemical procedures were carried out using a polyclonal anti-FOXM1 antibody (Santa Cruz Biotechnology; 1:200 dilution) and a polyclonal anti-PLAUR antibody (American Diagnostic Inc.; 1:100 dilution). Similar tissue sections immunostained with normal immunoglobulin G (IgG) were used as negative controls. The staining results were scored by 2 pathologists blinded to the clinical data as described previously (39).

**Cell lines and culture conditions**

The human colon cancer cell lines SW480 and SW620 were purchased from the American Type Culture Collection. All cells were maintained at 37°C in 5% CO2 in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS.

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Western blot analysis

Whole-cell lysates were prepared from the colon cells as described previously (39). Standard Western blot analysis of the lysates was conducted with an antibody against FOXM1 (Santa Cruz Biotechnology) or against PLAUR (American Diagnostic Inc.) and a second anti-IgG antibody (Amersham Life Sciences). The membranes were then stripped and blotted with an anti-β-actin antibody (Sigma Chemical Co.) and used as loading controls. The probe proteins were detected using an enhanced chemiluminescence system (Amersham Life Sciences) according to the manufacturer’s instructions.

Transient transfection of colon cancer cells

To generate the pcDNA3.1-FOXM1 plasmid, full-length human FOXM1 was subcloned into the pcDNA3.1 (Invitrogen), which was released by EcoRI and XhoII digestion of the cytomegalovirus human FOXM1 cDNA. In addition, 4 FOXM1-siRNAs were designed and synthesized by Qiagen to generate an effective FOXM1-siRNA oligonucleotides for gene knockdown studies. A siRNA with the sequence CUCUUCUCCCUCAGAUAUAdTdT was determined to be the most effective siRNA in inhibiting FOXM1 expression. SW480 cells were transfected with pcDNA3.1-FOXM1 or control vector with the use of Lipofectamine 2000 Transfection Reagent (Invitrogen) according to the manufacturer’s instructions.

Animals

Female athymic nude mice were purchased from The Jackson Laboratory. The mice were housed in laminar flow cabinets under specific pathogen-free conditions and used when they were 8-weeks old. The animals were maintained in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care International in accordance with the current regulations and standards of the U.S. Department of Agriculture, Department of Health and Human Services, and NIH.

Tumor growth and metastasis

To prepare tumor cells for inoculation into the mice, cells in the exponential growth phase were harvested by brief exposure to a 0.25% trypsin/0.02% EDTA solution (w/v). Cell viability was determined using Trypan blue exclusion, and only single-cell suspensions that were more than 95% viable were used. Tumor cells (1 × 10^6 per mouse) were then injected into the subcutis or cecum wall of nude mice in groups of 5. The animals were killed 60 days after the tumor-cell injection or when they had become moribund. Next, the resulting primary colon tumors were weighed. Also, each mouse’s liver was removed and fixed in Bouin’s solution for 24 hours to differentiate neoplastic lesions from the organ parenchyma; liver metastases were determined (double-blinded) as described previously (16).

Analysis of PLAUR promoter activity

The activity of full-length pPLAUR1103, pPLAUR627, and mutant pPLAUR627 promoter constructs was analyzed as described previously (17). To examine the transcriptional regulation of the PLAUR promoter by FOXM1, SW480, and SW620 cells were seeded to approximately ~80% confluence in 6-well plates (in triplicate) and transiently transfected with full-length or minimum PLAUR reporter plasmid and pcDNA3.1-FOXM1 plasmid or FOXM1-siRNAs as indicated in each experiment by using Lipofectamine 2000 Transfection Reagent (Invitrogen) according to the manufacturer’s instructions. The cells were then washed with PBS and harvested in Passive Lysis Buffer (Promega Corp.) 24 hours after transfection. Quantitation of firefly and Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega Corp.). The PLAUR promoter activity was normalized by cotransfection with a Renilla-actin luciferase reporter containing a full-length Renilla luciferase gene (39).

Statistical analysis

The two-tailed χ² test was used to determine the significance of the difference among the covariates. Survival durations were calculated using the Kaplan–Meier method. The log-rank test was used to compare the cumulative survival rates in the patient groups. A Cox proportional hazards model was used to calculate univariate and multivariate HRs for the study variables. The FOXM1 expression level, patient age, disease stage (American Joint Committee on Cancer system), tumor differentiation, and distant metastasis were included in the model. The significance of the in vitro data was determined using the Student t test (two-tailed). In all of the tests, P values less than 0.05 were considered statistically significant. The SPSS software program (version 12.0; SPSS Inc.) was used for statistical analyses.

Results

FOXM1 overexpression and its direct association with poor prognosis in patients with colon cancer

To determine the effect of FOXM1 expression on colon-cancer progression and metastasis, we first determined the expression of FOXM1 protein in the 203 primary colon tumor and paired adjacent normal colon mucosa specimens as well as the 66 lymph node metastasis specimens in a TMA. We observed FOXM1-positive staining in the cytoplasm and nuclei of the cancer cells with FOXM1-negative or weak FOXM1-positive staining in adjacent normal colon mucosa (Supplementary Fig. S1A). Interestingly, immunostaining results showed that metastatic lymph nodes had significantly higher levels of FOXM1 expression than did primary cancerous tissue in 66 paired cases (Fig. 1A and Supplementary Table S1). These results indicated that FOXM1 is commonly overexpressed in human colon cancer, particularly in metastases.

We further analyzed the relationship between clinicopathologic features and FOXM1 expression levels in colon cancer cases. Our study revealed that increasing
FOX1 expression correlated with disease stage ($P < 0.001$), pT classification ($P < 0.001$), regional lymph node metastasis ($P < 0.001$), distant metastasis ($P = 0.001$), and vessel invasion ($P = 0.014$; Supplementary Table S2). These findings strongly indicated that FOX1 expression plays a critical role in colon cancer development and progression and is a valuable biomarker for this disease.

To assess the clinical significance of FOX1 overexpression in colon cancer, we analyzed the relationship between the level of FOX1 expression and patient survival. The 5-year OS was significantly lower in patients with FOX1-positive tumors than in those with FOX1-negative tumors [49.6% vs. 85.9%; HR 3.62; 95% confidence interval (CI), 1.86–9.24; $P = 0.001$; Fig. 1B, right; Supplementary Table S3]. FOX1 expression was an independent marker for both OS and metastasis-free survival (MFS; Supplementary Table S4). Thus, these results strongly indicate that FOX1 expression has a direct association with metastasis diseases of patients with colon cancer.

**PLAUR overexpression and its direct association with poor prognosis in patients with colon cancer**

We further evaluated PLAUR expression in the same cohort of TMA specimens. We observed PLAUR-positive staining in the cytoplasm of the cancer cells (Supplementary Fig. S1B). It showed an increasingly positive staining activity of PLAUR between normal colon mucosa, primary cancer tissue, and lymph node metastasis (Fig. 1C and Supplementary Table S5). PLAUR overexpression correlated with disease stage ($P < 0.001$), pT classification ($P = 0.001$), regional lymph node metastasis ($P < 0.001$), distant metastasis ($P < 0.001$), and is a valuable biomarker for this disease.
metastasis ($P = 0.001$), and vessel invasion ($P = 0.005$; Supplementary Table S6).

Positive PLAUR expression was inversely associated with OS and MFS ($P < 0.001$; Fig. 1D) and was an independent predictor of poor OS ($P = 0.038$; Supplemental Table S7). Strikingly, positive PLAUR expression in primary colon cancer is associated with distant metastasis after colectomy (Supplementary Table S8).

Close association of FOXM1 expression with PLAUR expression in human colon cancer

Given that both FOXM1 and PLAUR are predictive of poor patient survival and closely related to invasive and metastatic diseases of patients with colon cancer, we sought to evaluate the relationship between FOXM1 expression and PLAUR expression in the primary colon cancer specimens. We observed that FOXM1 expression was significantly correlated with PLAUR expression by analyzing consecutive primary colon cancer sections (Fig. 2A and Supplementary Table S9). We confirmed these findings by analyzing FOXM1 and PLAUR expression in 4 cases of colon cancer specimens. FOXM1 expression pattern was consistent with the PLAUR expression pattern (Fig. 2B and C). These data provided clinical evidence supporting our hypothesis that aberrant activation of FOXM1 is associated with PLAUR expression and increased colon cancer invasion and metastasis.

Altered FOXM1 expression affected colon cancer cell migration and invasion in vitro

To assess the functional role of FOXM1 on migration and invasion of colon cancer cells, FOXM1-transfected and
FOXM1-siRNA–transfected colon cancer cells were wounded by scratching and maintained at 37°C for 12 hours. Forced FOXM1 expression strongly promoted the flattening and spreading of SW480 cells (Supplementary Fig. S2A), whereas downregulation of FOXM1 attenuated the flattening and spreading of SW620 cells (Supplementary Fig. S2B). This result was also confirmed by migration and invasion assay, we found that the levels of both migration and invasion of FOXM1–transfected SW480 cells were significantly higher than those of control cells (Fig. 3A), whereas the migratory and invasive ability were attenuated in FOXM1-siRNA–transfected SW620 cells (Fig. 3B).

**Direct impact of altered FOXM1 expression on the tumorigenicity and metastasis of human colon cancer cells in vivo**

To determine whether FOXM1 plays an important role in the tumorigenicity and metastasis of colon cancer cells, we injected FOXM1–transfected SW480 cells into the subcutis or ileocolic vein of nude mice in groups of 5. Consistent with the effect of altered FOXM1 expression on migration and invasion of colon cancer cells in vitro, overexpression of FOXM1 significantly promoted tumor growth (Fig. 4A and B). Conversely, downregulation of FOXM1 notably inhibited tumor growth (Fig. 4C and D). Moreover, in the orthotopic models, overexpression of FOXM1 significantly increased liver metastasis of SW480 cells (Fig. 5A and B), whereas knockdown of FOXM1 abrogated liver metastasis of SW620 cells (Fig. 5C and D). These data suggested that FOXM1 promotes tumorigenicity and metastasis of colorectal cancer.

**Transcriptional activation of PLAUR expression in colon cancer cells by FOXM1**

Our study has shown a direct correlation between FOXM1 and PLAUR expression in colon cancer cell lines (Fig. 6A, left). To further explore the molecular mechanisms of regulation of PLAUR expression by FOXM1, we first examined the effects of altered FOXM1 expression on PLAUR expression in SW480 cells and SW620 cells. Restored FOXM1 expression significantly upregulated PLAUR expression in SW480 cells, whereas silencing FOXM1 expression markedly downregulated PLAUR expression in SW620 cells (Fig. 6A, right and B). Furthermore, to determine the role of FOXM1 in regulation of PLAUR transcription, we generate 2 PLAUR promoters (pPLAUR1103 and pPLAUR627), which have the FOXM1 potential binding site #2 (Fig. 6C, left). FOXM1 clearly bound the site #2 in SW620 cells as determined by the Chromatin immunoprecipitation (ChIP) assay (Fig. 6C, right). Transfection of FOXM1 in SW480 cells significantly increased the PLAUR promoter activity (pPLAUR1103), whereas mutation of PLAUR promoter (pPLAUR627M) significantly decreased this activity (Fig. 6D, left). Conversely, knockdown FOXM1 expression by transfected FOXM1-siRNA in SW620 cells suppressed the PLAUR promoter activity (pPLAUR1103), also mutation of PLAUR promoter (pPLAUR627M) significantly decreased the PLAUR promoter activity comparing with the wild-type of PLAUR promoter (pPLAUR627; Fig. 6D, right). These data strongly suggested that FOXM1 regulates PLAUR expression most likely at the transcriptional level, and the FOXM1-binding site was site #2 in the PLAUR promoter. To confirm this result, we added FOXM1 inhibitor, siomycin A, into SW480 and SW620 cells. Siomycin A inhibited both the expression of FOXM1 and PLAUR (Supplementary Fig. S3A). Next, we carried out a ChIP assay and luciferase to determine whether siomycin A can affect the regulation of PLAUR by FOXM1. The result showed that siomycin A significantly inhibited the binding affinity between FOXM1 and PLAUR (Supplementary Fig. S3B), also the activity of PLAUR promoter was decreased remarkably in SW480 and SW620 cells (Supplementary Fig. S3C and S3D). Collectively, these finding showed that FOXM1 positively regulates PLAUR transcription by binding at PLAUR promoter.
Discussion

In this study, we discovered 4 lines of evidence supporting a critical role for FOXM1–PLAUR signaling in colon cancer pathogenesis. First, we observed an elevated FOXM1 expression and concomitant PLAUR overexpression, which directly correlated with colon tumor progression and metastasis. Both FOXM1- and PLAUR-positive staining of colon cancer cells could be used to identify a greatly increased risk of metastasis in patients after colectomy, which might serve as valuable prognosis markers for colon cancer. Second, overexpression of FOXM1 enhanced the tumorigenicity and metastasis of human colon cancer cells in animal models, whereas reduced expression of FOXM1 did the opposite, indicating that targeting of FOXM1 could constitute a potential therapeutic strategy for colon cancer. Third, genetically enforced FOXM1 overexpression led to increased PLAUR expression in and metastatic potential of human colon cancer cells, whereas knockdown of FOXM1 expression did the opposite. Fourth, FOXM1 directly regulated the expression of the PLAUR gene at the transcriptional level via binding to the PLAUR promoter. Therefore, abnormal expression of FOXM1 during the initiation and development of colon tumors contributed to abnormal PLAUR expression and activation. This novel FOXM1–PLAUR signaling critically contributes to colon tumor metastatic and aggressive colon cancer biology.

It has been reported that nearly 50% of the patients with colon cancer show metastasis after curative colectomy, which is the major cause of death in patients with colon cancer (3). Studies of prognosis for patients with colon cancer and of prognostic factors to predict the risk of metastasis for individual patients with colon cancer are intriguing and could affect clinical practice. MSI and BRAF status are of predictive significance for response of therapy and survival as showed by Funkhouser and colleagues (41). Recently, it is very provocative to show that the frequencies of CIMP-high, MSI-high, and BRAF mutations in cancer increased gradually along colorectum subsites from the rectum to ascending colon. Those novel data challenge the common conception of discrete molecular features of proximal versus distal colorectal cancers, which might be used for optimizing therapeutic strategies in future (42, 43). In the present study, we found a lowest positive expression of both FOXM1 and PLAUR in sigmoid colon cancer, whereas it did not reach a significant difference due to the sample size that was not large enough. It will also be very interesting to further study our investigation on the correlation of both MSI and BRAF status with FOXM1 and PLAUR expression.

Our previous evidence has shown that Fox transcription factor FOXM1 may serve as a novel prognostic biomarker involved in the pathogenesis and metastasis of several malignancies. However, the potential role of FOXM1 involved in the colon cancer still remains unclear. In the present study, we found direct clinical evidence of a strong correlation among FOXM1 overexpression and unfavorable clinicopathologic variables, such as advanced clinical stage, lymph node metastasis, and metastasis of colon cancer. FOXM1 expression conversely associated with poor OS and MFS as an independent prognostic factor. More importantly, FOXM1 has a potential clinical value to predict metastasis after colectomy. Considering the treatment heterogeneity within groups of different clinical stages, we further investigated the prognostic significance of FoxM1/PLAUR expression in stage I/II or stage III/IV subgroups and found that FOXM1/PLAUR–positive expression is significantly correlated with poorer survival in patients with early stage and advanced stage (data not shown). This means that at the time of initial diagnosis of colon cancer, FOXM1 expression can be used not only to design optimal, individualized treatment, but also to distinguish patients who would benefit from close monitoring after surgery from those who would not.

Our both in vitro and in vivo studies also revealed that altered FOXM1 expression affected migration and invasion in vitro and growth and liver metastases in vivo of colon cell lines. The clinicopathologic variables, such as advanced clinical stage, lymph node metastasis, and metastasis of colon cancer, indicated that targeting FOXM1 and PLAUR expression could be a novel therapeutic strategy for colon cancer patients.
cancer cells. Enforced expression of FOXM1 in colon cancer cells exhibited much higher live metastatic ability than control cells in mouse model, whereas knockdown of FOXM1 expression did the opposite. Consistent with our findings, FOXM1 overexpression led to the acquisition of EMT phenotype by activation of mesenchymal cell markers in pancreatic cancer cells and ovarian cancer cells (44, 45). Knockdown of FOXM1 suppresses cell viability, clonogenicity, and carcinogenesis in medulloblastoma, lung cancer, and breast cancer (46, 47). These studies further support the hypothesis that FOXM1 serve as a novel target for cancer therapy. It would be interesting and significant to further screen and validate drugs that target FOXM1 signaling.

Abnormal expression of FOXM1 could transactivate the downstream molecules including VEGF, Caveolin, and MMP2 (15–20), thus mediated multiple aspects of cancer metastatic biology, including angiogenesis, invasiveness, EMT. Providing a crucial role of FOXM1 involved in the metastasis of malignances, it is still necessary to elucidate the underlying mechanism of FOXM1-mediated downstream signaling molecules in colon cancer. PLAUR is overexpressed in tumor-stromal invasive microenvironment in several human cancers (48, 49). In our study, we showed that PLAUR-positive group has a much poorer MFS relative to PLAUR-negative group in Kaplan–Meier analysis ($P = 0.011$), whereas no statistical significant difference was observed in multivariate Cox regression analysis. One of the possible reasons could be due to the smaller sample size in our current study. Role of PLAUR in tumor progression and angiogenesis has been established (50–52). Targeting PLAUR has shown a promising antitumor effect in treatment of PLAUR-overexpressing tumors. Previous study has also noted that hypoxia-inducible factor 1 (HIF-1)–mediated regulation of PLAUR expression plays a role in the invasiveness of colorectal cancer (53). Tumor hypoxia has been implicated in the mechanisms of resistance to chemoradiotherapy in rectal adenocarcinomas (54), the process of which is mediated by the essential regulator HIF-1α, whose overexpression is associated with poor prognosis in colorectal cancers (55). It has been reported that transcriptional upregulation of FOXM1 in response to hypoxia is mediated by HIF-1α provide a new insight into how tumor cells overcome hypoxic stress and survive (56). However, the precise mechanisms of PLAUR overexpression in the colon cancer still remain largely unknown. The present study is the first to show that FOXM1 activation correlates with PLAUR expression in human colon tumor specimens, suggesting a link between FOXM1 activation and PLAUR overexpression. By using well-established FOXM1 overexpression and knockdown systems, we were able to show that altered FOXM1 expression significantly affected PLAUR expression in colon cancer cells. For example, inhibition of FOXM1 expression significantly suppressed PLAUR expression in and the metastatic phenotype of colon cancer cells. We also noted that FOXM1 expression is involved in mediating PLAUR promoter activity in colon cancer cells. Specifically, we identified 2 potential FOXM1-binding sites on PLAUR promoters. Mutations of these sites significantly attenuated FOXM1-mediated transactivation of PLAUR promoters. Therefore, these putative binding sites are functional, which we confirmed using a ChIP assay, which showed active recruitment of FOXM1 to specific binding sites in PLAUR promoter.

Besides its role in metastasis, lines of evidence suggest that FOXM1 is essential for cell-cycle progression, further supporting the critical role of FOXM1 in colon cancer.
progression (57). In fact, FOXM1 regulates the expression of various proteins that stimulate cell proliferation in various cancer types (28, 32), suggesting that FOXM1 is required for proliferative expansion during tumor progression. Consistently, present study also showed overexpression of FOXM1 promoted the tumor growth in vivo, whereas FOXM1 silencing did the opposite, supporting a critical role for FOXM1 in cell proliferation. Given the shown critical roles of FOXM1 in these 2 critical aspects of cancer biology, promotion of colon cancer growth and metastasis by activation of FOXM1 is conceivable, further supporting our finding that FOXM1 is a useful prognostic factor for colon cancer.

Finally, the mechanism underlying FOXM1 overactivation is currently unknown. FOXM1 expression can be induced by diverse stimuli, such as liver regeneration, keratinocyte growth factor expression, and oxidative stress (58, 59). It will be interesting and significant to explore the molecular mechanisms that result in FOXM1 overactivation, which may not only shed more light on abnormal FOXM1 activation, but also help better understand the value of FOXM1 as a prognostic factor and aid in the development of effective therapies that target FOXM1 to reverse the chemoradiotherapy resistance.

In summary, this study provided both clinical and mechanistic evidence supporting the regulatory role of FOXM1 in PLAUR expression and the critical role of this novel FOXM1–PLAUR signaling in colon cancer progression and metastasis. Our study not only undercover a novel

Figure 6. Transcriptional activation of PLAUR expression in colon cancer cells by FOXM1. A, Western blot analysis of FOXM1 and PLAUR protein expression in colon cancer cell lines (left). Real-time PCR analysis of FOXM1 and PLAUR mRNA expression in SW480 and SW620 cells (right). B, the total protein lysates of FOXM1 transfected SW480 cells or FOXM1-siRNA transfected SW620 cells were harvested; FOXM1 and PLAUR protein level were determined using Western blot analysis. C, schematic structure of the PLAUR promoter (left); pPLAUR1103 promoter has 2 potential binding sites (top), pPLAUR627 promoter has 1 potential binding site (bottom). ChIP assay using chromatin isolated from SW620 cells (right). D, pPLAUR1103 promoter and pcDNA3.1-FOXM1 or FOXM1-siRNA were transfected into SW480 (left) or SW620 cells (right) in triplicate; pPLAUR627 promoter (WT) and mutation of it in FOXM1 binding site 2 were transfected into SW480 or SW620 cells in triplicate; the relative PLAUR promoter activities were measured 24 hours after transfection. *statistic significance.

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molecular mechanism for pancreatic cancer progression and metastasis, but also identified this aberrant FOXM1–PLAUR signaling as a promising new molecular target for designing novel therapeutic modalities to control colon cancer progression and metastasis.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: D. Li, P. Wei, Z. Peng, H. Tang, S. Huang, K. Xie
Development of methodology: D. Li, P. Wei, Z. Jia, X. Le, S. Huang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D. Li, P. Wei, Z. Peng, C. Huang, H. Tang, Z. Jia, J. Cui, X. Le
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D. Li, P. Wei, Z. Peng, C. Huang, H. Tang, Z. Jia, Jiujie Cui, X. Le, S. Huang, K. Xie

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