

ORIGINAL ARTICLE

Splicing factor PRPF6 upregulates oncogenic androgen receptor signaling pathway in hepatocellular carcinoma

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Abstract

Androgen receptor (AR) signaling is considered to be crucial for the pathogenesis of hepatocellular carcinoma (HCC) with obvious sexual dimorphism. Pre-mRNA processing factor 6 (PRPF6) was identified as a coactivator of AR. However, the molecular mechanism underlying the modulation function of PRPF6 on AR-mediated transcriptional activity in HCC needs to be further clarified. In this study, we analyzed data from The Cancer Genome Atlas to show that PRPF6 is highly expressed in HCC. Our data indicated that PRPF6 interacts with AR/AR splice variants (AR-Vs) and upregulates AR/AR splice variant 7-mediated transcriptional activity even without dihydrotestosterone treatment. We observed that AR is obviously induced by androgen treatment and is mainly expressed in the nucleus in HCC-derived cell lines. Moreover, overexpression of PRPF6 enhances AR expression accompanied with the increase of AR-Vs expression. We provided evidence that PRPF6 participates in upregulating AR self-transcription. PRPF6 facilitates the recruitment of AR to the androgen responsive element region of the AR gene. Finally, PRPF6 depletion inhibits cell proliferation in HCC cells and mouse xenografts. Taken together, our results suggest that PRPF6 as a splicing factor enhances AR self-transcription, thereby coactivating oncogenic AR/AR-Vs actions in HCC.

KEYWORDS

androgen receptor, hepatocellular carcinoma, PRPF6, splicing factor, transcriptional regulation

Abbreviations: aa, amino acid; AF, activation function; AR, androgen receptor; ARE, androgen responsive element; CCRK, cell cycle-related kinase; DHT, dihydrotestosterone; HCC, hepatocellular carcinoma; IHC, immunohistochemistry; PRC1, protein regulator of cytokinesis 1; PRPF6, pre-mRNA processing factor 6; qPCR, quantitative real-time PCR; TCGA, The Cancer Genome Atlas; TPR, tetratricopeptide repeat.

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1 | INTRODUCTION

Hepatocellular carcinoma is the predominant type of liver cancer. It is a highly invasive malignancy and a main cause of cancer-related death worldwide. A large number of risk factors for HCC have been identified, including hepatitis B virus, hepatitis C virus, alcohol abuse, genetic metabolic diseases, and cirrhosis.¹⁻³ Although early diagnosed tumors can be treated through liver resection and transplantation, the early diagnosis of HCC is challenging. Limited treatment options and low survival rates are still the awkward problems to be faced for HCC. Sorafenib and lenvatinib, inhibitors of multityrosine kinase, are currently recognized as first-line drugs for the treatment of advanced HCC. However, the efficacy of these 2 inhibitors is limited and side-effects are serious.⁴ Therefore, the early diagnosis biomarkers and the new therapeutic strategies for HCC are urgently required.

The epidemiological feature of HCC is especially more prevalent in men than in women, with a male-to-female ratio of 2-4:1, and AR plays a central role in this gender preference.⁵⁻⁷ Androgen receptor, as a member of the nuclear receptor superfamily, is a transcription factor activated by ligand (androgen, DHT) to induce a series of target genes. Growing evidence reveals that AR-induced target genes transcription is important for the development of HCC progression, although the effect of antiandrogen therapy is not satisfactory in clinical liver cancer.⁸⁻¹¹ This indicates that the AR signaling pathway and the regulation of the oncogenic AR action would be more complicated in HCC. A recent study has reported that AR regulates the AKT-mTOR pathway in HCC in a ligand-independent manner.¹² During the process of transactivation induced by AR, a series of coregulators are usually recruited to participate in modulating AR-induced transcriptional activity to exert its biological function in prostate cancer.^{13,14} Moreover, AR-Vs possesses a constitutively active function to be essential for prostate cancer and HCC progression.^{11,15} However, how the AR/AR-Vs actions are regulated and the molecular mechanism of the coregulators of AR in HCC are still elusive.

Splicing of precursor mRNA is necessary for the maturation of almost all mRNA. It has also been reported that splicing is a complicated process coupling transcription and epigenetic modification.¹⁶ Recent studies have shown that splicing facilitates the recruitment of methyltransferase to enhance the level of H3K36me3, which is considered as a signature mark for exons and transcriptional activation.¹⁷⁻²⁰ It has been reported that inhibition of expression of the splicing factor SAP130 reduced levels of H3K36me3.¹⁷ In addition, inhibition of the activity of the U2 small nuclear ribonucleoprotein component SF3B1 results in loss of the H3K36me3 mark.²¹ These results indicate that splicing factors are also involved in maintenance of epigenetic marks, leading to alteration of chromatin structure. Pre-mRNA processing factor 6 as a splicing factor plays an important role in the formation of spliceosomes.^{22,23} It contains 941 amino acids with a molecular weight of 102 kDa, including 19 TPR elements, 2 LxxLL motifs, and 1 leucine zipper motif. We have previously identified PRPF6 as a novel AR coactivator with multiple functional domains.^{24,25} It has been recently reported that PRPF6 drives colon cancer proliferation by preferential splicing of genes

associated with growth regulation, implying an important role of PRPF6-mediated splicing in cancer growth.²⁶ However, it needs to be further clarified whether PRPF6 as a cofactor of AR plays an essential role in HCC, and what the molecular mechanism underlying the modulation function of PRPF6 on AR-mediated transcriptional activity in HCC cells is.

In this study, we have shown that PRPF6 associates with AR/AR-Vs and enhances AR-induced transactivation in a ligand-independent manner in HCC-derived cell line (HCCLM3 cells). Importantly, PRPF6 enhances constitutive AR-AF1 or AR-V7-mediated transactivation. In addition, we have found that ectopic expression of PRPF6 increases AR expression accompanied by the increase of AR-Vs expression. We provided evidence to show that PRPF6 upregulates AR self-transcription. PRPF6 is recruited to the ARE region of the AR gene, and facilitates the recruitment of AR to the same region. We also detected that PRPF6 depletion subsequently abrogates the level of H3K36me3 modification at the ARE region of the AR gene. Interestingly, we observed that AR is obviously induced by androgen treatment and is mainly expressed in the nucleus in HCC-derived cell lines. Functionally, PRPF6 depletion inhibits cell growth/proliferation in HCC cells. Additionally, PRPF6 is highly expressed in HCC, and the higher expression of PRPF6 is positively correlated with poor prognosis. Taken together, these results suggest a function of PRPF6 on upregulating AR self-transcription, thereby enhancing AR/AR-Vs actions to promote the progression of HCC. Our study could provide a potential target for HCC therapy.

2 | MATERIALS AND METHODS

2.1 | Antibodies

The Abs used in this study were: anti-PRPF6 (23929-1-AP, Proteintech; and A302-773A, Bethyl Laboratories), anti-Flag (GNI4110-FG, GNI), anti-AR441 (MA5-13426, Thermo Fisher Scientific), anti-AR (22089-1-AP, Proteintech), anti-CCRK (HPA027401, Sigma), anti-Ki-67 (sc-15402, Santa Cruz Biotechnology), anti-GAPDH (AC002, ABclonal Technology), anti-FKBP5 (#12210S, Cell Signaling Technology), and anti-trimethyl H3K36 (ABE435, Millipore).

2.2 | Cell culture, siRNA transfection, and lentiviral infection

The detailed experimental procedures of this section are described in Appendix S1. The sequences of siPRPF6 used in siRNA transfection are shown in Table S1.

2.3 | Quantitative real-time PCR

Total RNA was isolated using the TRIzol reagent (Invitrogen). Reverse transcription was performed using PrimeScript RT Master Mix (Perfect

Real Time) (Takara). Quantitative real-time PCR was carried out using the SYBR Premix Ex Taq II (Takara) on a QuantStudio3 instrument (Applied Biosystems). The sequences of the forward and reverse primers were shown in Table S2. Gene expression levels were calculated relative to the housekeeping gene *18S* using the $2^{-\Delta\Delta CT}$ method.

2.4 | Chromatin immunoprecipitation

Chromatin immunoprecipitation was carried out as previously described.^{14,27} The DNA fragments were extracted with phenol-chloroform and precipitated in absolute ethanol. The DNA was dissolved in TE buffer and analyzed by qPCR. Results are shown as the percentage of input chromatin. The primers used in qPCR are shown in Table S3.

2.5 | Dual luciferase reporter assay

A detailed description of this section is available in Appendix S1.

2.6 | Immunohistochemistry

A detailed description of this section has been included in Appendix S1.

2.7 | Xenograft tumor growth

HCCLM3 cells carrying shPRPF6 or shCtrl (5×10^6 cells/mouse) were suspended in 100 μ L sterile PBS with half Matrigel (BD Biosciences) and were injected s.c. into 4-week-old male BALB/C-null mice (Vital River Laboratories). Tumor diameter was measured every week with electronic calipers. Tumor volume was calculated according to the formula: volume (mm^3) = (short diameter)² \times long diameter/2.²⁸ Tumor-bearing mice were killed in keeping with the policy of the humane treatment of animals after 4 weeks. All procedures involved in animal experiments were approved by the Animal Ethics Committee of China Medical University.

2.8 | The Cancer Genome Atlas data

Clinical and gene expression quantification data for PRPF6 in liver cancer were downloaded from the UALCAN database (<http://ualcan.path.uab.edu/>).

2.9 | Cell viability, colony formation, Transwell, and scratch assays

A detailed description of this section is available in Appendix S1.

2.10 | Statistical analysis

All statistical analyses were undertaken using SPSS statistics 22.0 software. The data presented in bar graphs are means \pm SD of at least 3 independent experiments. The statistical analyses were carried out with Student's 2-tailed *t* test. For analysis of clinical specimens, one-way ANOVA and the χ^2 test were used. **P* < .05, ***P* < .01, and ****P* < .001 were considered to be statistically significant.

3 | RESULTS

3.1 | Pre-mRNA processing factor 6 is highly expressed in HCC, which is associated with tumor progression and poor prognosis

To determine the role of PRPF6 in liver cancer, we used the UALCAN database based on data from TCGA to analyze the expression of the *PRPF6* gene in primary HCC and normal liver tissues. The results showed that *PRPF6* mRNA expression was significantly higher in tumors than that in normal liver tissues (Figure 1A), and the expression level increased with the increase of tumor pathological grades and clinical stages (Figure 1B,C). In addition, higher expression of the *PRPF6* gene was positively correlated with poor prognosis in HCC (Figure 1D). To verify these results, we examined PRPF6 protein expression by IHC in 75 cases of HCC samples and 33 cases of adjacent noncancerous liver tissues. The results are shown in Table 1, the expression of PRPF6 protein was significantly higher in HCC than that in adjacent noncancerous liver tissues (*P* < .001), and the expression intensity of PRPF6 protein was gradually enhanced with the increase of pathological grades (Figure 1E,F). We further analyzed the relationship between PRPF6 protein expression and clinical features of HCC in the high and low PRPF6 expression groups based on the results of IHC analysis. The data showed that PRPF6 expression was positively correlated with pathological grade, and there was no significant association with the other clinical features (Table 2). To further address the question whether HCC is more prevalent in men than in women with regards to PRPF6 expression, western blotting experiments were carried out in 10 human HCC samples, from 5 men and 5 women, to detect the expression of PRPF6. The results indicated that PRPF6 expression in HCC samples from men was comparable to that from women (Figure 1G). Taken together, our results indicate that PRPF6 is highly expressed in HCC tumors, and the expression level of PRPF6 is correlated with advanced disease stage and poor prognosis, suggesting that PRPF6 could play an important role in the progression of HCC.

3.2 | Pre-mRNA processing factor 6 associates with AR in HCC cells

We have previously identified PRPF6 as a coactivator of AR,²⁴ which is also considered to play a crucial role in pathogenesis of HCC. We

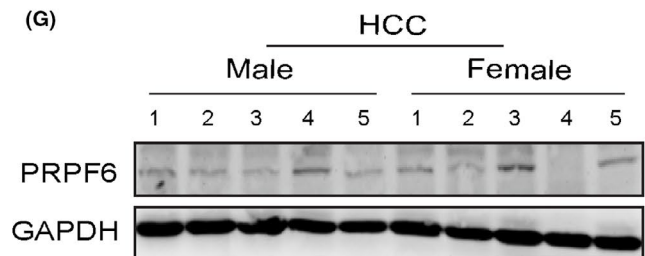
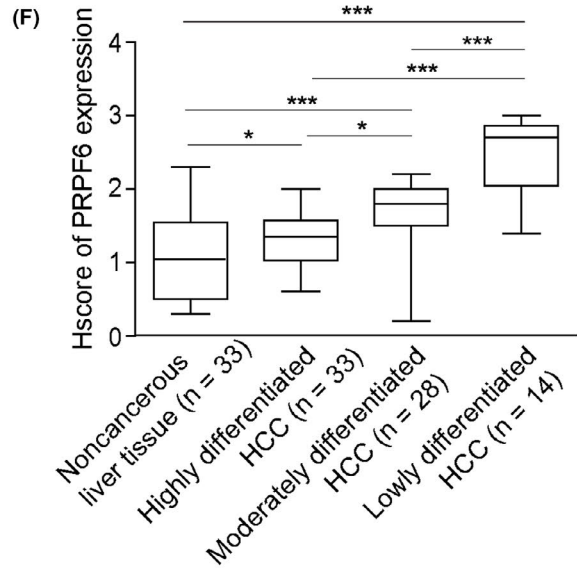
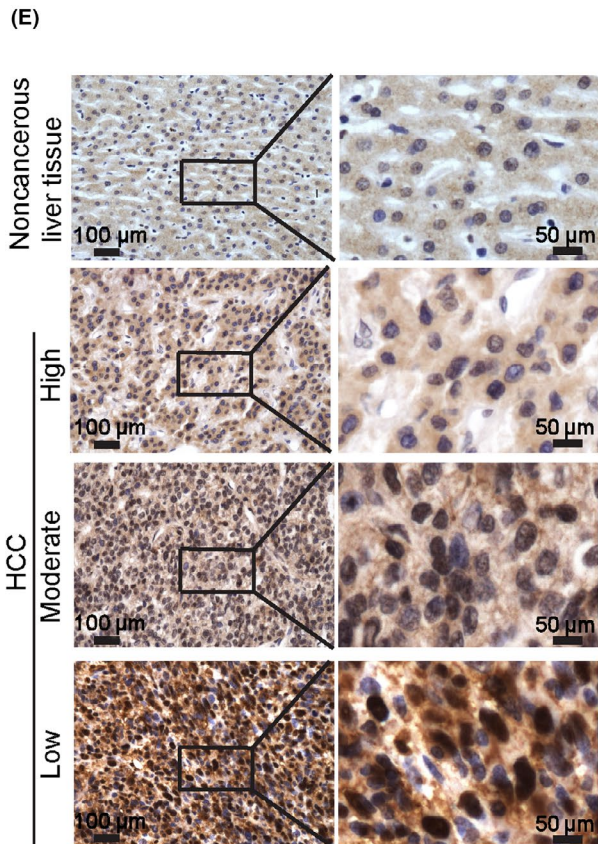
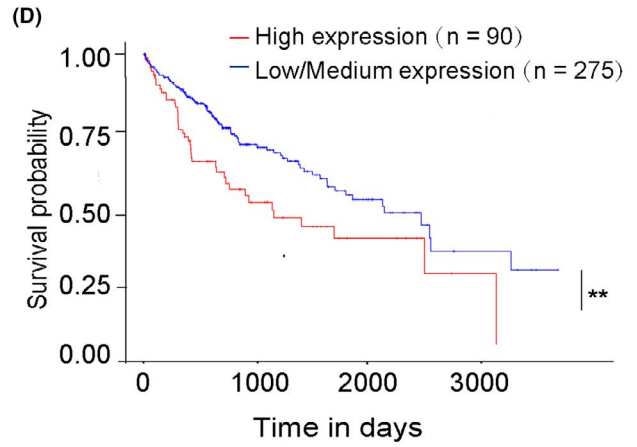
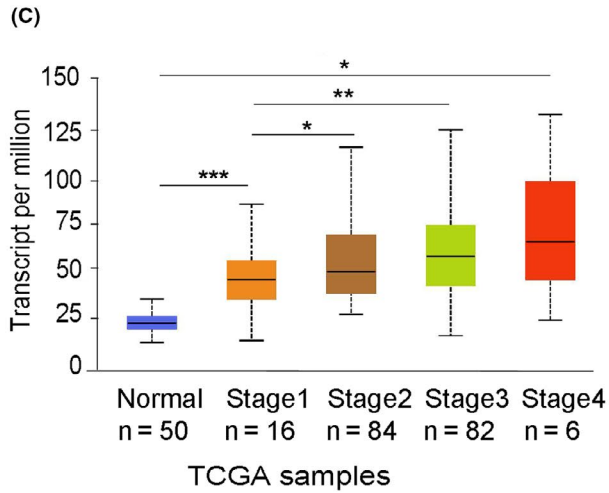
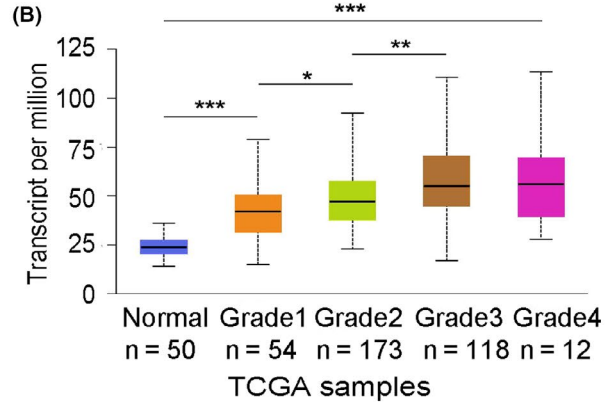
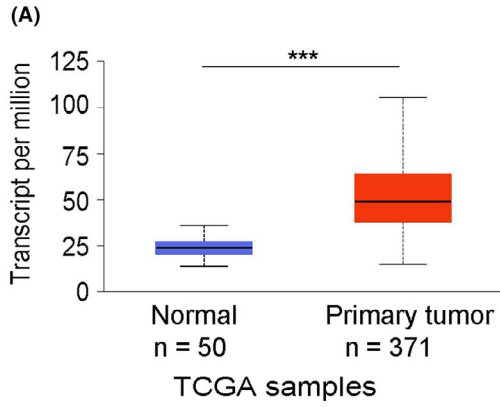


FIGURE 1 Pre-mRNA processing factor 6 (PRPF6) is highly expressed in hepatocellular carcinoma (HCC). A, PRPF6 mRNA is overexpressed in HCC. B, C, PRPF6 mRNA expression correlates with pathological grades and clinical stages of HCC. D, Overall survival curves of HCC patients with high and low PRPF6 mRNA expression. Data in A-D were downloaded from the UALCAN database, based on data from The Cancer Genome Atlas (TCGA). E, Representative immunohistochemical staining of PRPF6 in adjacent noncancerous liver tissues and primary HCC. Magnification, 200 \times and 400 \times . F, PRPF6 expression in different pathological grades of HCC compared with adjacent noncancerous liver tissues. One-way ANOVA was used. G, Expression of PRPF6 in 10 human HCC samples (from 5 men and 5 women) were detected by western blotting. * $P < .05$, ** $P < .01$, and *** $P < .001$

TABLE 1 Pre-mRNA processing factor 6 (PRPF6) expression in hepatocellular carcinoma (HCC) and adjacent noncancerous liver tissues

Group	Cases n = 108 (%)	PRPF6 expression		P value
		Low	High	
HCC	75 (69.4)	27	48	<.001
Noncancerous liver tissues	33 (30.6)	24	9	

thus examined the association between PRPF6 and AR in HCC-derived cell lines. The protein expression of PRPF6 or AR in different HCC cell lines was detected. The results showed that AR was significantly more highly expressed in HCCLM3 cells and PRPF6 was ubiquitously expressed in each cell line (Figure 2A). We then generated the PLC5 cell line with stable overexpression of AR (PLC5-AR) (Figure 2B). Coimmunoprecipitation experiments were carried out to detect the interaction between PRPF6 and AR in HCCLM3 cells. The results showed that endogenous PRPF6 associated with AR and AR-Vs in a DHT-independent manner, and treatment with DHT enhanced their interaction (Figure 2C). Similarly, PRPF6 also interacted with exogenous AR in PLC5-AR cells (Figure 2D).

To identify the interaction domain in PRPF6, which is required for association between PRPF6 and AR, we constructed expression plasmid encoding the full-length PRPF6 (PRPF6-FL) tagged with Flag and a series of truncated mutants (Figure 2E). HEK293 cells were cotransfected with plasmids expressing AR and PRPF6-FL or its truncated mutants. Coimmunoprecipitation results indicated that AR individually interacted with PRPF6-FL, PRPF6-C, PRPF6-5TPR, PRPF6-10TPR, or PRPF6-15TPR, but did not interact with PRPF6-N (Figure 2F), indicating that the interaction between PRPF6 and AR requires the first 5 TPR motifs (309-463 aa fragment) in PRPF6. Furthermore, immunofluorescence staining of HCCLM3 cells results showed that endogenous PRPF6 was distributed in the nucleus. Interestingly, endogenous AR was predominantly expressed in the nucleus even in the absence of DHT, indicating that AR colocalized with PRPF6 in the nucleus in HCCLM3 cells (Figure 2G). We further examined the exogenous subcellular localization of PRPF6 and AR in HEK293 cells. As reported above, PRPF6-FL or its truncated mutants PRPF6-N, PRPF6-5TPR, PRPF6-10TPR, and PRPF6-15TPR showed a diffuse distribution throughout the nucleus; PRPF6-C was not concentrated in the nucleus, but diffusely distributed throughout both the nucleus and cytoplasm, suggesting that the nuclear localization signal was mainly located in the N-terminus of PRPF6. In addition, AR was colocalized with full-length or truncated mutants of PRPF6 in the nucleus with

TABLE 2 Relationship between pre-mRNA processing factor 6 (PRPF6) expression and clinicopathologic characteristics of 75 patients with hepatocellular carcinoma

Characteristic	Cases, n = 75 (%)	PRPF6 expression		P value
		Low n = 27	High n = 48	
Gender				
Male	55 (73.3)	22	33	.231
Female	20 (26.7)	5	15	
Age (y)				
<55	41 (54.7)	13	28	.395
\geq 55	34 (45.3)	14	20	
Pathologic grades				
Well	33 (44)	19	14	.002
Moderate	28 (37.3)	6	22	
Poorly	14 (18.7)	2	12	
AFP				
Negative	12 (16)	4	8	.834
Positive	63 (84)	23	40	

Bold values represent $P < .05$.

AFP, alpha fetoprotein.

DHT treatment in HEK293 cells (Figure 2H). Collectively, our results showed that PRPF6 physically associates with AR in cultured cells.

3.3 | Pre-mRNA processing factor 6 upregulates AR-mediated transactivation in a ligand-independent manner in HCC cells

Having shown PRPF6 associates with AR in HCC cells in the absence or presence of DHT, we next explored the effect of PRPF6 on the transactivation function of AR in HCC cells. To this end, a series of luciferase assays were carried out. As shown in Figure 3A, PRPF6 and its truncated mutants, including PRPF6-5TPR, PRPF6-10TPR, and PRPF6-15TPR, enhanced AR-induced transcriptional activity even in the absence of DHT, and the upregulation effect was more significant in the presence of DHT. In contrast, truncated PRPF6-N and PRPF6-C had no obvious effect on AR action. Collectively, these results suggest that the N-terminus (309-463 aa) in PRPF6 may contain the functional domain for coactivation of AR action. As a transcription factor, AR mainly contains ligand-independent AF1 and AF2, required for cognate ligand

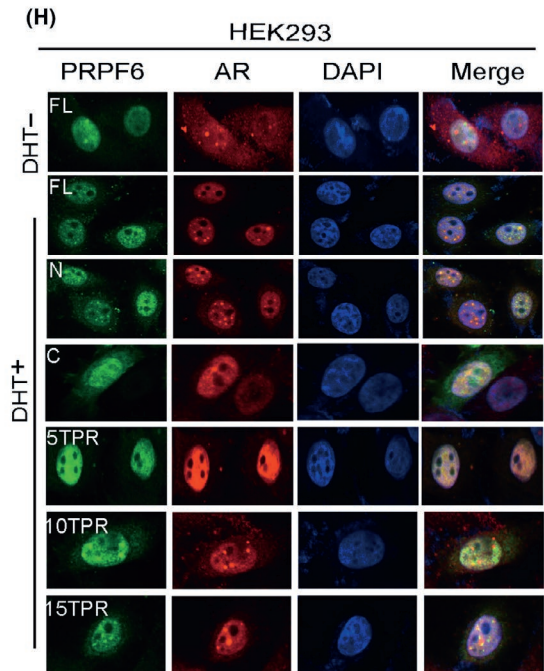
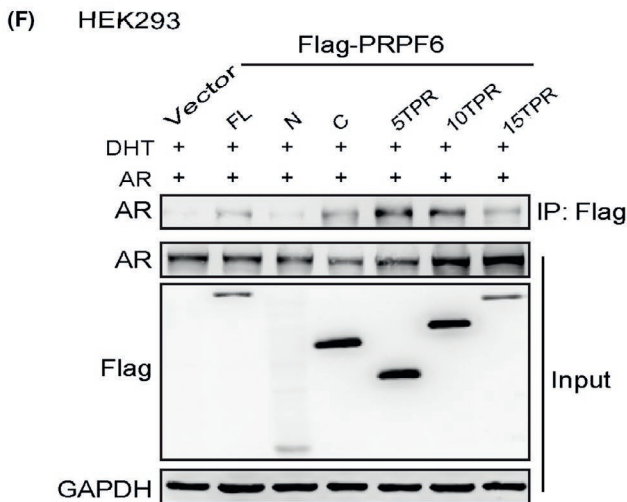
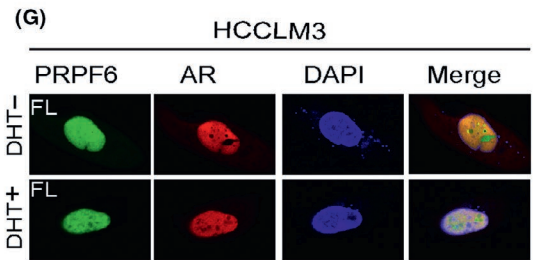
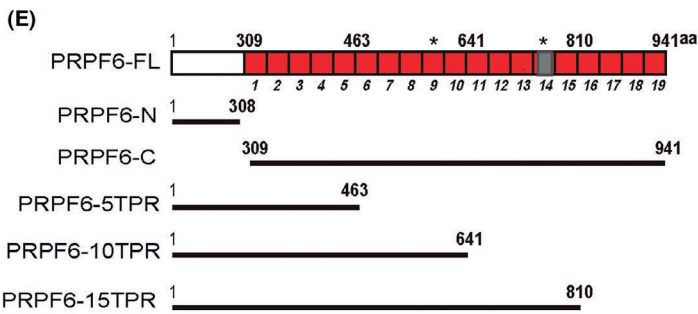
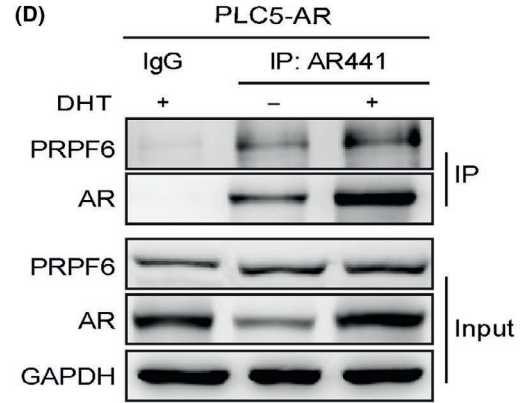
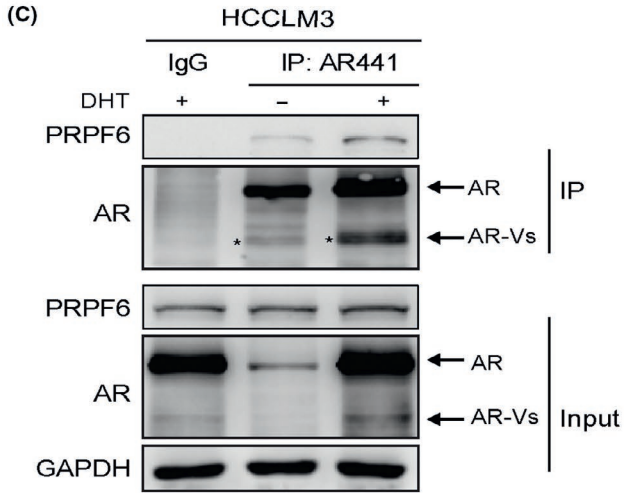
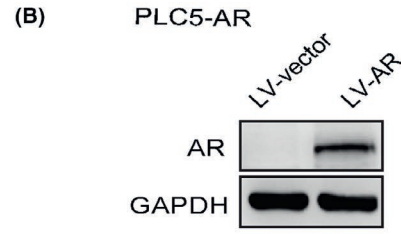
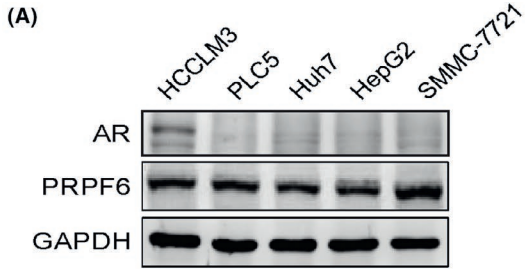


FIGURE 2 Pre-mRNA processing factor 6 (PRPF6) interacts with androgen receptor (AR) and colocalizes in the nucleus. A, PRPF6 or AR protein expression in different hepatocellular carcinoma cells. B, PLC5-AR cell line was successfully constructed by infected with the lentivirus carrying AR (LV-AR) in AR-negative PLC5 cells. LV-vector was used as a negative control. C, Endogenous PRPF6 interacts with AR and AR variants (AR-Vs) in HCCLM3 cells. *Location of AR-Vs protein. D, PRPF6 interacts with exogenous AR in PLC5-AR cells. E, Schematic diagrams of PRPF6 and its truncated mutants as indicated. PRPF6 possesses 19 tetratricopeptide repeat (TPR) elements in a tandem fashion (red open box), 2 LXXLL motifs (asterisks), and 1 leucine zipper motif (gray box). F, Exogenous PRPF6 or its truncated mutants interact with AR in HEK293 cells. G, PRPF6 and AR are predominantly colocalized in the nucleus of HCCLM3 cells. H, Confocal fluorescence image of subcellular localization for exogenous PRPF6 and AR in HEK293 cells. Blue, nucleus; green, PRPF6; red, AR (60 \times) (G, H). DHT, dihydrotestosterone; IP, immunoprecipitation

binding. In addition, it has been reported that AR-V7 is a well described variant in prostate cancer and HCC.^{11,13} We thus turned to examine the effect of PRPF6 on the transactivation mediated by AR-AF1, AR-AF2, or AR-V7. The results showed that PRPF6 significantly increased the constitutive transactivation function mediated by AR-AF1 or AR-V7. However, the coactivation function of PRPF6 on the ligand-dependent transactivation mediated by AR-AF2 was not observed (Figure 3B). These results suggest that PRPF6 as a coactivator of AR/AR-V7 participates in upregulation of AR/AR-V7-mediated transcriptional activity without DHT in HCC cells.

To further examine whether PRPF6 upregulates the endogenous AR target genes in HCC cells, 3 siRNAs against PRPF6 (siPRPF6#1, #2, and #3) were transfected into HCCLM3 cells to test the efficiency of PRPF6 knockdown (Figure 3C). siPRPF6#1 was selected for further studies to detect the influence of PRPF6 on the mRNA expression of AR-regulated genes, including *CCRK*,⁸ *PRC1*,⁸ *VEGFA*,²⁹ and *FKBP5*¹² by qPCR in HCCLM3 cells. As shown in Figure 3D, the mRNA expression of *CCRK* and *PRC1* was significantly reduced by PRPF6 knockdown without or with DHT, and the mRNA expression of *VEGFA* and *FKBP5* had no obvious alteration. Interestingly, the mRNA expression of *AR* was also decreased by the depletion of PRPF6. Western blotting results indicated that depletion of PRPF6 obviously suppressed the protein expression of AR as well as *CCRK*, but did not inhibit the expression of *FKBP5* in HCCLM3 or PLC5-AR cells (Figure 3E). These results suggest that PRPF6 partially upregulates the transcription of AR target genes, including *CCRK*, which is required for cell cycle regulation. Moreover, ectopic expression of PRPF6 increased AR expression, thereby enhancing *CCRK* or AR-Vs expression in HCCLM3 cells (Figure 3F). Similar results were observed in PLC5-AR cells, but not in AR-negative PLC5 cells (Figure 3G), indicating that the upregulation of PRPF6 on *CCRK* was dependent on AR. According to the previous report,⁸ bioinformatics analysis showed that there was a putative ARE (5'-AGAAGG-3') located at approximately 768 bp downstream of transcription start site on the *CCRK* gene (Figure 3H). Thus, ChIP assays were further carried out to determine the recruitment of PRPF6 or AR to the ARE of the *CCRK* gene. The results showed that PRPF6 or AR was recruited to the ARE site with or without DHT, and PRPF6 depletion reduced AR recruitment on the ARE region of *CCRK* in HCCLM3 cells (Figure 3I). Taken together, the results indicate that PRPF6 might be involved in upregulation of AR self-transcription, subsequently enhancing AR/AR-Vs-induced transactivation. Moreover, PRPF6 was

recruited to the ARE region of *CCRK*, thereby promoting the recruitment of AR to the same region in HCC cells.

3.4 | Pre-mRNA processing factor 6 enhances AR self-transcription induced by androgen

Having established that PRPF6 depletion reduced the mRNA and protein expression of AR (Figure 3D,E), we thus asked whether PRPF6 participates in AR self-transcription in HCC cells. During the experiments, we also observed that AR self-transcription was significantly increased with DHT treatment of HCCLM3 cells carrying endogenous AR expression or PLC5-AR cells carrying exogenous AR expression, but not in PLC5 cells without endogenous AR expression (Figure 4A,B), indicating that AR transcription is induced by its own ligand (DHT) and the AR gene could be its own target gene in HCC cells. In addition, our data revealed that ectopic expression of PRPF6 enhanced AR expression in a dose-dependent manner (Figure 4C). Therefore, we speculated that PRPF6 might associate with AR to be involved in androgen-induced AR self-transcription. A previous report³⁰ and bioinformatics analysis showed that there are 2 putative AREs in the coding region of the AR gene, including a half site composed of a 6-bp sequence similar to the palindrome common hormone response element (ARE I, 5'-TGTCCT-3') and a part of the same sequence as the androgen response region found in the *probasin* gene promoter of rat (ARE II, 5'-AGTACTCC-3') (Figure 4D). We then assessed the recruitment of AR, PRPF6, and the epigenetic changes at chromatin encompassing the loci. As expected, AR recruitment was increased with the stimulation of DHT at the ARE I region compared with the control group, indicating that AR might be its own target gene in HCC cells. In addition, PRPF6 was obviously recruited to the ARE I site (Figure 4E). Knockdown of PRPF6 decreased AR recruitment and the modification level of histone H3K36me3 at the ARE I region of the AR gene (Figure 4F). The results indicate that PRPF6 or AR is able to be recruited to the AREs of the AR gene, thereby regulating the histone H3K36me3 level to maintain the active chromatin state.

3.5 | Depletion of Pre-mRNA processing factor 6 reduces cell proliferation in HCC cells

To further clarify the potential biological function of PRPF6 in HCC progression, we analyzed the influence of PRPF6 expression on cell

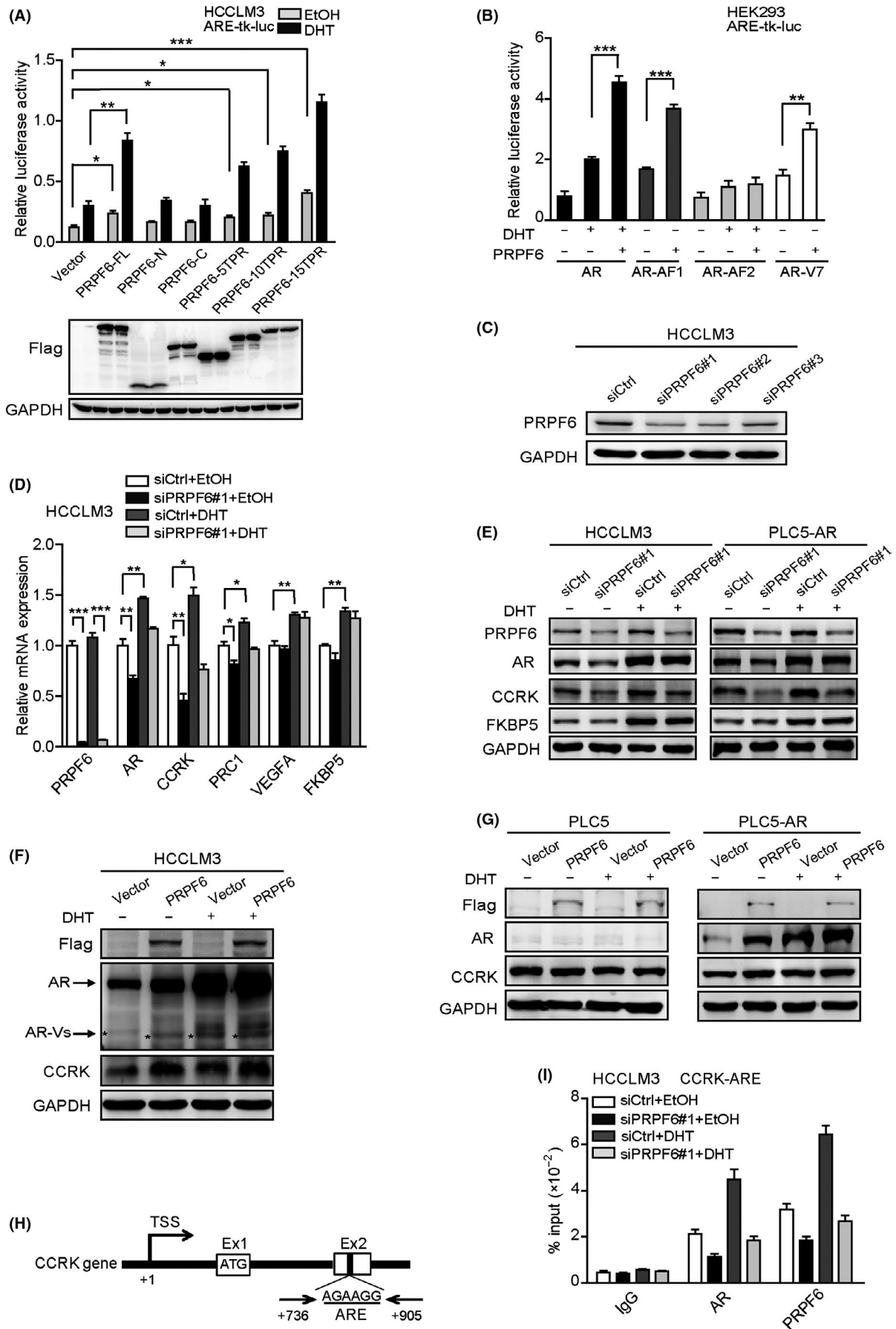
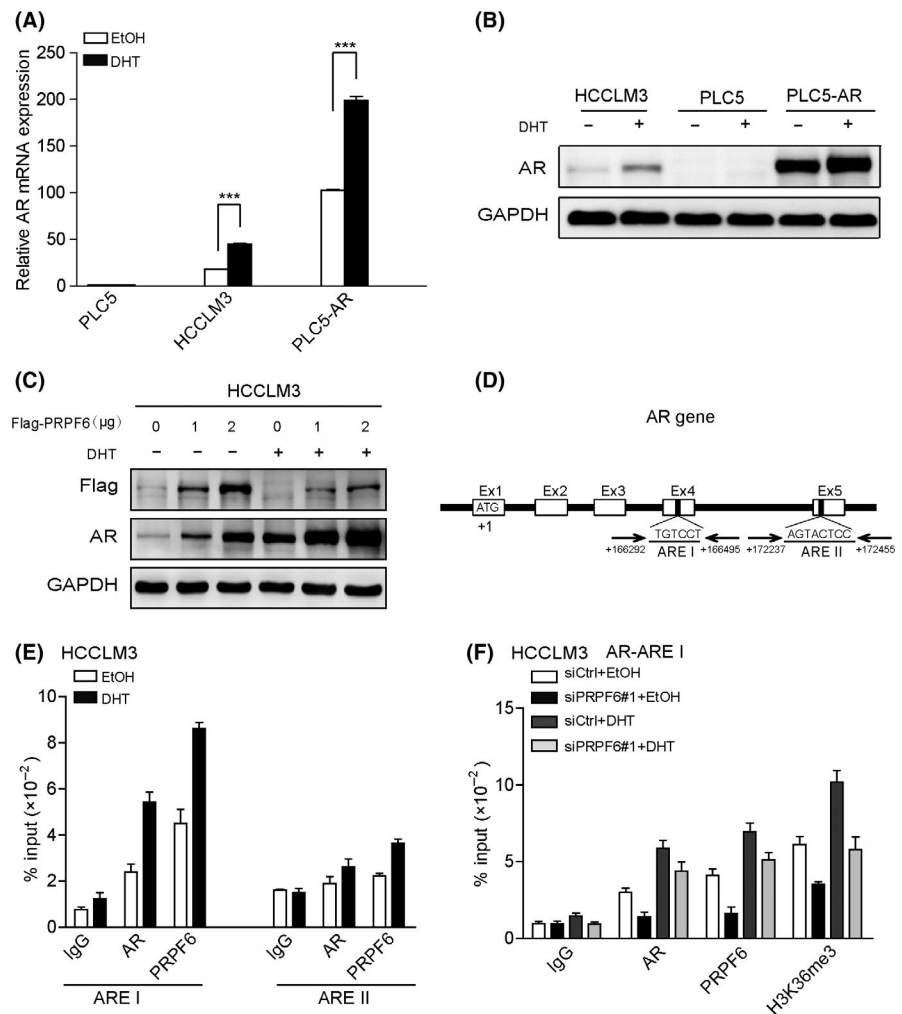


FIGURE 3 Pre-mRNA processing factor 6 (PRPF6) upregulates androgen receptor (AR)-mediated transactivation in an androgen-independent manner. A, The effect of PRPF6 or truncated mutants on the AR-mediated transcriptional regulation. The expression of PRPF6 truncated mutants were detected by western blotting. B, PRPF6 enhances AR, AR-activation function 1(AF1)- or AR-variant 7 (V7)-mediated transactivation. C, Detection of knockdown efficiency of PRPF6 siRNA (siPRPF6#1, #2, and #3). D, Quantitative PCR analysis the influence of PRPF6 on the mRNA expression of AR-regulated genes. E, PRPF6 depletion reduces AR and CCRK protein expression in AR-positive HCC cells. F, Overexpression of PRPF6 enhances AR expression, thereby enhancing CCRK or AR-Vs expression in HCCLM3 cells. *Location of the AR-Vs protein. G, Ectopic expression of PRPF6 increases AR or CCRK expression in PLC5-AR cells, not in AR-negative PLC5 cells. H, Schematic representation of putative androgen responsive elements (ARE) region downstream of the transcription start site in the CCRK gene as indicated. I, PRPF6 affects AR recruitment at ARE of the CCRK gene. In the above experiments, * $P < .05$, ** $P < .01$, *** $P < .001$. CCRK, cell cycle-related kinase; DHT, dihydrotestosterone; EtOH, ethyl alcohol; Ex, exon; PRC1, protein regulator of cytokinesis 1; VEGFA, vascular endothelial growth factor A

FIGURE 4 Pre-mRNA processing factor 6 (PRPF6) promotes androgen receptor (AR) gene self-transcription in hepatocellular carcinoma (HCC) cells. A, Quantitative PCR analysis was undertaken to detect mRNA expression of AR gene in HCC cells. *** $P < .001$. B, AR protein expression was induced by dihydrotestosterone (DHT) treatment in AR-positive HCC cells. C, Ectopic expression of PRPF6 enhances AR protein level in a dose-dependent manner. D, Schematic diagram of 2 androgen responsive elements (ARE) sites in the AR gene. E, Recruitment of AR or PRPF6 to ARE I/II regions on the AR gene. F, PRPF6 depletion reduced the recruitment of AR and histone H3K36me3 level at ARE I region of the AR gene. EtOH, ethyl alcohol; Ex, exon; siCtrl, control



proliferation. First, knockdown efficiency of PRPF6 by shPRPF6 in HCCLM3 and PLC5-AR cells was tested by western blotting (Figure 5A,B). Next, we undertook an MTS assay to analyze the effect of PRPF6 on cell proliferation. The results indicated that PRPF6 depletion attenuated cell proliferation even in the absence of DHT, whereas the effect of suppression of cell growth was stronger in the presence of DHT in HCCLM3 or PLC5-AR cells (Figure 5C,D). Similar results were observed in the colony formation assay (Figure 5E,F). However, the effect of PRPF6 depletion on colony formation in AR-negative HCC cell (PLC5 cells) was not obviously observed (Figure 5G). As

PRPF6 regulates the transcription of some AR target genes, including CCRK and PRC1, which are involved in the regulation of the cell cycle, we then assessed the influence of PRPF6 on cell cycle progression. Flow cytometry results indicated that PRPF6 knockdown significantly delayed G₁-S phase transition in HCCLM3 cells (Figure 5H). To examine the effect of PRPF6 on cell migration ability in HCC cells, scratch and Transwell experiments were carried out. The results showed that PRPF6 had no influence on cell migration in HCC cells (Figure 5I,J). Taken together, our data suggest that PRPF6 participates in the promotion of cell growth and proliferation in HCC cells.

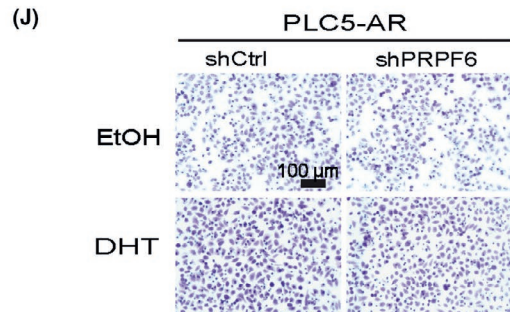
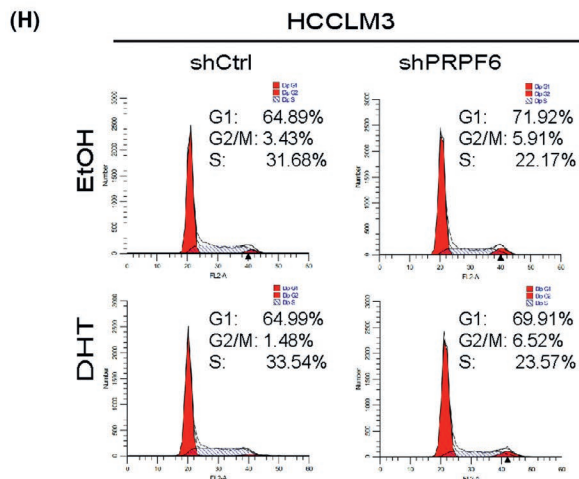
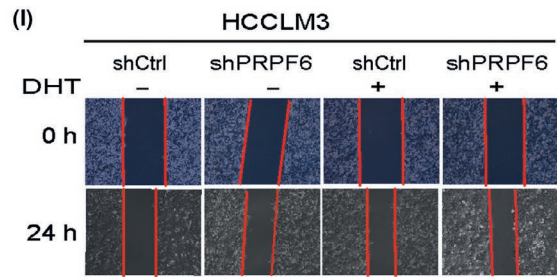
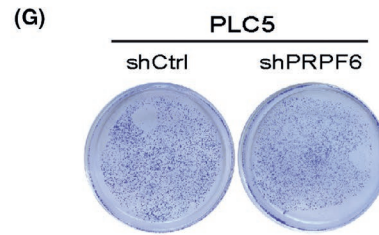
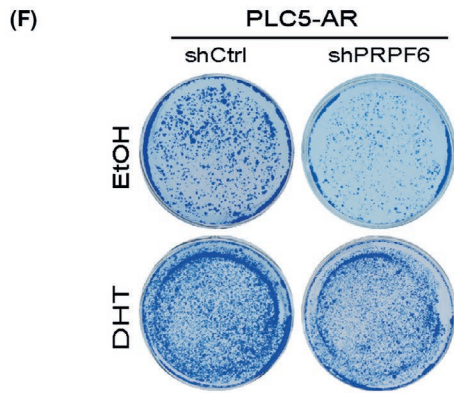
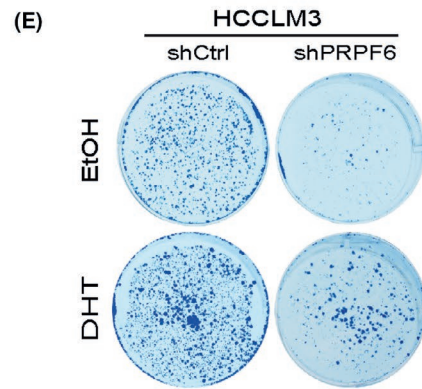
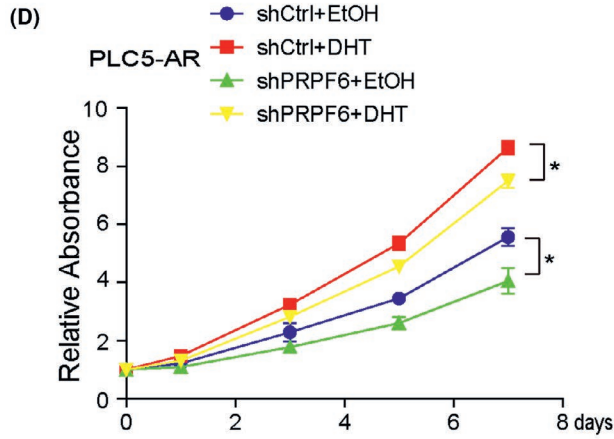
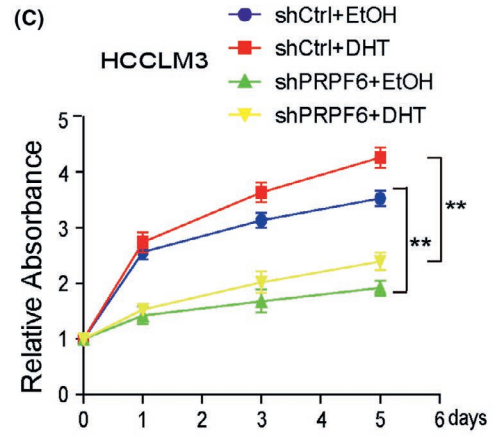
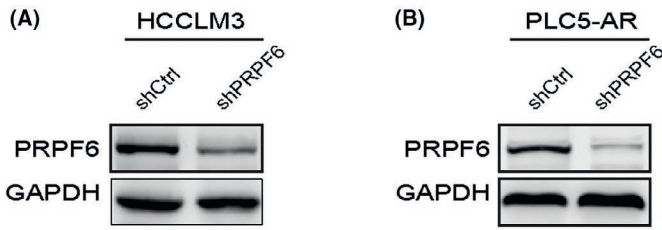


FIGURE 5 Pre-mRNA processing factor 6 (PRPF6) depletion inhibits hepatocellular carcinoma (HCC) cell growth and proliferation. A, B, Detection of knockdown efficiency of PRPF6 shRNA in HCCLM3 and PLC5-androgen receptor (AR) cells. C, D, Knockdown of PRPF6 inhibits cell proliferation in HCC cells by MTS assay. * $P < .05$, ** $P < .01$. E-G, Knockdown of PRPF6 significantly inhibits cell growth in HCCLM3 and PLC5-AR cells, but not in AR-negative PLC5 cells by colony formation assay. H, PRPF6 knockdown delayed G₁-S phase transition in HCCLM3 cells, by flow cytometry assay. I, PRPF6 knockdown has no effect on cell migration in HCCLM3 cells, by scratch assay. J, PRPF6 depletion has no obvious effect on cell migration in PLC5-AR cells, by Transwell assay. DHT, dihydrotestosterone; EtOH, ethyl alcohol; shCtrl, control

3.6 | Depletion of Pre-mRNA processing factor 6 inhibits tumor growth of HCC in mouse xenograft

Having established that PRPF6 participates in promotion of the proliferation of HCC cells, we next turned to explore the function of PRPF6 on cell growth in HCC cells in vivo. Mice xenograft experiments were carried out. HCCLM3 cells carrying shPRPF6 or shCtrl were individually injected into the right (shPRPF6) or left (shCtrl) flank of 4-week-old male BALB/C-null mice (Figure 6A). After 4 weeks, the mice were killed and the tumors were removed. As shown in Figure 6B, the size of tumors grown from shPRPF6-HCCLM3 cells were much smaller than those from shCtrl-HCCLM3 cells. The tumor volume of shPRPF6-HCCLM3 cells showed a much slower growth rate than those of shCtrl-HCCLM3 cells (Figure 6C), and the tumor weight of shPRPF6-HCCLM3 cells was also lower than that of control cells (Figure 6D). Furthermore, the results from IHC experiments indicated that the expression levels of PRPF6 were significantly reduced, accompanied with the reduction of Ki-67 expression in tumor tissues from the shPRPF6 group (Figure 6E). In addition, western blotting results showed that the protein expression levels of PRPF6 were significantly lower in tumors from shPRPF6-HCCLM3 cells than those from the shCtrl group (Figure 6F). Collectively, these results indicate that PRPF6 is involved in promotion of cell growth and proliferation in HCC.

4 | DISCUSSION

Hepatocellular carcinoma is the most common primary malignant tumor of the liver, and the incidence is predominant in men. Previous studies have shown that AR, as a sexual hormone receptor, exerts an oncogenic function in HCC progression through the transactivation of its target genes, including *CCRK*, *VEGFA*, and *FKBP5*.^{6,8,12,29,31} In addition, constitutive active AR-Vs have been found to participate in the promotion of HCC development. However, modulation of AR action is largely unknown in HCC. Here, we found that PRPF6, a precursor mRNA splicing factor, associates with AR/AR-Vs and enhances AR self-transcription, thereby enhancing AR/AR-V7-mediated transcriptional activity even in the absence of DHT. Subsequently, PRPF6 promotes cell growth/proliferation in HCC (Figure 6G).

Zhang et al¹² found that AR is primarily localized in the nucleus in high AR-expressing HCC cells. In agreement with this finding, our results showed that AR is predominantly expressed in the nucleus of HCCLM3 cells even in the absence of DHT. We also provided evidence that PRPF6 interacts with AR/AR-Vs in the

nucleus of HCC cells without or with DHT. The interaction domain of PRPF6 with AR is mainly located in the 309-463 aa fragment of PRPF6, which consists of the first 5 TPR motifs. The results suggest that the first 5 TPR motifs, as the putative protein-protein interaction motifs, are required for the interaction between PRPF6 and AR. Furthermore, the luciferase assay results showed that PRPF6 enhances AR/AR-V7-induced target gene transcription in HCC cells. The functional domain in PRPF6 for upregulating AR action is in the 309-463 aa fragment, which is similar to the binding domain of PRPF6 with AR.

Previous studies have shown some nuclear receptor coregulators, as RNA binding proteins, play dual functions in the regulation of transcription and RNA splicing.³²⁻³⁴ In this study, our results indicated that PRPF6 as a splicing factor participates in upregulating AR self-transcription. Depletion of PRPF6 not only decreased AR mRNA expression level, but also reduced partial AR target genes, including *CCRK*. Notably, ectopic expression of PRPF6 enhanced AR expression accompanied with the increase in AR-Vs expression, although we did not study the splicing function of PRPF6 on the AR gene. H3K36me3 is highly related to the active transcription process as well as transcription extension and splicing.^{35,36} Our results indicated that PRPF6 depletion reduced AR recruitment and the levels of histone H3K36me3 at the ARE I region of the AR gene, suggesting that PRPF6 involvement in AR self-transcription might be due to the recruitment of other factors, thereby altering H3K36me3 levels to maintain the active chromatin state, subsequently promoting the transcription of the AR gene. In the future, it would be valuable to identify the factors that are recruited together with PRPF6 to regulate AR transcription.

Studies have shown that splicing factors as oncoproteins or tumor suppressors play crucial roles in carcinogenesis and tumor development.^{37,38} Recent studies have reported that PRPF6 promotes the proliferation of colon cancer cells.²⁶ Here, we provided evidence, by TCGA analysis and IHC assay, to show that PRPF6 is highly expressed in HCC tissues, and the higher expression of PRPF6 is positively correlated with tumor progression and poor prognosis. Our results have further shown that PRPF6 depletion reduces cell growth/proliferation in AR-positive HCC cells, but not in AR-negative HCC cells, suggesting that the function of PRPF6 on HCC growth is at least partially related with oncogenic AR signaling. In summary, our study suggests that PRPF6 as a splicing factor is involved in enhancing AR gene self-transcription, thereby coactivating the oncogenic AR/AR-Vs signaling to participate in the promotion of HCC progression. The function of PRPF6 on the upregulation of AR gene transcription would provide a novel therapeutic strategy for AR-targeted treatment in HCC.

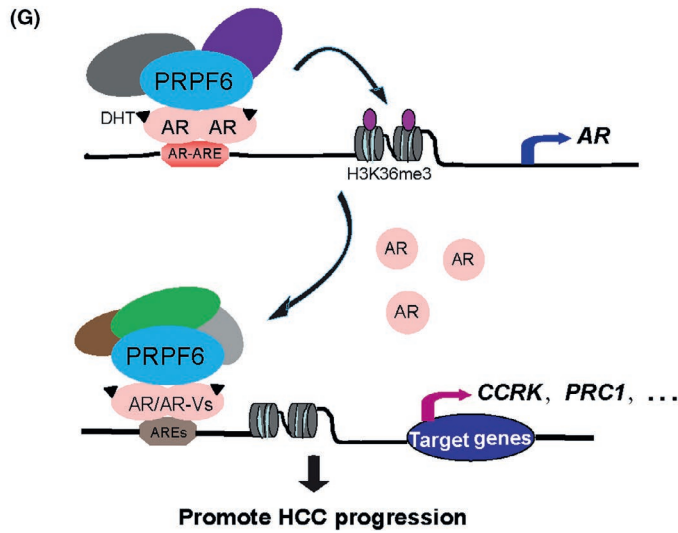
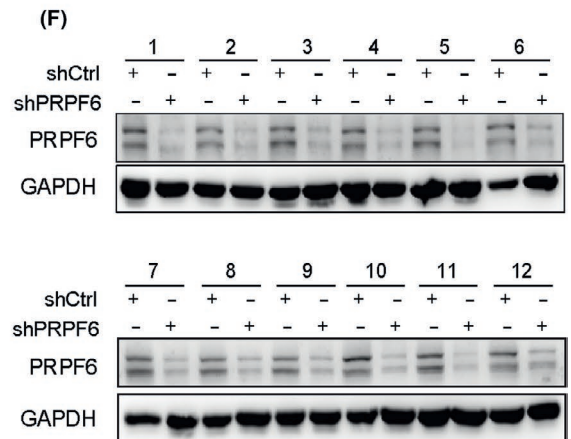
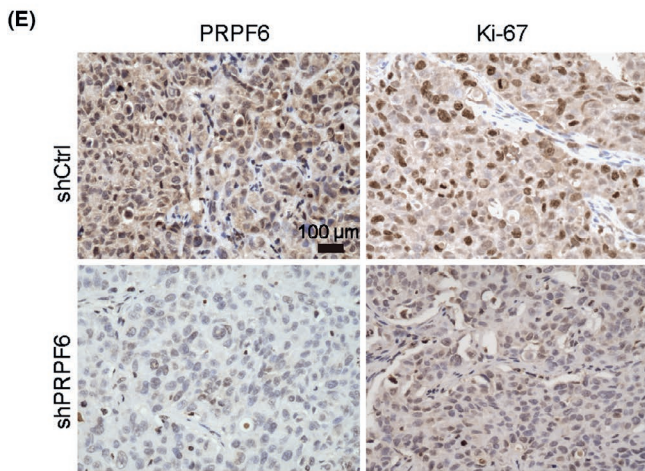
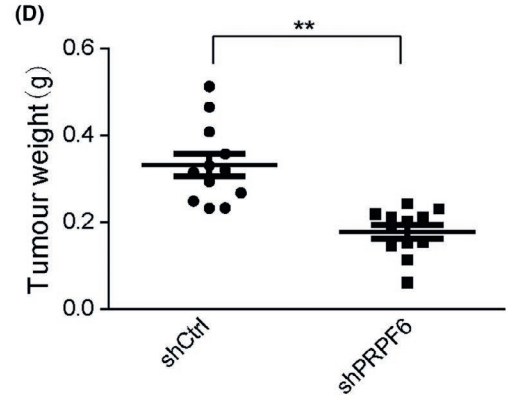
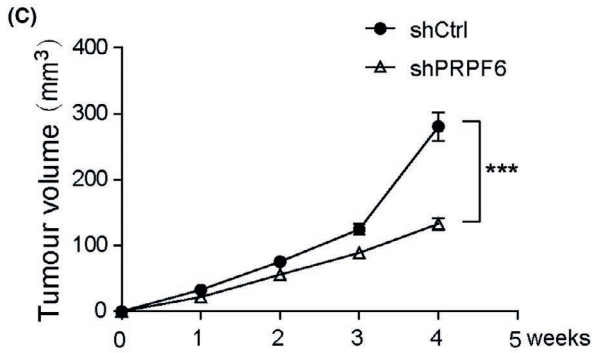
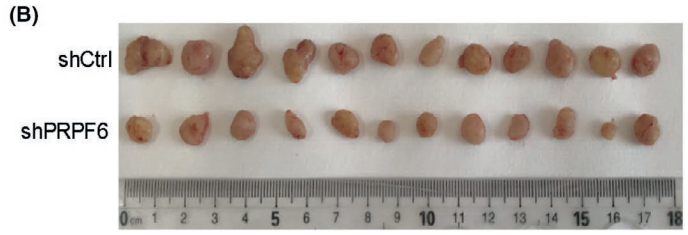
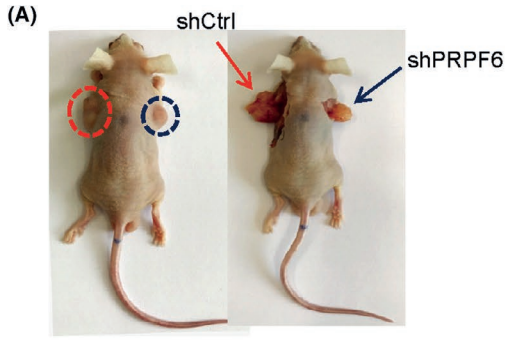


FIGURE 6 Pre-mRNA processing factor 6 (PRPF6) depletion inhibits hepatocellular carcinoma (HCC) tumor growth in mouse xenograft. A, Representative photographs showing xenograft tumors in male BALB/C-null mice. Left, control (shCtrl); right, shPRPF6. B, PRPF6 depletion significantly inhibits tumor growth in vivo. C, PRPF6 depletion significantly reduces HCC tumor burden. *** $P < .001$. D, PRPF6 depletion significantly decreases HCC tumor weight. ** $P < .01$. E, Expression of PRPF6 and Ki-67 in xenograft tumors was analyzed by immunohistochemistry (200 \times). F, Protein expression of PRPF6 in xenograft tumors was detected by western blotting. G, Schematic representation of upregulation function of PRPF6 on AR self-transcription, thereby coactivating androgen receptor (AR)/AR variant (AR-Vs)-induced transactivation to promote the progression of HCC. ARE, androgen responsive element; DHT, dihydrotestosterone

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DISCLOSURE

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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