

## ORIGINAL ARTICLE

# TCOF1 coordinates oncogenic activation and rRNA production and promotes tumorigenesis in HCC

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## Abstract

Treacle ribosome biogenesis factor 1 (TCOF1) is a nucleolar factor that regulates ribosomal DNA (rDNA) transcription in the nucleolus. TCOF1 has been previously reported to be implicated in Treacher Collins–Franceschetti syndrome (TCS), a congenital disorder of craniofacial development. Except TCS, TCOF1 has not been reported to be involved in other diseases so far. Here, we show that TCOF1 expression is aberrantly elevated in human hepatocellular carcinoma (HCC) and correlates with HCC progression and poor outcome. In vitro and in vivo studies reveal oncogenic roles of TCOF1 in HCC. Mechanistically, TCOF1 regulates KRAS-activated genes and epithelial-mesenchymal transition (EMT) genes in HCC and is required for the increased ribosomal RNA (rRNA) production, a hallmark of cancer. Interestingly, our analysis reveals an inverse correlation between TCOF1 expression and tumor infiltration of antitumor immune cells, suggesting that TCOF1 may also have an important impact on antitumor immune responses in HCC. Together, our findings support a model in which TCOF1 coordinates oncogenic activation and rRNA production to promote HCC tumorigenesis. The inverse correlation between TCOF1 expression and the infiltration of antitumor immune cells opens a new avenue to understanding the promoting role of TCOF in HCC tumorigenesis.

## KEYWORDS

HCC, immune infiltration, KRAS, rRNA production, TCOF1

## 1 | INTRODUCTION

Treacle ribosome biogenesis factor 1 (TCOF1, also called Treacle) is a nucleolar factor which acts in collaboration with upstream binding factor (UBF) to regulate ribosomal DNA (rDNA) transcription in eukaryotes.<sup>1</sup> Haploinsufficiency of *TCOF1* in mice leads

to ribosome biogenesis perturbation, cell cycle arrest, increased cell death, and reduced proliferation and migration of neural crest cells.<sup>2,3</sup> Haploinsufficiency of *TCOF1* also impairs the migration of human neural crest cells and mesenchymal stem cells.<sup>4</sup> The increased cell death in TCOF1-deficient cells, which is independent of TCOF1 deficiency's effect on ribosome biogenesis,

**Abbreviations:** EMT, epithelial-mesenchymal transition; HCC, hepatocellular carcinoma; TCOF1, treacle ribosome biogenesis factor 1; TCS, Treacher Collins–Franceschetti syndrome.

Chaoshen Wu, Dian Xia, and Dian Wang contributed equally to this work.

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is attributed to the stabilization of the tumor suppressor p53.<sup>3</sup> Reduced ribosomal RNA (rRNA) transcription, stabilization of p53, and increased cell death were also observed in TCOF1-depleted zebrafish model.<sup>5</sup>

Treacle ribosome biogenesis factor 1 is also involved in DNA damage response (DDR).<sup>6–10</sup> Mechanistically, DNA double-strand breaks (DSBs) in rDNA repeats activate ATM kinase to phosphorylate TCOF1, and the phosphorylated TCOF1 recruits the MRE11-RAD50-NBS1 (MRN) complex through the NBS1 subunit. Subsequently, rDNA transcription is suppressed, and the rDNA is translocated into nucleolar caps. Besides its role in rDNA transcription, TCOF1 is also involved in transcription events outside nucleolus. A recent report demonstrated that TCOF1 exits nucleolus and is recruited to telomeres by TRF2, where it acts to suppress telomere transcription by pol II.<sup>11</sup> In another study, a genome-wide analysis in a murine neuroblastoma cell line showed that TCOF1 depletion leads to dramatic change in a wide range of genes.<sup>12</sup> Genes that promote proliferation are likely to have similar expression patterns to that of TCOF1. These studies suggest that the regulatory role of TCOF1 in transcription is beyond rDNA transcription.

Treacle ribosome biogenesis factor 1 mutations are closely associated with Treacher Collins–Franceschetti syndrome (TCS), a congenital disorder of craniofacial development.<sup>13,14</sup> The majority of mutations result in nonsense-mediated mRNA decay or truncated proteins. Except TCS, there is no report of involvement of TCOF1 in other diseases. Here, we report that TCOF1 plays oncogenic roles in human hepatocellular carcinoma (HCC). Mechanistically, TCOF1 coordinates rDNA transcription, oncogenic activation, and immune infiltration to promote tumorigenesis in HCC.

## 2 | MATERIALS AND METHODS

### 2.1 | Data source and statistical analyses

The data for mRNA expressions (mRNA SeqV2) and clinical phenotypes of human liver HCC were obtained from TCGA (<http://portal.gdc.cancer.gov/>) database and the UCSC Xena TCGA hub (<https://tcga.xenahubs.net>). Statistical analyses were performed with R 3.6.1 software (<http://www.r-project.org/>). Log-rank test was used to assess the difference between the survival curves. Gene set enrichment analysis (GSEA) was performed using the Bioconductor package clusterProfiler (version 3.14.0) based on Hallmark gene sets from GSEA MSigDB. To quantify infiltration levels of immune cells in each of the HCC samples, single-sample gene set enrichment analysis (ssGSEA) was performed using Bioconductor package gsva (version 1.34.0) with default settings. ssGSEA is a projection methodology to project each sample within a dataset onto a space of gene set enrichment scores.<sup>15</sup> The gene set of human immune cell subtypes used in this study was described elsewhere.<sup>16</sup>

### 2.2 | Cell culture and gene silencing

Human HCC cell lines were originally obtained from the American Type Culture Collection (ATCC). Cells were cultured as previously described.<sup>17</sup> Mycoplasma contamination was regularly examined, and no contamination occurred during this study. The shRNAs targeting TCOF1 (TCOF1-sh1: GAGGAAGAAGCAGAAGCCGAA; TCOF1-sh2: CAGCAGAGCCCTCAGCAAATA) and a negative control shRNA with scrambled sequence (shControl: TTCTCCGAACGTGTACAGT) were constructed into GV493 lentivirus vector (GeneChem). Lentiviruses were produced by the transfection of 293T cells with plasmids using packaging Mix (GeneChem). For gene silencing, HCC cells were infected with the lentiviruses and selected with puromycin for 2 weeks.

### 2.3 | RNA extraction, reverse transcription, and quantitative real-time PCR

RNA extraction, reverse transcription, and quantitative real-time PCR were performed as previously described.<sup>17</sup> Primers used quantitative real-time PCR are as follows:  $\beta$ -actin forward, 5'-CTGGAACGGTGAAGGTGACA-3',  $\beta$ -actin reverse: 5'-AAGGGACTTCCTGTAACAACGCA-3'; pre-rRNA forward, 5'-GGCGGTTTGAGTGAGACGAGA-3', pre-rRNA reverse: 5'-ACGTGCGCTCACCGAGAGCAG-3'.  $\beta$ -actin was used as internal control for normalization.

### 2.4 | Western blotting, immunofluorescence, and antibodies

Western blotting and immunofluorescence were performed as previously described.<sup>17</sup> Primary antibodies used in this study are as follows: anti-TCOF1 (Proteintech, 11003-1-AP), anti-NPM1 (ThermoFisher, FC-61991), anti-Vimentin (Proteintech, 10366-1-AP), anti-E-cadherin (Proteintech, 20874-1-AP), and anti- $\beta$ -Tubulin (Proteintech, 66240-1-Ig).

### 2.5 | Cell Counting Kit 8, colony formation, wound healing, and transwell assays

Cell Counting Kit 8 (CCK-8) assay, colony formation assay, wound healing assay, and transwell cell invasion assay were performed as previously described.<sup>17</sup> Three independent experiments were performed for statistical analysis.

### 2.6 | Flow cytometry analysis

Cells were grown in a six-well plate to 80% confluence. Then, trypsin without EDTA was used to treat cells to obtain

single-cell suspension. For cell cycle analysis, single-cell suspension ( $1 \times 10^6$  cells/mL) was fixed in 70% ice-cold ethanol overnight, and then washed and stained in dark in PI/RNase staining buffer at room temperature for 30 minutes following the manufacturer's manual (KeyGen, KGA512). For apoptosis analysis, cell suspension ( $5 \times 10^5$  cells/mL) in  $1 \times$  binding buffer was stained with Annexin V-kFluor647 and PI following the manufacturer's manual (KeyGen, KGAV115). Three independent experiments were performed for statistical analysis.

## 2.7 | Chromatin immunoprecipitation

Cells were crosslinked for 10 minutes with 1% formaldehyde in PBS and quenched with glycine. Cells were then lysed in RIPA buffer and subjected to sonication to shear chromatin. After centrifugation, the supernatant was precleared with Protein G magnetic beads (CST, #9006) and then incubated with rabbit anti-POLR1A antibody (Proteintech, 20595-1-AP) or normal rabbit IgG. Antibody-bound protein/DNA complexes were then purified using Protein G magnetic beads followed by elution with elution buffer (1% SDS and 0.1 M NaHCO<sub>3</sub>). To reverse formaldehyde crosslinking, the eluates were incubated in 0.2 M NaCl for 5 hours at 65°C. After proteinase K digestion, the immunoprecipitated DNA was purified by phenol-chloroform extraction and ethanol precipitation and subjected to analysis by qRT-PCR.

## 2.8 | data analysis

RNA sequencing (RNA-seq) library construction and RNA-seq were completed at BGI Company. Paired-end 150-bp sequencing was carried out on BGISEQ-500. The quality control for the sequencing data was accomplished with FASTQC version 0.11.8. Clean reads were aligned to human reference genome GRCh38 with HISAT2 version 2.1.0. The aligned reads were combined, and the number of reads for each gene was counted with Subread featureCounts pipeline version 1.6.4. Differential gene expression analysis was performed by the R package DESeq2 version 1.26.0.

## 2.9 | Mice xenograft model

Equal number of TCOF1 knockdown cells ( $5 \times 10^6$  cells) and negative control cells were subcutaneously injected into the axillary fossae of 4-week-old male BALB/c nude mice. Tumor volume was calculated with the formula  $TV = \text{length} \times \text{width}^2 \times 0.5$ . At the end point, all mice were sacrificed, and tumors were excised and weighed. All animal experiments were approved by the Animal Care Committee of the Xuzhou Medical University.

## 3 | RESULTS

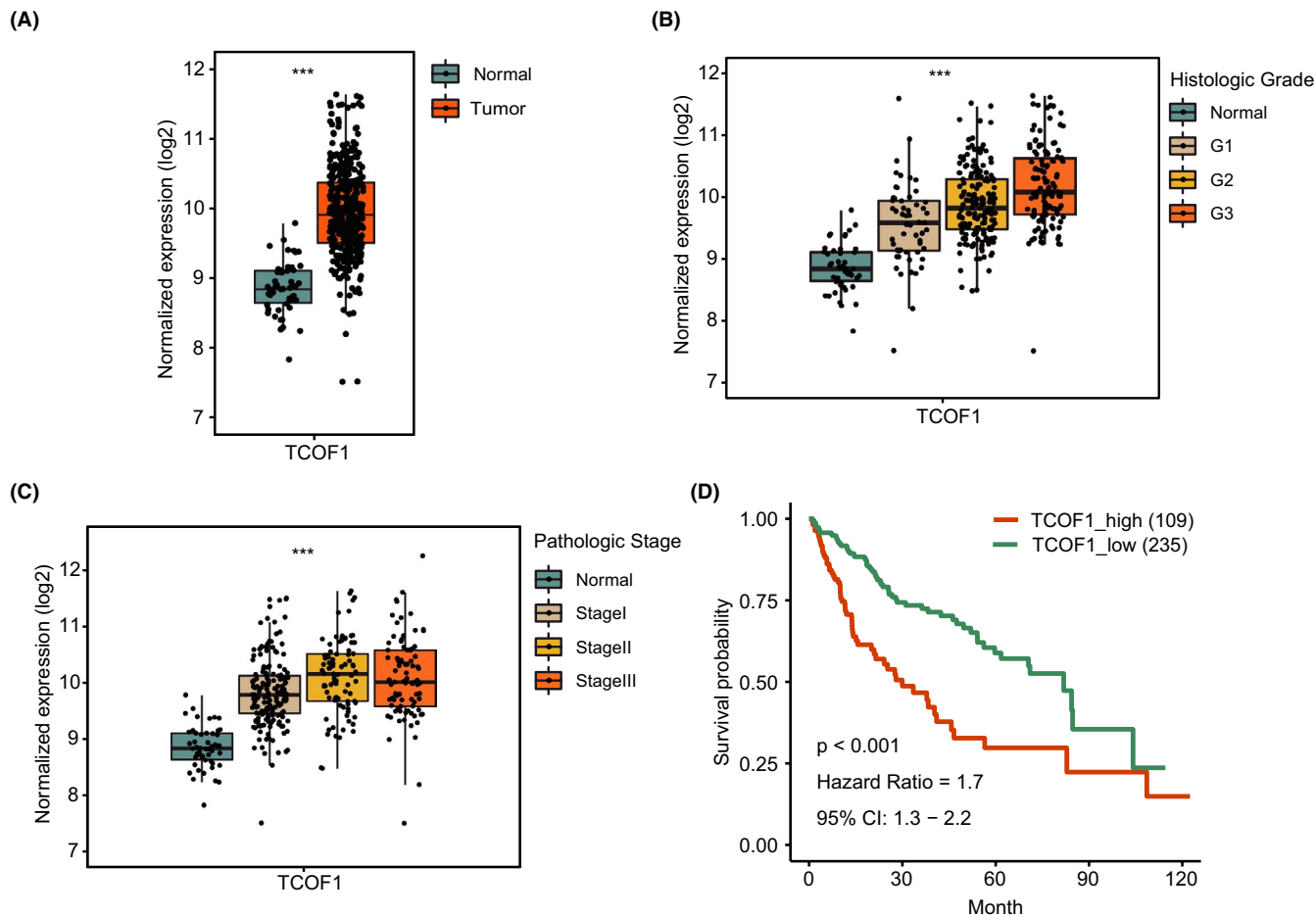
### 3.1 | TCOF1 expression is aberrantly elevated in HCC and correlates with HCC progression and poor outcome

To investigate the potential role of TCOF1 in HCC, we initially performed a survey of TCOF1 expression in HCC samples and normal liver tissue samples utilizing the RNA-seq data from the TCGA dataset. The results revealed that the TCOF1 was markedly overexpressed in HCC samples relative to normal liver tissue samples (Figure 1A). To investigate this further, we also analyzed TCOF1 expression in HCC samples of different pathologic stages and histologic grades. Cases lacking histologic grade information or pathologic stage information were excluded from these analyses. The results demonstrated that TCOF1 expression positively correlated with advanced pathologic stages and histologic grades, suggesting its positive correlation with cancer progression (Figure 1B,C).

Next, we continued to examine the clinical significance of TCOF1 expression in HCC. The expression levels of TCOF1 were categorized into high- and low-expression groups using the optimal cutoff value determined by the `surv_cutoff` function of the R package `survminer`. Log-rank test was performed to assess the difference between the survival curves. As seen in Figure 1D, high TCOF1 expression was associated with significantly worse overall survival probability in patients with HCC. These above data collectively suggest that TCOF1 may play an important role in human HCC.

### 3.2 | TCOF1 silencing inhibits HCC cell proliferation and induces cell cycle arrest and apoptosis

To investigate the regulatory role of TCOF1 in HCC, we knocked down the expression of TCOF1 in two separate HCC cell lines (Figure 2A) and examined its effect on HCC cell proliferation by CCK8 analysis. As seen in Figure 2B, upon TCOF1 knockdown, cell proliferation was significantly inhibited in both cell lines. We also performed colony formation assay because the cell capacity to form cell clones may reflect the potential for tumor growth. As shown in Figure 2C,D, the capacity of both cell lines to form cell clones was dramatically reduced following TCOF1 silencing. Next, we examined the role of the TCOF1 in cell cycle regulation and apoptosis by flow cytometry. The results showed that TCOF1 silencing led to cell cycle arrest at G<sub>0</sub>/G<sub>1</sub> phase and resulted in elevated apoptosis rate (Figure 2E-H). The effect of TCOF1 silencing on HCC cell apoptosis was further confirmed by Western blot results which showed that cleaved Caspase 3 levels increased in TCOF1-depleted HCC cells (Figure S1). Together, these data suggest that TCOF1 may play a promoting role in HCC tumorigenesis.



**FIGURE 1** Treacle ribosome biogenesis factor 1 (TCOF1) expression is aberrantly elevated in hepatocellular carcinoma (HCC) and associated with cancer progression and poor prognosis. A, TCOF1 expression was analyzed in HCC samples ( $n = 371$ ) and normal liver tissue samples ( $n = 50$ ). B, C, TCOF1 expression was correlated with cancer progression in HCC. Cases lacking histologic grade information or pathologic stage information were not included in these analyses. D, High expression of the TCOF1 was associated with poor prognosis of HCC patients. The number of cases analyzed in each group is indicated. \*\*\* $P < .001$ , log-rank test

### 3.3 | TCOF1 silencing inhibits migration and invasion of HCC cells

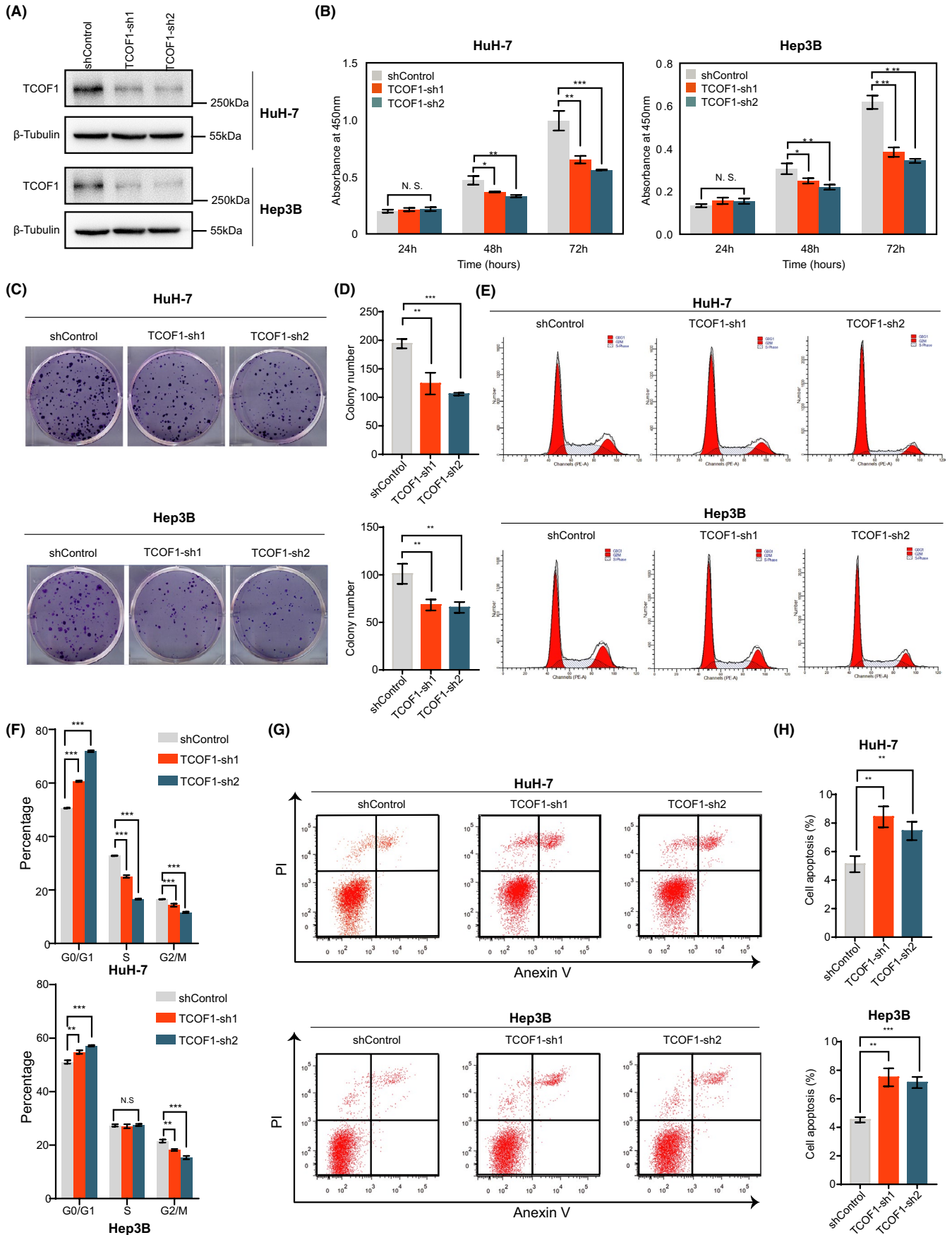
Next, we investigated the role of TCOF1 in HCC cell migration and invasion. Cancer cell capacity of migration and invasion reflects its potential in cancer metastasis. Wound healing assay was performed to assess the effect of TCOF1 silencing on HCC cell migration. As shown in Figure 3A,B, TCOF1 knockdown in HCC cells significantly inhibited cell migration. To investigate the role of TCOF1 in HCC cell invasion, transwell cell invasion assay was carried out. Consistent with the wound healing assay results, TCOF1 silencing markedly reduced HCC cell invasion (Figure 3C). These data demonstrated that TCOF1 plays a promoting role in HCC cell migration and invasion. To further substantiate our findings, we also examined

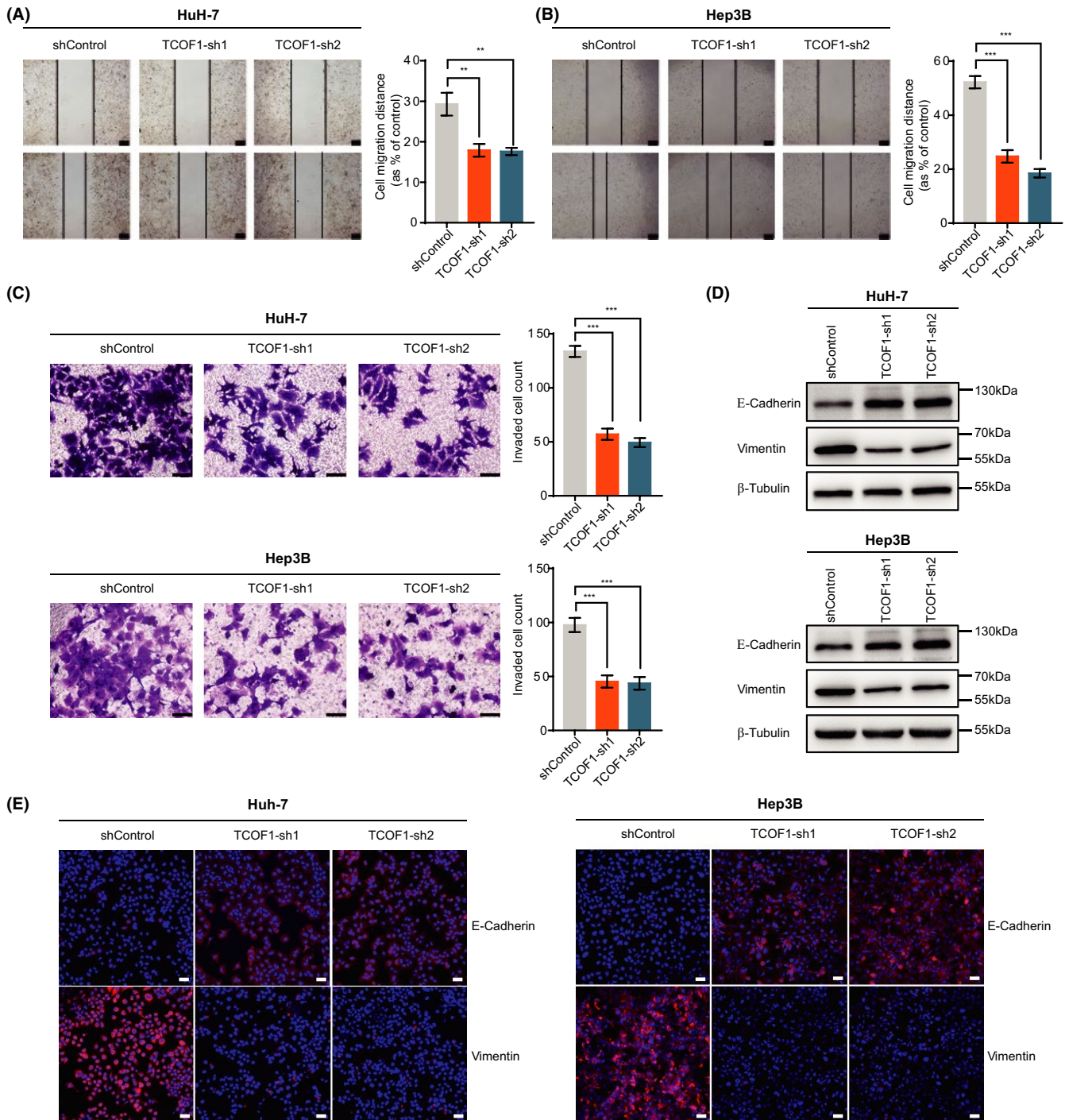
the change in the expression of epithelial mesenchymal transition (EMT) marker genes in HCC cells following TCOF1 knockdown. In order to do so, Western blotting assays and immunostaining assays were performed. The results showed that HCC cells underwent a mesenchymal-epithelial transition (MET)-like change upon TCOF1 silencing, indicating that TCOF1 may promote EMT in HCC cells (Figure 3D,E, Figure S2).

### 3.4 | TCOF1 silencing mitigates HCC tumorigenicity in vivo

To investigate the role of TCOF1 in regulating HCC tumorigenesis in vivo, we established a mouse xenograft model using

**FIGURE 2** Treacle ribosome biogenesis factor 1 (TCOF1) depletion inhibits hepatocellular carcinoma (HCC) cell proliferation and induces cell cycle arrest and apoptosis. A, Knockdown efficiency of TCOF1 in HCC cells was verified by Western blotting assays.  $\beta$ -tubulin was used as loading control. B, CCK-8 assay was employed to evaluate the effect of TCOF1 depletion on HCC cell proliferation. C, D, Colony formation assays were carried out to examine the effect of TCOF1 depletion on colony-forming ability of HCC cells. E-H, TCOF1 depletion induces cell cycle arrest and apoptosis of HCC cells. Flow cytometry analyses were carried out to evaluate the effect of TCOF1 depletion on cell cycle progression and apoptosis of HCC cells. For statistical analyses, three independent experiments were carried out and the results were quantified and represented as mean + SD \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ , unpaired  $t$  test





**FIGURE 3** Treacle ribosome biogenesis factor 1 (TCOF1) depletion inhibits hepatocellular carcinoma (HCC) cell migration and invasion. A, B, TCOF1 depletion inhibits HCC cell migration. Wound healing assay was carried out to assess the effect of TCOF1 depletion on HCC cell migration. Three independent experiments were carried out, and the results were quantified and represented as mean + SD  $**P < .01$ ,  $***P < .001$ , unpaired *t* test. Scale bar: 100  $\mu$ m. C, TCOF1 depletion inhibits HCC cell invasion. Transwell invasion assay was employed to evaluate the effect of TCOF1 depletion on HCC cell invasion. Three independent experiments were carried out and the results were quantified and represented as mean + SD  $**P < .01$ ,  $***P < .001$ , unpaired *t* test. Scale bar: 100  $\mu$ m. D, E, TCOF1 depletion impacts the expression of epithelial-mesenchymal transition (EMT) marker genes. Expression levels of EMT marker genes were measured by Western blotting and immunofluorescence staining. Scale bar: 50  $\mu$ m

TCOF1-knockdown cells and negative control cells. Each mouse was monitored regularly, and the tumor volume was measured. Tumor derived from the TCOF1-knockdown cells showed slower growth

and smaller volume compared with that derived from the control cells. Consistently, at the end point of the study, significant differences in tumor volume and tumor weight between the two groups

were observed (Figure 4). The *in vivo* mouse xenograft model results demonstrate that TCOF1 promotes HCC growth *in vivo*, corroborating our *in vitro* experiments results.

### 3.5 | TCOF1 regulates oncogenic transcriptional programs in HCC

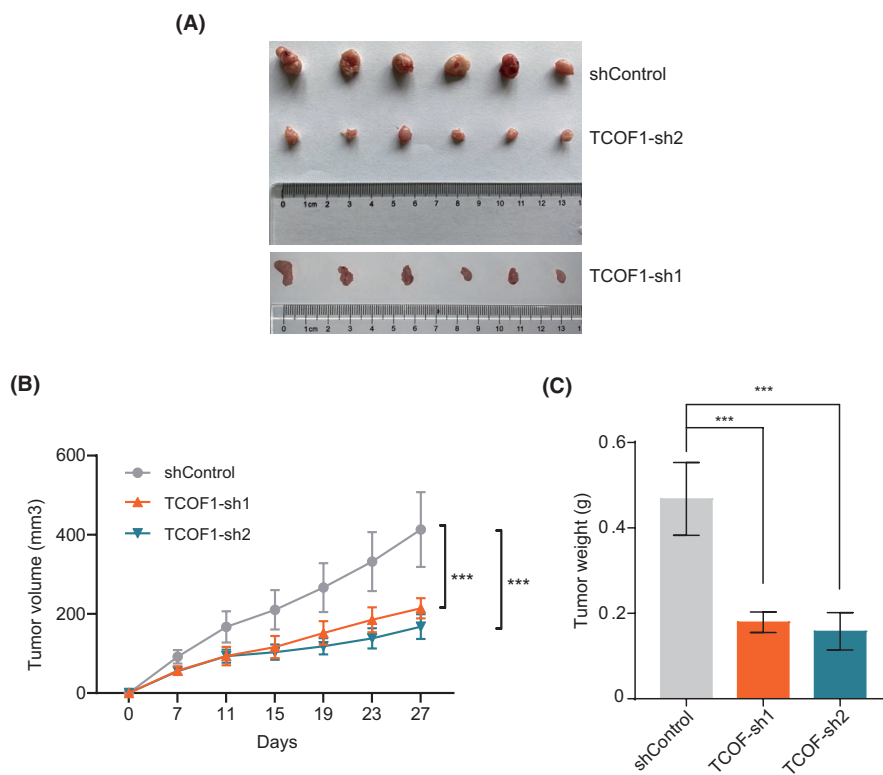
Treacle ribosome biogenesis factor 1 is well known as a regulator in rDNA transcription. However, mounting evidence demonstrates that TCOF1 is also involved in transcription elsewhere in the genome.<sup>11,12</sup> Thus, to understand the mechanisms by which TCOF1 promotes HCC tumorigenesis, we carried out RNA-seq to identify genes affected by TCOF1 depletion. The Pearson correlation of the three biological replicates within each group was greater than 0.99, indicating this dataset provides a high-quality profile of the TCOF1-associated transcriptome in HCC cells (Figure 5A). Differential gene expression analysis with DESeq2 software showed that the TCOF1-sh1 group and the TCOF1-sh2 group showed similar gene expression patterns, which are different from that of the shControl group (Figure 5B). Of these differentially expressed genes (DEGs), 2447 genes are commonly found in both the TCOF1-sh1 and TCOF1-sh2 groups (Figure 5C). To eliminate the off-target effect, we only used these common DEGs for the subsequent analyses.

To gain insight into the TCOF1-regulated gene expression profile, gene set enrichment analysis (GSEA) was performed using the Bioconductor package clusterProfiler based on hallmark gene sets from the Molecular Signatures Database. The results showed that

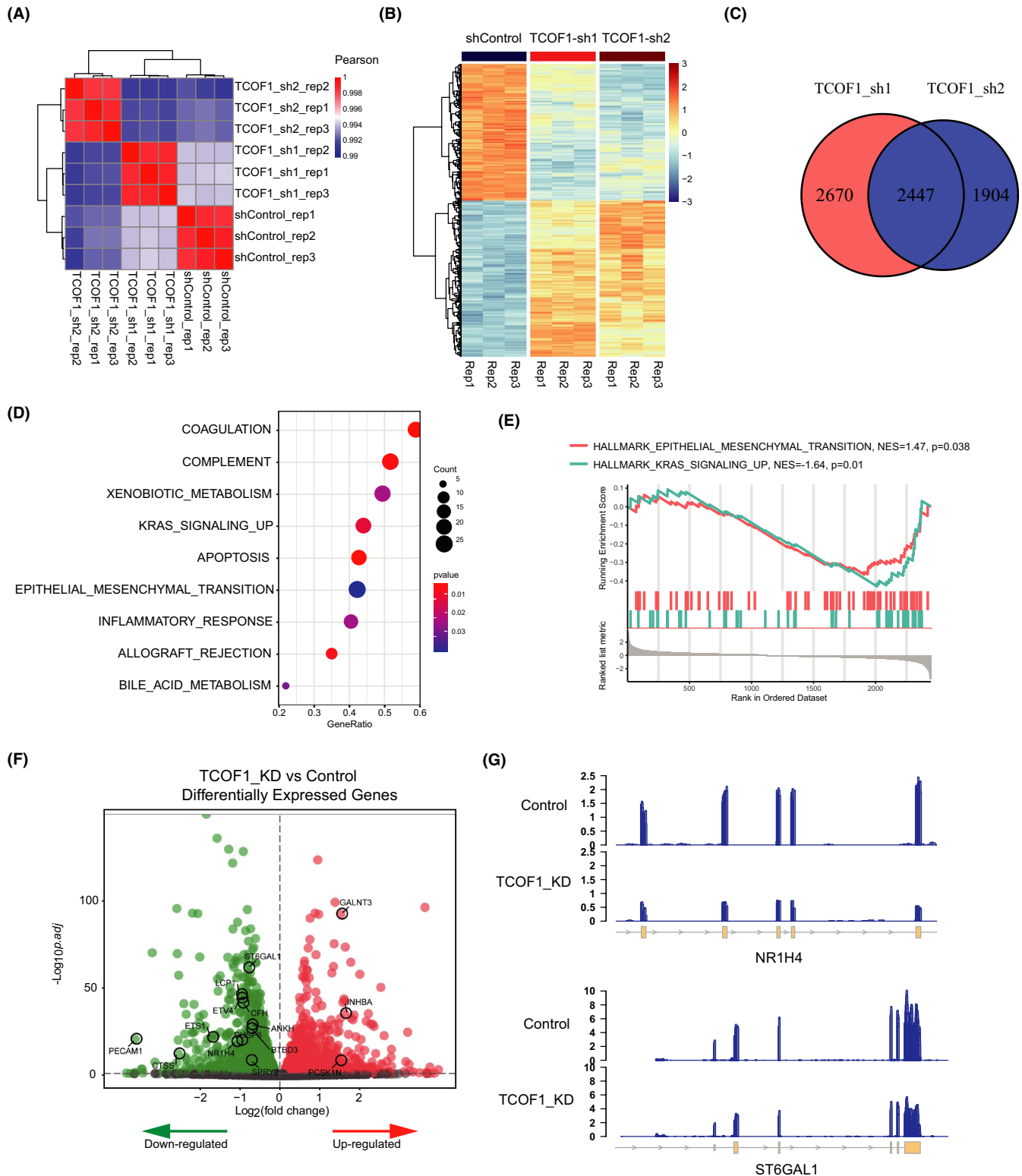
the genes upregulated by KRAS activation were significantly suppressed upon TCOF1 silencing, supporting a requirement for TCOF1 in KRAS activation-induced oncogenesis (Figure 5D-G). Moreover, the suppression of EMT genes was also observed upon TCOF1 depletion, consistent with our above-described results (Figure 5D,E). Together, these data demonstrate that TCOF1 plays essential roles in oncogenic activation in HCC.

### 3.6 | TCOF1 is required for high levels of rRNA production in HCC

Increased rDNA transcription is a well-known hallmark of cancer and high expression levels of rRNA genes (rDNA) are required for enhanced cancer cell proliferation. Therefore, we next sought to examine the effect of TCOF1 silencing on rRNA production in HCC cells. To this end, we first examined the subcellular localization of TCOF1 proteins. The results showed that TCOF1 proteins predominantly reside in the nucleolus of HCC cells supporting its involvement in rRNA production in HCC cells (Figure 6A). Next, we extracted total RNA from the TCOF1-depleted HCC cells and the control cells, and the same amounts of those total RNAs were used for agarose gel electrophoresis (Figure 6B). The amounts of 28S rRNA and 18S rRNA were then quantified, and the results demonstrated that rRNA production was significantly impaired in HCC cells following TCOF1 depletion (Figure 6C). To further confirm this result, we went on to measure pre-rRNA levels using quantitative real-time PCR. Pre-rRNA levels reflect rRNA production at the transcription level. As shown, a reduction in pre-rRNA levels was observed upon TCOF1



**FIGURE 4** Treacle ribosome biogenesis factor 1 (TCOF1) depletion suppresses hepatocellular carcinoma (HCC) growth *in vivo*. A, Tumors derived from TCOF1-depleted HCC cells and the negative control HCC cells in six male nude mice are presented. B, Tumor volume of both groups was measured at the indicated time points, and the data were presented as growth curves. \*\*\* $P < .001$ , analysis of variance (ANOVA). C, Tumor mass of both groups was weighed and graphed. \*\*\* $P < .001$ , unpaired t test



**FIGURE 5** Treacle ribosome biogenesis factor 1 (TCOF1) is crucial for activation of oncogenic transcriptional programs in hepatocellular carcinoma (HCC). A, Heatmap of Pearson correlations among RNA-seq samples. B, Hierarchical clustering of differentially expressed genes (DEGs). Columns represent individual experiments and rows correspond to individual genes. C, Venn diagram shows that 2447 DEGs were commonly identified in both TCOF1-depleted groups. D, Gene set enrichment analysis (GSEA) enrichment plot for hallmark terms that were significantly affected by TCOF1 depletion. E, Enrichment score (ES) plots show that genes upregulated by KRAS activation and genes involved in epithelial-mesenchymal transition (EMT) are downregulated upon TCOF1 depletion. F, Volcano plot showing genes differentially expressed between the TCOF1-depleted group and the control group. Significantly dysregulated genes with false discovery rate (FDR) < 0.05 are shown as red dots (upregulated) and green dots (downregulated). The representative KRAS-activated genes are labeled. G, RNA-Seq analysis results of two KRAS-activated genes. RNA-Seq analysis showing reduced expression of NR1H4 and ST6GAL1 upon TCOF1 depletion

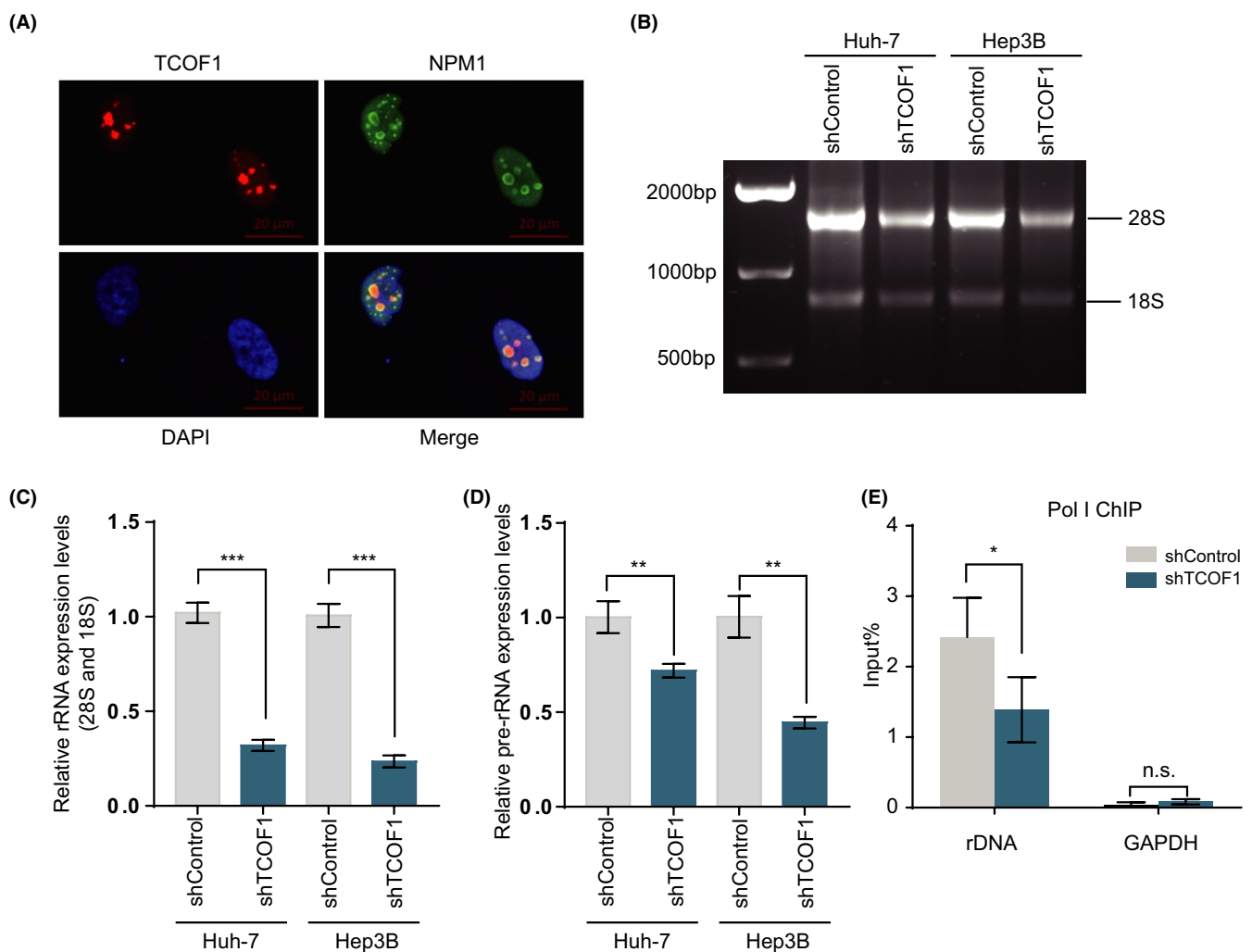


depletion, while the transcription of *GAPDH*, which served as a negative control, was not affected (Figure 6D, Figure S3). In addition, TCOF1 depletion also reduced Pol I occupancy at the rRNA gene (Figure 6E). Taken together, these results demonstrate that TCOF1 is required for the hyperactivation of pol I transcription of rRNA genes in HCC cells.

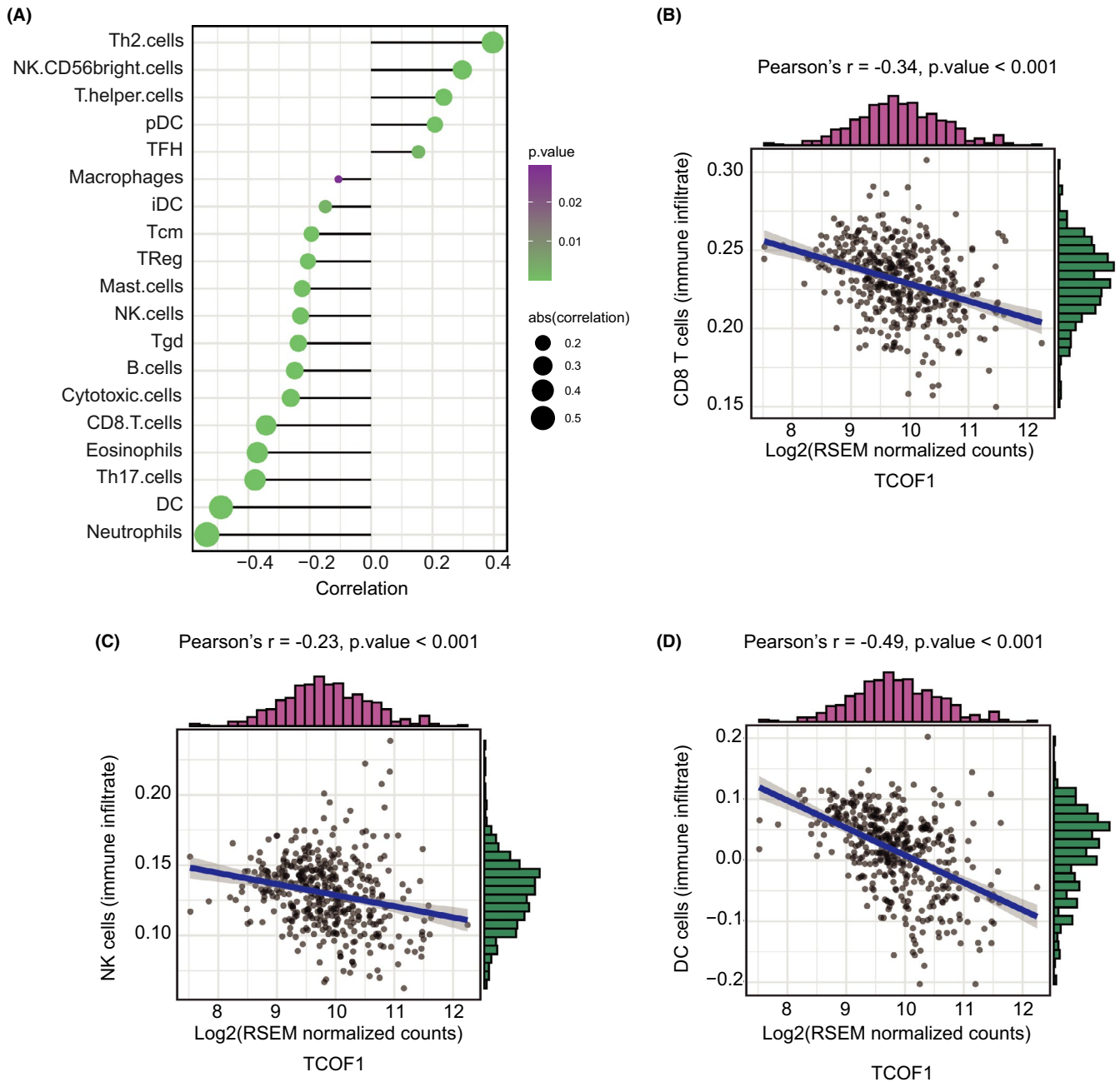
### 3.7 | TCOF1 expression closely correlates with anticancer immune infiltration in HCC

The interplay between tumors and their immunologic microenvironment is complex and difficult to decipher, but understanding it is of great importance in developing novel therapeutic strategies. In this

study, we were interested in investigating the impact of high TCOF1 expression on tumor immune microenvironment in HCC. To do so, we first quantified infiltration levels of different immune cell types in each HCC sample using ssGSEA in the R package *gsva*. The gene signatures expressed by immune cell populations<sup>16</sup> were applied in ssGSEA for each HCC sample. Then, we analyzed the correlation between TCOF1 expression and the infiltration levels of different immune cell types. The results showed that TCOF1 expression inversely correlates with CD8<sup>+</sup> T cell, NK cell, and DC cell infiltration in HCC (Figure 7). CD8<sup>+</sup> T cells, NK cells, and DC cells are crucial immune cell types in antitumor responses and cancer immunotherapy. Our results indicate that TCOF1 may facilitate HCC tumorigenesis by two ways, promoting tumor growth and inhibiting anticancer immune infiltration.



**FIGURE 6** Treacle ribosome biogenesis factor 1 (TCOF1) is required for high levels of ribosomal RNA (rRNA) production in hepatocellular carcinoma (HCC). A, Immunostaining experiments showing subcellular localization of TCOF1 in HCC cells. Scale bar: 20  $\mu$ m. B, C, RNA gel electrophoresis showing reduced rRNA production upon TCOF1 depletion. Three independent experiments were carried out, and the results were quantified and represented as mean + SD \*\*\* $P$  < .001, unpaired  $t$  test. D, Real-time quantitative PCR showing reduced pre-rRNA expression levels upon TCOF1 depletion. Three independent experiments were represented as mean + SD \*\* $P$  < .01, unpaired  $t$  test. E, ChIP-qPCR showing reduced occupancy of pol I at rDNA repeat upon TCOF1 depletion. Three independent experiments are represented as mean + SD \* $P$  < .05, unpaired  $t$  test



**FIGURE 7** Treacle ribosome biogenesis factor 1 (TCOF1) expression closely correlates with anticancer immune infiltration in hepatocellular carcinoma (HCC). A, Correlations between TCOF1 expression and immune infiltrate. Immune cell infiltration levels in each of the HCC samples were quantified using single-sample gene set enrichment analysis (ssGSEA) in R package gsva. The gene signatures expressed by immune cell populations<sup>16</sup> were used in ssGSEA for each HCC sample. B-D, Correlations between TCOF1 expression and the infiltration levels of CD8<sup>+</sup> T cells, NK cells, and DC cells in HCC

## 4 | DISCUSSION

Mutations in TCOF1 result in deficient ribosome biogenesis and have been associated with TCS, a severe congenital craniofacial disorder. Except TCS, TCOF1 has not been reported to be involved in other diseases. In the present study, our analyses reveal that TCOF1 is aberrantly overexpressed in human HCC, and the expression levels positively correlate with HCC progression and poor outcome. Further investigation

demonstrates that TCOF1 promotes HCC tumorigenesis both in vitro and in vivo. Mechanistically, we show that TCOF1 regulates rRNA production and oncogenic activation in HCC cells. Interestingly, our results also reveal an important impact of high TCOF1 expression on HCC immune microenvironment. TCOF1 expression inversely correlates with infiltration of key antitumor immune cell types, CD8<sup>+</sup> T cells, NK cells, and DC cells. Altogether, our results suggest that TCOF1 plays a promoting role in HCC tumorigenesis via coordinating oncogenic activation, rRNA production, and anticancer immune infiltrate.

Ribosomal RNA production upregulation is a hallmark of tumor cells.<sup>18</sup> RNA Pol I transcription of rDNA is crucial for driving ribosome biogenesis and increased protein synthetic capacity to promote tumor cell proliferation. Small-molecule inhibitors of RNA Pol I transcription have been shown to be able to selectively kill malignant cells in vivo and to treat cancer.<sup>19,20</sup> Our results showed an essential role of TCOF1 in the hyperactivation of RNA Pol I transcription of rDNA in HCC cells. We reasoned that this may be one of the mechanisms by which TCOF1 promotes tumorigenesis of HCC. Except rDNA transcription, recent studies have also shown that TCOF1 regulates transcription elsewhere in the genome.<sup>11,12</sup> In this study, our data demonstrate that TCOF1 regulates oncogenic transcriptional programs in HCC, supporting a regulatory role of TCOF1 in RNA pol II transcription. However, we do not know whether this is a direct regulation or indirect regulation at this time. Future study of a genome-wide chromatin-binding profile of TCOF1 in HCC cells would help answer this question.

Tumor-infiltrating lymphocytes are key elements of the tumor microenvironment, and high immune infiltration has been associated with improved clinical outcomes in many human solid tumors. Investigation of the factors regulating tumor-immune microenvironment interplay is of seminal importance in developing effective immunotherapy strategies. Here, we show that TCOF1 expression inversely correlates with key tumor-infiltrating lymphocytes, CD8<sup>+</sup> T cells, NK cells, and DC cells, indicating it may suppress anticancer immune cell infiltration thus to facilitate tumorigenesis in HCC.

## 5 | CONCLUSIONS

Together, our findings support a model in which TCOF1 coordinates oncogenic activation and rRNA production to promote HCC tumorigenesis. The inverse correlation between TCOF1 expression and the infiltration of antitumor immune cells opens a new avenue to understanding the promoting role of TCOF1 in HCC tumorigenesis. Future studies of detailed mechanisms by which TCOF1 exerts its influence in the tumor-immune microenvironment interplay are of great importance.

## ACKNOWLEDGMENTS

The anti-NPM1 antibody was a gift from Prof Yong Liu (Cancer Institute, Xuzhou Medical University). We thank all the members in Dr Zhang's lab for insightful scientific discussion.

## DISCLOSURE

The authors declare that they have no competing interests.

## ETHICAL APPROVAL

All animal experiments were approved by the Animal Care Committee of the Xuzhou Medical University. All institutional and national guidelines for the care and use of laboratory animals were followed.

## DATA AVAILABILITY STATEMENT

RNA-seq data have been deposited into Sequence Read Archive (SRA accession PRJNA703328: <https://www.ncbi.nlm.nih.gov/sra/PRJNA703328>) and will be made publicly available upon acceptance of this manuscript.

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#### SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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