

ORIGINAL ARTICLE

Noncoding telomeric repeat-containing RNA inhibits the progression of hepatocellular carcinoma by regulating telomerase-mediated telomere length

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Abstract

Telomeric repeat-containing RNA (TERRA) is closely involved in the regulation of telomere length, which plays critical roles in tumorigenesis. However, the biological significance of TERRA in hepatocellular carcinoma (HCC) remains largely unknown. In this study, we found that HCC cells show a frequent downregulation of TERRA and its positive regulator TTAGGG repeat binding factor-1 (TRF1), whereas the negative regulator TTAGGG repeat binding factor-1 (TRF2) was upregulated. We found that TERRA, TRF1, and TRF2 contributed to poor prognosis of HCC patients. Importantly, we found that the downregulation of TERRA significantly promoted HCC cell growth and metastasis in vitro and in vivo, whereas the upregulation of TERRA showed an opposite effect. Mechanistically, downregulation of TERRA significantly increased telomerase activity and promoted telomere elongation. Moreover, the inhibitory effects of TERRA overexpression on the growth and metastasis of HCC cells were reversed by treatment with TA-65 that activates telomerase activity. In contrast, the protumor effect of TERRA downregulation was reversed by treatment with TMPyP4 that inhibits telomerase activity. Our findings reveal that TERRA plays a critical role in HCC cell growth and metastasis, indicating that TERRA is a potential therapeutic target for HCC.

KEYWORDS

hepatocellular carcinoma, telomerase activity, telomere length, TERRA, TRF1/2

Abbreviations: DFS, disease-free survival; HCC, hepatocellular carcinoma; IHC, immunohistochemistry; lncRNA, long noncoding RNA; OS, overall survival; qPCR, quantitative PCR; TERRA, telomeric repeat-containing RNA; TRF, TTAGGG repeat binding factor.

Haiyan Cao, Yulong Zhai and Xiaoying Ji contributed equally to this work.

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1 | INTRODUCTION

Telomeres are dynamic structures capping the physical end of linear eukaryotic chromosomes. They are essential for ensuring genome stability by protecting the chromosome ends from degradation and erroneous recombination.^{1,2} A series of studies have reported that telomere shortening resulted in chromosomal instability, which drives the initiation of cancers, including HCC.³

Telomeric repeat-containing RNA, an lncRNA transcribed from the subtelomeric sequences, is characterized by telomeric repeats of UUAGGG at its 3'-end. Telomeric repeat-containing RNA is involved in the maintenance and regulation of telomere homeostasis by inhibiting telomerase activity and altering the length of telomeres.⁴⁻⁷ It has also been reported that at long telomeres, the telomere protein Rif2 recruits RNase H2 to the telomere, which subsequently degrades TERRA and the R-loop of the telomere, leading to a reduction in telomere length. However, the protein expression level of Rif2 and RNase H2 were significantly reduced at short telomeres.⁸ It has been observed that TERRA inhibits telomerase activity at chromosome ends in a telomere length-dependent manner by mimicking RNA oligonucleotide (UUAGGG)₃.⁹ Additionally, TERRA is upregulated by TRF1 through an interaction between TRF1 and RNA polymerase II¹⁰ and is downregulated by TRF2 through its homodimerization domain, which was previously reported to induce chromatin compaction and to prevent the early steps of DNA damage response activation.¹¹

Telomeric repeat-containing RNA has been found to be closely related to tumorigenesis. The expression level of TERRA was frequently downregulated in various types of tumor tissues compared with normal tissues.¹²⁻¹⁴ In contrast, TERRA is upregulated and significantly associated with poor prognosis of patients with medulloblastoma.¹⁵ These findings indicate that the expression level of TERRA varies during tumor progression, possibly in a tumor type-specific manner. In addition, TERRA has been found to be a direct target of important tumor suppressor genes, such as *p53*. The expression level of TERRA is upregulated by *p53* to protect genome integrity.^{12,16} Another study by Atkinson MJ (*Sci Rep*. 2017 Feb 7) has shown that the expression of TERRA is decreased in cells with knockdown of tumor suppressor gene *Rb1* and this effect is reversed after the restoration of *Rb1* expression.

In many Asian and African countries, the incidence and mortality of HCC are the highest among various kinds of tumors. Although many key signal transduction pathways in HCC have been elucidated, including the PI3K/Akt/mTOR pathway, Jak/Stat pathway, and WNT- β -catenin pathway, the mechanism underlying pathogenesis is not completely understood.¹⁷ Recently, telomere shortening has been strongly proposed as a genetic risk factor for chronic liver disease and HCC¹⁸ and a number of lncRNAs are dysregulated in HCC.¹⁷ However, there is still limited understanding of whether TERRA, an lncRNA closely related to telomere shortening, is deregulated in HCC cells, the functional roles of TERRA, and its mechanism in HCC progression.

In the present study, we investigated the expression of TERRA and its role in regulating telomere length in HCC cells. Moreover, the

effects of TERRA on HCC cell growth and metastasis, as well as the underlying molecular mechanisms, were systematically explored. Our study provides supporting evidence for the potential application of TERRA in HCC treatment.

2 | MATERIALS AND METHODS

2.1 | Antibodies and reagents

The primary Abs used in this study and their working concentration are listed in Table S1. TA-65, which activates telomerase activity, and TMPyP4, which inhibits telomerase activity, were purchased from Selleck. The hybridization in situ kit and fluorescent probe were purchased from Roche. The TERRA fluorescent probe sequence is listed in Table S2.

2.2 | Cell culture and tissue collection

Human HCC cell lines SNU-739, SNU-368, HLE, HLF, SNU-878, and normal liver cell line Bel-7702 were purchased from Shanghai Cell Bank of the Chinese Academy of Sciences. The cell lines were authenticated using short tandem repeat DNA testing by the FMMU Center for DNA Typing in 2018. The HCC cells were routinely cultured. In addition, 176 human HCC tissue samples and clinical data were described in our previous study (permission number: KY20173189-1; date issued: 6 March 2017).¹⁹ To avoid the contamination of tumor cells, peritumor liver tissues were dissected 2-5 cm away from HCC tissue, which were further histologically confirmed by H&E staining.

2.3 | Knockdown and overexpression of target genes and cell transfection

For the generation of shRNA and overexpression vectors, specific sequences targeting the human *TRF1* and *TRF2* mRNA sequence were cloned into the pSilencer 3.1-H1 puro vector (Ambion) and pcDNA 3.1(+). Small interfering RNA was used for knockdown of the human TERRA. All shRNAs and siRNAs were synthesized by GenePharma and the sequences are provided in Table S2. For overexpression, the coding sequences of *TRF1* and *TRF2* were amplified from cDNA derived from SNU-368 by PCR assay. The primers used are listed in Table S2. We added the sticky ends to the shRNA/overexpression vectors and control vectors and constructed them by double enzyme (*EcoRI* and *BamHI*) digestion, and then connected to pSilencer 3.1-H1 puro vector or pcDNA 3.1(+) expression vector by T4 DNA ligase.

All expression plasmids were transfected with Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. Cell lines stably expressing *TRF1*-sh and *TRF2*-sh in SNU-739/SNU-368 were established by G418 (Sigma) treatment after transfection.

For the transient knockdown of TERRA, siRNA against TERRA was designed and transfected with Lipofectamine 2000. Scrambled siRNA was used as control.

2.4 | Tissue and cell hybridization in situ

In situ hybridization was undertaken using the RNAscope 2.0 HD Brown Chromogenic Reagent Kit according to the manufacturer's instructions (Advanced Cell Diagnostics). Target probes with proprietary sequences of TERRA²⁰ were designed and synthesized by Roche. The probe sequences are provided in Table S2.

Briefly, tissues and cells were immobilized with paraformaldehyde and incubated with protease, and then incubated with pre-hybridization solution for 1 hour and incubated with target probes overnight at 4°C. Finally, samples were imaged on an Olympus FV 1000 laser-scanning confocal microscope. At least 4 fields (image magnification, 200×) were captured for each sample. Positive foci (most likely resembling single positive cells) were counted manually and calculated by GraphPad Prism 6 software for statistical analysis.

2.5 | Immunohistochemistry

Paraffin-embedded tissues were sectioned and immersed in boiled citrate-disodium hydrogen phosphate buffer (pH 6.0) with high pressure for 5 min for antigen retrieval. All tissues were assessed by H&E staining to select suitable regions for IHC examination. After hydrogen peroxide block, antigen retrieval, and normal goat serum block, the sections were incubated with the primary Ab at 4°C overnight. After washing, IHC was undertaken with the Envision+ 2-step system (Dako). Paraffin sections were treated with 3,3'-diaminobenzidine and counterstained with hematoxylin. The primary Abs of TRF1/TRF2/Ki-67 used in this study and their working concentrations are listed in Table S1. A proportion score was assigned, which represented the estimated proportion of positively stained tumor cells (0, less than 10%; 1, 10%-25%; 2, 26%-50%; 3, 51%-75%; and 4, more than 75%). An intensity score was also assigned, which represented the average intensity of the positive tumor cells (0, none; 1, weak; 2, intermediate; and 3, strong). The proportion and intensity scores were then multiplied to obtain a total score, which ranged from 0 to 12. Total scores were defined as: less than 2, negative (-); 2 to less than 5, weak positive (+); 5 to less than 8, moderate positive (++); and 8 or more, strong positive (+++).

2.6 | Cell viability assay

Hepatocellular carcinoma cells were cultured in 96-well plates at a density of 1×10^4 cells/well. Cells were transfected as described above for 48 hours and were incubated with 0.5% (w/v) MTS for another 2 hours at 37°C. Then 150 μ L DMSO was added into each

well to solubilize the formazan in the cells. The OD value at 490 nm was measured using a spectrophotometer.

2.7 | Cell proliferation assay

The proliferation ability of HCC cells was detected by EdU incorporation assay kit (Ribobio) following the instructions. Cells were incubated with EdU and reacted with 1× Apollo reaction cocktail for 30 minutes when cells were transfected after 48 hours. Cells were then treated with Hoechst 33342 for 10 minutes at room temperature and visualized under a fluorescent microscope.

2.8 | In vitro cell apoptosis assay

Cell apoptosis was determined by an annexin V-FITC detection kit (BB-4101; BestBio) according to the manufacturer's protocol when cells were transfected after 48 hours. The percentages of total apoptotic cells (both early and late), defined as the annexin V-FITC positive fraction, were determined by flow cytometry.

2.9 | Cell apoptosis assay in xenograft tissue

We used TUNEL assay (Roche Applied Science) to analyze cell apoptosis in xenograft tissues according to the manufacturer's protocol. Images of TUNEL/DAPI-stained sections were grabbed by a confocal laser scanning microscope FV1000 (Olympus). The apoptosis ratio was calculated as the percentage of both TUNEL and DAPI positive nuclei after at least 500 cells were counted.

2.10 | In vitro migration and invasion assays

Cell invasion and migration abilities were assessed by Transwell assays (Millipore) and cell scratch assay. For cell scratch assay, cells were cultured in 6-well plates, the cross lines were drawn with a medium pipette tip after culturing for 24 hours. Wound closure was photographed under a microscope and then measured 48 hours after scratch. Transwell assay was carried out as previously described.²¹ Cell migration and invasion assays were undertaken using Transwell chambers (BD Biosciences) equipped with 8 μ m porosity polyvinylpyrrolidone-free polycarbonate filters that were precoated with 10% FBS for the cell migration assay, or with 50 μ L, 1 μ g/ μ L Matrigel (BD Biosciences) for the invasion assay, according to the manufacturer's instructions. Cells in 100 μ L serum-free medium were loaded in the upper chamber. After incubation for 24 hours, nonmigrated or noninvaded cells were removed with cotton swabs, while cells moved onto the lower surface of the filter were fixed and stained with trypan blue and then photographed. For each filter/Matrigel, the number of cells in 5 randomly chosen fields was counted with a microscope and the counts were averaged.

2.11 | In vivo metastasis model

Six-week-old male nude mice (BALB/c) were randomly divided into groups (6 mice/group) and were anesthetized with 2.5% sodium pentobarbital (40 mg/kg; Sigma-Aldrich). Then 2×10^6 SNU-739-shTRF1 (transfected with pcDNA3.1 TRF1), SNU-739-shTRF2 (transfected with pcDNA3.1 TRF2), and SNU-739-shCtrl (transfected with pcDNA3.1 empty vector) cells were suspended in 50 μ L Matrigel and orthotopically inoculated into the left hepatic lobe of nude mice. After 2 weeks, the mice with SNU-739-shTRF1 cells were divided into 2 groups, 1 group injected with TMPyP4 and the other with PBS through venules 3 times a week. The mice with SNU-739-shTRF2 cells were divided into 3 groups, 1 injected with TA-65, another with siRNA targeted TERRA, and the third with PBS through venules 3 times a week. After 8 weeks, the mice were killed and the number of intra-hepatic and lung metastatic foci were counted by double-blind evaluation and H&E staining. All animal experiments in this study were carried out according to the guidelines of the Institutional Animal Care and Use Committee of the Fourth Military Medical University (permission number: IACUC-20170105; date issued: 1 January 2017).

2.12 | Measurement of telomerase activity

Hepatocellular carcinoma cells (1×10^5) and tumor-forming tissue from mice were lysed and centrifuged at 16 000 *g* for 20 minutes at 4°C. The supernatant was gently collected and the protein concentration in the supernatant was determined by a BCA protein assay kit (Pierce Biotechnology). A quantitative TeloTAGGG Telomerase PCR ELISA plus kit (Roche Applied Science) was used to evaluate telomerase activity according to the manufacturer's recommendation. Results were obtained with at least 3 replicates.

2.13 | Measurement of telomere length

Telomere length was measured with a validated qPCR system as described previously.²² Briefly, the cycle for qPCR is as follows: 2 minutes at 95°C, 15 seconds at 94°C, 15 seconds at 49°C, and 35 cycles of 15 seconds at 94°C, 10 seconds at 60°C, 20 seconds at 74°C, 10 seconds at 84°C, and 20 seconds at 88°C with signal acquisition. The telomere primers (900 nmol/L) were used for the telomere signal and the 36B4 single-copy gene was used as reference (300 nmol/L). The primer sequences are listed in Table S2. The telomere (T) signal was normalized to the signal from the single-copy (S) gene to generate a T/S ratio indicative of the relative telomere length. The amount of DNA to be used for each reaction was calculated from the standard curve obtained from the dilution series of a mixed sample DNA pool. All samples were assayed in triplicate and performed on the same run.

2.14 | Statistical analysis

Experiments were repeated 3 times, where appropriate. Data represent mean \pm SEM. SPSS 17.0 software (SPSS) was used for all statistical analyses and the detailed method is provided in Supporting Information.

See Supporting information for additional data.

3 | RESULTS

3.1 | Downregulated expression of TERRA significantly contributes to poor prognosis of HCC patients

To investigate the functional roles of TERRA in human HCC, we first examined the expression level of TERRA in paired HCC and peritumor tissues by FISH. Our results showed that the expression of TERRA was significantly decreased in HCC tissues ($n = 176$) when compared with the corresponding peritumor tissues (Figure 1A). We further found that HCC patients with low expression of TERRA ($C/P < 1$, where C is the foci of TERRA in cancer tissues, and P is the foci of TERRA in peritumor tissues) had significantly poorer DFS and OS than those with high expression of TERRA ($C/P > 1$) (log-rank, $P < .01$) (Figure 1B). Previous studies have shown that the expression of TERRA is mainly regulated by TRF1 and TRF2.¹¹ Therefore, we assessed the expression of both molecules and the relationship between TERRA and TRF1 and TRF2. The IHC staining analysis of TRF1 and TRF2 showed that the expression of TRF1 was downregulated in HCC tissues, whereas TRF2 was upregulated in HCC tissues when compared with adjacent nontumor tissues (Figure 1C). Moreover, we found that HCC patients with low TRF1 expression had significantly poorer DFS and OS than those with high TRF1 expression (log-rank, $P < .01$) (Figure 1D). In contrast, patients with high TRF2 expression had significantly poorer DFS and OS than those with low TRF2 expression (log-rank $P < .05$) (Figure 1E). Finally, we found that TERRA expression was positively correlated with TRF1 expression ($r = .403$, $P < .01$) but was inversely correlated with TRF2 expression ($r = -.226$, $P < .01$) (Figure 1F). These results reveal that the downregulation of TERRA significantly correlated with the poor prognosis of HCC patients.

3.2 | Expression of TERRA is regulated by TRF1 and TRF2

To explore the expression of TERRA in HCC cells, we systematically detected the expression level of TERRA in 5 HCC cell lines (SNU-739, SNU-368, HLE, HLF, and SNU-878) and 1 normal liver cell line (BEL-7702) by FISH. Our results further confirmed that the expression of TERRA in HCC cells was significantly lower than that in normal liver cells (Figure 2A). Then we constructed HCC cell lines with stable TRF1 and TRF2 knockdown and overexpression, which were

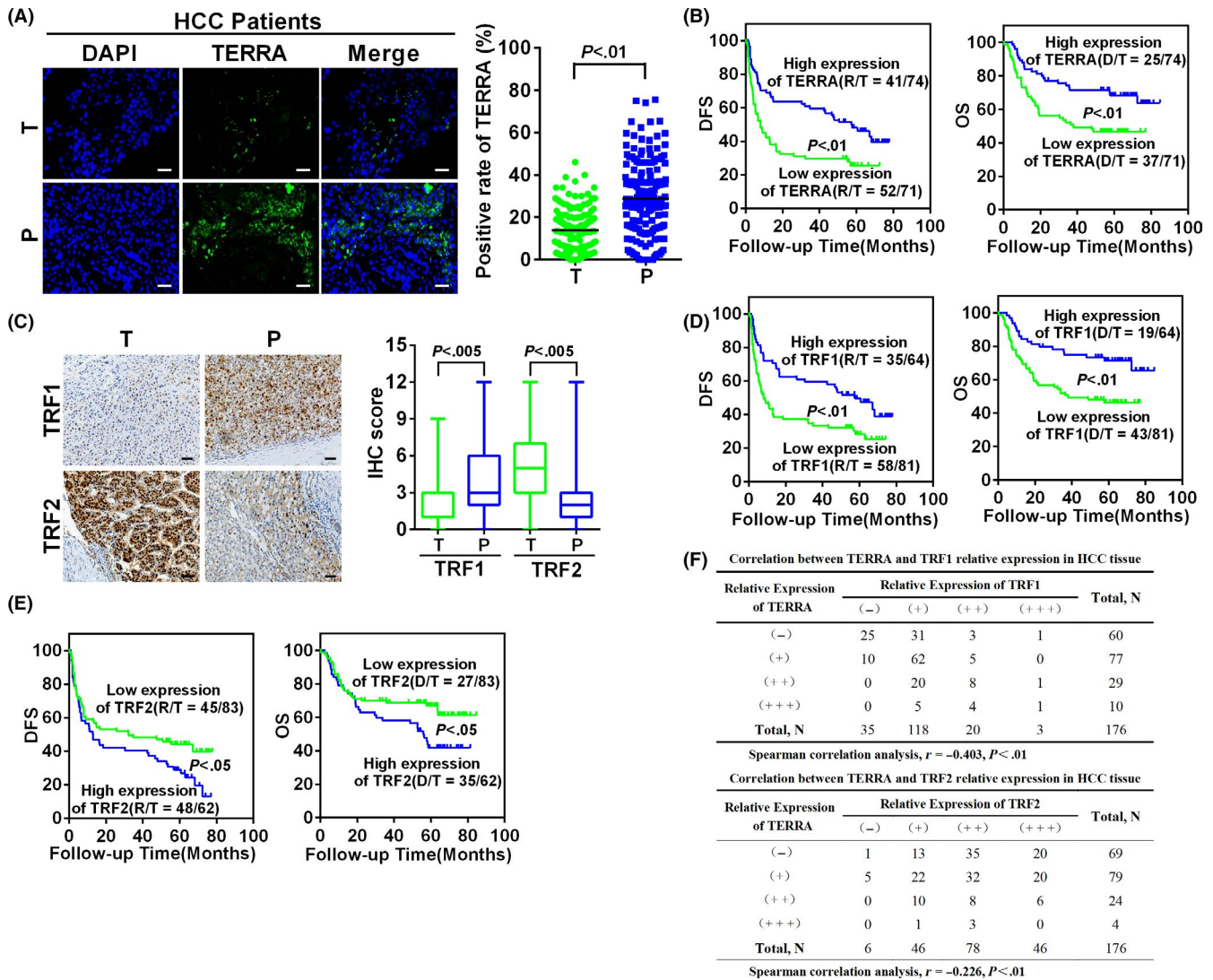


FIGURE 1 Downregulated telomeric repeat-containing RNA (TERRA) significantly contributed to poorer prognosis of hepatocellular carcinoma (HCC) patients. A, FISH of TERRA in paired tissues from HCC ($n = 176$). P, peritumor; T, tumor. B, Kaplan-Meier curve of disease-free survival (DFS) and overall survival (OS) in 176 HCC patients based on TERRA expression in HCC tissues ($P < .01$). D/T, death/total; R/T, recurrence/total. C, Representative immunohistochemical (IHC) staining images of TTAGGG repeat binding factor (TRF1) and TRF2 in paired HCC tissues ($n = 176$) and IHC score of TRF1 and TRF2. Scale bar = 100 μm . D, E, Kaplan-Meier curve of DFS and OS in 176 HCC patients based on TRF1 and TRF2 expression of HCC tissues ($P < .01$ and $P < .05$). F, Spearman correlation analysis for expression correlation between TRF1/TRF2 and TERRA in HCC tissues. TRF1 and TRF2 expression levels were detected by IHC, and TERRA expression was detected by FISH ($n = 176$, $r = -0.403$, $P < .01$; $r = -0.226$, $P < .01$)

confirmed by western blot analysis (Figure S1A), to elucidate the role of TRF1 and TRF2 in regulating the expression of TERRA. As shown in Figure 2B, the expression of TERRA was positively correlated with TRF1 expression but negatively correlated with TRF2 expression. These results indicate that the expression of TERRA is mainly regulated by TRF1 and TRF2 ($*P < .05$; $**P < .005$).

3.3 | Telomeric repeat-containing RNA inhibits HCC cell survival and metastasis in vitro

To explore the biological function of TERRA in HCC cells, we constructed a cell model with TERRA knockdown using transient siRNA

transfection (Figure S2A). In vitro viability, cell proliferation, and apoptosis of HCC cells with different TERRA expression levels were analyzed. As shown in Figure 3A-C, both cell viability and cell proliferation ability were significantly increased, whereas cell apoptosis was significantly inhibited, in HCC cells with TERRA knockdown compared with the control group. Furthermore, cell models with downregulated or upregulated expression of TERRA were also established by stable knockdown of TRF1 or TRF2, respectively. As shown in Figure 3D,E, cell viability and cell proliferation abilities were significantly increased in HCC cells with TRF1 knockdown, whereas opposite effects were observed in HCC cells with TRF2 knockdown. In contrast, cell apoptosis was significantly inhibited by TRF1 knockdown in both SNU-368 and SNU-739 cells, whereas it

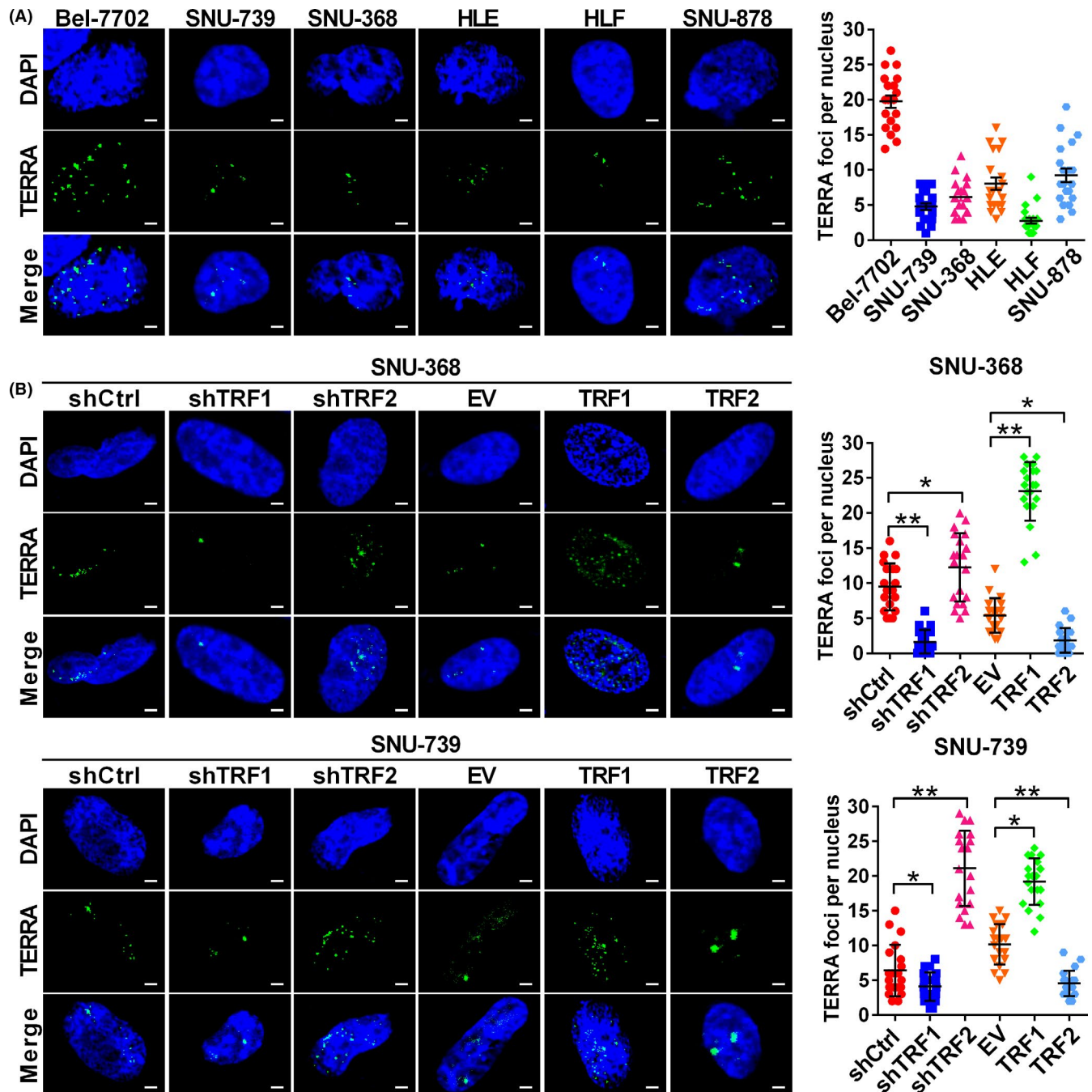


FIGURE 2 Expression of telomeric repeat-containing RNA (TERRA) is regulated by TTAGGG repeat binding factor (TRF)1 and TRF2. A, Representative images of RNA-FISH for TERRA (green) in 1 normal liver cell line and 5 hepatocellular carcinoma cell lines. Quantification of TERRA in 6 cell lines was scored by positive TERRA foci per cell. Nuclei were stained with DAPI (blue). B, Representative images of RNA-FISH for TERRA (green) in SNU-368 and SNU-739 cells treated as indicated. Quantification of TERRA was scored by positive TERRA foci per cell. Nuclei were stained with DAPI (blue). Data shown are mean \pm SD from 3 independent experiments. * $P < .05$; ** $P < .01$. EV, empty vector; shCtrl, control shRNA; shTRF, shRNA against TRF; TRF1 or TRF2, expression vector encoding TRF1 or TRF2

was increased by TRF2 knockdown (Figure 3F). Therefore, these results indicate that TERRA expression is correlated with the viability of HCC cells (* $P < .05$; ** $P < .005$).

Subsequently, we evaluated the role of TERRA in HCC cell metastasis. The wound-healing assay showed that the migration rate of HCC cells with TERRA knockdown was significantly higher than those in control cells (Figure 4A). Transwell migration and

invasion assays also indicated that the TERRA knockdown significantly increased the migration and invasion capabilities of HCC cells when compared with controls (Figure 4B,C). Furthermore, our results showed that the migration and invasion capabilities of HCC cells with downregulation of TERRA mediated by TRF1 knockdown were remarkably higher than those in control cells, whereas upregulation of TERRA mediated by TRF2 knockdown

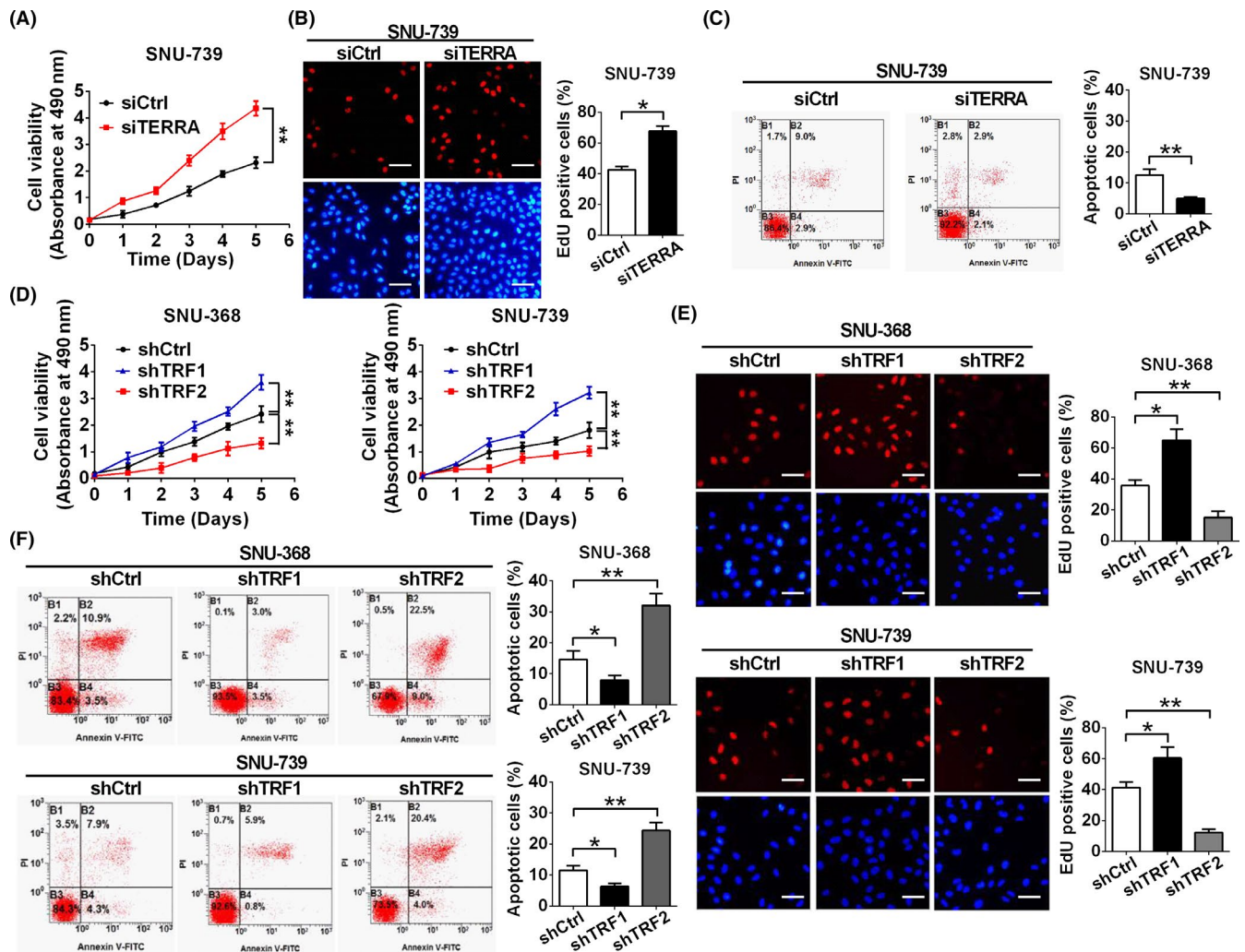


FIGURE 3 Telomeric repeat-containing RNA (TERRA) inhibited hepatocellular carcinoma (HCC) cell survival in vitro. A, D, HCC cells were transfected with siRNA or shRNA as indicated. Cells were reseeded for MTS cell viability assay 48 h after transfection. B, E, Cell proliferation was evaluated by EdU incorporation assay in SNU-368 and SNU-739 cells with treatment as indicated. Cells were reseeded for EdU incorporation assay 48 h after transfection. Scale bar = 50 μ m. Data shown are mean \pm SEM from 3 independent experiments. C, F, Flow cytometry analysis of apoptosis by annexin V (indicator of apoptosis) and propidium iodide staining in both SNU-368 and SNU-739 with treatment as indicated. Cells were collected for flow cytometry analysis 48 h after transfection. Data shown are mean \pm SD from 3 independent experiments. * P < .05; ** P < .01. shCtrl, control shRNA; siCtrl, control siRNA; shTRF1 or shTRF2, shRNA against TTAGGG repeat binding factor (TRF)1 or TRF2; siTERRA, siRNA against TERRA

remarkably inhibited the migration and invasion abilities of HCC cells (Figure 4D-F). Taken together, our data suggested that the increased expression of TERRA promoted metastasis of HCC cells (* P < .05, ** P < .005).

3.4 | Telomeric repeat-containing RNA inhibits telomere elongation by decreasing telomerase activity

To further explore the molecular mechanism underlying the inhibition of HCC cell survival and metastasis by TERRA, we assessed the effect of TERRA expression level on telomere length and telomerase activity in HCC cells that were transfected with siTERRA, using qPCR and a quantitative TeloTAGGG Telomerase PCR ELISA plus kit, respectively (Figure 5). TMPyP4 and TA-65 were used to

suppress^{23,24} and activate^{25,26} telomerase activity, respectively, in HCC cells transfected with siTERRA. The ability of TMPyP4 and TA-65 in repressing and enhancing telomerase activity, respectively, was validated in HCC cells (Figure S3A). Our results showed that the telomere length was significantly elongated and telomerase activity was significantly increased by TERRA knockdown in HCC cells; these effects were reversed by TMPyP4 treatment (Figure 5A,B). Moreover, the effect of TRF1/2 on telomere length and telomerase activity was detected. As shown in Figure 5C,D, similar results were obtained in HCC cells with TRF1 knockdown. However, the telomere length was remarkably shortened and telomerase activity was significantly decreased by TRF2 knockdown; these effects were reversed by TA-65 treatment or TERRA knockdown. Our data indicate that TERRA plays an important role in the regulation of telomere length by regulating telomerase activity (* P < .05; ** P < .005).

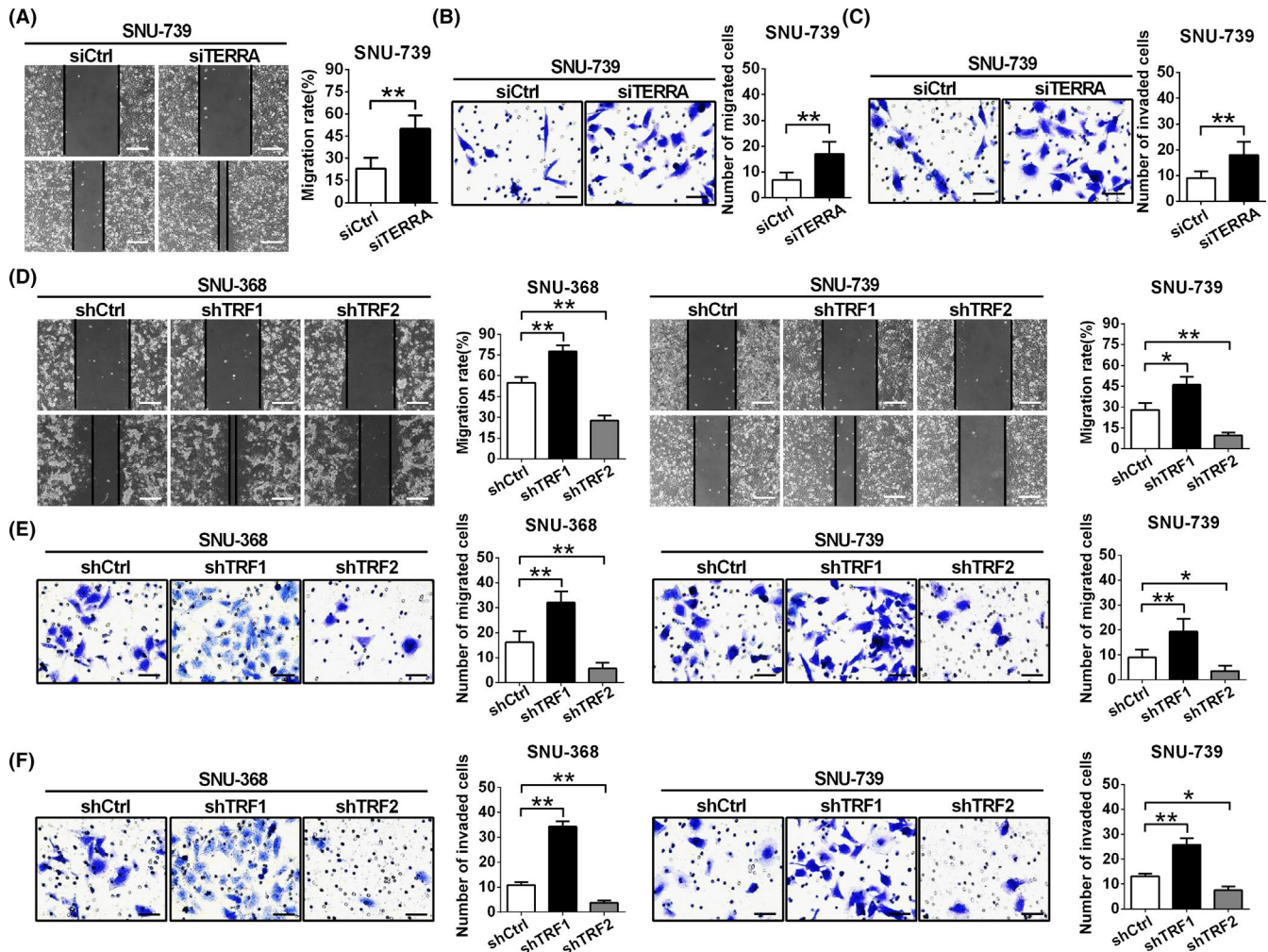


FIGURE 4 Telomeric repeat-containing RNA (TERRA) inhibited hepatocellular carcinoma (HCC) cell metastasis in vitro. A, D, Representative images of the wound-healing assay in SNU-368 and SNU-739 cells with treatment as indicated. B, E, Transwell migration analysis for migration ability of HCC cells treated as indicated. C, F, Transwell invasion analysis for invasion ability of HCC cells treated as indicated. Data shown are mean \pm SD from 3 independent experiments. * $P < .05$; ** $P < .01$. shCtrl, control shRNA; shTRF1 or shTRF2, shRNA against TTAGGG repeat binding factor (TRF)1 or TRF2; siCtrl, control siRNA; siTERRA, siRNA against TERRA. Scale bar: 50 μ m

3.5 | Telomeric repeat-containing RNA inhibits HCC cell survival and metastasis by telomerase-mediated telomere elongation

We further validated the important role of telomerase-mediated telomere elongation in the regulation of HCC cell survival and metastasis by TERRA. Our results showed that the inhibition of telomerase-mediated telomere elongation by TMPyP4 significantly reversed the proliferation-promoting and apoptosis-inhibiting effect of TERRA knockdown in HCC cells (Figures 6A,B and S4A,B). In addition, increased invasion and migration abilities of HCC cells mediated by TERRA knockdown were significantly reversed by the inhibition of telomerase-mediated telomere elongation by TMPyP4 (Figures 6C-E and S4C-E).

Consistently, our results showed that TMPyP4 treatment significantly reversed the proliferation-promoting and apoptosis-inhibiting effects of TRF1 knockdown on HCC cells, whereas both TA-65 treatment and siTERRA significantly reversed the proliferation-inhibiting and apoptosis-promoting effects of TRF2 knockdown

(Figures 6F,G and S4F,G). Moreover, similar results of TMPyP4 and TA-65 on the invasion and migration effects of HCC cells were obtained (Figures 6H-J and S4H-J).

3.6 | Telomeric repeat-containing RNA inhibits HCC cell survival and metastasis in vivo

We next examined the effect of TERRA on HCC growth and metastasis in vivo by constructing a xenograft nude mouse model using HCC cell lines with stable TRF1 and TRF2 knockdown. As shown in Figure 7A-C, xenograft tumors developed from SNU-739 cells with stable TRF1 knockdown and TRF2 knockdown showed a significant increase and decrease in growth capacity, respectively, when compared with control cells. In addition, TMPyP4 (5 mg/kg, once/2 days for 1 month) treatment significantly reversed the tumor growth-promoting effect of TRF1 knockdown, whereas TA-65 treatment or TERRA knockdown significantly reversed the tumor growth-inhibiting effect

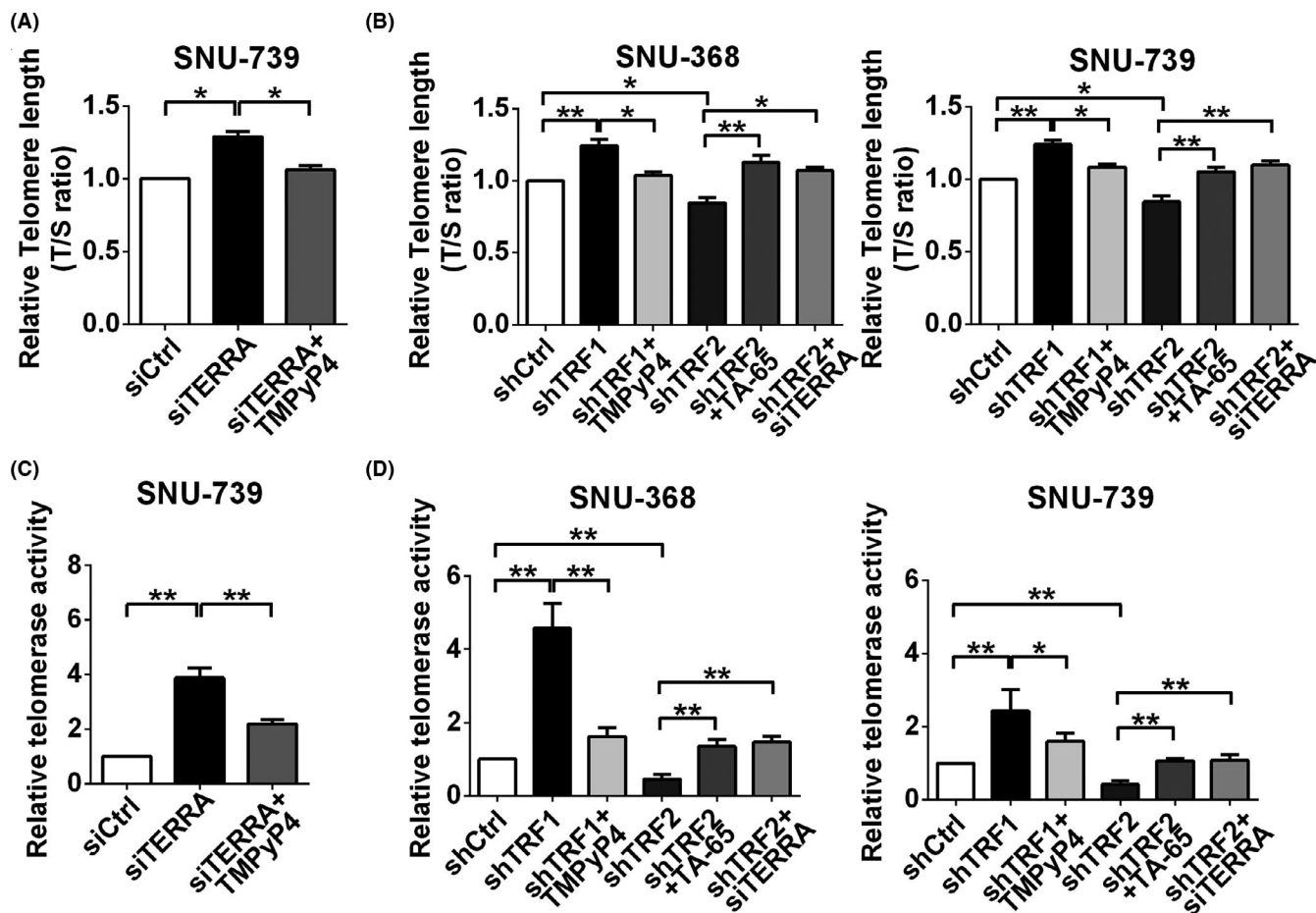


FIGURE 5 Telomeric repeat-containing RNA (TERRA) inhibited telomere elongation by reducing telomerase activity. A, C, Quantitative RT-PCR analysis for the telomere length of hepatocellular carcinoma cells treated as indicated. T/S, telomere (T) signal normalized to signal from single-copy (S) gene. B, D, Telomerase activity was measured using a telomerase activity kit in SNU-368 and SNU-739 cells stably transfected with shRNA as indicated: TMPyP4 (50 $\mu\text{mol/L}$, for 48 h), TA-65 (10 $\mu\text{mol/L}$, for 48 h), or cells were transfected with siRNA for 48 h. Data shown are mean \pm SD from 3 independent experiments. * $P < .05$; ** $P < .01$. shCtrl, control shRNA vector; shTRF1 or shTRF2, shRNA vector against TTAGGG repeat binding factor (TRF)1 or TRF2; siCtrl, control siRNA; siTERRA, siRNA against TERRA

of TRF2 knockdown. We then found that the telomere length and telomerase activity were significantly increased by TRF1 knockdown in xenograft tumors. Opposite results were obtained with TMPyP4 treatment. However, the telomere length and telomerase activity were remarkably decreased by TRF2 knockdown; these effects were remarkably reversed by TA-65 (25 mg/kg, once/2 days for 1 month) treatment or TERRA knockdown (Figure 7D,E). We also examined the effect of TERRA on the expression of Ki-67 in xenograft tumors. Immunohistochemical analysis showed that TMPyP4 treatment significantly decreased the positive rate of Ki-67, which was increased by TRF1 knockdown, whereas TA-65 treatment or TERRA knockdown significantly increased the positive rate of Ki-67, which was inhibited by TRF2 knockdown (Figure 7F). In contrast, the percentage of apoptotic cells, which was decreased by TRF1 knockdown, was significantly increased following TMPyP4 treatment. However, TA-65 treatment or TERRA knockdown in HCC cells resulted in decreased percentage of apoptotic cells, which was increased by TRF2 knockdown (Figure 7G).

We next examined the role of TERRA in HCC metastasis in vivo. Our results indicated that TERRA downregulation mediated by

TRF1 knockdown significantly increased the incidence of intrahepatic metastasis and lung metastasis compared with control mice. These effects caused by TERRA downregulation were significantly reversed by TMPyP4 treatment. In contrast, the incidence of intrahepatic metastasis and lung metastasis were remarkably decreased by TRF2 knockdown in mice with xenografts developed from SNU-739 cells. Opposite results were obtained in xenograft mice treated with TA-65 or TERRA knockdown (Figure 8A-E).

Our findings indicated that the downregulation of TERRA mediated by TRF1 significantly promoted telomerase activity and telomere elongation, which plays a critical role in the modulation of HCC cell survival and metastasis, and provides strong evidence for this process as a drug target in HCC treatment (Figure 8F).

4 | DISCUSSION

Growing evidence has shown that the dysregulation of TERRA has been implicated in many types of cancers, such as osteosarcoma,

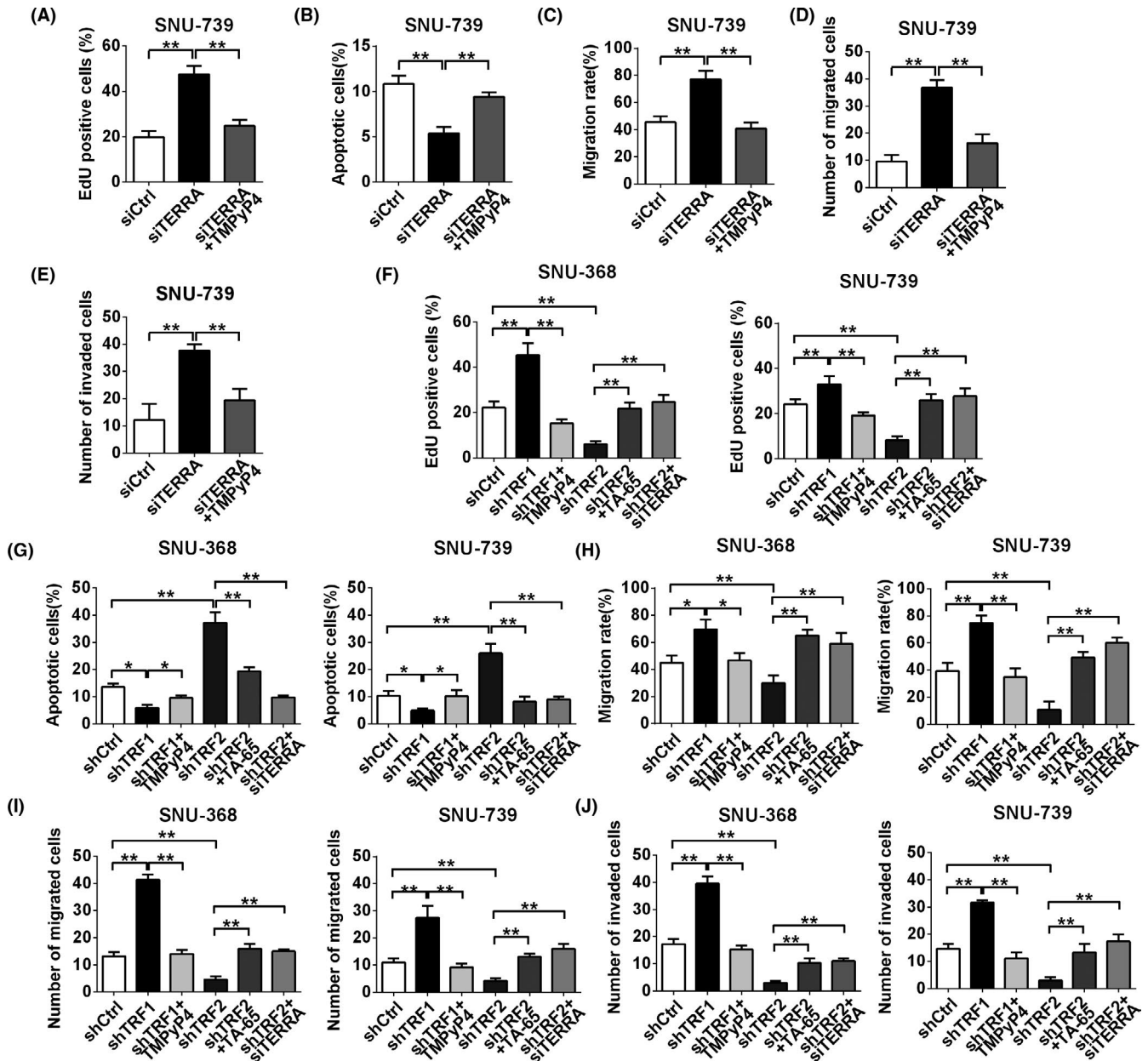


FIGURE 6 Telomeric repeat-containing RNA (TERRA) inhibited hepatocellular carcinoma (HCC) cell survival and metastasis by inhibiting telomerase-mediated telomere elongation. A, F, Cell proliferation was evaluated by EdU incorporation assay in SNU-368 and SNU-739 cells with treatment as indicated. Scale bar = 50 μ m. Data shown are mean \pm SEM from 3 independent experiments. B, G, Cell apoptosis was investigated by flow cytometry in SNU-368 and SNU-739 cells with treatment as indicated. C, H, Representative results of wound-healing assay in SNU-368 and SNU-739 cells with treatment as indicated. D, I, Transwell migration analysis for migration ability of HCC cells treated as indicated. E, J, Transwell invasion analysis for invasion ability of HCC cells treated as indicated. Data shown are mean \pm SD from 3 independent experiments. * P < .05; ** P < .01. shCtrl, control shRNA vector; shTRF1 or shTRF2, shRNA vector against TTAGGG repeat binding factor (TRF)1 or TRF2; siCtrl, control siRNA; siTERRA, siRNA against TERRA; TA-65, activating telomerase activity; TMPyP4, inhibiting telomerase activity

medulloblastoma, stomach adenocarcinoma, head and neck cancer, and cervical carcinoma.^{12-15,27,28} However, the functional role of TERRA in hepatocarcinogenesis is largely unknown. In this study, the expression levels of TERRA and its regulators (TRF1 and TRF2) were investigated in HCC. We found that downregulated TERRA significantly contributed to the poor prognosis of HCC patients, and we provided evidence supporting that TERRA was regulated by TRF1 and TRF2 in HCC cells. Subsequently, we found that TERRA suppressed cell proliferation and metastasis abilities, as well as induced cell apoptosis in

HCC by inhibiting telomere elongation. These findings indicated the intriguing possibility that TERRA could be a great potential biomarker and therapeutic target in the clinical management of HCC patients.

Gonzalez-Vasconcellos et al have also reported that TERRA plays a critical role in the condensation of telomeric chromatin and the appropriate histone modifications of telomeric DNA,¹² which is essential for telomere homeostasis. Furthermore, the deregulation of TERRA transcription has been proved to impair the telomere and contribute to tumorigenesis. For example, head and neck squamous

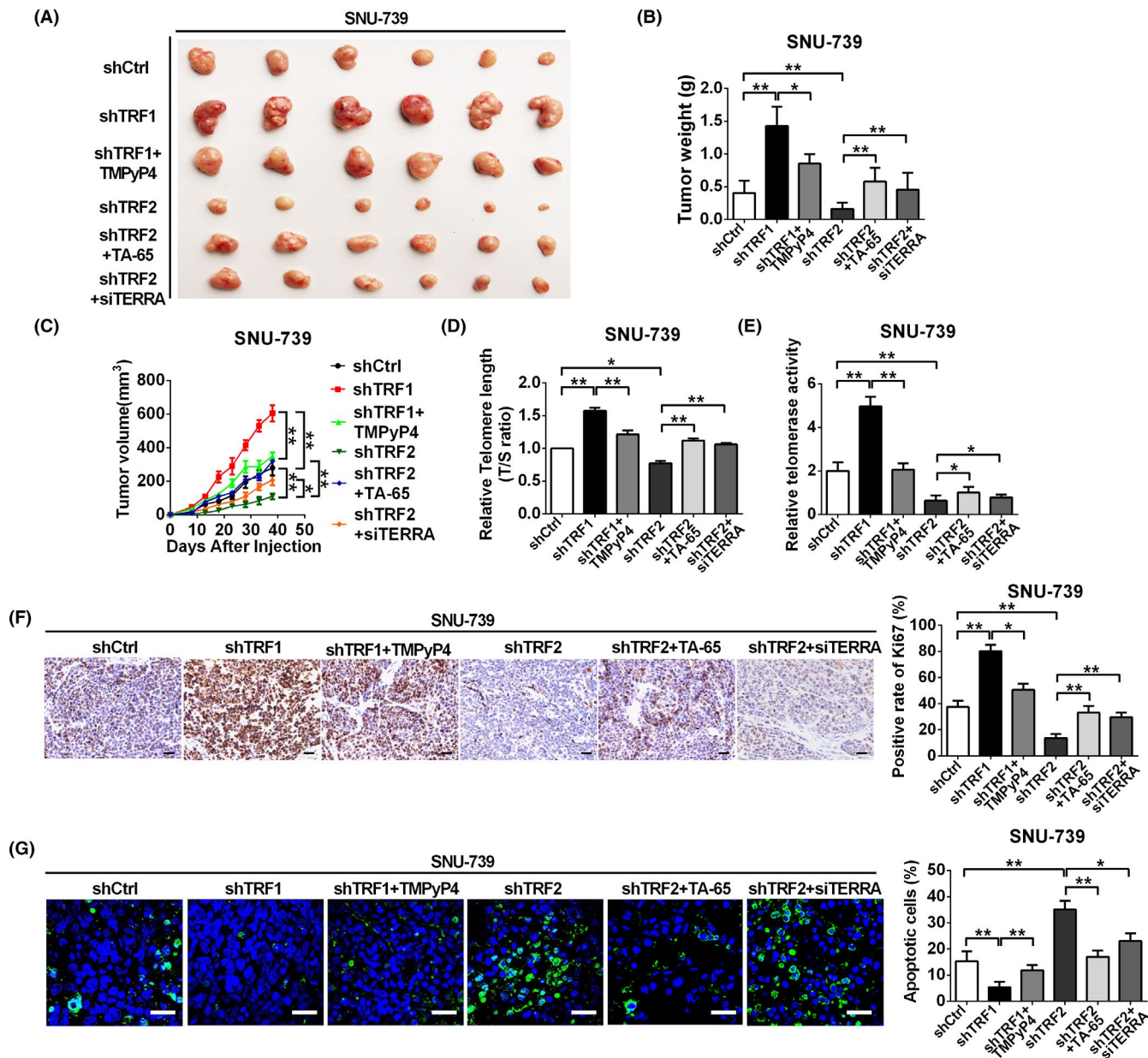
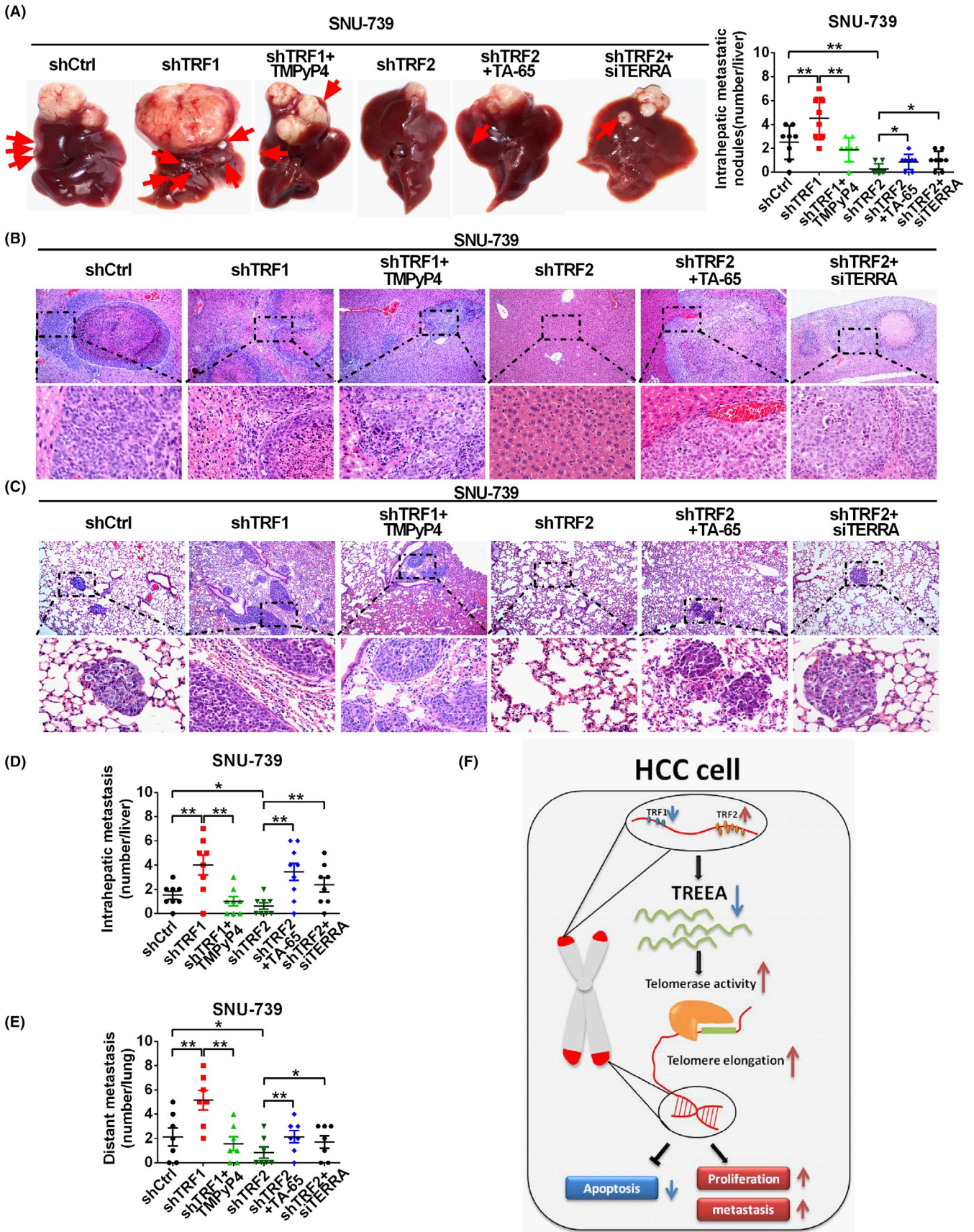


FIGURE 7 Telomeric repeat-containing RNA (TERRA) inhibited hepatocellular carcinoma (HCC) cell survival by inhibiting telomerase activity in vivo. A, Tumors from sacrificed subcutaneous xenograft mice were dissected 30 d after transplantation. B, Tumor size including tumor length (L) and width (W) was measured every 4 d from day 7 after transplantation. Tumor volumes were calculated according to the formula $(L \times W^2)/2$ and presented as mean \pm SEM. C, Tumor growth curves of s.c. xenograft tumor model developed from HCC cells with different treatment as indicated. D, Telomere length of s.c. xenograft tumor developed from HCC cells with different treatment as indicated. E, Telomerase activity of s.c. xenograft tumor developed from HCC cells with different treatment as indicated. F, Representative immunohistochemical staining images of Ki-67 in xenograft tumors developed from SNU-739 cells with different treatment as indicated. G, TUNEL staining in tumor tissues of nude mice xenograft developed from different HCC cells with different treatment as indicated. Blue, Hoechst 33342; green, TUNEL positive nucleus. Data shown are mean \pm SD from 3 independent experiments. * $P < .05$; ** $P < .01$. shCtrl, control shRNA vector; shTRF1 or shTRF2, shRNA vector against TTAGGG repeat binding factor (TRF)1 or TRF2; siTERRA, siRNA against TERRA; TA-65, activating telomerase activity; TMPyP4, inhibiting telomerase activity

cell carcinoma patients with low-level expression of TERRA have poorer clinical outcomes than patients with high-level expression of TERRA.¹³ In addition, the expression of TERRA is downregulated in malignant glioma tissues and negatively correlated with advanced clinical grade.¹⁴ Consistently, we found that TERRA was significantly downregulated in HCC tissues and the downregulated

TERRA contributed to the poor prognosis of HCC patients. However, contrasting results have been reported, indicating that TERRA expression is increased in several types of human cancers, such as ovarian cancer and medulloblastoma.¹⁵ These data suggest that the functional roles of TERRA could be cancer type-specific, which needs comprehensive investigation in future studies.



There are 6 individual proteins that are associated with telomeric DNA, known as the shelterin complex. They are essential in preventing the recognition of telomere as single- or double-strand breaks by

forming a closed configuration T-loop structure to hide the natural ends of the telomere. In addition, the shelterin complex plays critical roles in telomere length homeostasis.²⁹ Telomeric repeat binding

FIGURE 8 Telomeric repeat-containing RNA (TERRA) inhibited hepatocellular carcinoma (HCC) cell metastasis by inhibiting telomerase activity in vivo. A, Representative liver tissues from different experimental groups to show the incidence of intrahepatic metastasis in orthotopic implantation nude mouse tumor models. Arrowheads indicated metastatic foci. B, Histological analyses of intrahepatic metastatic nodules from HCC metastasis nude mouse model. Images showing representative H&E staining of liver tissue samples from different experimental groups (n = 8 per group). C, Histological analyses of lung metastatic nodules from HCC metastasis nude mice model. Images showing representative H&E staining of lung tissue samples from different experimental groups (n = 8 per group). D, E, Statistical results of intrahepatic metastatic nodules and lung metastatic nodules from H&E staining. F, Schematic depicting of TTAGGG repeat binding factor (TRF)1 and TRF2-mediated TERRA downregulation promoting HCC proliferation and metastasis through telomere elongation. Data shown are mean \pm SD from 3 independent experiments. * $P < .05$; ** $P < .01$. shCtrl, control shRNA vector; shTRF1 or shTRF2, shRNA vector against TTAGGG repeat binding factor (TRF)1 or TRF2; siTERRA, siRNA against TERRA; TA-65, activating telomerase activity; TMPyP4, inhibiting telomerase activity

factor 1 and 2 are the constituent parts of the complex, which directly bind the double-stranded repeat regions. Despite their similarities, TRF1 and TRF2 exert different effects on the regulation of TERRA levels. Consistent with these studies, we also found that TRF1 knockdown or TRF2 overexpression significantly decreased the expression of TERRA in HCC cells, whereas TERRA expression was remarkably increased by TRF1 overexpression or TRF2 knockdown in HCC cells. These findings indicate that TRF1 and TRF2 play reverse roles in regulating TERRA transcription in HCC cells.

Replicative senescence can be delayed by restoring telomerase expression and activity, which replenishes telomeres.³⁰ When telomerase activity is diminished, significant apoptosis of epithelial cells is initiated. With further disruption of telomerase activity, apoptosis occurs at significantly higher levels.³¹ Telomeric repeat-containing RNA binds telomerase core components, telomerase RNA component (TERC) and telomerase reverse transcriptase (TERT). It is generally accepted that TERRA can inhibit telomerase activity.⁷ We found that TERRA inhibited telomerase activity to prevent telomere elongation, which is consistent with previous results.³²⁻³⁴ The inactivation of telomerase acts as a tumor-suppressing mechanism. Its reexpression in human fibroblasts facilitates the bypassing of senescence. As a result, cellular immortalization occurs. Accordingly, telomerase activity is reactivated to stabilize telomere length in 90% of human cancer cells.³⁵

Emerging evidence indicates that telomeres diversely function in many of the stages that define the metastatic cascade. To date, many studies have examined the association between cancer progression and telomere homeostasis, and they concluded that differences in either telomere length or telomerase activity are correlated with tumor metastatic progression.³⁵⁻³⁷ It has been accepted that telomere homeostasis represents a critical determinant of metastatic capability in cancer cells. Telomerase-active cancer cells become more prone to disseminate from primary tumor sites and form overt metastases in distant organs.³⁸ Consistently, our study showed that TERRA knockdown significantly promoted telomerase activity and telomere elongation, leading to the increased metastasis of HCC cells. Our results, for the first time, indicate that downregulated TERRA contributes to HCC cell metastasis by activating telomerase activity.

In the present work, our data show that TRF1/TRF2-mediated TERRA transcription plays a key role in the regulation of HCC cell survival and metastasis by inhibiting telomerase activity, which regulated the elongation of the telomere. In conclusion, our findings indicated that TERRA played a vital role in malignant transformation

in HCC cells, and could be a potential therapeutic target for HCC treatment.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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SUPPORTING INFORMATION

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

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