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Circular RNA hsa_circ_0002938 (circCRIM1) promotes the progression of esophageal squamous cell carcinoma by upregulating transcription factor 12

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Growing evidence has indicated that circular RNAs (circRNAs) play crucial roles in the tumorigenesis and progression of diverse malignancies. However, the majority of circRNAs involved in esophageal squamous cell carcinoma (ESCC) remain undefined and the exact functions and underlying mechanisms of circRNAs in ESCC still need further exploration. In this study, we identified a novel onco-circRNA hsa_circ_0002938, derived from the exons of cysteine-rich transmembrane BMP regulator 1 (CRIM1) pre-mRNA, referred to as circCRIM1. We found that the expression of circCRIM1 was higher in ESCC tissues, compared to para-carcinoma tissues. Increased expression of circCRIM1 was positively correlated with clinical parameters of ESCC patients including tumor-node-metastasis (TNM) stage, tumor invasion range, and lymph node metastasis. Functionally, the results from the experiments *in vitro* showed that the knockdown of circCRIM1 suppressed proliferation, migration, invasion, and epithelial-mesenchymal transition (EMT) in ESCC cells. By conducting bioinformatics algorithms analyses and microRNA (miRNA) rescue experiments, we found that circCRIM1 could act as a competing endogenous RNA (ceRNA) to sponge miR-342-3p in ESCC cells, and thereby upregulated the expression of transcription factor 12 (TCF12), a key regulator promoting the EMT process. Taken together, circCRIM1 facilitates the progression of ESCC by sponging miR-342-3p to regulate TCF12 and promote EMT, and the circCRIM1/miR-342-3p/TCF12 axis may be regarded as a potential predictive biomarker and therapeutic target for treating ESCC.

Key words: circCRIM1; miR-342-3p; transcription factor 12; esophageal squamous cell carcinoma; epithelial-mesenchymal transition

Esophageal cancer is one of the most prevalent gastrointestinal malignancies, accounting for the sixth leading cause of cancer-related mortality worldwide [1]. Squamous cell carcinoma (SCC) is the predominant histological subtype of esophageal cancer, accounting for 85% of cases [2]. Surgery still acts as the cornerstone of treatment for potentially resectable esophageal squamous cell carcinoma (ESCC) [3]. However, the majority of ESCC patients are initially diagnosed at an advanced stage of the disease, thereby losing the precious opportunity for surgery [4]. Unfortunately, despite the improvement in multidisciplinary anticancer therapy, the 5-year survival rate of ESCC patients is below 20% [5]. Therefore, aiming at further improving the prognosis of patients, it is urgent to identify novel molecules involved in ESCC progression.

Circular RNAs (circRNAs), a class of single-stranded non-coding RNA originating from precursor mRNA back-splicing, are characterized by a covalently closed loop structure without 5' caps and 3' poly(A) tails [6]. Since circRNAs are relatively resistant to exonucleases and can exert a long-lasting effect, they have great potential to serve as stable biomarkers for diagnosing diseases and predicting the prognosis [7]. circRNAs participate in the regulation of gene expression via multiple mechanisms, such as sponging microRNAs (miRNAs), interacting with RNA binding proteins (RBPs) and encoding peptides [8]. Among these functions, acting as competing endogenous RNAs (ceRNAs) to sponge miRNAs is the most well-studied mechanism. As miRNAs play a major role in the regulation of gene expression at the post-transcriptional level by either decreasing mRNA stability or inducing translation repression, their binding with circRNAs neutralizes the suppressing effect on target genes [9]. Emerging studies have revealed that numerous circRNAs affect the proliferation, metastasis, metabolism, and immune

evasion of cancer cells by acting as ceRNAs [10]. For instance, circSDHC promoted the proliferation and metastasis of renal cell carcinoma by sponging miR-127-3p via the CDKN3/E2F1 axis [11]. circWAC increased WW domain-containing ubiquitin E3 ligase 1 (WWP1) expression and activated the PI3K/AKT signaling pathway by acting as a miR-142 sponge, consequently developing drug resistance in triple-negative breast cancer [12]. circARHGAP29 enhanced aerobic glycolysis by increasing and stabilizing the mRNA of lactate dehydrogenase A (LDHA), thereby triggering docetaxel resistance in prostate cancer [13]. However, how circRNAs regulate the progression of ESCC is largely unknown and the exact mechanisms need further exploration.

In the present study, we identified a potential tumor-promoting circRNA which derived from 8–13 exons of cysteine-rich transmembrane BMP regulator 1 (CRIM1) pre-mRNA, referred to as circCRIM1 (circBase ID: hsa_circ_0002938). We found that circCRIM1 was significantly upregulated in ESCC tissues and cell lines. In addition, high expression of circCRIM1 was positively correlated with the tumor-node-metastasis (TNM) stage, tumor invasion range, and lymph node metastasis of ESCC patients. circCRIM1 promoted the proliferation, migration, invasion, and epithelial-mesenchymal transition (EMT) in ESCC cells. Mechanistically, based on the bioinformatic analysis as well as luciferase reporter assay, circCRIM1 was revealed to be a sponge of miR-342-3p, and miR-342-3p could target the 3'-UTR of transcription factor 12 (TCF12) to negatively regulate its expression. Therefore, circCRIM1 could probably promote the progression of ESCC through the circCRIM1/miR-342-3p/TCF12 axis. In summary, this study shows that circCRIM1 may serve as a useful biomarker and therapeutic target for ESCC patients.

Patients and methods

Patients and specimens. Cancer and matched para-carcinoma tissue specimens from 50 ESCC patients who underwent surgery at the Fourth Hospital of Hebei Medical University (Shijiazhuang, China) were collected. None of the patients received preoperative treatments. All specimens were immediately snap-frozen in liquid nitrogen and stored at –80 °C. All written informed consents were obtained before surgery and the research was approved by the ethics committee of the Fourth Hospital of Hebei Medical University.

Cell culture. Human ESCC cell lines (TE-1, KYSE30, KYSE410, KYSE150) were obtained from Procell Life Science & Technology Co., Ltd. (Wuhan, China) and cultured in RPMI-1640 medium (Servicebio, Wuhan, China) containing 10% fetal bovine serum (FBS) (Solarbio, Beijing, China) and 1% penicillin/streptomycin (P/S). Human normal esophageal epithelial cell line (Het-1A) was purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in bronchial epithelial cell growth medium (BEGM, BulletKit, Lonza, MD). All cells were maintained in a humidified incubator with 5% CO_2 at 37 °C.

Quantitative real-time PCR (qRT-PCR). Total RNA from tissues and cells was extracted by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed using the RevertAid™ First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). SYBR Green PCR Master Mix (YEASEN, China) was used for the qRT-PCR. As for miRNA detection, special primers were used in reverse transcription. Primers used in this study were designed and synthesized by Tsingke (Wuhan, China) and RiboBio (Guangzhou, China). GAPDH and U6 were used to be the internal normalization and the data were calculated using the $2^{-\Delta\Delta CT}$ method. The primer sequences are listed in Table 1.

Confirmation of circular structure. The sequence of circCRIM1 was verified using Sanger sequencing. The linear and circular transcripts of CRIM1 were amplified by convergent and divergent primers in both complementary DNA (cDNA) and genomic DNA (gDNA) extracted from ESCC cells. Agarose gel was used to separate the PCR products. Total RNA (2 μg) was incubated with or without 2 U/μg of RNase R (Geneseed, Guangzhou, China) for 25 min at 37 °C. The expression of circular and linear CRIM1 was measured by qRT-PCR.

Cytoplasmic and nuclear fraction isolation. The cytoplasmic and nuclear fractions of ESCC cells were separated by using the Minute™ kit (INVENT). The cells were collected and centrifuged at 4 °C 500 rpm for 5 min. Then, 500 μl fractionation buffer was added and mixed gently. After centrifugation again, the supernatant was the cytoplasmic fraction and the precipitation was the nuclear fraction. RNA was extracted and qRT-PCR analysis was performed. U6 and GAPDH were used as internal controls for the nuclear and cytoplasm, respectively.

Table 1. Oligos used in this study.

Gene	Primer sequence (5'-3')
CRIM1	Forward: TGTCCCGCAATAACAGCGTA
(divergent)	Reverse: GTGATGATGGTTGGTTCTATGCAG
CRIM1	Forward: TGGAAACGCACCCACTTACA
(convergent)	Reverse: TAGCACTTTGGGCATCGGTT
miR-342-3p	RT Primer: CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTT-GAGACGGGTGC
	Forward: ACACTCCAGCTGGGTCTCACA-CAGAAATCGC
	Reverse: TGGTGTCGTGGAGTCG
TCF12	Forward: GGACCTTCCACCAGTTTGCCT
	Reverse: CTTCCCGATGAGTTCCAACCA
GAPDH	Forward: GGAAGCTTGTCATCAATGGAAATC
	Reverse: TGATGACCCTTTTGGCTCCC
U6	Forward: CTCGCTTCGGCAGCACA
	Reverse: AACGCTTCACGAATTTGCGT

Cell transfection. Small interfering RNAs (siRNAs) targeting the back-splicing junction of circCRIM1 (si-circCRIM1#1, si-circCRIM1#2) and negative control siRNA (si-NC) were synthesized by GenePharma (Shanghai, China). The sequences of siRNAs were listed as follows: si-circCRIM1#1, 5'-CCUACUGCAUAGAACCAACTT-3'; si-circCRIM1#2, 5'-GGCATGAAAGGTGCAAACCCA-3'. miR-342-3p mimic/inhibitor and matched control (mimic NC/inhibitor NC) were obtained from RiboBio (Guangzhou, China). The ESCC cells were transfected with Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The transfection efficiency was confirmed by qRT-PCR.

Cell counting kit-8 (CCK-8) assay. Cell vitality was detected by a CCK-8 detection kit (Solarbio). The ESCC cells were plated in 96-well plates at a density of 1×10^3 cells/well. At 0, 24, 48, 72, and 96 h, 10 μl CCK-8 reagent was added to each well and incubated at 37 °C for 2 h. Then, a microplate reader RT-6000 (Rayto, China) was utilized to measure the absorbance of each well at 450 nm wavelength.

Colony formation assay. Transfected cells were seeded in 6-well plates (1×10^3 cells/well) and cultured for 10–14 days. Then, the colonies were fixed with 4% paraformaldehyde for 20 min and stained with crystal violet for 20 min. The number of colonies (≥50 cells) in each group was calculated.

Wound-healing experiment. Transfected cells were plated and cultured in 6-well plates. Cell monolayers were scraped linearly by a 200 μl pipet tip and incubated with serum-free RPMI-1640 medium. Images were obtained at 0 h and 24 h after the injury. The migration distance of the cells in each group was calculated and normalized to the 0 h time point.

Transwell invasion assay. For invasion assays, Transwell chambers (Corning, USA) were coated with Matrigel (Corning, USA). About 5×10^4 cells were seeded in the upper chambers within 200 μl of serum-free medium, while 600 μl medium supplemented with 10% FBS was added to the lower chambers. After incubation for 36 h, the cells on the lower surface of the chambers were fixed with paraformaldehyde and stained with crystal violet. The invading cells were counted under at least five random microscopic fields.

Fluorescence in situ hybridization (FISH). The biotin-labeled circCRIM1 probes and Dig-labeled miR-342-3p probes were designed and synthesized by Servicebio (Wuhan, China). The probe sequences were as follows: circCRIM1, 5'-TGATGATGGTTGGTTCTATGCAGTAGGGACA-3'; and miR-342-3p, 5'-ACGGGTGCGATTTCTGTGTGAGA-3'. ESCC cells were cultured on coverslips for 24 h in 24-well plates and then fixed with paraformaldehyde. After prehybridization in phosphate-buffered saline with 0.5% Triton X-100, cells were hybridized with circCRIM1 and miR-342-3p probes overnight at 37 °C. Cy3 and FAM were utilized to detect the biotin-labeled and Dig-labeled probes, respectively. The nuclei were stained by DAPI. Signal images were observed using a fluorescence microscope (Nikon, Tokyo, Japan).

Dual-luciferase reporter assay. The pmirGLO luciferase reporter vectors for the wide-type and mutate-type of circCRIM1 (circCRIM1-WT/MUT) or TCF12 3'-UTR (TCF12-3'-UTR-WT/MUT) were synthesized by GenePharma. ESCC cells (5×10^4) were seeded in 24-well plates and co-transfected with corresponding plasmids and miR-NC or miR-342-3p mimic by using Lipofectamine 3000. After 48 h incubation, the relative luciferase activity was examined according to the manufacturer's instructions for the dual-luciferase reporter gene assay system (Promega, USA).

RNA immunoprecipitation (RIP). For RIP assays, BersinBio™ RIP Kit (Guangzhou, China) was used according to the manufacturer's instructions. Myc-tagged Argonaute-2 (AGO2) vector and miR-342-3p mimic were co-transfected into the ESCC cells. After 48 h, about 2×10^7 cell samples were collected and washed with 4 ml PBS, centrifuged at 1000 rpm at room temperature for 5 min. Next, 1.7 ml polysome lysis buffer, 17 μl protease inhibitor, and 7.5 μl RNase inhibitor were mixed and added to the cell pellet. The mixtures were placed on ice for 10 min and put in the −80 °C freezer for 5 min after the vortex. Then, cell lysates were thawed at room temperature. Myc antibody (Abcam, ab9106) (5 μg) or a negative control IgG antibody (5 μg) was incubated with cell lysates on a rotator overnight at 4 °C. The next day, magnetic beads protein A/G were added into the samples and rotated for 1 h. Then, the beads were washed with cold RIP washing buffer. After purification, the RNA was extracted and measured by qRT-PCR.

Western blotting. The proteins were extracted from the cells by using RIPA lysis buffer (Beyotime, Shanghai, China), and the concentration was measured by a BCA protein assay kit (Beyotime, Shanghai, China). The cell lysates were separated by 10% SDS-PAGE electrophoresis and then transferred to PVDF membranes (Millipore, Billerica, MA, USA). After blocking with 5% BSA for 1 h, the membranes were incubated with the following primary antibodies overnight at 4 °C: anti-E-cadherin (GB11868, Servicebio), anti-N-cadherin (GB11135, Servicebio), anti-Vimentin (GB11132, Servicebio), anti-TCF12 (A4146, ABclonal), and anti-GAPDH (GB11002, Servicebio). The next day, the membranes were washed 3 times for 15 min in TBST and incubated with species-matched secondary antibodies for 2 h at room temperature. The blots were visualized by ECL chemiluminescent reagent (Biosharp Life Sciences, Hefei, China).

Statistical analysis. The measurement data were presented as mean ± SD of at least three independent experiments. Results were analyzed and statistical graphs were drawn by using GraphPad Prism 9.0 (GraphPad Software, La Jolla, CA, USA). Two-tailed student's t-test or one-way ANOVA was utilized to analyze data fitting the normal distribution, and the Mann-Whitney U test was used for those without equal variance. Chi-square test was used to determine the correlation between circCRIM1 expression and clinicopathological parameters in ESCC patients. The correlation between

circCRIM1 and miR-342-3p expression in ESCC tissue specimens was calculated by using Pearson correlation analysis. A p-value <0.05 was considered statistically significant.

Results

Circular RNA circCRIM1 is upregulated in ESCC. Circular RNA hsa_circ_0002938 referred to as circCRIM1, is formed by the back-splicing of exons 8–13 from the host gene CRIM1 on chromosome 2 (chr2:36726361-36749456) (Figure 1A). The back-splicing junction of circCRIM1 was verified by Sanger sequencing (Figure 1B). Next, we collected 50 pairs of ESCC tissues and adjacent normal tissues to analyze the expression of circCRIM1 by qRT-PCR. The results showed that circCRIM1 was remarkably elevated in ESCC tissues compared to the adjacent normal tissues (Figure 1C). These 50 ESCC patients were divided into circCRIM1 high-expression and low-expression groups with a median value as the cut-off threshold. The correlations between circCRIM1 expression and a variety of clinicopathological parameters were analyzed in these patients. High expression of circCRIM1 was positively correlated with tumor invasion range, lymph node metastasis, and TNM stage, while not related to gender and age (Table 2). Furthermore, the expression levels of circCRIM1 in four ESCC cell lines (TE-1, KYSE30, KYSE410, KYSE150) were higher than that in the normal human esophageal epithelial cell line (Het-1A), especially in TE-1 and KYSE30 cell lines (Figure 1D).

Based on these results, we selected TE-1 and KYSE30 cells with higher circCRIM1 expression for our further study. To investigate the circular structure of circCRIM1, divergent and convergent primers were designed to amplify circCRIM1 and linear CRIM1. Results confirmed that circCRIM1 was only amplified from cDNA by divergent primers, while linear CRIM1 could be detected using convergent primers in both cDNA and gDNA (Figure 1E). Afterward, total RNA extracted from TE-1 and KYSE30 cells was treated with RNase R. qRT-PCR results demonstrated that the expression level of linear CRIM1 mRNA was apparently degraded after RNase R digestion, while there was no distinct difference in circCRIM1 expression (Figure 1F). Furthermore, nuclear-cytoplasmic separation assays and FISH revealed that circCRIM1 was mainly located in the cytoplasm of TE-1 and KYSE30 cells (Figures 1G, 1H). The results above confirmed that circCRIM1 was a bona fide circular RNA, which was upregulated in ESCC and mainly localized to the cytoplasm.

Table 2. Correlations between circCRIM1 expression with clinical characteristics of ESCC patients.

	Total	n (%)		p-value
		High expression	Low expression	
Gender				
Male	33	16 (48.48%)	17 (51.52%)	0.765
Female	17	9 (52.94%)	8 (47.06%)	
Age				
<60	16	10 (62.50%)	6 (37.50%)	0.225
≥60	34	15 (44.12%)	19 (55.88%)	
TNM stage				
I+II	24	7 (29.17%)	17 (70.83%)	0.005
III+IVA	26	18 (69.23%)	8 (30.77%)	
Invasion range				
T1+T2	20	6 (30.00%)	14 (70.00%)	0.021
T3+T4	30	19 (63.33%)	11 (36.67%)	
Lymph node metastasis				
Negative	22	6 (27.27%)	16 (72.73%)	0.004
Positive	28	19 (67.86%)	9 (32.14%)	

Note: *p<0.05 is statistically significant
Abbreviation: TNM stage-tumor node metastasis stage

circCRIM1 promotes the proliferation, migration, invasion, and EMT of ESCC cells. To explore the biological function of circCRIM1 in ESCC cells, two small interfering RNAs (si-circCRIM1#1, si-circCRIM1#2) targeting the back-splicing sequence of circCRIM1 were transfected into TE-1 and KYSE30 cells. The results indicated that circCRIM1 siRNA remarkably inhibited the expression of circCRIM1 without affecting the expression of CRIM1 mRNA (Figure 2A). circCRIM1 knockdown significantly decreased the cell viability and cloning formation capability compared to the control group in ESCC cells (Figures 2B, 2C). Furthermore, the migration and invasion abilities of ESCC cells were also restrained after circCRIM1 downregulation (Figures 2D, 2E). On the basis of higher efficiency, si-circCRIM1#1 was selected for further transfection experiments. As we know, EMT is a fundamental event in the progression and metastasis of cancer cells. We speculated whether circCRIM1 could regulate ESCC cells' EMT phenotype. Then, the expression of EMT-related proteins was measured by western blotting analysis. Results demonstrated that the downregulation of circCRIM1 in ESCC cells markedly enhanced the expression of E-cadherin, an epithelial marker, while the expression of mesenchymal markers Vimentin and N-cadherin was reduced (Figure 2F). Taken together, these results provided evidence that circCRIM1 silencing repressed ESCC cells' progression with EMT.

circCRIM1 acts as a competing endogenous RNA (ceRNA) for sponging miR-342-3p in ESCC cells. Next, we wanted to explore the detailed mechanism of circCRIM1-mediated ESCC progression. circRNAs, especially cytoplasmic circRNAs, frequently act as miRNA sponges to regulate gene expression [14]. According to the evidence above, circCRIM1 was mainly located in the cytoplasm of ESCC cells, and this led us to speculate whether circCRIM1 could act as a miRNA sponge. The bioinformatics algorithms miRanda, RNAhybrid, and starBase were utilized to screen miRNAs that might bind to circCRIM1. According to the binding force score and minimum free energy, we found miR-342-3p as a potential miRNA (Figure 3A). Besides, the binding site between circCRIM1 and miR-342-3p belongs to the 8mer site type with a higher specificity than other

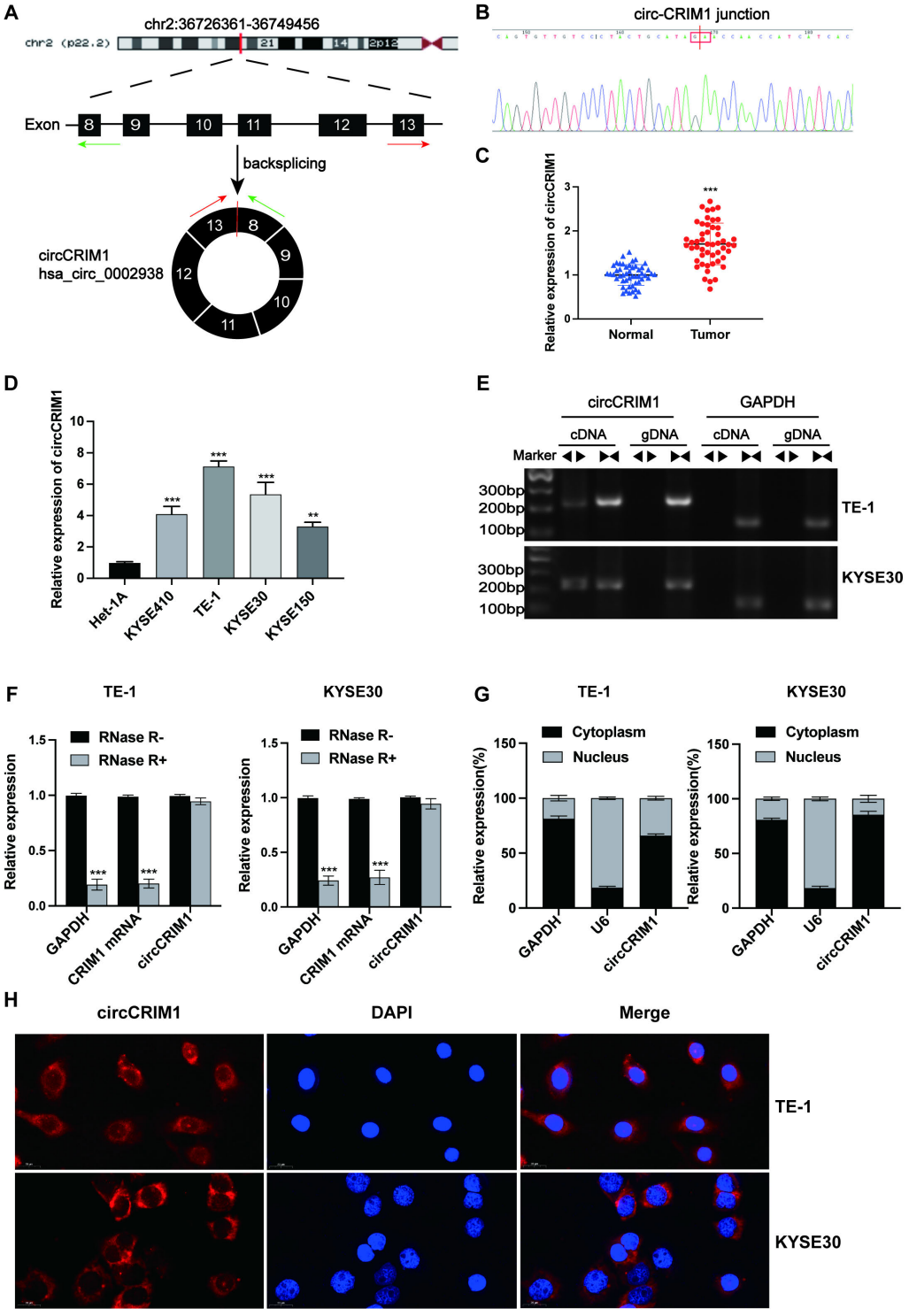

Figure 1. Circular RNA circCRIM1 is upregulated in ESCC. A) Schematic illustration of the hsa_circ_0002938 (circCRIM1) formation from the CRIM1 gene in the chromosome. B) The back-splicing junction was verified by Sanger sequencing. C) Relative expression of circCRIM1 in 50 paired ESCC tissues and adjacent non-cancer tissues D) The expression levels of circCRIM1 in different ESCC cell lines (TE-1, KYSE30, KYSE410, KYSE150) and normal human esophageal epithelial cell line (Het-1A) were measured by qRT-PCR. E) The existence of circCRIM1 was confirmed in two ESCC cells by gel electrophoresis. Divergent primers amplified circCRIM1 in cDNA but not gDNA. GAPDH was used as a linear control. F) The expression levels of circCRIM1 and CRIM1 mRNA in both TE-1 and KYSE30 cells were detected by qRT-PCR in the presence or absence of RNase R. G) The relative expression levels of circCRIM1 in the cytoplasm and nuclear by nuclear-cytoplasmic fractionation assay in TE-1 and KYSE30 cells. H) FISH assay showed that circCRIM1 was mainly located in the cytoplasm. *p<0.05, **p<0.01, ***p<0.001

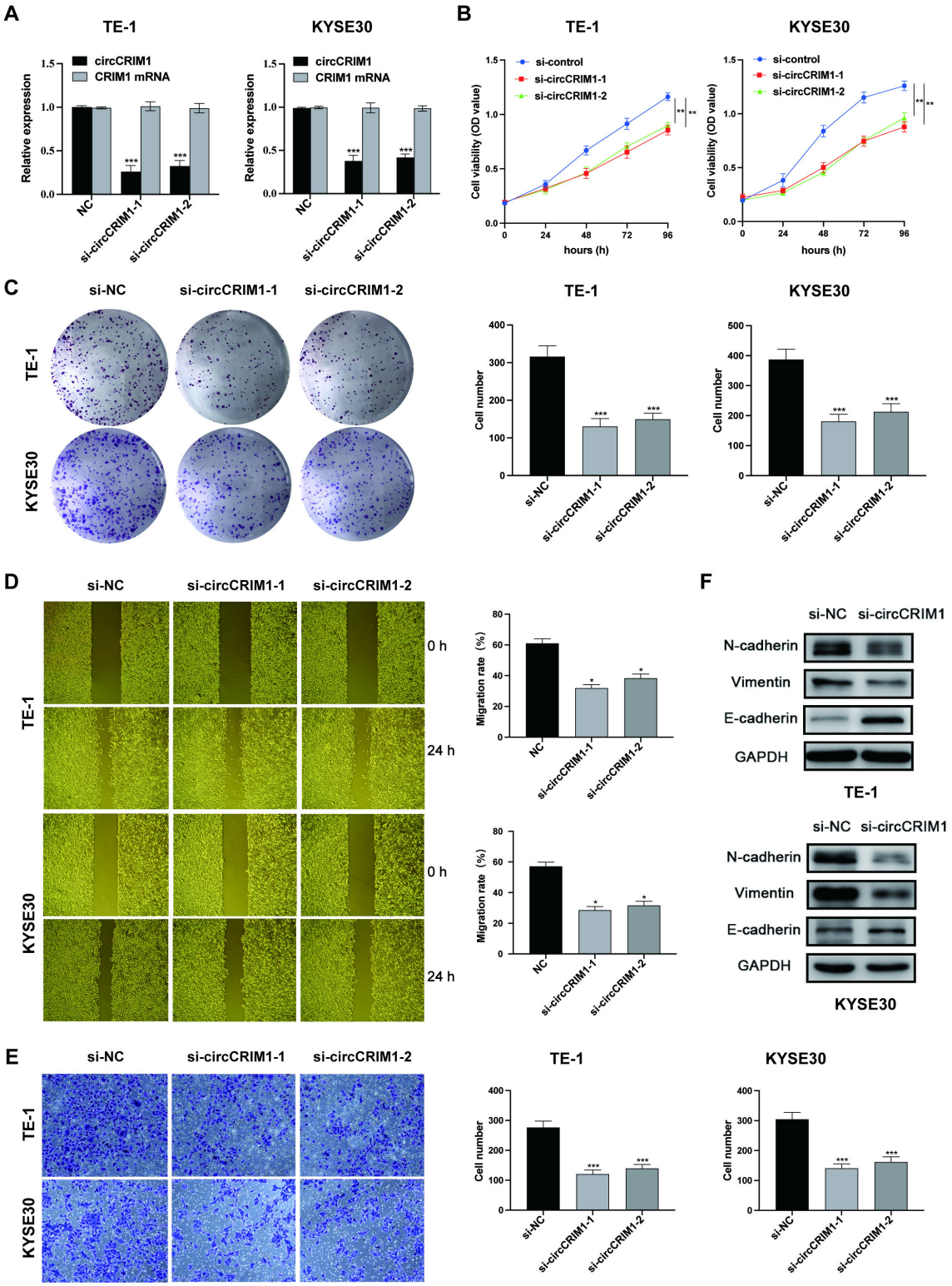

Figure 2. circCRIM1 promotes the proliferation, migration, invasion, and EMT of ESCC cells. A) The knockdown efficiency was measured by qRT-PCR in TE-1 and KYSE30 cells. B, C) The proliferation viability and colony formation ability of TE-1 and KYSE30 cells transfected with circCRIM1 siRNA or NC siRNA were evaluated by CCK-8 assay and colony formation assay. D, E) The migration and invasion abilities of TE-1 and KYSE30 cells transfected with circCRIM1 siRNA or NC siRNA were evaluated by wound healing assay and Transwell invasion assay. F) Western blotting analysis of E-cadherin, N-cadherin, and Vimentin in TE-1 and KYSE30 cells with or without circCRIM1 depletion. *p<0.05, **p<0.01, ***p<0.001

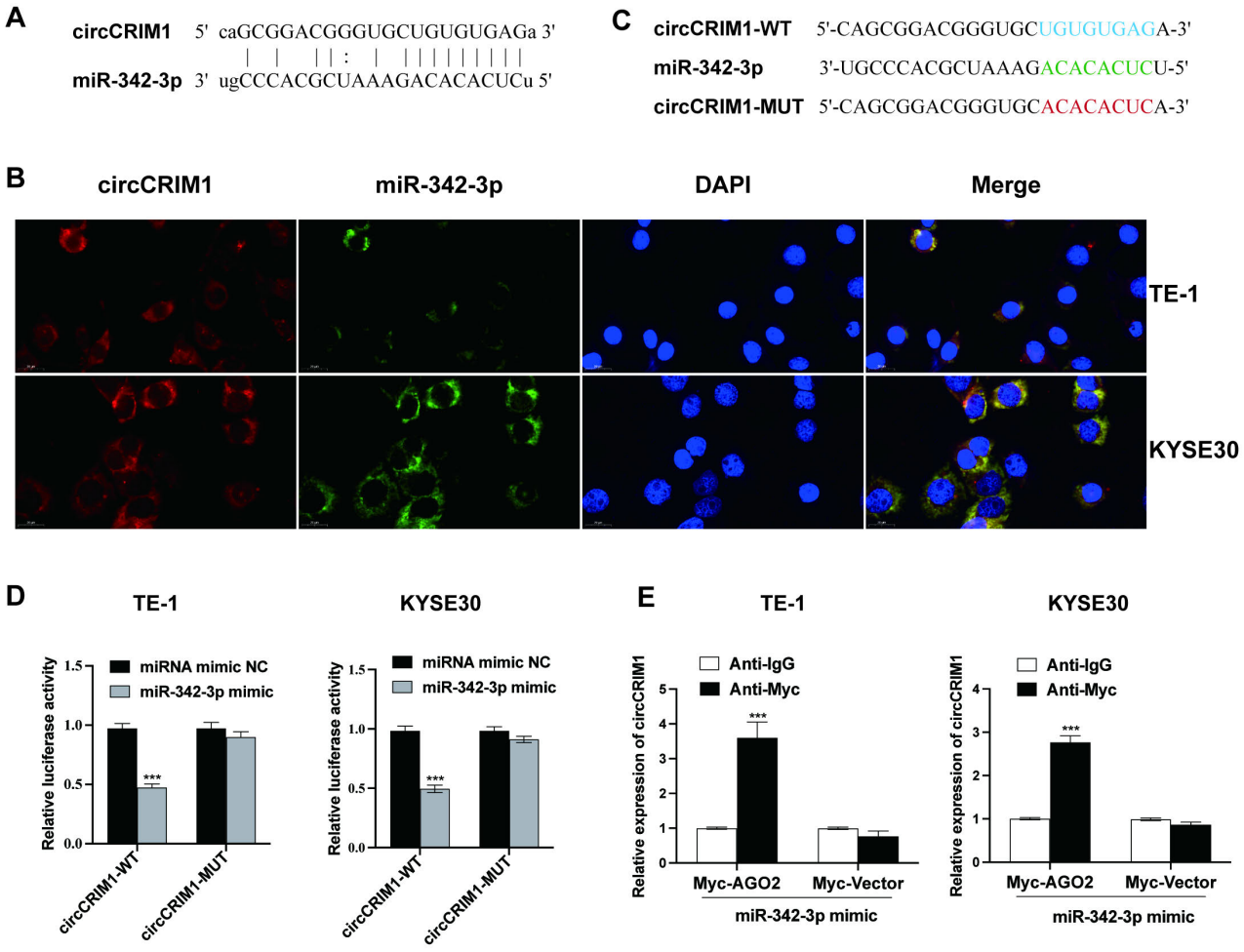

Figure 3. circCRIM1 acts as a competing endogenous RAN (ceRNA) for sponging miR-342-3p in ESCC cells. A) The potential binding site between circCRIM1 and miR-342-3p was predicted by databases. B) FISH results showed that both circCRIM1 and miR-342-3p were predominantly colocalized in the cytoplasm of TE-1 and KYSE30 cells. C) The sequences of circCRIM1-WT and circCRIM1-MUT. D) miR-342-3p mimic decreased the luciferase activity in TE-1 and KYSE30 cells transfected with circCRIM1-WT but not in the cells transfected with circCRIM1-MUT. E) Immunoprecipitation of Myc-tagged AGO2 from TE-1 and KYSE30 cells transfected with either Myc-AGO2 or Myc-vector negative control plus miR-342-3p mimic. The enrichment of circCRIM1 was detected by qRT-PCR. ***p<0.001

types of sites. To further evaluate the potential binding of circCRIM1 and miR-342-3p, their subcellular locations in ESCC cells were detected by FISH. Results showed that both circCRIM1 and miR-342-3p were predominantly co-localized in the cytoplasm of ESCC cells, suggesting the sponge effect between them (Figure 3B). Moreover, luciferase assay utilizing pmiR-circCRIM1 reporter was performed after transfecting with miR-342-3p mimic. The results showed that miR-342-3p mimic obviously reduced the luciferase activity of circCRIM1 transfected with circCRIM1-WT, while no distinct difference in that of circCRIM1-MUT (Figures 3C, 3D). Subsequently, the RIP assay further verified that circCRIM1 was significantly enriched by anti-Myc in ESCC cells co-transfected with Myc-AGO2 and miR-342-3p mimic (Figure 3E). Collectively, the above findings suggested that circCRIM1 may act as a miR-342-3p sponge in ESCC cells.

miR-342-3p suppresses the proliferation, migration, and invasion of ESCC cells. It is currently unknown whether miR-342-3p plays a role in the development and progression of ESCC tumors. We then detected the expression of miR-342-3p in ESCC tissues using qRT-PCR (Figure 4A). According to the correlation analysis, the expression level of miR-342-3p was negatively correlated with that of circCRIM1 in ESCC tissues (Figure 4B). Then, we explored the effects of miR-342-3p on the malignant behaviors to confirm whether it acted as a tumor-suppressive miRNA in ESCC. The expression of miR-342-3p in four kinds of ESCC cell lines (TE-1, KYSE30, KYSE410, KYSE150) was lower than that in the normal esophageal epithelial cell line (Het-1A) (Figure 4C). Next, we used a miR-342-3p mimic to detect its effects on the malignant behaviors in TE-1 and KYSE30 cells. CCK-8 assay and colony formation assay demonstrated that miR-342-3p

mimic obviously suppressed the proliferation and colony formation abilities of ESCC cells (Figures 4D, 4E). Wound-healing experiment and Transwell assay verified that the miR-342-3p mimic also inhibited the migration and invasion of ESCC cells (Figures 4F, 4G). These results suggested that miR-342-3p could inhibit malignant behaviors of ESCC cells and might be a tumor-suppressing gene.

TCF12 is a target of the circCRIM1/miR-342-3p axis in ESCC cells. In order to detect the molecular mechanism of the circCRIM1/miR-342-3p axis in ESCC, the bioinformatics algorithms miRWALK, miRDB, starBase, and TargetScan were used to identify the potential target genes. By overlapping the analysis, we screened out 53 candidate mRNAs that had the potential to be downregulated by miR-342-3p in ESCC (Figure 5A). Among these 53 mRNAs, TCF12 caught our attention due to its tumor-promoting effect on multiple malignant tumors, including hepatocellular carcinoma, glioma, melanoma, meningioma, and lung adenocarcinoma [15–19]. However, the role of TCF12 in ESCC is elusive. As Figure 5B demonstrates, there is a potential binding site on TCF12 for interacting with miR-342-3p. To prove the interaction between miR-342-3p and TCF12, we constructed wild-type and mutant-type 3'-UTR-TCF12 reporter vectors for dual-luciferase reporter assays (Figure 5C). Results demonstrated that miR-342-3p mimic observably reduced the luciferase activity of the wild-type 3'-UTR-TCF12 reporter, but not that of the reporter carrying mutant-type 3'-UTR-TCF12 (Figure 5D). Additionally, miR-342-3p mimic markedly decreased the expression levels of TCF12 mRNA in TE-1 and KYSE30 cells (Figure 5E). Western blotting assays revealed that miR-342-3p mimic simultaneously reduced the expression of TCF12 protein in TE-1 and KYSE30 cells (Figure 5F). The above evidence confirmed that TCF12 was a direct target of miR-342-3p in ESCC cells.

circCRIM1 promotes the progression of ESCC by regulating the circCRIM1/miR-342-3p/TCF12 axis. To investigate whether circCRIM1 promoted ESCC progression by regulating the miR-342-3p/TCF12 axis, we performed rescue experiments using a miR-342-3p inhibitor in TE-1 and KYSE30 cells. Results showed that knockdown of circCRIM1 inhibited cell proliferation, migration, and invasion, while miR-342-3p inhibition rescued these effects (Figures 6A–6D). The expression of TCF12 protein was reduced by the knockdown of circCRIM1, while this effect could be neutralized via the downregulation of miR-342-3p. A previous study reported that TCF12 served as a transcriptional repressor of E-cadherin in colorectal cancer and was associated with EMT and cancer metastasis [20]. We hypothesized that the circCRIM1/miR-342-3p axis might affect EMT-related proteins by regulating TCF12 in ESCC cells. The western blotting analysis demonstrated that circCRIM1 suppression enhanced E-cadherin expression while decreasing the levels of N-cadherin and Vimentin, but co-transfection of miR-342-3p inhibitor neutralized those effects (Figure 6E). Taken together, these findings were consistent with the hypothesis that circCRIM1 likely exerted the oncogenic properties by antagonizing the tumor-suppressive effect of miR-342-3p on TCF12 in ESCC cells (Figure 6F).

Discussion

circRNAs are a novel type of highly stable and abundant endogenous transcripts with limited coding potential [10]. With the advances in high-throughput sequencing and the emergence of bioinformatics, the number of circRNAs involving in the development and progression of cancers is rapidly increasing [21]. Generally, the circRNAs are relatively resistant to the digestion of exonuclease because of their special circular structure [22], and usually exhibit tissue- and developmental-stage-specific expression pattern [23]. Moreover, the abundance of circRNAs in cells and extracellular fluids like saliva, blood, and urine are usually sufficient for liquid biopsy, a minimally invasive biopsy method allowing for real-time monitoring [24]. Therefore, circRNAs are regarded as one of the most ideal biomarkers in diverse malignancies [25]. Recent studies indicate that circRNAs exert critical roles in the regulation of gene expression, and the dysregulation of circRNAs may seriously influence the development and progression of cancers [9]. In summary, screening out the key circRNAs and revealing their mechanisms are essential for circRNA-based diagnosis and therapy. Recently, emerging studies have reported that several circRNAs have been verified to be aberrantly expressed between ESCC and adjacent normal tissues, indicating their crucial roles in ESCC development, progression, and prognosis [26, 27]. However, the majority of circRNAs involved in ESCC are largely unknown and the exact functions and underlying mechanisms of circRNAs in ESCC still need further research.

In this study, we first identified hsa_circ_0002938, referred to as circCRIM1, as a crucial onco-circRNA in ESCC by detecting the specimens. circCRIM1 is a transcript originating from exons 8–13 of its parent gene CRIM1. As another circRNA originated from the CRIM1 gene, hsa_circ_0002346, which is derived from exons 2–4 of CRIM1 pre-mRNA, has been well studied. hsa_circ_0002346 promoted metastasis and chemotherapeutic resistance by upregulating FOXQ1 in nasopharyngeal carcinoma [28]. In addition, hsa_circ_0002346 facilitated proliferation and angiogenesis by upregulating SKP2 in hepatocellular carcinoma [29]. Likewise, hsa_circ_0002346 upregulated ZEB2 to promote cell viability, migration, invasion, and inhibit cell apoptosis in ovarian cancer [30]. Besides the tumor-promoting roles, hsa_circ_0002346 was also proved to act as a tumor-suppressing gene in some cancers, such as lung adenocarcinoma [31]. However, to our best knowledge, the roles of hsa_circ_0002938 in cancers have not been determined.

By comparing the ESCC tissues to the matched normal esophageal epithelium specimens, we found that circCRIM1

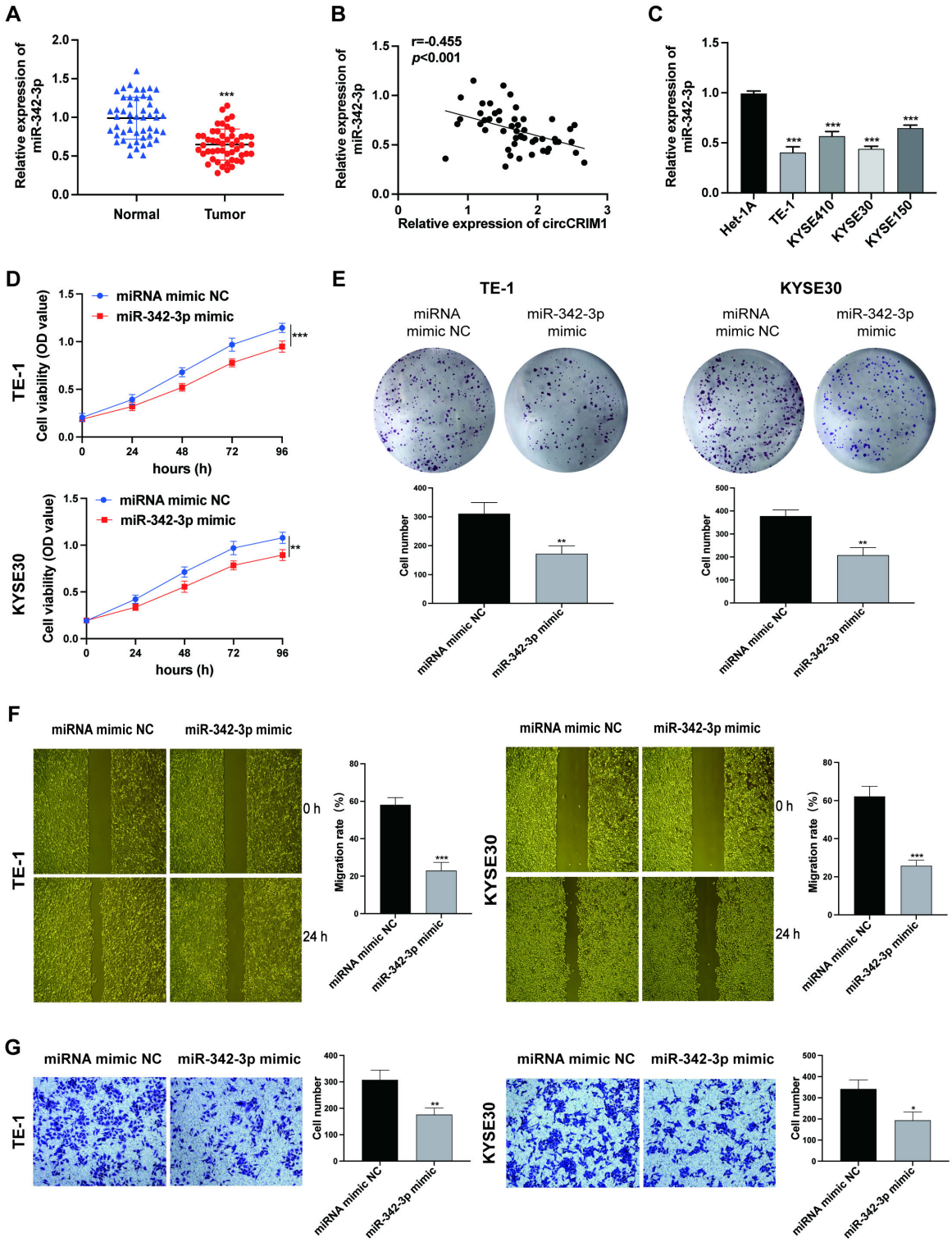

Figure 4. miR-342-3p suppresses the proliferation, migration, and invasion of ESCC cells. A) The expression levels of miR-342-3p in ESCC tissues and adjacent non-cancer tissues were detected by qRT-PCR (n = 50). B) Correlation between circCRIM1 and miR-342-3p expression in ESCC tissues. C) The relative expression of miR-342-3p in ESCC cell lines (TE-1, KYSE30, KYSE410, KYSE150) and normal human esophageal epithelial cell line (Het-1A). D, E) The proliferation viability and colony formation ability of TE-1 and KYSE30 cells transfected with miR-342-3p mimic or miRNA mimic NC were evaluated by CCK-8 assay and colony formation assay. F, G) The migration and invasion abilities of TE-1 and KYSE30 cells transfected with miR-342-3p mimic or miRNA mimic NC were evaluated by wound healing assay and Transwell invasion assay. The experiments were conducted in triplicate. *p<0.05, **p<0.01, ***p<0.001

was significantly upregulated in ESCC. In addition, high expression of circCRIM1 was correlated with TNM stage, tumor invasion range, and lymph node metastasis of the patients, indicating that circCRIM1 might be a potential onco-circRNA in ESCC. Moreover, the expression levels of circCRIM1 in four ESCC cell lines (TE-1, KYSE30, KYSE410, KYSE150) were verified to be higher than that in the normal human esophageal epithelial cell line (Het-1A). To reveal the biological functions of circCRIM1, we knocked down circCRIM1 expression with siRNA and evaluated the alteration in the malignant behaviors in ESCC cells. Consequently, circCRIM1 knockdown suppressed the proliferation, colony formation, migration, and invasion in ESCC cells, significantly. Furthermore, circCRIM1 knockdown altered the expression of some EMT-related proteins in ESCC cells. Specifically, the knockdown of circCRIM1 upregulated the expression of E-cadherin and downregulated the expression of N-cadherin and Vimentin. EMT is a key step in inducing tumor metastasis, during which the epithelial cells lose cell-to-cell adhesion and cell polarity, and gain motility properties to become mesenchymal stem cells. The initiation of EMT was featured by the downregulation of epithelial

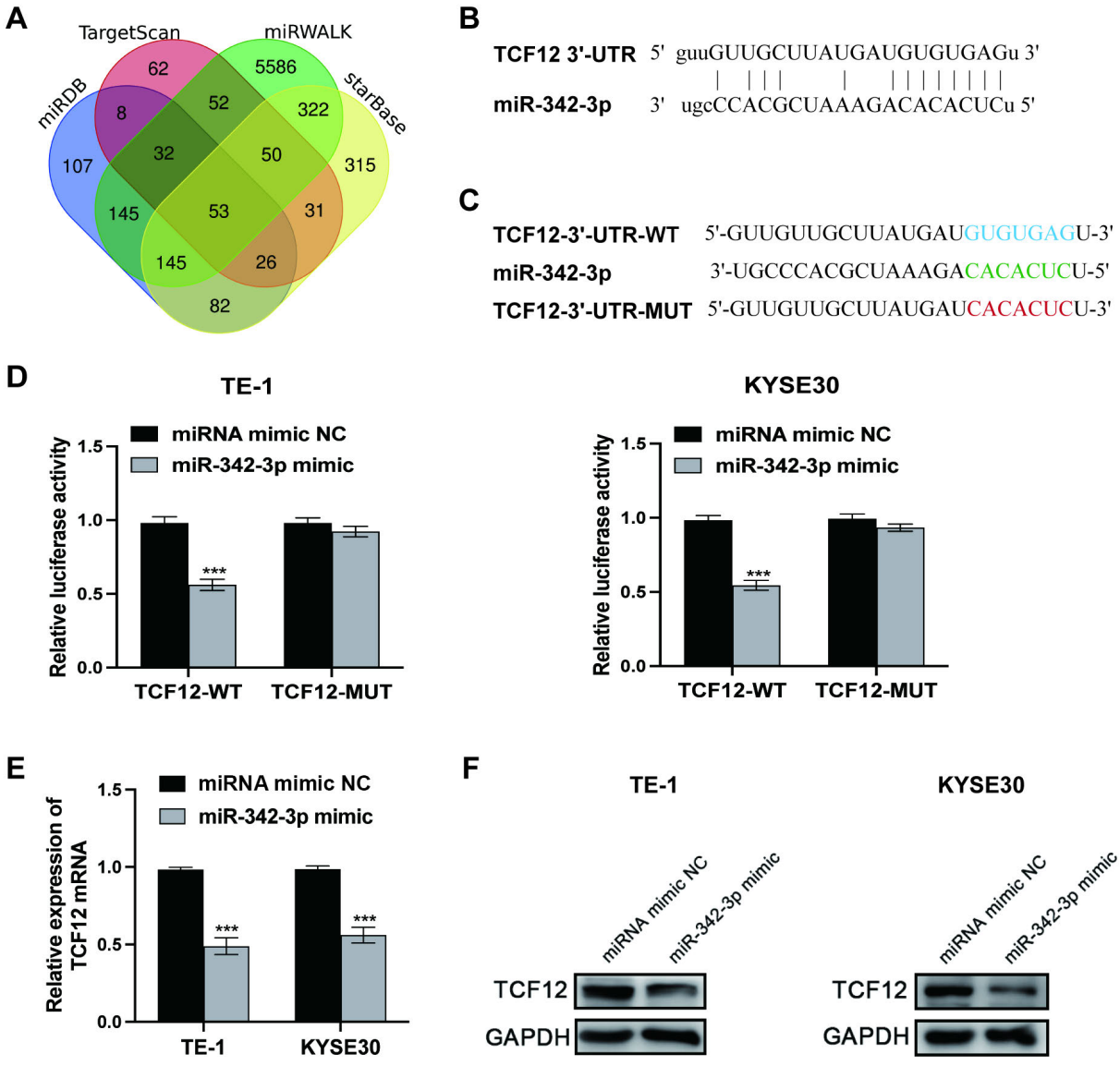

Figure 5. TCF12 is a target of the circCRIM1/miR-342-3p axis in ESCC cells. A) TCF12 was contained in the overlapped results from the bioinformatics algorithms miRWALK, miRDB, starBase, and TargetScan. B) The predicted binding sites between miR-342-3p and TCF12-3'-UTR. C) The sequences of TCF12-3'-UTR-WT and TCF12-3'-UTR-MUT. D) miR-342-3p mimic decreased the luciferase activity of ESCC cells transfected with TCF12-3'-UTR-WT but not in the cells transfected with TCF12-3'-UTR-MUT. E) miR-342-3p mimic decreased the expression of TCF12 mRNA in ESCC cells. F) miR-342-3p mimic decreased the expression of TCF12 protein in ESCC cells. The experiments were conducted in triplicate. ***p<0.001

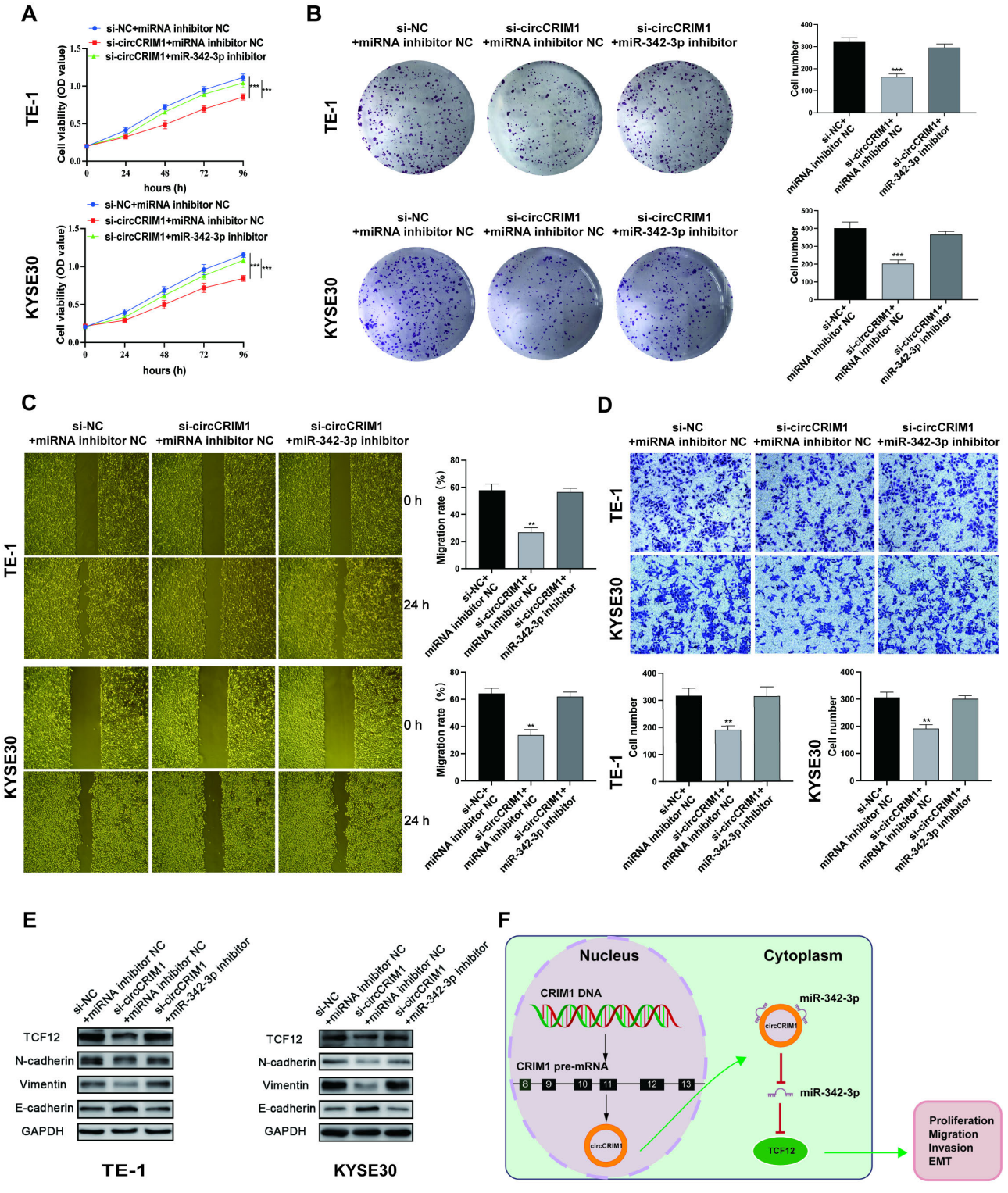

Figure 6. circCRIM1 promotes the progression of ESCC by regulating circCRIM1/miR-342-3p/TCF 12 axis. A, B) The effects of the circCRIM1/miR-342-3p/TCF12 axis on the proliferation and clone formation abilities of ESCC cells. C, D) The effects of the circCRIM1/miR-342-3p/TCF12 axis on the migration and invasion abilities of ESCC cells. E) The effects of the circCRIM1/miR-342-3p axis on the expression of TCF12 and EMT-related proteins. The experiments were conducted in triplicate. F) Hypothetical model for the role of circCRIM1 in ESCC. circCRIM1 promotes ESCC progression via the circCRIM1/miR-342-3p/TCF12 axis. **p<0.01, ***p<0.001

markers (i.e., E-cadherin) and the upregulation of mesenchymal markers (i.e., N-cadherin and Vimentin, etc.) [32]. In summary, circCRIM1 functioned as an onco-circRNA in ESCC cells by promoting proliferation, colony formation, migration, invasion, and EMT.

Despite the exon-intron and intron-derived circRNAs dominantly function in the cell nucleus by affecting the transcription of their parent genes, the circRNAs derived from exons commonly have no significant effects on their parent genes, due to their cytoplasmic location [8]. Sponging miRNAs to regulate the expression of downstream genes is one of the most well-studied functional mechanisms of cytoplasmic circRNAs in cancers [9]. microRNAs (miRNAs) are a class of conserved small non-coding RNAs consisting of 20–22 nucleotides [33]. miRNAs can bind to the 3'-UTR of target mRNAs and suppress their translation or degrade them [34]. circRNAs have been reported to competitively sponge miRNAs to neutralize their effects on the biological processes of cancer cells [35]. By analyzing the bioinformatics algorithms including miRanda, RNAhybrid, and starBase, we focused on the miR-342-3p which had been proven to be a tumor-suppressing miRNA in numerous cancers including ESCC [36]. Then, we performed FISH and confirmed that the subcellular locations of circCRIM1 and miR-342-3p were both in the cytoplasm of ESCC cells. Next, by performing dual-luciferase reporter and RIP assays, we verified that circCRIM1 and miR-342-3p occupied the same AGO2 protein to form an RNA-induced silencing complex (RISC) in ESCC cells. Furthermore, we applied the bioinformatics algorithms including miRDB, miRWALK, starBase, and TargetScan to predict the mRNAs that were potentially regulated by miR-342-3p. By overlapping the analysis, we screened out 53 candidate mRNAs that had the potential to be downregulated by miR-342-3p in ESCC. Among these 53 mRNAs, TCF12 caught our attention due to its elusive role in ESCC. TCF12 has been reported to be an oncogene in multiple malignant tumors, including hepatocellular carcinoma, glioma, melanoma, meningioma, and lung adenocarcinoma [15–19]. Specifically, TCF12 was reported as a key regulator in EMT by transcriptionally repressing the expression of E-cadherin in colorectal cancer [20], and TCF12 was also verified to facilitate the EMT process by activating the PI3K/AKT signaling pathway in glioma [15]. By conducting dual-luciferase reporter assays and rescue experiments, we confirmed the existence of the circCRIM1/miR-342-3p/TCF12 axis and its promoting effect on the EMT process in ESCC cells.

In conclusion, this study shows that circular RNA circCRIM1 is upregulated in ESCC and can promote proliferation, migration, invasion, and EMT of ESCC cells. Mechanically, circCRIM1 acts as an oncogene to exert the malignant biological behaviors in ESCC through the circCRIM1/miR-342-3p/TCF12 axis. These findings provide novel insights into ESCC progression and a promising strategy for ESCC treatment.

To our knowledge, this is the first study that investigates the expression pattern and functions of circCRIM1 in ESCC. Our results shed light on the existence of the circCRIM1/miR-342-3p/TCF12 axis in ESCC. circCRIM1/miR-342-3p/TCF12 axis facilitated proliferation, colony formation, migration, and invasion in ESCC by promoting EMT. These findings might provide a novel insight into the mechanism underlying the circRNA-induced progression of ESCC and circCRIM1 has the potential for acting as a predictive biomarker and therapeutic target for treating ESCC.

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