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Long non-coding RNA MEG3 represses cholangiocarcinoma by regulating miR-361-5p/TRAF3 axis

W.-X. LU

Department of General Surgery, Jiangsu Province Hospital, Nanjing, Ch

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Abstract. – OBJECTIVE: The aim of this study was to investigate the effect of long non-coding RNA MEG3 (MEG3) and microRNA-361-5p (miR-361-5p) on cholangiocarcinoma cells and to explore the molecular mechanisms.

PATIENTS AND METHODS: The level of MEG3 and miR-361-5p was detected using quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The relationship between miR-361-5p and MEG3/TNF receptor-associated factor 3 (TRAF3) was confirmed by the Dual-Luciferase Reporter Assay. 3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and cytometry analysis were used to determ viability and cell apoptosis. Moreover, the in level of TRAF3, p65, and p-p65 was measur Western blot assay.

RESULTS: We found that MEG3 was downre ulated in CCA tissues and cell especially in TFK1 and QBC939 cells. M bind to miR-361-5p, which was hig xpres in CCA tissues and cell lines. F r analy dicated that MEG3-plasmid c hibit and induce cell apopt is i , but these effects were signifi y rev by miR-361-5p mimic. Moreov e proved RAF3 was a direct target 361-5p and down-regulated in C and cell lin . In addition, we fo that 361-5p downregulation signifi ntly inhibite cell viability and induced apoptosis, and effects were elimina by the knockdown RAF3. Further functi analy showed that the knockdown of T 3 upr ated the expression of p-p65 dec d b R-361- hibitor in CCA cells.

CON NS: O esults suggested that G3 rep d ch giocarcinoma by down-ing h expression. Meanwhile, the pressio miR-361-5p might improve CCA rvival by targeting TRAF3 and inhibiting the ay, which might help to develop n trate for CCA therapy.

Words:
angiocarcinoma, MiR-361-5p, LncRNA-MEG3, TR

roduction

Cholangiocarcino CA), known as bile duc r, is a hepat lar carcinoma that o ates in the bile ducts epithelial cells[1,2]. CCA d easily infilt te into adjacent organs includ- liver and por ein due to its high aggressive ty[3]. Althou surgery, chemotherapy, and c liver t plantation are methods for the treat A[4-6], the incidence of CCA has significantly increased[7,8], and the 5-year survival te of CCA patients is still very low[9-12]. Therefore, it is important and urgent to develop new diagnostic or treatment strategies for CCA. In this work, we investigated the molecular mechanism correlated with the tumorigenesis and progression of CCA and hoped to seek more novel therapeutic targets for CCA.

MicroRNAs (miRNAs), a group of small non-coding RNAs with 20-22 nucleotides in length, can regulate the target gene expression by binding with the 3'-untranslated region (3'-UTR) of mRNAs[13-15]. MiR-361-5p, one of the miRNAs, was found to function as a tumor suppressor in various tumors. Sun et al[16] have indicated that the downregulation of miR-361-5p significantly inhibited tumor growth, and miR-361-5p played essential roles in the development and progression of cancers via NF-κB signaling pathway. However, whether miR-361-5p can regulate CCA cells and its regulatory functions is still unclear.

Long non-coding RNAs (lncRNAs), a group of RNAs, have more than 200 nucleotides seldom encoding proteins[17]. Previous reports[18,19] have suggested that lncRNAs act as promoter or inhibitor in cancer development by targeting oncogenes or tumor suppressors. LncRNA-MEG3 (MEG3) is located on chromosome 14q32[20,21]. Various researches have indicated that MEG3 suppressed cancer cell proliferation by activating the relative

Corresponding Author: Wenxiong Lu, MD; e-mail: luwenxiong0216@163.com

signaling pathway, such as NF-κB signaling pathway[22]. Also, MEG3 upregulation has been identified to inhibit cell proliferation in cancer cells[23-25]. However, the underlying mechanism of MEG3 in cholangiocarcinoma remains unexplored.

Therefore, the aim of the study was to investigate the effect of MEG3 and miR-361-5p on the progression of CCA and to explore the potential mechanism. In the present investigation, we detected the expression of MEG3 and miR-361-5p in CCA tissues and cell lines, investigated the effect of MEG3 and miR-361-5p on CCA cells, and further explored the molecular mechanism. We hope to provide therapeutic targets and more theoretical basis for the treatment of CCA.

Patients and Methods

Clinical Specimens Collection

A total of 20 cholangiocarcinoma tissues and 20 corresponding adjacent normal tissues were obtained from 20 cholangiocarcinoma patients (age range: 32-61 years old; 12 males, 8 female) who underwent surgical treatment at the Ji... Province Hospital. No patient received ... therapy or radiotherapy before surgery. ...se specimens were immediately snap-frozen i... uid nitrogen and preserved at -80°C until ne... All the patients enrolled in the present repo... were ≥18 years old, had no ...cer, and were not taking nonsteroid ...ti-in...matory drugs or proton pump inh...rs. The ...dy was approved by the Institu... Ethic... of the Jiangsu Provinc... Hosp... atients provided the informed ...sent a... informed about the use of t... specimens in ...search.

Cell Culture

Human ...CCA cell l... CCLP1, SG231, HUCCT1, ...K1, QBC939 ... he human intrahepa...ile duct epithelial cell line HiBECs, were ...chased ... the American Type Culture Co...n (A..., Mana...s, VA, USA). All cell lines ...ured i...swell Park Memorial Institute (...) me... 1640 (Gibco, Carlsbad, ...SA), s...ted with 10% fetal bovine seru... FBS, Ca...d, CA, USA), 1% penicillin/ stre...ycin and incubated at 37°C in a humidified ...of 5% CO_2.

...l Transfection and Reagents

...MEG3 sequence was synthesized (based on t... MEG3 sequence) and then sub-cloned into the pCDNA3.1 vector (GeneChem, Shanghai, China) (MEG3-plasmid). The empt... vector was used as a control (con... plasmi...). The TFK1 and QBC939 cells w... seeded into the 6-well plates, miR-361-5p ... (5′-ACGCCUGGAGAUUCUGAUAAUU-3... ic control (5′-UUCUCCGAAC...GUCA...T-3′), control-plasmid, MEG3... smid, ME... mid+miR-361-5p mim... nhibit... control... No. CS8005; Biomi... otech... gy, Nantong, China), miR-361-5p ...itor... t No. C...9153; Cohesion, Guan...ou, ..., TRA...hRNA (Cat No. sc-2...-V; Sa...uz... otechnology, Santa ..., CA, USA)... ol-shRNA (Cat No. ... Santa Cruz ...otechnology, Santa C..., CA, ...) or miR-361-5p inhibitor+TRAF3-shRNA ... ransfected into TFK1 and ...39 cells resp...ly using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, ...A) according t... the manufacturer's protocol. ...er 48 h of t...fection, the cells were harvested for further ...periments. The transfection e...cy was ...tected by qRT-PCR and/or Wes...lysis.

...iferase Reporter Assay

...Sta...ase (http://starbase.sysu.edu.cn/) and TargetScan Release 7.1 (www.targetscan.org/vert_71) were used to predict the binding sites between MEG3 and miR-361-5p, and the binding sites between miR-361-5p and TRAF3, respectively. The results indicated the binding sites between miR-361-5p and MEG3/TRAF3. As miR-361-5p and MEG3 for example, the fragment of the MEG3 containing the target sequence of miR-361-5p was amplified by qRT-PCR and then inserted into a pmirGLO vector (Promega, Madison, WI, USA) to form the reporter vector lncMEG3-wild-type (MEG3-WT). Another expressing vector was also constructed by inserting mutated binding site and was named as lncMEG3-mutated-type (MEG3-MUT). MEG3-WT or MEG3-MUT and miR-361-5p mimic or mimic control were co-transfected into 293T cells using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) respectively and incubated for 48 h. Then, the relative Luciferase activity was detected by Dual-Luciferase® Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions. We used the consistent assay to investigate whether TRAF3 was a direct target of miR-361-5p. The experiment was performed at least three times.

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Quantitative Real-Time Polymerase Chain Reaction (QRT-PCR)

The total cellular RNA was extracted from CCA tissues and cell lines using TRIzol reagent (Thermo Fisher Scientific, Inc. Waltham, MA, USA) following the manufacturer's instructions. NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Waltham, MA, USA) was used to measure the RNA concentrations at 260 and 280 nm (A260/280). PrimeScript™ RT-PCR Kit (TaKaRa, Otsu, Shiga, Japan) was used to perform the reverse transcription experiment and qPCR was performed using a Prism 7000 Real Time-PCR system with SYBR Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan) to detect the level of miRNA/mRNA. The amplification conditions were as follows: 35 cycles of denaturing at 94°C for 60 sec, annealing at 60°C for 60 sec, and chain extension at 72°C for 1 min, followed by a final extension step at 72°C for 10 min. GAPDH and U6 were used as internal loading controls for mRNA and miRNA respectively. The primers were provided by Sangon Biotechnology (Shanghai, China) and the primer sequences were listed as follows: GA[illegible]H, forward 5′-TGTTGCCATCAATGACCC[illegible], reverse 5′-CTCCACGACGTACTCAGCG-[illegible]6, forward 5′-GCTTCGGCAGCACATATAC[illegible] AAAT-3′; reverse 5′-CGCTTCACGAATT[illegible] CGTGTCAT-3′; lncMEG3, forward 5′-CTGC[illegible] CCATCTACACCTCACG-3′; [illegible]CTCTC-CGCCGTCTGCGCTAGGC[illegible]-3′; [illegible]361-5p, forward 5′-ATAAAGRC[illegible]GACAC[illegible]CAGA-TAGTG-3′; reverse 5′-[illegible]GTA[illegible] GCGGT-3′; TRAF3 forw[illegible]AGCAAG-GAGGCTACAAGC[illegible]3′; re[illegible]5′-CATG-CAGCTCTCGCA[illegible]AC-3′. The re[illegible]level of miR-361-5p, M[illegible]TRAF3 mR[illegible]A were calculated by [illegible]$2^{-\Delta\Delta}$[illegible]thod. All the experiments were repeated at le[illegible]imes.

Western Blot Analysis

CC[illegible]cells w[illegible]washed with ice-cold PBS an[illegible]a lys[illegible]with R[illegible]io Immunoprecipitation [illegible]PA) b[illegible] (Beyotime Biotechnology, S[illegible]ai, C[illegible]), centrifuged at 12,000 r[illegible] 30 [illegible]C to get the total proteins. The [illegible]tein co[illegible]ration was determined with a bi[illegible]choninic acid (BCA) protein kit (Beyotime[illegible]ogy, Shanghai, China). The equal [illegible]unt of proteins was separated by 10% Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and then transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were then blocked with 5% nonfat milk for 1 h at room tempe[illegible] incubated at 4° C overnight resp[illegible]ely w[illegible] the primary antibody: TRAF3 [illegible]No. 61095, dilution ratio: 1:1000; Cell Sign[illegible]Technology, Inc., Danvers, MA, USA), p-p65 [illegible]o. 3033; dilution ratio: 1:1000; Cell [illegible]naling [illegible]logy, Inc., Danvers, MA, US[illegible]-65 (Cat N[illegible] dilution ratio: 1:1000; C[illegible]Signali[illegible]Techno[illegible], Inc., Danvers, MA[illegible]) and [illegible]ctin (Cat No. 4970; dilution ratio[illegible]; C[illegible]Signali[illegible]Technology, Inc., Da[illegible], M[illegible]A). Aft[illegible]at, the membranes w[illegible]ashed [illegible]es[illegible]BST and then incuba[illegible]ith horseradi[illegible]idase-conjugated a[illegible]IgG secondar[illegible]ntibody (cat No. 707[illegible]lutio[illegible]: 1:2,000; Cell Signaling Technology, Inc., D[illegible]MA, USA) for 2 h at r[illegible]mperature. F[illegible], the protein bands w[illegible]isualized using an enhanced chemiluminescence Western blotting substrate (Millipore, erica, MA, [illegible]) according to the manufac-'s instructio[illegible]

MTT

QBC939 cells and TFK-1 cells were seed-[illegible]into 96-well plates in triplicate and incubat-[illegible]ight. Then, the culture medium was [illegible]mov[illegible]and control-plasmid, MEG3-plasmid, MEG3-plasmid+miR-361-5p mimic, inhibitor control, miR-361-5p inhibitor, or miR-361-5p inhibitor+TRAF3-shRNA with 100 µl of fresh medium were added into 96-well plates and cultured for 48 h at 37°C. Then, MTT solution (10 µl) was added to each well and incubated for further 4 h. After that, the solution was removed and 100 µl DMSO was added to each well for 20 min to solubilize the formazan products. At last, the optical density (OD) was measured at 490 nm by a micro-plate reader (Bio-Rad, Hercules, CA, USA) after 15 min of vibration mixing. The relative cell viability was normalized with the control group using optical density values.

Flow Cytometry

In order to determine cell apoptosis, TFK1 and QBC939 cells were transfected with MEG3-plasmid, control-plasmid, MEG3-plasmid+miR-361-5p mimic, inhibitor control, miR-361-5p inhibitor, or miR-361-5p inhibitor+TRAF3-shRNA and cultured in triplicate in 24-well plates. After 48 h, the cells were trypsinized and double stained with fluorescein isothiocyanate (FITC)-Annexin V and propidium iodide (PI) according to the manufacturer's instructions (Cat No. 70-AP101-100;

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MultiSciences, Hangzhou, China). Cell apoptosis was analyzed by flow cytometer (BD Biosciences, Bedford, MA, USA). The data were analyzed by applying the FlowJo7.6 analysis software.

Statistical Analysis

The data were expressed as the mean±standard deviation (SD) of at least three independent experiments performed in triplicate. The statistical analyses were carried out using the Statistical Product and Service Solution 18.0 software package (SPSS, Inc., Chicago, IL, USA). The significance of differences between the groups were estimated by the Student's *t*-test or One-way analysis of variance (ANOVA) followed by Tukey's test. All the *p*-values <0.05 were considered as statistically significant.

Results

MEG3 Was Downregulated in CCA Cells and Tumor Tissues

Firstly, the expression level of MEG3 in 20 CCA tissues and 20 normal tissues was de[tected] by qRT-PCR. Our results showed that expression was remarkably downregula[ted] in CCA tissues compared with the normal ti[ssues] (Figure 1A). Compared with human normal trahepatic bile duct epithelial cell line HiBEC[s], MEG3 was significantly dow[nregulated] in CCA cell lines (TFK1; QBC93[9]; CCL[P1]; SG231; HUCCT1) (Figure 1B). As the downregulation of MEG3 was more evidently observed in QBC939 cells, these two cell lines [were] used in our following experiments. Thes[e data] indicated that MEG3 was downregulate[d in] CCA tissues and cell lines.

MiR-361-5p Interacts Directly with MEG3

To verify whether MEG3 co[uld] function as a competing endogen[ous] RNA [for] a certain miR-NA, we used th[e bioinformatics] tool [to] count for candidate miRNAs. There[fore], the putative MEG3 targets were predicted by bioinformatics analysis Starbase (http://starbase.sysu.edu.cn/). The predicted results sh[owed] the binding sites between miR-361-5p and MEG3 (Figure 2A). Moreover, we [conducted] a Luciferase Reporter Assay to [confirm] the predicted binding sites between MEG3 and miR-361-5p, and the results confirmed the direct targeting relationship between miR-361-5p and MEG3 (Figure 2B).

MiR-361-5p Was Up-Expressed in CCA Cells and Tumor Tissues

Having learned the relationship between miR-361-5p and MEG3, we then explored the expression of miR-361-5p in cholangiocarcinoma cells and tumor tissues by qRT-PCR analysis. The results demonstrated that the expression level of miR-361-5p was significantly upregulated in CCA cancer tissue samples. (Figure 3A) Moreover,

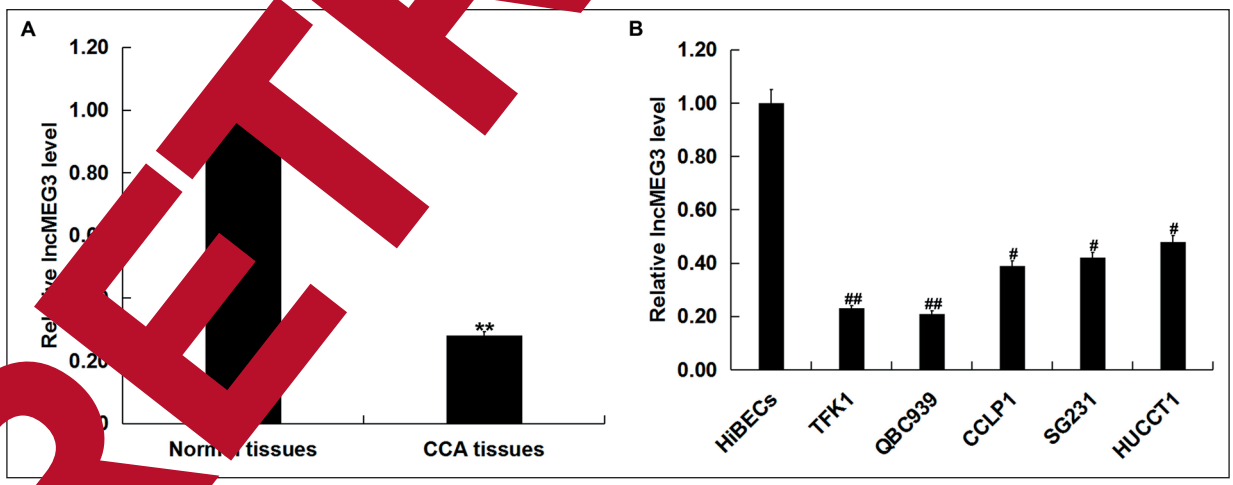

Figure 1. The low expression of MEG3 in cholangiocarcinoma tissues and cells. ***A,*** The expression of MEG3 in 20 cholangiocarcinoma tissues and 20 corresponding adjacent normal tissues of 20 cholangiocarcinoma patients was detected by qRT-PCR assay. ***B,*** MEG3 expression in cholangiocarcinoma cell lines (CCLP1; SG231; HUCCT1; TFK1; QBC939) and in the intrahepatic bile duct epithelial cell line (HiBECs) was detected by qRT-PCR assay. The data were expressed as the mean ± SD; **0.01 *vs.* normal tissues; #, ##p<0.05, 0.01 *vs.* HiBECs.

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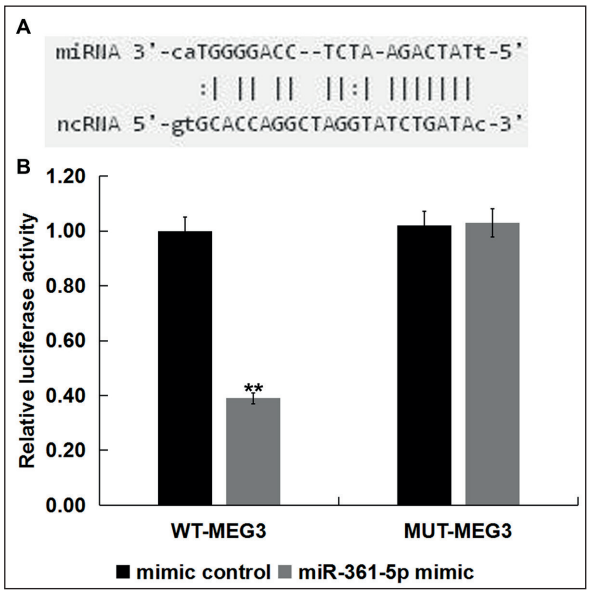

Figure 2. MEG3 binds to miR-361-5p. ***A,*** The binding sites between MEG3 and miR-361-5p. ***B,*** Dual-Luciferase Reporter Assay was used to confirm the binding sites between MEG3 and miR-361-5p. The data were expressed as the mean±SD; **p<0.01 *vs.* mimic control.

miR-361-5p was also increased in CCA c
(TFK1; QBC939; CCLP1; SG231; HU 1)
compared with that in human normal intrah
ic bile duct epithelial cell line HiBECs, nota
higher in TFK1 and QBC939 cell (Figure 3E
In summary, our data elucidat EG3 and
miR-361-5p might play an i tant in CCA
cells.

MEG3-Plasmid Inhibited the MiR-361-5p Expression

To investigate the effect of ME on C
cells, control-plasmid, MEG3-p id, mimic
control, miR-361-5p mimic, r control, or
MEG3-plasmid+miR-361-5p mi trans-
fected into TFK1 and QBC cells r The
transfection efficiency measured
PCR assay. As presen n Fig 4A an
MEG3 level was sig antly i ased in TFK1
and QBC939 cells cte th ME plas-
mid compared th grou addi-
tion, the leve miR-36 as nificantly
higher in th -361-5p mi compared
with the c p (Figures 4C nd D). Mean-
while, 3-pla transfection significantly
reduced the level o 361-5p in CCA cells,
and ect was signi y reversed by miR-
3 mimic (Figures 4E and 4F). We found
MEG3 could egatively regulate miR-361-5p
ression in C ells.

M Influe d Cell Viability and Apo CA Cells Through Down-Regulating MiR-361-5p Expression

plasmid was used to examine the
ecti influences of MEG3 upregulation on
CCA cell viability. Our results indicated that
the cell viability was significantly decreased by
MEG3-plasmid in CCA cells. However, the ef-
fect of MEG3-plasmid on CCA cell viability
was eliminated by miR-361-5p mimic (Figures
5A and B). Next, the increased apoptosis rates

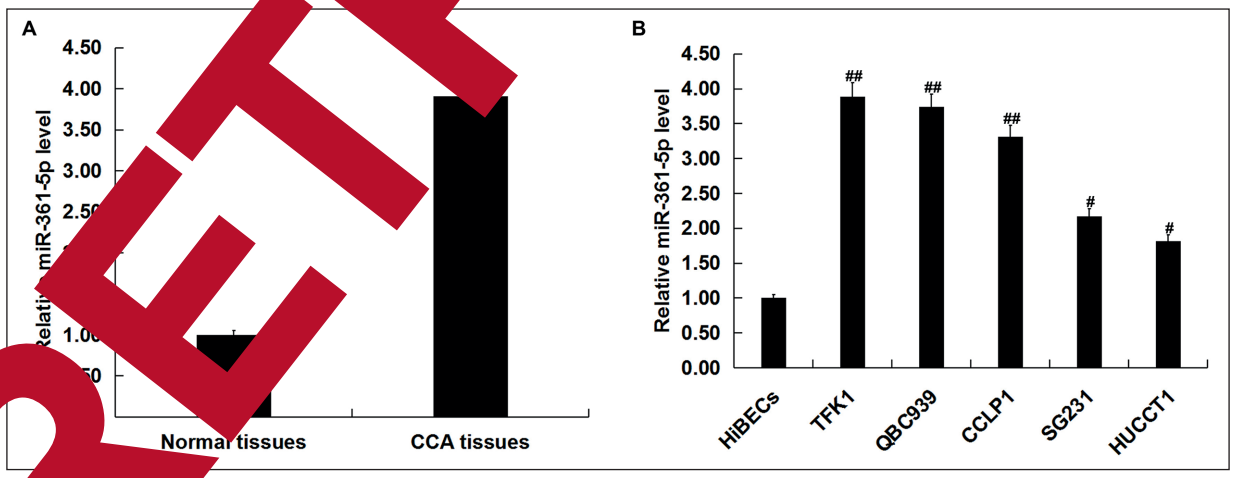

Figure 3. The high expression of miR-361-5p in cholangiocarcinoma tissues and cells. ***A,*** The expression of miR-361-5p in cholangiocarcinoma tissues and 20 corresponding adjacent normal tissues of 20 cholangiocarcinoma patients was detected T-PCR assay. ***B,*** MiR-361-5p expression in cholangiocarcinoma cell lines (CCLP1; SG231; HUCCT1; TFK1; QBC939) a intrahepatic bile duct epithelial cell line (HiBECs) was detected by qRT-PCR assay. The data were expressed as the mean D; **p<0.01 *vs.* normal tissues; #, ##p<0.05, 0.01 *vs.* HiBECs.

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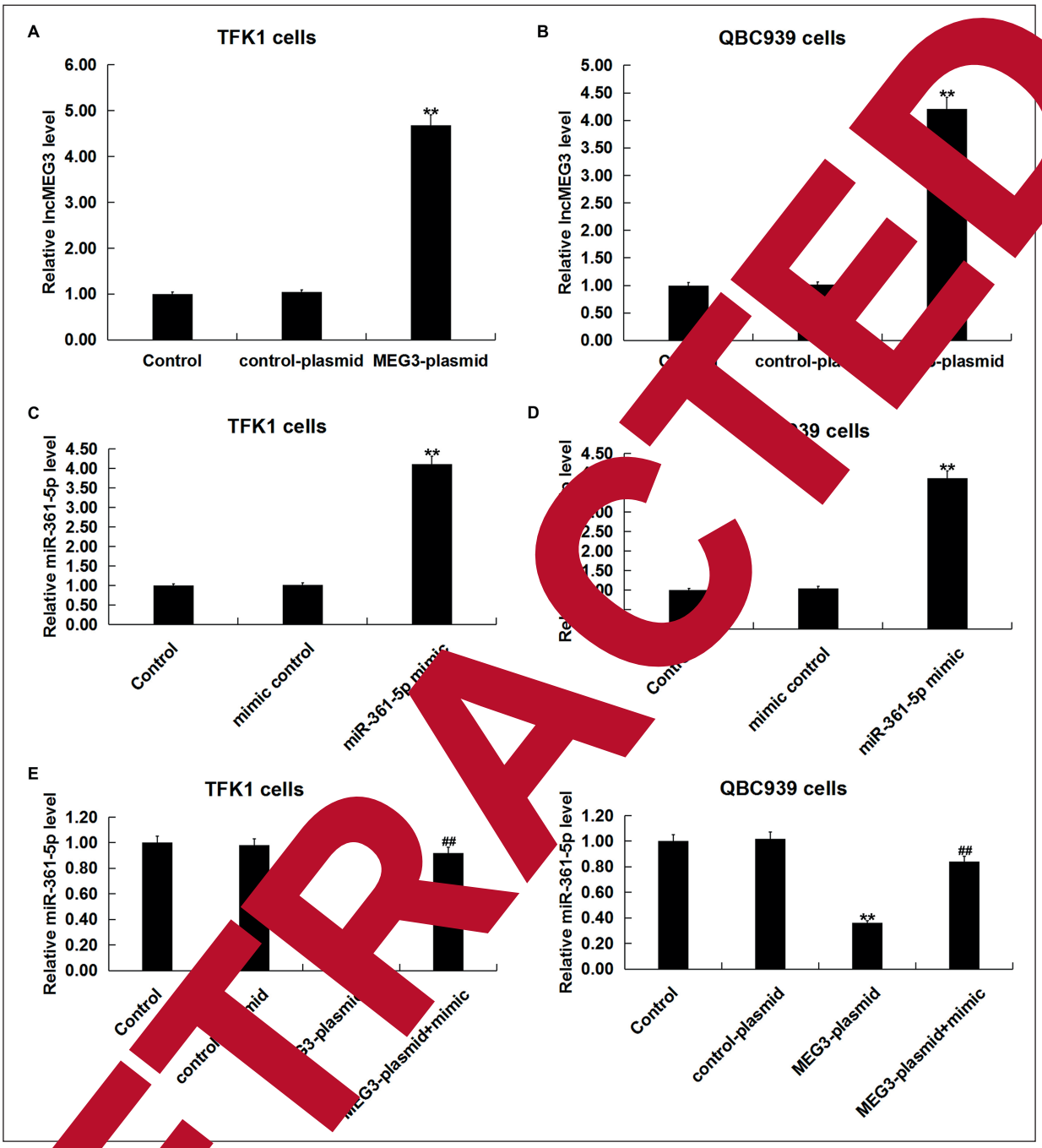

Fig MEG tively r lates miR-361-5p expression in CCA cells. ***A,*** and ***B,*** TFK1 and QBC939 cells were transfected with r l for 48 n, the expression of MEG3 in ***(A)*** TFK1 and ***(B)*** QBC939 cells was detected by qRT-PCR analysis. TFK1 BC939 cells were transfected with miR-361-5p mimic for 48 h, then, the expression of miR-361-***(C)*** TF ***(D)*** 39 cells was detected by qRT-PCR analysis. ***E,*** and ***F,*** The relative expression of miR-361-5p was d by qR ***(E)*** TFK1 and ***(F)*** QBC939 cells transfected with MEG3-plasmid or MEG3-plasmid+miR-361-5p mim e results presented as mean±SD. **$p<0.01$ *vs.* control; ##$p<0.01$ *vs.* MEG3-plasmid.

CA cells were measured by flow cytometry ysis after MEG3-plasmid transfection (Fig- and D), and the effect was reverse by miR -5p mimic co-transfection in TFK1 and QBC939 cells. According to all above findings, we confirmed that MEG3 inhibited cell viability and induced cell apoptosis by reducing the level of miR-361-5p.

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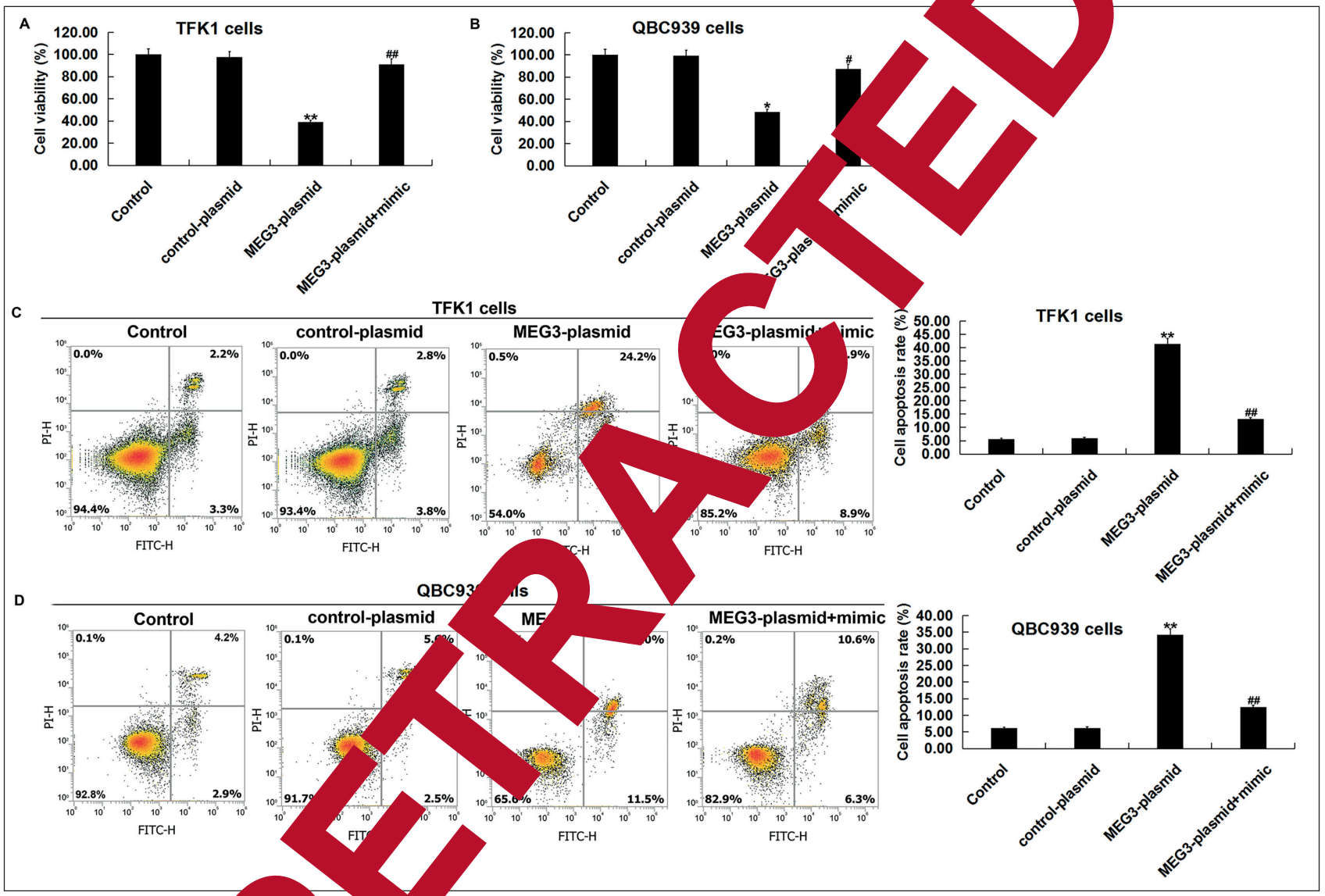

Figure 5. Effect of MEG3 on c... cel... ility an... tosis. ***A,*** and ***B,*** The cell viability of TFK1 cells and QBC939 cells was measured to evaluate the roles of MEG3-plasmid in CCA cells through MTT a... ***C,*** and ... Flow cytometry was performed to determine the percentages of apoptosis in TFK1 cells and QBC939 cells. Each bar in the histogram represented the mean±SD; ...<0.05 ... ol; #, ##p<0.05, 0.01 *vs.* MEG3-plasmid.

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TRAF3 Was a Target of MiR-361-5p

TRAF3 is found effective in various cancers, but few reports clarified how it works in cholangiocarcinoma. TargetScan Release 7.1 (www.targetscan.org/vert_71) was used to predict the targets of miR-361-5p, and the complementary sites of TRAF3 in miR-361-5p were firstly predicted. The results showed the binding sites between TRAF3 and miR-361-5p (Figure 6A). Then, the Luciferase Reporter Assay was used to confirm this prediction. As shown in Figure 6B, miR-361-5p mimic led to a significant decrease in the Luciferase activity in Wild-type 3'-UTR of TRAF3 reporter (WT-TRAF3) compared with the control group, while we detected no evident influence on TRAF3 3'UTR-mut reporter (MUT-TRAF3) (Figure 6B). All the results above illustrated the fact that TRAF3 was a direct target of miR-361-5p.

The Expression of TRAF3 Was Reduced in CCA Cells and Tumor Tissues

The expression of the TRAF3 in CCA cells and tumor tissues were further detected using qRT-PCR and Western blot assay. The fi[illegible]s indicated that TRAF3 significantly decr[illegible] CCA tumor tissues compared with the [illegible]al tissues (Figures 7A and 7B). Besides, the ex[illegible]sion of TRAF3 was found to be downregulate[illegible] CCA cell lines (TFK1; QBC939; CCLP1; SG23[illegible]

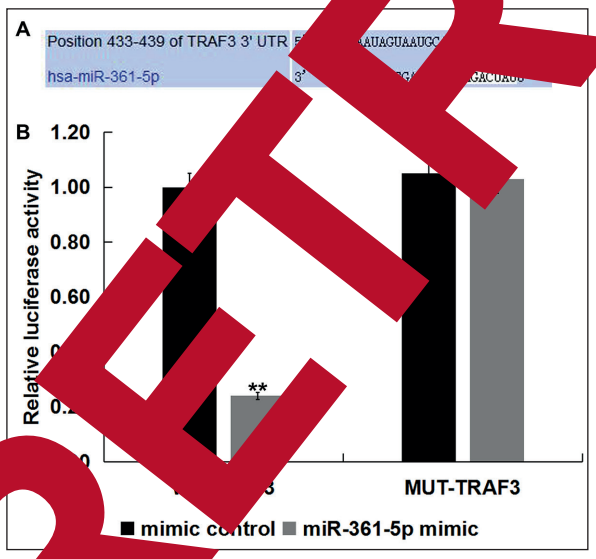

Figure 6. TRAF3 is a target of miR-361-5p. ***A,*** The binding sites between miR-361-5p and TRAF3 was predicted. Dual-Luciferase Reporter Assay was used to confirm the binding sites between miR-361-5p and TRAF3. The data were expressed as the mean±SD; $^{**}p<0.01$ *vs.* mimic control.

HUCCT1) compared with the human normal intrahepatic bile duct epithelial cell li[illegible] (Figure 7C and D), and the down[illegible]ation of TRAF3 was more evidently obs[illegible]d in TFK1 and QBC939 cells.

MiR-361-5p Downregu[illegible]ion E[illegible]ed the Expression of TR[illegible] in CCA [illegible]

To determine the [illegible] of miR-361-5p [illegible] TRAF3 in CCA cel[illegible]e tra[illegible]ted TFK and QBC939 cells wit[illegible]ito[illegible]ntrol, [illegible]-361-5p inhibitor, TR[illegible]3-sh[illegible]ontrol[illegible]NA, or miR-361-5p in[illegible]or and [illegible]3-sh[illegible]A for 48 h. qRT-RCR[illegible]y showed tha[illegible]61-5p level was reduc[illegible]-361-5p inhib[illegible]r transfected with CC[illegible]cells [illegible]s 8A and 8B). Furthermore, TRAF3-shRN[illegible]d significantly reduce the [illegible]ion of TRAF[illegible]FK1 and QBC939 c[illegible]Figures 8C and 8D). Additionally, miR-361-[illegible]inhibitor signi[illegible]cantly promoted the mRNA [illegible]el and the p[illegible]n expression of TRAF3 in [illegible]1 and QBC[illegible]cells, and this effect was re-v[illegible]byTRAF[illegible]RNA (Figures 8E-8H). Based on t[illegible]we confirmed that TRAF3-shRNA might reverse the tumor-repressive effects of miR-361-5p inhibitor on CCA cell lines.

Knockout TRAF3 Reversed the Tumor-Repressive Effects of MiR-361-5p Inhibitor in CCA Cells

To investigate the effect of miR-361-5p and TRAF3 on the biological behaviors of CCA cells, we performed some experiments on cell apoptosis and cell viability. The results demonstrated that the downregulation of miR-361-5p significantly reduced the cell viability of TFK1 and QBC939 cells (Figures 9A and B). In addition, the apoptosis assay results revealed that miR-361-5p inhibitor induced the apoptosis of CCA cells (Figures 9C and 9D). While all the effects of miR-361-5p inhibitor on CCA cells were eliminated by TRAF3-shRNA co-transfection.

Knockout TRAF3 Reversed the Inhibitory Effect MiR-361-5p Inhibitor on the Activation of NF-κB Signal Pathway

To further explore the signal pathway underlying the disincentive role in CCA cells, the protein levels of p65 and p-p65 on the NF-κB pathway were measured by Western blotting after transfection. Our results demonstrated that miR-361-5p inhibitor significantly depressed the p-p65 protein expression compared with the control group, and this effect was significantly elimi-

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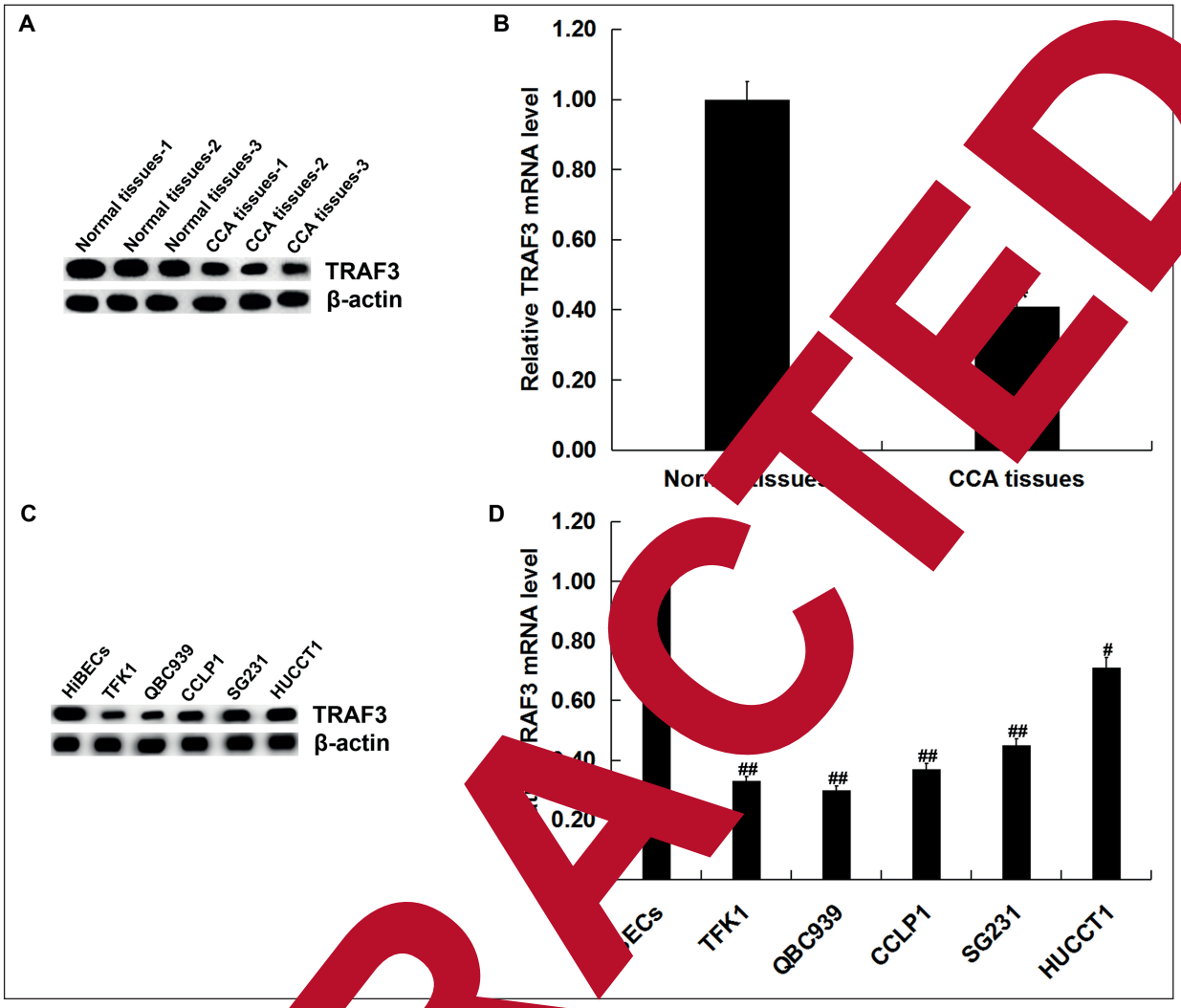

Figure 7. The low expression [illegible]F3 in [illegible]a tissues and cells. ***A,*** and ***B,*** The protein (in three representatives of CCA tissues and human [illegible]) and the mRNA expression of TRAF3 in 20 cholangiocarcinoma tissues and 20 corresponding adjacent [illegible] tissues from cholangiocarcinoma patients was detected by Western blotting and qRT-PCR assay. ***C,*** and ***D,*** The [illegible] and mRNA [illegible]ression of TRAF3 in cholangiocarcinoma cell lines (CCLP1; SG231; HUCCT1; TFK1; QBC939) and [illegible] intrahepatic bile [illegible]ithelial cell line (HiBECs) was detected by Western blotting and qRT-PCR assay. The data were [illegible]d as the mean ± [illegible] **$p<0.01$ *vs.* normal tissues; #, ##$p<0.05$, 0.01 *vs.* HiBECs.

nated by [illegible]AF3-shRNA ([illegible] 9E and 9F). These [illegible]lts indicated that [illegible]EG3 repressed CCA [illegible] inhibi[illegible] the NF-κB signaling pathway [illegible]down[illegible]ulating [illegible]iR-361-5p expression in CC[illegible]

[illegible]scussion

[illegible]erging as vital regulatory molecules in tumor suppressing and oncogenic pathways have been reported to be correlated with [illegible] physiological processes, such as cell prol[illegible]ation and apoptosis[26]. Previous studies have shown that MEG3 acted as a tumor suppressor in various tumors. However, how MEG3 functions in CCA nosogenesis remains unknown. MiRNAs have obtained increasing attention as they are involved in tumor progression. Many reports[27] suggested that miRNAs acted as tumor suppressors or oncogenes and are involved in post-translational regulation of gene expression.

In the present work, we demonstrated that MEG3 functioned as a tumor suppressor in CCA. We firstly found that MEG3 was downregulated in CCA tissues and cell lines, indicating the critical roles in CCA development. The Dual-Luciferase Reporter Assay combined with RNA binding as-

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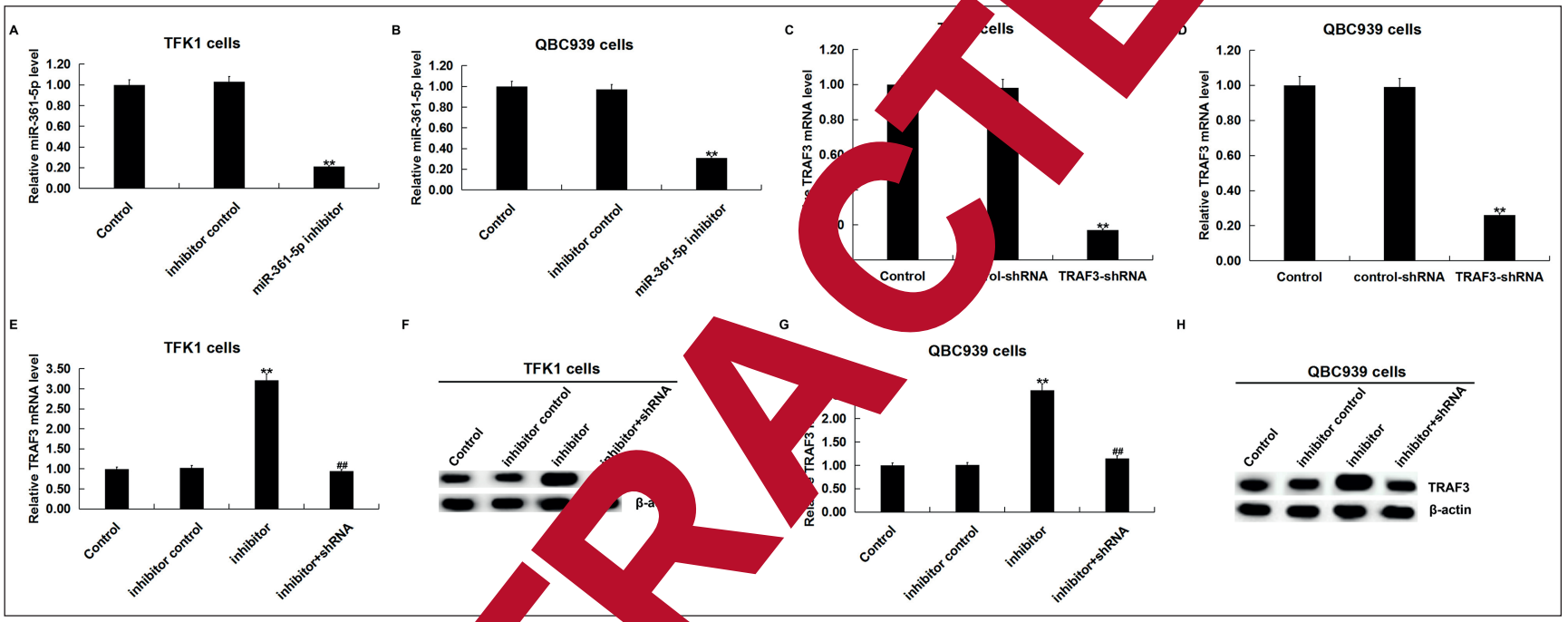

Figure 8. MiR-361-5p negatively regulates TRAF[illegible]ion in CCA c[illegible] ***A,*** and ***B,*** TFK1 and QBC939 cells were transfected with miR-361-5p inhibitor for 48 h, then, the expression of miR-361-5p in ***(A)*** TFK1 and ***(B)*** Q[illegible]39 c[illegible]s detected by qRT-PCR analysis. ***C,*** and ***D,*** TFK1 and QBC939 cells were transfected with TRAF3-shRNA for 48 h, then, the mRNA expression of TRAF3 in ***(C)*** TFK1 and [illegible]BC939 cells was detected by qRT-PCR analysis. ***E,-H,*** Relative mRNA (***E*** and ***G***) expression of TRAF3 and TRAF3 protein (***F*** and ***H***) expression in TF[illegible]nd QBC939 cel[illegible]sfected with miR-361-5p inhibitor or miR-361-5p inhibitor+TRAF3-shRNA were measured by qRT-PCR and Western blotting. The results were pr[illegible]ed as m[illegible]ean±SD. ***p*<[illegible].01 *vs.* Control; ##*p*<0.01 *vs.* inhibitor.

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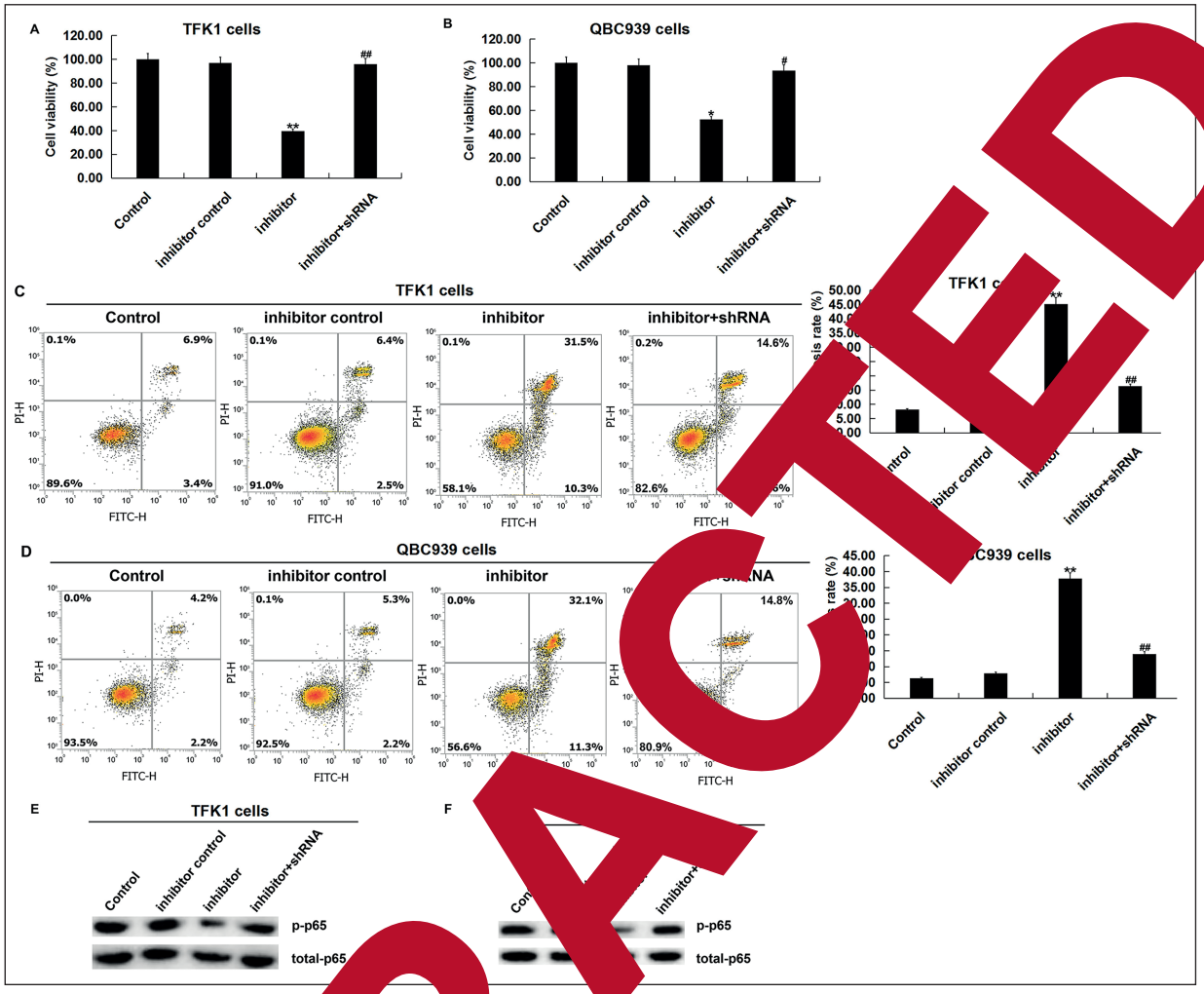

Figure 9. TRAF3 knockdow ersed the effects of miR-361-5p inhibitor on CCA cells. ***A,*** and ***B,*** The cell viability of TFK1 cells and Q alls tr R-361-5p inhibitor or miR-361-5p inhibitor+TRAF3-shRNA was measured through MTT as . ***C,*** cytometry was performed to determine the percentages of apoptosis in TFK1 cells and QBC939 cells fected -361-5p inhibitor or miR-361-5p inhibitor+TRAF3-shRNA. ***E,*** and ***F,*** Western blotting was performe etermine the ion of p65 and p-p65 in TFK1 cells and QBC939 cells transfected with miR-361-5p inhibitor or 5p inhibitor+T hRNA. Each bar in the histogram represents the mean±SD; *, **$p<0.05$, 0.01 *vs.* control; # 01 *vs.* inhibitor.

say provi urther support interaction of MEG3 -361-5 activity. Mea while, we found that 361-5p upregulated in CCA and it wa tivel related with MEG3 expression in CC e upre ion of MEG3 inhibited viabi pro d cell apoptosis in TFK1 C939 ch proved its tumor repressive ct and er revealed that MEG3 was able ad to positively downregulate the expressi p. Another very important finding was that miR-361-5p mimic could reverse the ts induced by MEG3-plasmid in CCA cells.

we speculated that miR-361-5p might be a cogene in CCA progression. To confirm this inference, a bioinformatics tool was used to predict the potential targets of miR-361-5p. Our results demonstrated that TRAF3 was a direct target of miR-361-5p in CCA. Next, we used qRT-PCR and Western blotting assay to measure the TRAF3 mRNA level and protein expression in CCA tissues and cell lines. We showed that TRAF3 was decreased in CCA tissues and CCA cell lines. Then, we especially focused on the effect of miR-361-5p inhibitor treatment on CCA cell lines. Here, our results indicated that TRAF3 expression was enhanced by miR-361-5p inhibitor treatment in CCA cells. We then further confirmed whether MEG3 regulated CCA cell

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growth via regulating miR-361-5p by targeting TRAF3, and we found that miR-361-5p inhibitor significantly inhibited CCA cell viability, induced cell apoptosis, and repressed NF-κB pathway. It was worth mentioning that all the effects of miR-361-5p inhibitor on CCA cells were eliminated by TRAF3 silencing.

Conclusions

LncRNA MEG3 served as a tumor suppressor in CCA development by regulating miR-361-5p/TRAF3/NF-κB pathway. This research better illuminated the pathogenesis and development of CCA and might provide novel clinical therapies for CCA treatment.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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