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Circular RNA circCNOT6L regulates cell development through modulating miR-384/FN1 axis in esophageal squamous cell carcinoma

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Abstract. – OBJECTIVE: In recent years, circular RNAs (circRNAs) and microRNAs (miRNAs) have been shown to be related to the development of esophageal squamous cell carcinoma (ESCC). However, their functional mechanisms remain to be investigated. Herein, we focus our research on the functions and mechanisms of circCNOT6L and miR-384 in ESCC.

MATERIALS AND METHODS: The levels of circCNOT6L, miR-384, and fibronectin 1 (FN1) were determined using quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). RNase R was used to investigate circCNOT6L stabilization. Cell proliferation and apoptosis were assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and flow cytometry, respectively. Western blot assay was employed to analyze the protein levels of FN1, proliferation-related genes, and iron metabolism-related genes. In addition, the interaction between miR-384 and circCNOT6L or FN1 was predicted by starBase3.0 and confirmed by the Dual-Luciferase reporter assay. Mouse xenograft was carried out to measure the effect of circCNOT6L on tumor growth *in vivo*.

RESULTS: CircCNOT6L and FN1 levels were upregulated, and miR-384 level was downregulated in ESCC tissues/cells. CircCNOT6L knockdown attenuated ESCC cell proliferation and iron metabolism disorder, as well as accelerated apoptosis. Notably, circCNOT6L targeted miR-384, and miR-384 targeted FN1. MiR-384 depletion and FN1 upregulation weakened the effects of circCNOT6L knockdown and miR-384 overexpression on ESCC cell progression, respectively. Besides, circCNOT6L knockdown inhibited tumor growth *in vivo*.

CONCLUSIONS: Our results demonstrated that circCNOT6L positively regulated the development of ESCC cells *via* modulating miR-384/FN1 axis. Our findings provided a theoretical basis for the therapy of ESCC patients.

Key Words:
CircCNOT6L, MiR-384, FN1, Cell development, ESCC.

Introduction

Esophageal squamous cell carcinoma (ESCC), with poor prognosis, is the main type of esophageal cancer (EC), which is also one of the leading reasons for tumor-related death[1]. According to the statistics, there are approximately 456,000 new cases and about 400,200 deaths of EC every year[2]. Although some methods are applied for the diagnosis and therapy of EC, the prognosis of patients is still unsatisfactory[3]. Therefore, it is of importance to investigate the mechanism of ESCC development.

Circular RNAs (circRNAs), identified as novel non-coding RNAs, have a closed-loop and exert pivotal function in human cancers[4,5]. They were involved in various cell progression, including proliferation, mobility, autophagy, and apoptosis[6-8]. CircCNOT6L, also known as circ_0006168, was less reported in human cancers. Only Shi et al[9] showed that circCNOT6L level was upregulated in ESCC tissues/cells, and circCNOT6L knockdown repressed ESCC cell proliferation and mobility by targeting miR-100. This data indicated that circ_0006168 was crucial for the development of ESCC. Therefore, it is essential to further explore the functional mechanism of circ_0006168 in ESCC cells.

MicroRNAs (miRNAs), considered as small non-coding RNAs with around 20-25 nucleotides, regulate the development of human cancers by modulating translation or degradation of message RNA (mRNA)[10]. Wei et al[11] indicated that miRNA exerted function as a tumor suppressor or an oncogene in cancers. MiR-30a-3p suppressed lung cancer cell proliferation, as well as cycle *via* regulating DNA methyltransferase 3a level[12]. MiR-233 accelerated the development of neuroblastoma cells by binding to forkhead box

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O1 (FOXO1)[13]. MiR-384, an endogenous miRNA with low expression in ESCC, was shown to suppress ESCC cell growth[14]. However, the detailed mechanism of miR-384 in ESCC is not fully reported.

Here, we found that miR-384 was a potential target of circCNOT6L and likely targeted fibronectin 1 (FN1). Then, the levels of circCNOT6L, miR-384, and FN1 were detected in ESCC tissues and cells. Also, we investigated the relationship among them and their function in ESCC cells. Furthermore, circCNOT6L acting as an oncogene to regulate ESCC cell growth was reported, and a new mechanism showing that circCNOT6L exerted function *via* modulating miR-384/FN1 axis was confirmed.

Materials and Methods

Tissues and Cell Culture

In this study, we collected ESCC tissues, as well as normal tissues (N=30), from the patients at the Affiliated Hospital of Traditional Chinese Medicine of Southwest Medical University and obtained informed consent from each patient. This research was approved by the Ethics Review Committees of the Affiliated Hospital of Traditional Chinese Medicine of Southwest Medical University.

One normal cell line (HET-1A) and two ESCC cell lines (TE1 and SKGT4) were obtained from Shanghai Institute for Biological Sciences (Shanghai, China). Roswell Park Memorial Institute-1640 (RPMI-1640) with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) was employed to incubate cells at an incubator (Thermo Fisher Scientific, Waltham, MA, USA) with 5% CO_2 at 37°C.

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

Total RNA was isolated with the use of an RNA extraction kit (Invitrogen, Carlsbad, CA, USA). Then, the PrimeScript RT kit (RR014A, TaKaRa Biotechnology Ltd., Dalian, China) and SYBR Premix Ex Taq II (Vazyme Biotech, Nanjing, China) were used to perform reverse transcription assay and qRT-PCR, respectively. Gene expression was normalized to U6 level or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) level and analyzed using the $2^{-\Delta\Delta Ct}$ method. The primers used in this search were as follows: CircCNOT6L, sense 5'-ACCAGCAGAACTAGGAAACA-3' and anti-sense 5'-TGGCATCCCTATTAGTCTTTC-3'; CNOT6L mRNA sense 5'-GGAAACATGGTGTCTCTCAGG-3' and anti-sense 5'-CGAGATTGTCAAGCATGAAGTT-3'; miR-384, sense 5'-TGTTAAATCAGGAATTTTAA-3' and anti-sense 5'-TGTTACAGGCATTATGAA-3'; FN1, sense 5'-GAGAATAAGCTGTACCATCGCAA-3' and anti-sense 5'-CGACCACATAGGAAGTCCCAG-3'; U6, sense 5'-CTCGCTTCGGCAGCACA-3' and anti-sense 5'-AACGCTTCACGAATTTGCGT-3'; GAPDH, sense 5'-GCACCGTCAAGCTGAGAAC-3' and anti-sense 5'-TGGTGAAGACGCCAGTGGA-3'; 18S rRNA, sense 5'-AACCTGGTTGATCCTGCCAGT-3' and anti-sense 5'-GGCACCAGACTTGCCCTC-3'.

Actinomycin D and RNase R Treatment

2 mg/ml Actinomycin D (Sigma-Aldrich, St. Louis, MO, USA) was applied to block transcription before RNase R treatment, dimethyl sulphoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) was used as control. 6 units of RNase R (Geneseed Biotechnology, Guangzhou, China) were applied to treat 2 μg RNA at 37°C for 20 min. Next, qRT-PCR was carried out to analyze the levels of circCNOT6L and CNOT6L mRNA.

Subcellular Fractionation Location

The PARIS Kit (Life Technologies, Carlsbad, CA, USA) was applied for cytoplasmic fraction based on the recommended instruction. In brief, ESCC cells were incubated with lysis buffer for 10 min and then, centrifuged at 4°C. Then, the supernatant and pellets were used to isolate cytoplasmic RNA and nuclear RNA, respectively. Finally, qRT-PCR was conducted to determine RNA level.

Cell Transfection

For upregulation or downregulation of circCNOT6L and miR-384, some products, including small interfering RNA against circCNOT6L (si-circCNOT6L), Small hairpin RNA against circCNOT6L (sh-circCNOT6L), miR-384 mimic (miR-384), miR-384 inhibitor (anti-miR-384), and their control (si-NC, miR-NC, anti-miR-NC, and sh-NC), were purchased from GenePharma (Shanghai, China). In addition, circCNOT6L or FN1 sequence was amplified and then inserted into pcDNA3.1 vector (GenePharma, Shanghai, China) to generate overexpression vector. Transfection assay was conducted with the application

of Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA).

3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) Assay

MTT kit (Promega, Madison, WI, USA) was used to analyze cell proliferation based on the user's manual. Briefly, transfected TE1 and SKGT4 cells were cultured for 24 h, 48 h, or 72 h. Subsequently, the cells were treated sequentially by MTT solution and dimethyl sulfoxide (DMSO). Finally, absorbance at 490 nm was examined by Multiskan FC (Thermo Fisher Scientific, Waltham, MA, USA).

Western Blot

Western blot was performed as previously described[15]. In brief, the proteins were obtained using lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA), separated by 10% sodium dodecyl sulfonate (SDS)-polyacrylamide gel electrophoresis (PAGE), and then, transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% non-fat skim, the membranes were incubated sequentially by the primary antibody (1:1,000, Abcam, Cambridge, MA, USA) against antigen KI-67 (Ki67), proliferating cell nuclear antigen (PCNA), iron regulator protein 1 (IRP1), IRP2, transferrin receptor (TfR1), ferritin light chain (FTL), FN1, or GAPDH and corresponding secondary antibody (1:2,000, Abcam). Finally, protein band was examined using enhanced chemiluminescence (ECL) kit (Bio-Rad, Hercules, CA, USA).

The Analysis of Apoptosis

Cell apoptosis was investigated by an Annexin-V-fluorescein fisothiocyanate (FITC) apoptosis detection kit (Beyotime Biotechnology, Shanghai, China) based on the recommended protocol. Briefly, TE1 and SKGT4 cells were transfected and cultured for 48 h. Next, Annexin V-FITC and propidium iodide (PI) were applied to treat the cells. Finally, apoptosis rate was determined using flow cytometer (BD Bioscience, Franklin Lakes, NJ, USA).

The Dual-Luciferase Reporter Assay

CircCNOT6L-WT/FN1 3'UTR-WT or circCNOT6L-MUT/FN1 3'UTR-MUT was cloned into psiCHECK-2 vector (Promega, Madison, WI, USA). Next, TE1 and SKGT4 cells were co-transfected with recombinant vector and miR-384 or miR-NC. 48 h later, Luciferase density was explored with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Mouse Xenografts

This experiment was conducted in line with the guidance of the National Animal Care and Ethics Institution and approved by the Animal Research Committee of the Affiliated Hospital of Traditional Chinese Medicine of Southwest Medical University. Briefly, BALB/c nude mice (5 weeks old) were subcutaneously injected with TE1 cells stably expressed with sh-circCNOT6L or sh-NC. For the measurement of the tumor, tumor volume (length × width^2 × 0.5) was calculated every week, and tumor weight was analyzed after 5 weeks of injection. Next, the levels of circCNOT6L, miR-384, and FN1 were determined in these tumors.

Statistical Analysis

The data were obtained from at least three independent biological repetitions, analyzed using Student's *t*-test, and then, exhibited as the means ± standard deviation (SD). The correlation between the levels of two genes was explored *via* analyzing Pearson's correlation coefficient. Difference was considered significant when $p<0.05$.

Results

CircCNOT6L Level Was Upregulated in ESCC Tissues and Cells

To explore the role of circCNOT6L in ESCC, circCNOT6L level in ESCC tissues was analyzed using qRT-PCR. The results suggested that circCNOT6L level was significantly upregulated in ESCC tissues compared with that in adjacent normal tissues (Figure 1A). Then, we investigated the level of circCNOT6L in ESCC cells. As shown in Figure 1B, circCNOT6L level was greatly high in ESCC cells (HET-1A) than that in normal cells (TE1 and SKGT4). Therefore, circCNOT6L might act as an oncogene in ESCC.

CircCNOT6L Mainly Existed in Cytoplasmic and Was Stable

It was well-known that circRNA was a circular RNA and mainly existed in cytoplasmic[4]. To confirm whether circCNOT6L had these biological characteristics, some experiments

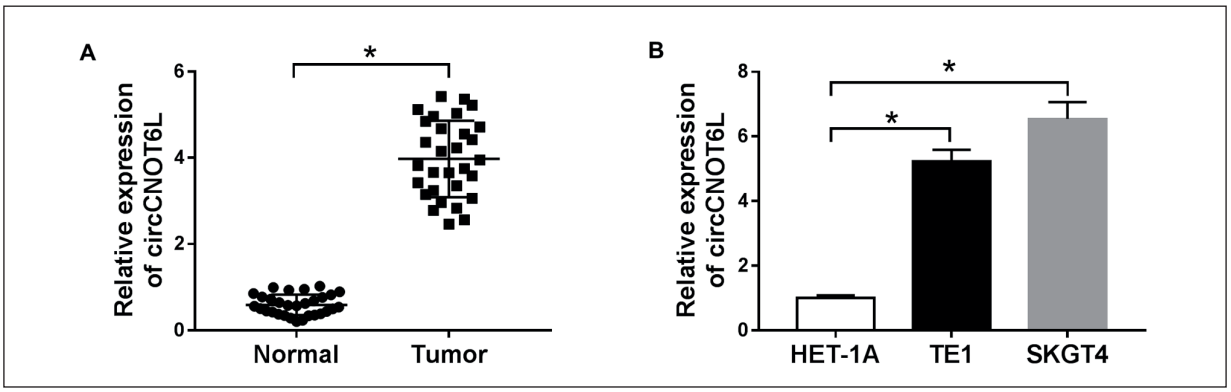

Figure 1. The level of circCNOT6L was detected in ESCC tissues and cells. **A,** The level of circCNOT6L was detected by qRT-PCR in ESCC tissues and normal tissues. **B,** The level of circCNOT6L was determined in ESCC cells (TE1 and SKGT4) and normal cells (HET-1A). *p<0.05.

were carried out in TE1 and SKGT4 cells. Firstly, total RNA was treated with RNase R after Actinomycin D treatment, and the levels of circCNOT6L and CNOT6L mRNA were determined. The results showed that CNOT6L mRNA level was dramatically reduced, while circCNOT6L level showed no change after RNase R treatment, meaning that circCNOT6L was resistant to RNase R (Figure 2A, B, D, and E). In addition, qRT-PCR demonstrated that circCNOT6L in cytoplasmic was remarkably higher than that in nuclear (Figure 2C and F). Therefore, circCNOT6L mainly existed in cytoplasmic and was stable.

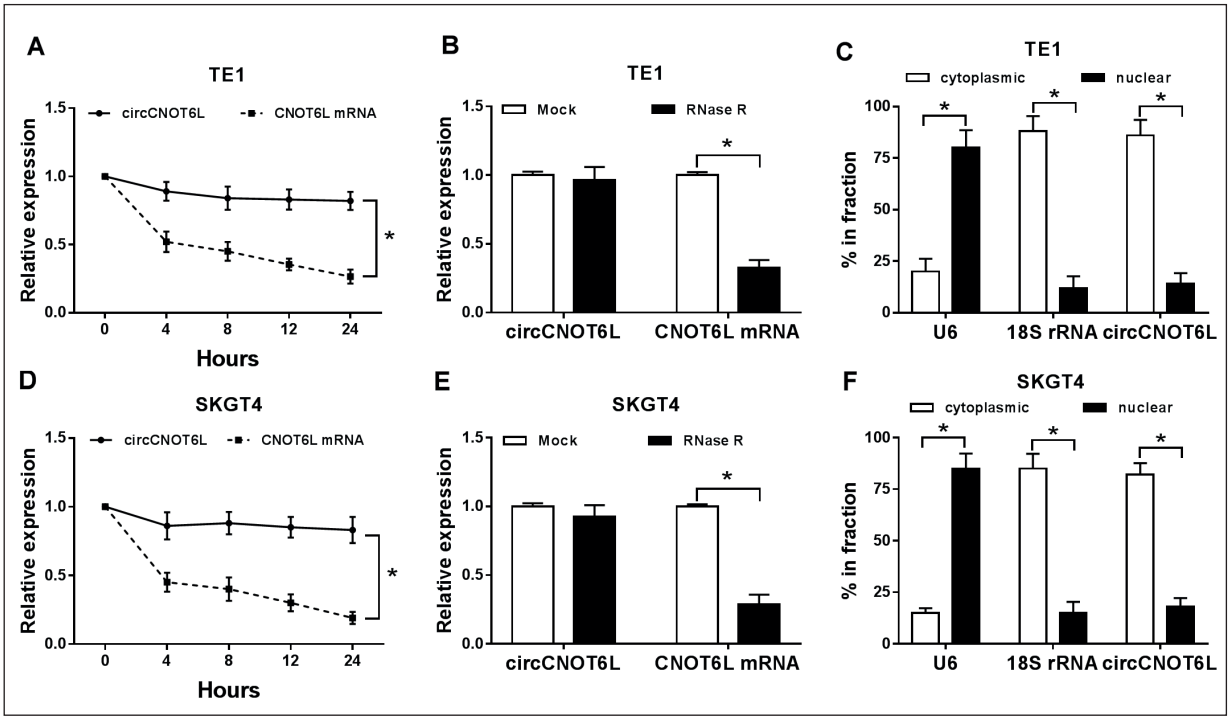

Figure 2. CircCNOT6L was mainly determined in cytoplasmic and was resistant to RNase R. **A,** and **B,** The levels of circCNOT6L and CNOT6L mRNA were examined in total RNA from TE1 cells after RNase treatment. **C,** The levels of U6, 18 rRNA and circCNOT6L were analyzed in cytoplasmic and nuclear of TE1 cells. **D,** and **E,** The levels of circCNOT6L and CNOT6L mRNA were determined in total RNA from SKGT4 cells after RNase treatment. **F,** The levels of U6, 18 rRNA and circCNOT6L were measured in cytoplasmic and nuclear of SKGT4 cells. *p<0.05.

CircCNOT6L Knockdown Repressed ESCC Cell Proliferation as well as IRE/IRP Regulatory System and Induced Apoptosis

To further investigate the effect of circCNOT6L on the cellular phenotype of ESCC, TE1 and SKGT4 cells were transfected with si-circCNOT6L or si-NC. Knockdown efficiency was confirmed by qRT-PCR (Figure 3A and B). Then, MTT assay was performed to analyze cell proliferation ability. The results showed that cell proliferation was significantly suppressed by circCNOT6L knockdown (Figure 3C and D). Meanwhile, we analyzed the levels of two proliferation-related markers (Ki67 and PCNA) and found that the levels of Ki67 and PCNA were remarkably downregulated by circCNOT6L knockdown (Figure 3E and F). Next, flow cytometry was used to determine cell apoptosis rate. As shown in Figure 3G, circCNOT6L knockdown dramatically induced the apoptosis of TE1 and SKGT4 cells. In addition, the effect of circCNOT6L on iron metabolism was explored by detecting the levels of IRP1, IRP2, TfR1, and FTL. IRPs can bind iron-responsive elements (IREs) to regulate gene expression and inversely modulated TfR1 as well as FTL[16]. In this study, we detected that the levels of IRP1, IRP2, and TfR1 were downregulated by circCNOT6L knockdown, and the level of FTL was upregulated by circCNOT6L knockdown (Figure 3H and I). Taken together, circCNOT6L knockdown repressed the growth of ESCC cells.

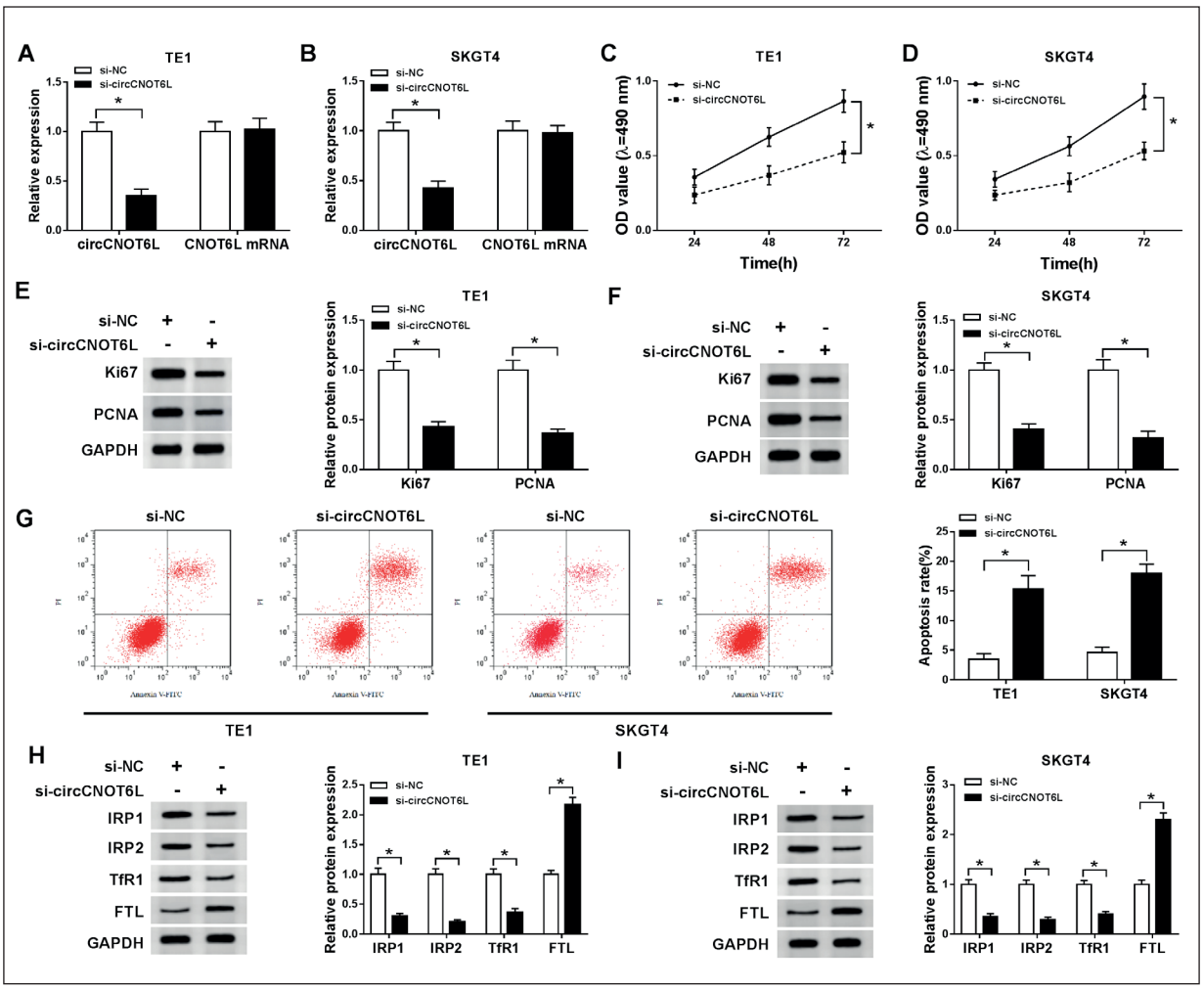

Figure 3. CircCNOT6L knockdown suppressed the growth of ESCC cells. **A,** and **B,** The levels of circCNOT6L and CNOT6L mRNA were determined in TE1 and SKGT4 cells transfected with si-NC or si-circCNOT6L. **C,** and **D,** MTT assay was carried out to assess cell proliferation. **E,** and **F,** The levels of Ki67 and PCNA were detected by Western blot assay. **G,** Flow cytometry was used to analyze cell apoptosis rate. **H,** and **I,** The levels of IRP1, IRP2, TfR1, and FTL were measured by Western blot assay. *p<0.05.

CircCNOT6L Targeted MiR-384

Through bioinformatics tool starBase3.0, we found that miR-384 was a potential target of circCNOT6L (Figure 4A). Then, the Dual-Luciferase reporter assay was performed to verify this interaction *via* co-transfecting circCNOT6L-WT or circCNOT6L-MUT and miR-384 or miR-NC into TE1 and SKGT4 cells. As shown in Figure 4B and C, the co-transfection with miR-384 and circCNOT6L-WT significantly reduced the Luciferase activity of the cells, while the co-transfection with miR-384 and circCNOT6L-MUT did not. These data confirmed that miR-384 interacted with circCNOT6L. Next, whether circCNOT6L affecting miR-384 expression was investigated. The results suggested that miR-384 expression was significantly upregulated by circCNOT6L knockdown and downregulated by circCNOT6L overexpression (Figure 4D and E). On the other hand, qRT-PCR was employed to analyze the level of miR-384 in ESCC. As expected, miR-384 level was lower in ESCC tissues/cells compared with that in normal tissues/cells (Figure 4F and G). Thus, circCNOT6L targeted miR-384 and negatively regulated its expression.

CircCNOT6L Regulated ESCC Cell Progression by Repressing MiR-384 Expression

To investigate whether the function of circCNOT6L was dependent on miR-384, TE1 and SKGT4 cells were transfected with si-NC, si-circCNOT6L, si-circCNOT6L + anti-miR-NC, or si-circCNOT6L + anti-miR-384, respectively. QRT-PCR confirmed that miR-384 expression was upregulated by circCNOT6L knockdown, and then, was downregulated due to the transfection with anti-miR-384 (Figure 5A and B). Later, MTT assay was employed to assess cell proliferation ability. As demonstrated in Figure 5C and D, circCNOT6L knockdown suppressed cell proliferation, whereas this action was abolished by miR-384 depletion. Moreover, the effect of circCNOT6L knockdown on the levels of Ki67 and PCNA was weakened by miR-384 depletion (Figure 5E and F). Next, cell apoptosis rate was explored using flow cytometry. The results indicated that circCNOT6L knockdown-induced cell apoptosis was inhibited by miR-384 depletion in TE1 and SKGT4 cells (Figure 5G and H). Besides,

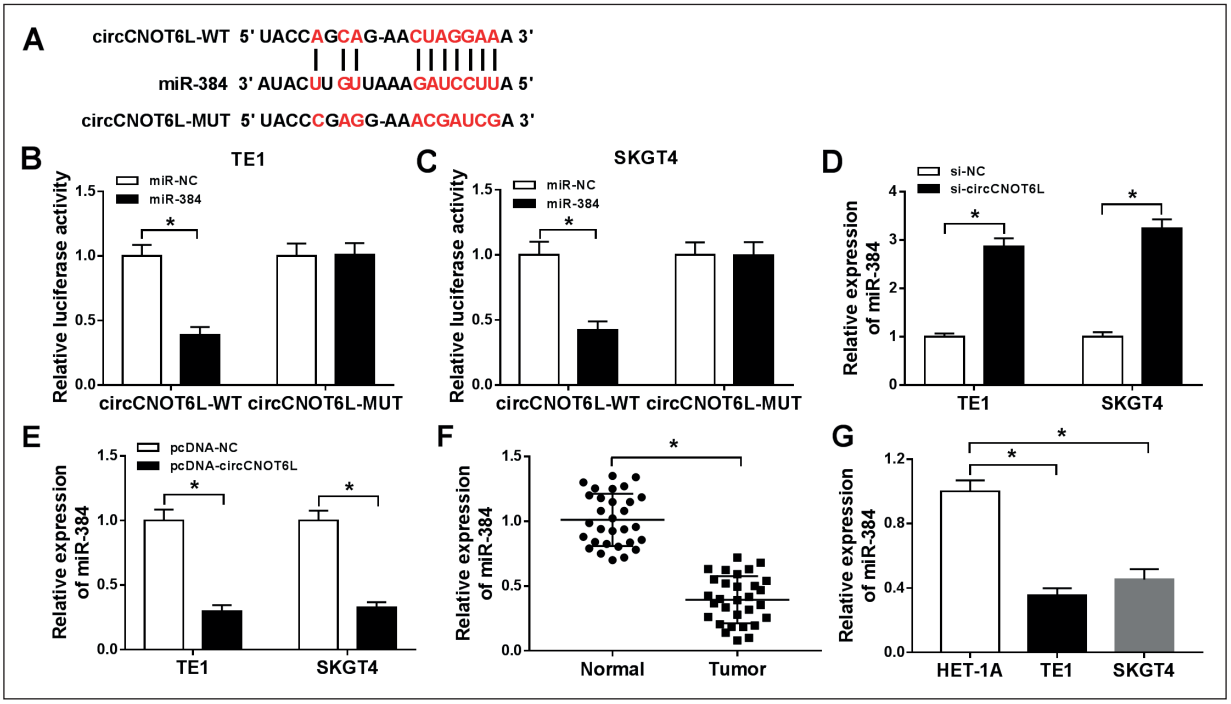

Figure 4. CircCNOT6L interacted miR-384. **A,** The interaction between circCNOT6L and miR-384 was predicted using starBase3.0. B, and **C,** The luciferase activity was determined in TE1 and SKGT4 cells transfected with circCNOT6L-WT or circCNOT6L-MUT and miR-384 or miR-NC. **D,** and **E,** MiR-384 expression was examined in TE1 and SKGT4 cells transfected with si-NC, si-circCNOT6L, pcDNA-NC, or pcDNA-circCNOT6L. **F,** and **G,** MiR-384 level was investigated in ESCC and normal tissues (**F**) as well as ESCC and normal cells (**G**). *p<0.05.

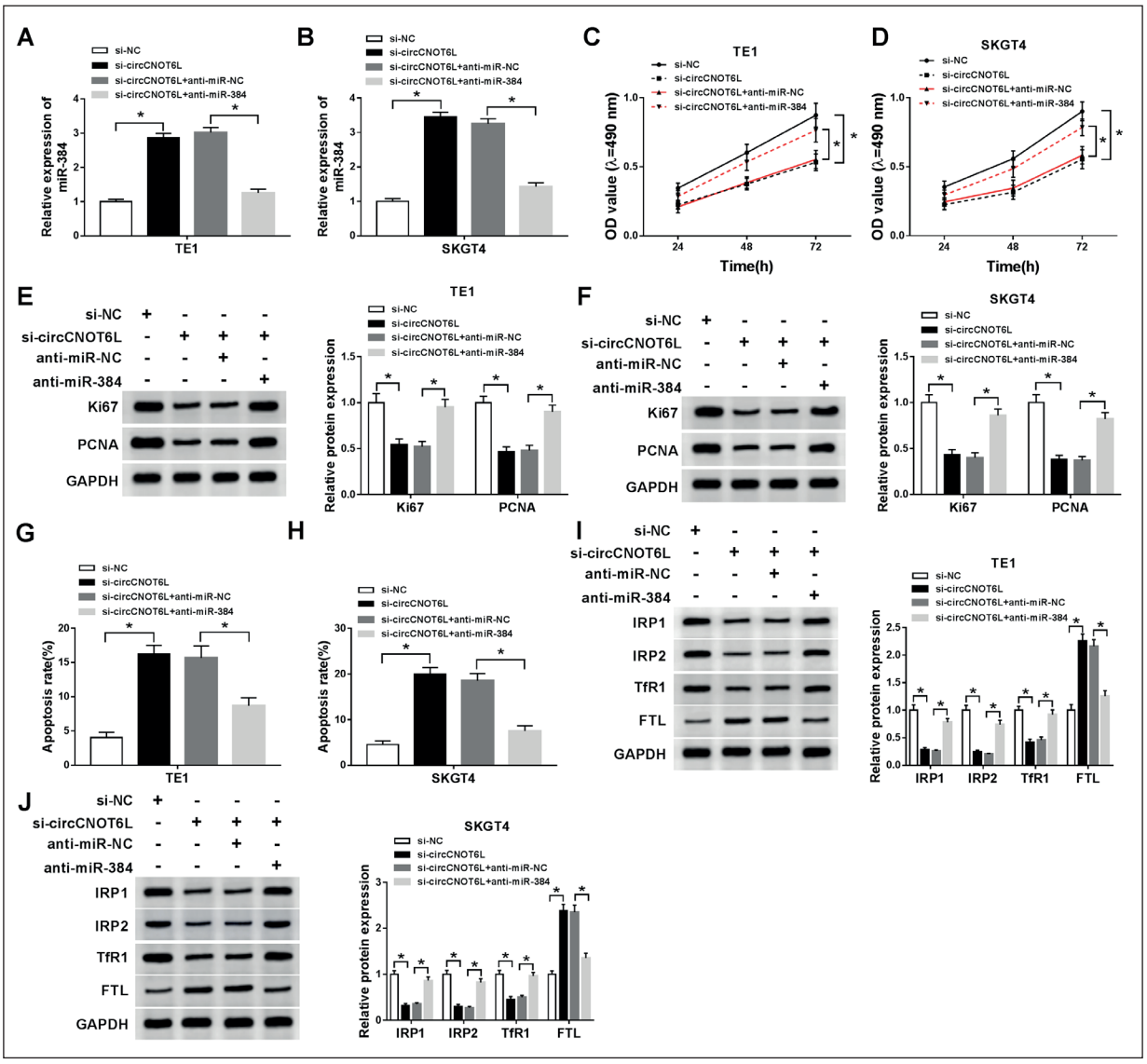

Figure 5. CircCNOT6L regulated miR-384 expression to affect ESCC cell development. **A,** and **B,** MiR-384 expression was detected in TE1 and SKGT4 cells transfected with si-NC, si-circCNOT6L, si-circCNOT6L + anti-miR-NC, or si-circCNOT6L + anti-miR-384, respectively. **C,** and **D,** Cell proliferation ability was assessed by MTT assay. **E,** and **F,** Western blot assay was performed to determine the levels of Ki67 and PCNA. **(G** and **H)** Flow cytometry was used to analyze cell apoptosis rate. **I,** and **J,** Western blot assay was carried out to examine the levels of IRP1, IRP2, TfR1, and FTL. **p*<0.05.

our data demonstrated that miR-384 depletion eliminated the effect of circCNOT6L knockdown on the levels of IRP1, IRP2, TfR1, and FTL (Figure 5I and J). Taken together, circCNOT6L repressed miR-384 expression to affect ESCC cell progression.

MiR-384 Targeted FN1

Bioinformatics tool starBase3.0 predicted that FN1 was a potential target gene of miR-384 (Figure 6A). To confirm the interaction, the Dual-Luciferase reporter assay was carried out. As demonstrated in Figure 6B and C, miR-384 significantly reduced the Luciferase activity of FN1 3'UTR-WT but did not affect the Luciferase activity of FN1 3'UTR-MUT, confirming the interaction between miR-384 and FN1. Next, the level of FN1 in ESCC was investigated. The results revealed that FN1 level was higher in ESCC tissues/cells than that in normal tissues/cells (Figure 6D-G). Next, we analyzed the effect of miR-384 on FN1 expression and found that

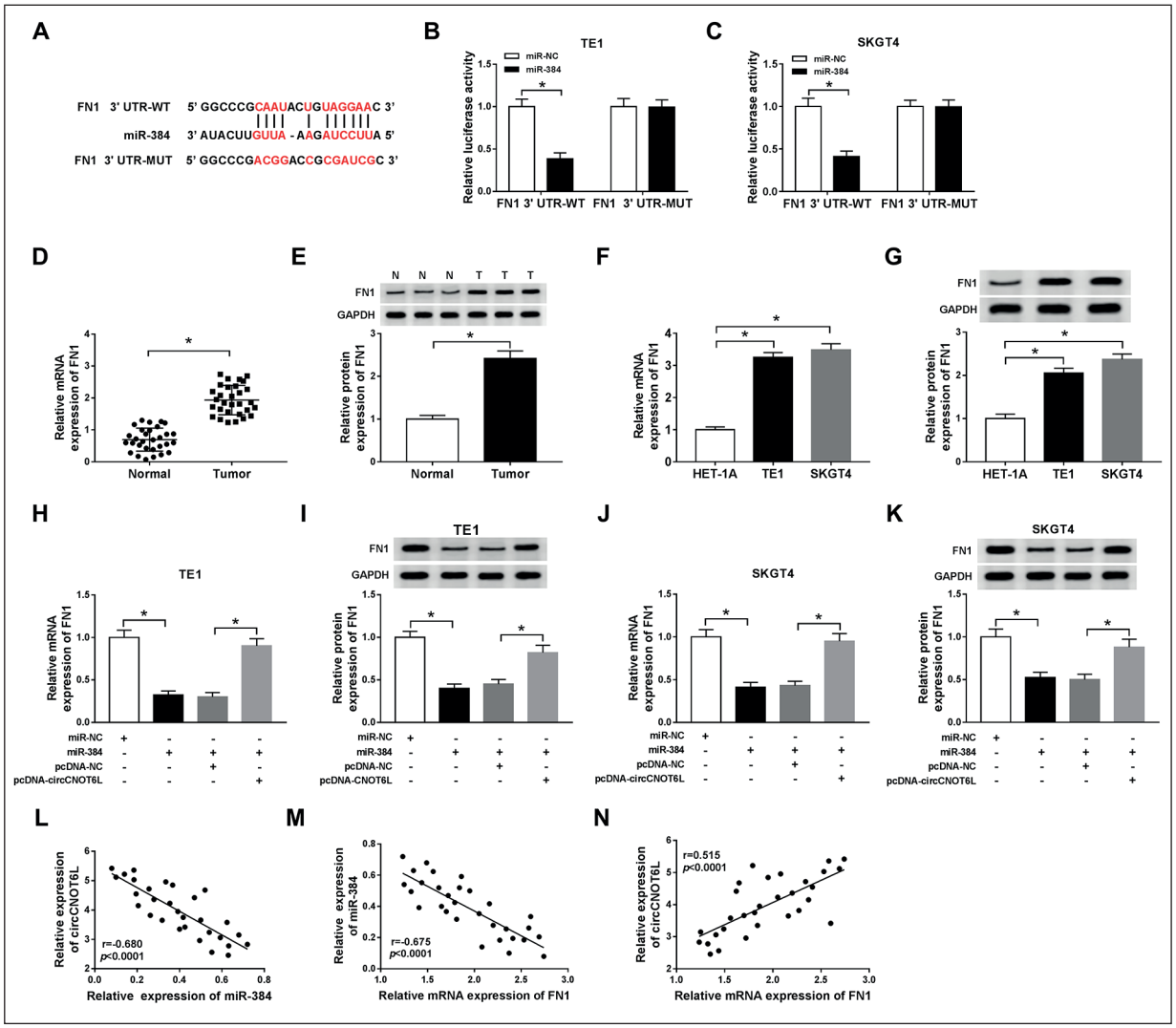

Figure 6. MiR-384 targeted FN1. **A,** The interaction between miR-384 and FN1 was predicted using starBase3.0. **B,** and **C,** The Luciferase activity was determined in TE1 and SKGT4 cells transfected with FN1 3'UTR-WT or FN1 3'UTR-MUT and miR-384 or miR-NC. **D-G,** FN1 level was determined in ESCC and normal tissues (**D** and **E**), as well as ESCC and normal cells (**F** and **G**). **H-K,** FN1 expression was examined in TE1 and SKGT4 cells transfected with miR-NC, miR-384, miR-384 +pcDNA-NC, or miR-384 + pcDNA-circCNOT6L, respectively. **L,** The relationship between miR-384 level and circCNOT6L level was analyzed. **M,** The relationship between FN1 level and miR-384 level was analyzed. **N,** The relationship between FN1 level and circCNOT6L level was analyzed. *$p<0.05$.

FN1 expression was downregulated by miR-384 overexpression in ESCC cells (Figure 6H-K). Moreover, the negative effect of miR-384 on FN1 expression was reversed due to circCNOT6L upregulation (Figure 6H-K). Finally, the relationship among circCNOT6L, miR-384, and FN1 was explored. MiR-384 expression was negatively correlated with circCNOT6L expression and FN1 expression (Figure 6L and M). CircCNOT6L expression was positively correlated with FN1 expression (Figure 6N). Taken together, MiR-384 repressed FN1 expression through interaction.

MiR-384 Modulated ESCC Cell Progression Via Suppressing FN1 Expression

To explore whether MiR-384 affected ESCC cell progression *via* regulating FN1 expression, TE1 and SKGT4 cells were transfected with miR-NC, miR-384, miR-384 +pcDNA-NC, or miR-384 +cDNA-FN1, respectively. QRT-PCR analysis and Western blot assay indicated that FN1 expression was downregulated by miR-384 upregulation, and then, upregulated by the transfection with pcDNA-FN1 (Figure 7A and B). Next, cell proliferation ability was determined

using MTT assay. As shown in Figure 7C and D, cell proliferation was repressed by miR-384 upregulation, and then, partly rescued due to FN1 overexpression. Similarly, FN1 overexpression reversed the effect of miR-384 upregulation on the levels of proliferation-related proteins (Figure 7E and F). Furthermore, we found that miR-384 upregulation accelerated the apoptosis of ESCC cells, whereas this action was impaired by FN1 overexpression (Figure 7G and H). Besides, the phenomenon that FN1 overexpression inhibited the effect of miR-384 upregulation on the levels of IRP1, IRP2, TfR1, and FTL was observed in TE1 and SKGT4 cells (Figure 7I and J). These data indicated that miR-384 regulated ESCC cell progression by suppressing FN1 expression.

CircCNOT6L Depletion Attenuated Tumor Growth In Vivo

To investigate whether circCNOT6L regulating tumor growth *in vivo*, TE1 cells transfected with sh-circCNOT6L or sh-NC were subcutaneously injected into BALB/c nude mice. Then, tumor size was analyzed every week. As shown in

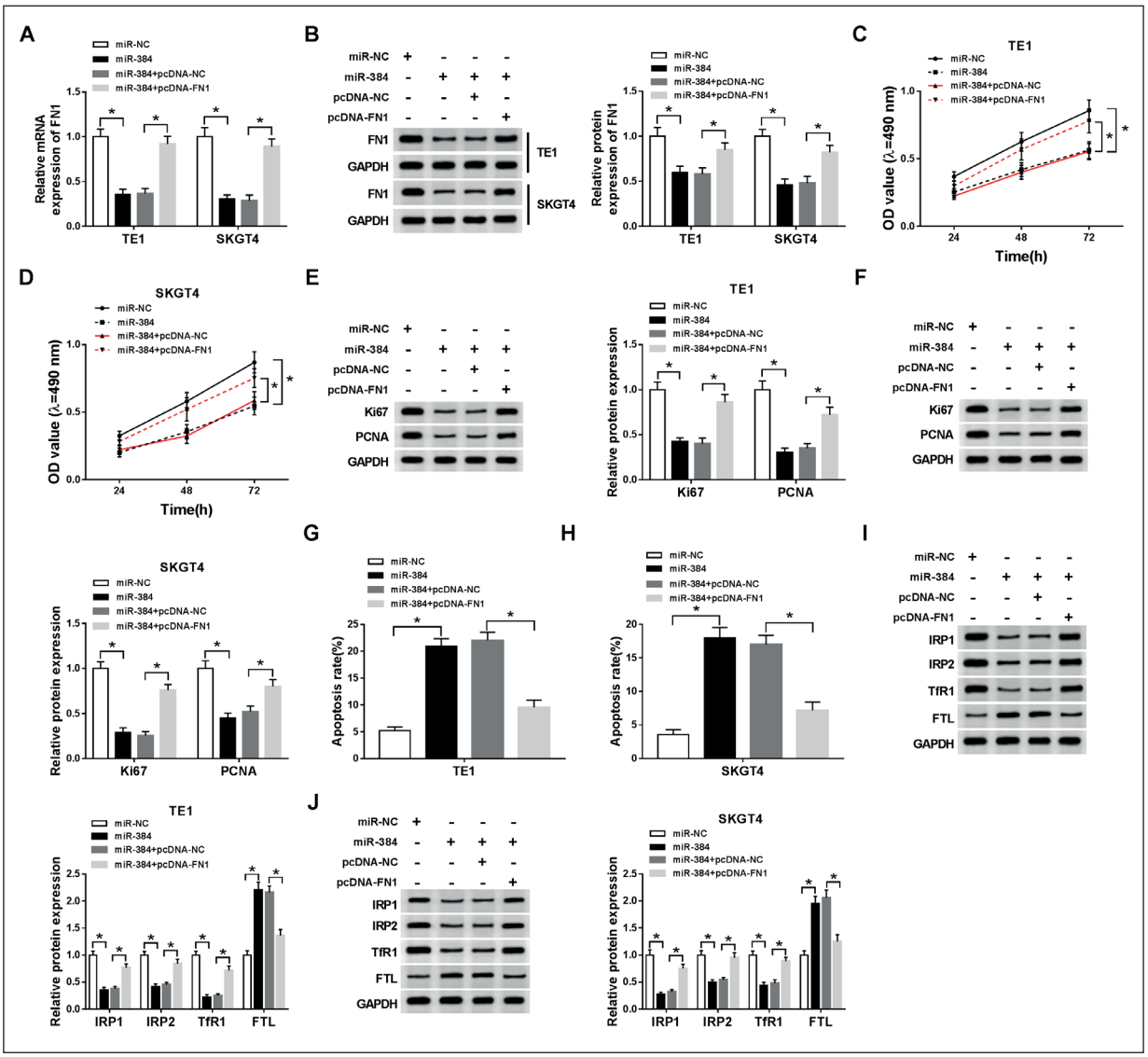

Figure 7. MiR-384 modulated FN1 expression to affect ESCC cell development. **A,** and **B,** FN1 expression was measured in TE1 and SKGT4 cells transfected with miR-NC, miR-384, miR-384 +pcDNA-NC, or miR-384 +pcDNA-FN1, respectively. **C,** and **D,** MTT assay was used to analyze cell proliferation ability. **E,** and **F,** The levels of Ki67 and PCNA were measured by Western blot assay. **G,** and **H,** Cell apoptosis rate was explored by flow cytometry. **I,** and **J,** The levels of IRP1, IRP2, TfR1, and FTL were analyzed using Western blot assay. *$p<0.05$.

Figure 8A, tumor size was significantly smaller in sh-circCNOT6L group than that in sh-NC group. Furthermore, we calculated tumor weight of the mice, and found that circCNOT6L depletion reduced tumor weight (Figure 8B). Subsequently, the levels of circCNOT6L, miR-384, and FN1 were determined in sh-circCNOT6L group and sh-NC group. As expected, the levels of circCNOT6L and FN1 were decreased, and the level of miR-384 was increased in the sh-circCNOT6L group (Figure 8C and D). Thus, circCNOT6L depletion attenuated tumor growth *in vivo*.

Discussion

ESCC is a cancer with a high mortality rate and low survival rate around the world [17,18]. CircRNAs, a novel non-coding RNAs related to the development of human cancers[19], have been reported to play important roles in ESCC development. Xing et al[20] confirmed that circ-FoxO3 suppressed ESCC cell proliferation, as well as mobility, by regulating miR-23a expression. Song et al[21] observed that circ_0000337 overexpression positively regulated the growth of ESCC cells by targeting miR-670-5p. Shi et al[22] revealed that circ-PRKCI exerted function as a sponge for miR-3680-3p to affect ESCC cell proliferation, as well as migration. Therefore, the analysis of circRNA function is essential for the therapy of ESCC patients.

Here, we demonstrated that circCNOT6L, a circRNA, was upregulated in ESCC tissues and cells. Furthermore, circCNOT6L knockdown repressed ESCC cell proliferation, iron metabolism disorder, and tumor growth, as well as accelerated apoptosis. Nowadays, the analysis of circCNOT6L function in human cancers was less reported. Only Shi et al[9] mentioned that circCNOT6L was highly expressed in ESCC and positively modulated ESCC cell growth. The results in both our research and this reporter were consistent. Up to date, there is no more literature about circCNOT6L.

MiRNAs, identified as another type of non-coding RNA, have been shown to play important roles in human cancers[23]. Su et al[24] indicates that circRNA can modulate cancer cell development by targeting miRNAs. In fact, circANKIB1 repressed miR-19b expression to regulate osteosarcoma cell development[25]. To explore the functional mechanism of circCNOT6L, StarBase3.0 was used to seek the potential targets of circCNOT6L. Next, miR-384, as a target of circCNOT6L, was chosen for further analysis since its level was significantly downregulated in ESCC tissues/cells among several checked targets. Moreover, miR-384 was reported to suppress ESCC cell development[14]. In addition, previous data revealed that miR-384 acted as a suppressor in other cancers, including laryngeal carcinoma[26], breast cancer[27], osteosarcoma[28], and prostate cancer[29]. In this study, we proved

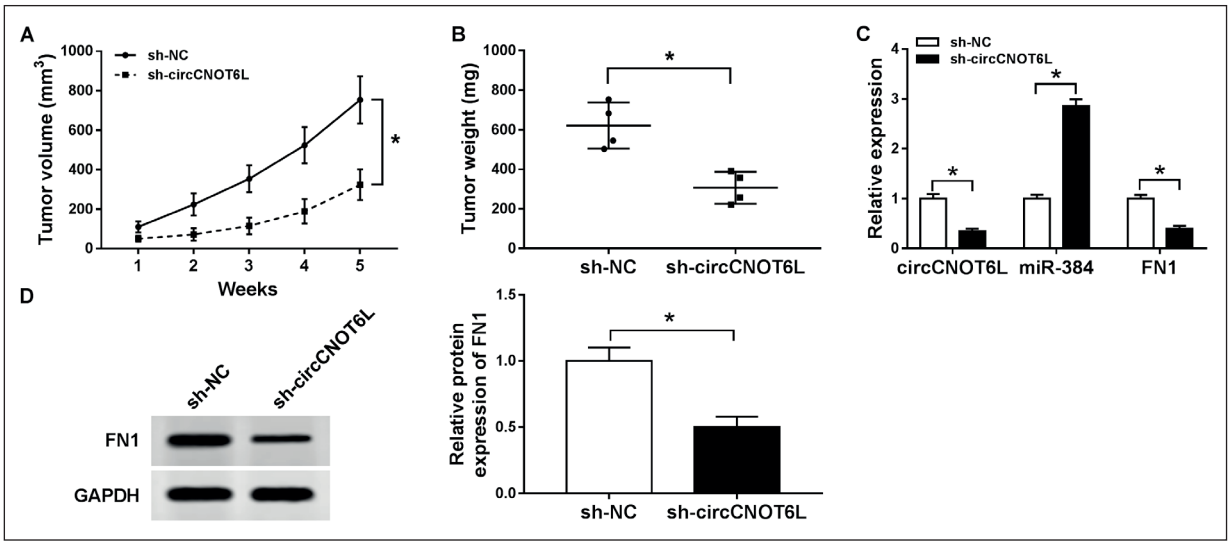

Figure 8. CircCNOT6L depletion repressed tumor growth *in vivo*. **A,** and **B,** Tumor volume and weight were calculated in sh-circCNOT6L group mice and sh-NC group mice. **C,** The RNA levels of circCNOT6L, miR-384, and FN1 were determined by qRT-PCR. **D,** The protein level of FN1 was detected by Western blot assay. *$p<0.05$.

that circCNOT6L directly downregulated miR-384 level in ESCC cells. Taken together, we speculated that circCNOT6L modulated ESCC cell development by repressing miR-384 expression. Then, a series of experiments were carried out. As expected, miR-384 depletion reversed the effect of circCNOT6L knockdown on ESCC cell progression. Therefore, miR-384 was a downstream component of circCNOT6L in ESCC development.

According to previous data, FN1 exerted an oncogenic role in various cancers, such as gastric cancer[30], prostate cancer[31], cervical cancer[32], and oral squamous cell carcinoma[33]. Furthermore, FN1 was highly expressed and reported to promote cell proliferation, as well as mobility in ESCC and EC [34,35]. Here, we found that FN1 was a target of miR-384 and was negatively modulated by miR-384. In addition, FN1 level was upregulated in ESCC tissues and cells. By studying the function of miR-384, we speculated that miR-384 affected ESCC cell development *via* suppressing FN1 expression. Then, this hypothesis was detected by our experiments. As expected, FN1 upregulation weakened the effect of miR-384 overexpression on ESCC cell progression. Thus, FN1 was a downstream component of miR-384 in ESCC. On the other hand, our results indicated that circCNOT6L repressed miR-384 expression to upregulate FN1 level, meaning that circCNOT6L could serve as a competing endogenous RNA (ceRNA) for miR-384 to modulate FN1 expression. Other studies[36,37] suggested that one circRNA can modulate multiple miRNA/mRNA axis. Therefore, circCNOT6L may regulate another miRNA/mRNA axis to promote ESCC cell development. Taken together, the novelties of this study are that it is the first time to demonstrate that circCNOT6L could actively regulate FN1 by sponging miR-384, thereby partly promoting ESCC cell progression. Moreover, more experiments should be carried out for analysis of circCNOT6L functional mechanism in ESCC cells.

Conclusions

We demonstrated that circCNOT6L knockdown suppressed cell development by modulating miR-384/FN1 axis in ESCC, providing a potential target for the therapy of ESCC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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