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CircRNA EPB41L2 inhibits tumorigenicity of lung adenocarcinoma through regulating CDH4 by miR-211-5p

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Abstract. – OBJECTIVE: Circular RNAs (circRNAs) can make contributions to cell proliferation, migration and invasion in lung adenocarcinoma (LUAD). Circ_Band 4.1-like protein 2 (circ_EPB41L2) was found to have anti-tumor roles in lung cancer. Herein, we investigated the roles of circ_EPB41L2 in LUAD tumorigenicity.

PATIENTS AND METHODS: The expression of circ_EPB41L2, microRNA (miR)-211-5p, and Cadherin-4 (CDH4) was measured using quantitative real-time polymerase chain reaction. Western blot was used to detect the levels of CDH4, Ki67, and E-cadherin. Cell p[illegible]ation, migration and invasion were ex[illegible] using 3-(4,5)-dimethylthiazol(-z-y1)-3,5-[illegible]henyl tetrazolium bromide (MTT) assay and [illegible]swell assay, respectively. The interaction[illegible]tween miR-211-5p and circ_EPB41L2 or C[illegible] was confirmed by Dual-Lucifera[illegible]eporter a[illegible]say. *In vivo* experiments wer[illegible]ed using the murine xenograft mode[illegible]

RESULTS: Circ_EPB41[illegible] and C[illegible]4 were down-regulated in LUA[illegible]es an[illegible] Overexpressed circ_EPB41[illegible] suppressor in the LU[illegible] cell pr[illegible]on, invasion and migration *in v*[illegible] MiR-211-5[illegible]tly bound to circRNA_EPB[illegible]nd CDH4 3'-U[illegible]d circ_EPB41L2 indire[illegible]ted CDH4 expression by binding to mi[illegible]1-5p [illegible]D cells. Furthermore, rescue ass[illegible] showed ci[illegible]41L2 played a protective ro[illegible]y repressing p[illegible]ation, migration and inv[illegible]on through regulati[illegible]CDH4 and miR-211-5[illegible] LUAD cells. Besides, *in vivo* experiments indi[illegible]d circ_[illegible]41L2 overexpression also inhibi[illegible]or[illegible]wth thr[illegible]ugh regulating miR-211-5p and [illegible]

CONC[illegible]S: C[illegible]_EPB41L2 functioned as [illegible]or sup[illegible] inhibit the proliferation, mi[illegible]on and i[illegible]on through regulating CDH4 by [illegible]-211-5p in LUAD cells, suggesting a new un[illegible]tanding on the tumorigenesis of LUAD a[illegible]ecular therapeutic targets.

[illegible]ords:
[illegible]PB41L2, CDH4, MiR-211-5p, LUAD, Tumorigeni[illegible].

[illegible]troduction

Lung cancer has [illegible]regarded as the leading caus[illegible] cancer-associ[illegible]ortality worldwide, w[illegible] highest inciden[illegible] and mortality rates [illegible]all cancers[1]. Lung adenocarcinoma (LUAD) [illegible]the main his[illegible]gical type of non-small cell [illegible] cancer (NS[illegible]C), accounting for around up to[illegible] of lung c[illegible]cer cases[2]. Currently, surgical rese[illegible] the most effective therapy for LUAD pa[illegible], while the survival rate and qual[illegible] of life after pneumonectomy are unsatisfac[illegible]ides that, most LUAD patients treated [illegible]dard cytotoxic chemotherapy ultimately become resistant to chemotherapy[4]. Thus, further investigations on the pathological mechanisms of LUAD to develop novel effective strategies or targeted agents are necessary.

Circular RNAs (circRNAs) are novel, endogenous, non-coding RNAs that have the covalently closed loop structures with high stability and conservativeness[5]. CircRNAs involve in normal developmental and pathological processes by functioning as oncogenes or antioncogenes, including cancers[6,7]. For example, circ-Foxo3 suppressed cell growth, migration, and invasion by regulating PTEN through miR-23a in esophageal squamous cell cancer[8]. Circ_0074027 promoted tumor malignancy by inducing cell proliferation and invasion in NSCLC through miR-185-3p/BRD4/MADD pathway[9]. In LUAD, circ-TSPAN4 interacted with miR-665 to facilitate cell metastasis via ZEB1[10]. Circ_0001946 contributed to cell growth through regulating miR-135a-5p/SIRT1 axis-mediated activation of Wnt/β-catenin signaling pathway[11]. All these studies suggest the potential application of circRNAs in targeted therapy in LUAD. Recently, it was showed that circ_Band 4.1-like protein 2 (circ_EPB41L2) was decreased in LUAD and had the diagnostic potential for LU-

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AD patients. However, its molecular role in the tumorigenicity of LUAD has not been clarified.

Cadherin superfamily is a transmembrane adhesion, calcium-dependent cell adhesion molecule family, which mediates cell-cell adhesion through homo- or heterotypic interactions[12]. Cell-cell adhesion plays a critical role in determining cell polarity, as well as in establishing and maintaining tissue homeostasis. Besides, this organized adhesion can be disturbed via genetic and epigenetic changes, which contribute to the loss of contact inhibition, changes in signaling, cell migration and stromal interactions during oncogenesis[13]. Thus, cadherins are important for the tumorigenesis of cancer cells[13,14]. Cadherin-4 (CDH4) (also known as retinal cadherin (R-cadherin)) is a classical cadherin from the cadherin superfamily and belongs to type I cadherins in cancer[14]. Recently, it was also discovered that CDH4 mRNA expression was down-regulated in lung cancer tissues, and was related to the grade and histotype, indicating the potential anti-tumor roles of CDH4 in lung cancer. Herein, we investigated the role of CDH4 in LUAD.

This research aimed to assess the [illegible]sion patterns and the roles of circ_E[illegible]1L2 and CDH4 in LUAD, as well as investi[illegible]d the potential regulatory relationship betw[illegible] circ_EPB41L2 and CDH4 in the tumorigene[illegible] of LUAD.

Patients [illegible]nd [illegible]s

Patients and S[illegible]imens

32 pairs of [illegible]ap[illegible]en LUAD t[illegible]ues and matched para[illegible]umor [illegible]l tissues were collected fro[illegible] patients who [illegible]ived surgical resections [illegible]Huaihe Hospital o[illegible]an University. All sp[illegible]mens were identified using histological and [illegible]ologic[illegible] diagnoses by two experienced path[illegible]s. [illegible]is inve[illegible]gation was permitted by the [illegible] Commi[illegible] of Huaihe Hospital of [illegible]n Uni[illegible] a[illegible]ll patients signed written [illegible]ed cons[illegible].

Ce[illegible]ulture and Transfection

[illegible]nchial epithelial cell line (HBE) [illegible] LUAD cell lines (A549, HCC827, H460, [illegible]Calu-3) were obtained from Shanghai Ac[illegible]y of Life Science (Shanghai, China), and were cultured in the Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA, USA) in accompany with 10[illegible] bovine serum (FBS), as well as 1[illegible] lin-streptomycin solution (Gibc[illegible] Rockville, MD, USA) at 37°C with 5% C[illegible].

The microRNA (miR)-211-5p [illegible] (miR-211-5p), miR-211-5p inhibitor (anti- mi[illegible]p), negative control (miR-NC) [illegible] inhibit[illegible]ative control (anti-miR-NC) w[illegible] obtained from [illegible]BIO (Guangzhou, Chi[illegible]. Small interfering [illegible] (siRNA) sequence[illegible]etin[illegible]circ_EPB41L2/CDH4 (si-circ_EPB41[illegible]-CDH4) [illegible]RNA negative contr[illegible]i-NC), [illegible]A-circ[illegible]PB41L2 overexpressi[illegible]ector (circ_[illegible]), control vector (NC[illegible]A-CDH4 ove[illegible]ression vector (CD[illegible] and [illegible]A empty vector (pcDNA) were designed an[illegible]thesized by Invitrogen (Carl[illegible] CA, USA). [illegible] oligonucleotides or v[illegible]ere transfected i[illegible] A549 and HCC827 [illegible]s using Lipofectamine 3000 (Invitrogen, [illegible]rlsbad, CA, [illegible]A) according to the standard [illegible]edure.

Nu[illegible]eparation and Quanti[illegible]e Real-Time Polymerase [illegible]hain Reaction (qRT-PCR)

[illegible]RNA was extracted using TRIzol re[illegible]ent [illegible]nvitrogen, Carlsbad, CA, USA). Genomic DNA was isolated with a QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA), and cytoplasmic and nuclear RNA isolations were carried out using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Waltham, MA, USA) following the recommendations of manufacturer. PrimeScript RT reagent Kit (TaKaRa, Dalian, China) was used to synthesize the complementary DNA (cDNA), and then quantitative PCR was conducted using SYBR Green methods on the ABI7500 system. The fold changes were normalized with U6 or GAPDH and qualified by $2^{-\Delta\Delta Ct}$ method. The specific primer sequences were listed as follows: circ_EPB41L2, F 5'-CCTCTGGATCGGAAGACTGA-3' and R 5'-TGCCAAGGGACAAGTGTTATTT-3'; miR-211-5p, F 5'-CGCTTCCCTTTGTCATCCT-3', and R 5'-TATGGTTTTTGACTGTGTGAT-3'; CDH4, F 5'-CGTCCATCATCAAAGTCAAGGT-3', and R 5'-GGTCGTAGTCCTGGTCCTCCT-3'; U6, F 5'-GCTTCGGCAGCACATATACTAAAAT-3', and R 5'-CGCTTCACGAATTTGCGTGTCAT-3'; GAPDH, F 5'-AAGAAGGTGGTGAAGCAGGC-3', and R 5'-GTCAAAGGTGGAGGAGTGGG-3'.

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Western Blot Assay

Transfected cells were collected and then lysed using the radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China). Then cell lysates were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), shifted onto polyvinylidene difluoride (PVDF) membranes, and blocked with 5% nonfat-milk. After that, the membranes were interacted with primary antibodies against CDH4 (1:20000, ab76011, Abcam, Cambridge, MA, USA), Ki67 (1:1000, ab16667, Abcam, Cambridge, MA, USA), E-cadherin (1:1000, ab15148, Abcam, Cambridge, MA, USA), as well as GADPH (1:10000, ab8245, Abcam, Cambridge, MA, USA), and followed by interaction with secondary horseradish peroxidase (HRP)-conjugated antibody (1:1000, ab9482, Abcam, Cambridge, MA, USA). Finally, protein bands were detected using an enhanced chemiluminescence (ECL) detection system (Thermo Fisher Scientific, Waltham, MA, USA).

RNase R Treatment

Total RNA (2 mg) was interacted [illegible] without 3 U/mg of RNase R (Qiagen) at 3[illegible] for 20 min. The resulting RNA was purified u[illegible] a RNeasy MinElute Cleanup Kit (Qiagen, Hi[illegible] Germany).

Cell Proliferation Assay

Transfected cells we[illegible]lated in [illegible] 96-well plate for overnight, 10 [illegible] (4,5[illegible] iazol-2-yl)-2,5-dipher[illegible]tetraz[illegible] bromide (MTT) solution (Si[illegible]a-Aldrich, [illegible]ouis, MO, USA) was suppl[illegible]ted into pe[illegible] Subsequently, the s[illegible] was resolve[illegible] at 4 h post-reaction, [illegible]nd foll[illegible] interaction with 200 μL dimet[illegible] sulfoxide ([illegible]: Sigma-Aldrich, St. Loui[illegible] MO, USA) to dis[illegible] the generated forma[illegible]. Finally, the absorbance was examined at 4[illegible]m on a[illegible]croplate reader.

Transw[illegible]say

[illegible]r mig[illegible]a[illegible], transfected cells were se[illegible] in the [illegible]ell upper chamber with 200 mL [illegible]um-free D[illegible]EM, and then 700 mL DMEM fixe[illegible] 10% FBS was added into the lower w[illegible]amber. After incubation for 24 h, [illegible]s on the lower face of the membranes were [illegible]and stained. Lastly, migrated cells were cou[illegible] in six randomly selected visual fields. For invasion assay, the upper transwell chambers were pre-coated with Matrigel (BD Bioscience, Franklin Lakes, NJ, USA), and other phi[illegible]by of measurement was similar to the ab[illegible] cell migration, except that.

Dual-Luciferase Reporter [illegible]

Wild (wt) type and mutant ([illegible]irc_EPB41L2 and CDH4 3' UTR [illegible]taining [illegible]tive binding sites of miR-21[illegible]p were ampli[illegible] inserted into the p[illegible]GLO Vector (Pro[illegible], Madison, WI, USA[illegible]549 a[illegible] HCC827 cells were co-transfected w[illegible]cted ve[illegible]s and miR-211-5p mi[illegible]s or m[illegible] using [illegible]pofectamine 3000 ([illegible]trogen) follo[illegible] protocol of manufactu[illegible]lly, a Dual-Lu[illegible]ase reporter assay ki[illegible]ro[illegible] Madison, WI, USA) was used to detect the re[illegible] Luciferase activity.

[illegible]nograft Experiments In Vivo

Athymic BA[illegible]/c nude mice (4-6-week-old, [illegible]) were purc[illegible]ed from Vital River Labora-to[illegible]nimal Te[illegible]ology (Beijing, China). The exp[illegible] permitted by the Animal Research Co[illegible]mittee of Huaihe Hospital of Henan [illegible]niversity and performed in compliance with [illegible]lines of the National Animal Care and [illegible]stitution. Six mice were divided into 2 groups for stable injection of HCC827 cells (NC or circ_EPB41L2). The tumor size in mice was detected every 3 days. After injection for 28 days, the tumor masses were weighed and harvested for further analysis.

Statistical Analysis

Data were expressed as the mean ± standard deviation (SD). Statistical differences between groups were compared using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test or Student's *t*-test. The correlation analysis was analyzed using Spearman's correlation test. All statistical analysis was performed using GraphPad Prism 7 (GraphPad Inc., La Jolla, CA, USA). *p*-values less than 0.05 suggested statistically significant.

Results

The Expression of Circ_EPB41L2 and CDH4 Is Down-Regulated In LUAD Tissues

To identify whether the circ_EPB41L2 and CDH4 played crucial roles in LUAD tumori-

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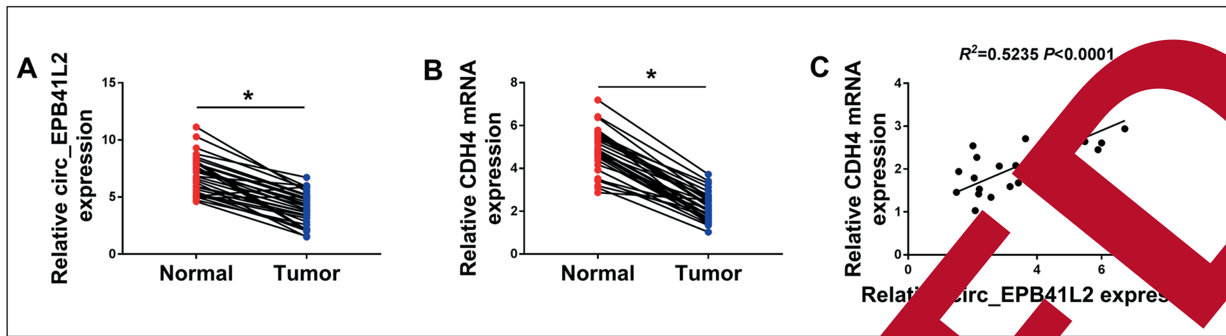
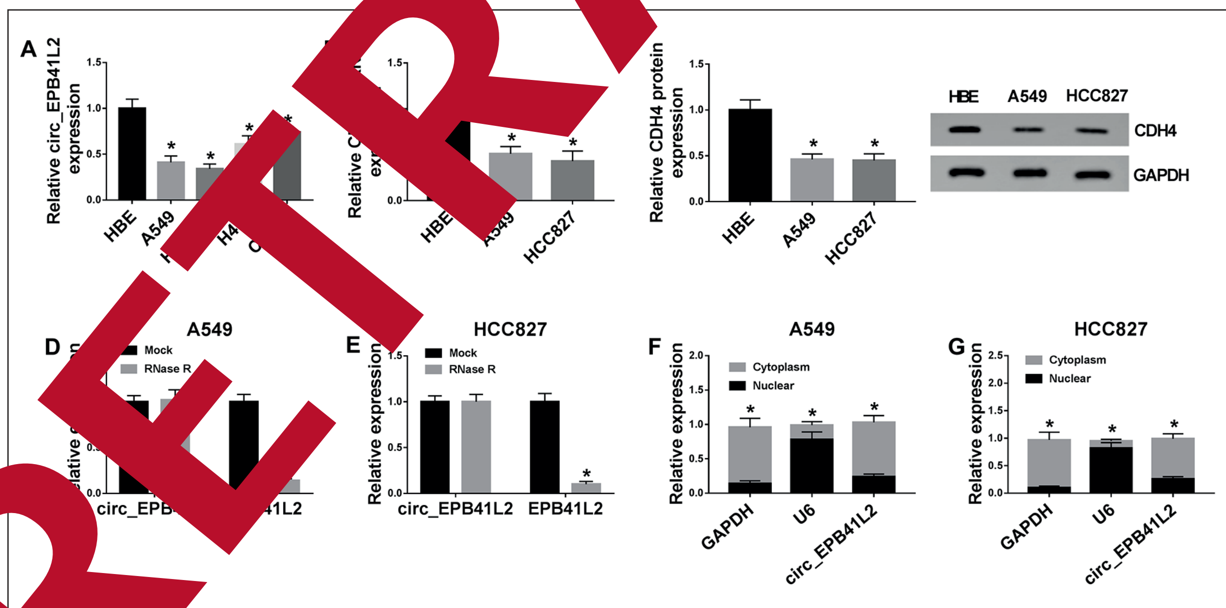


Figure 1. The expression of circ_EPB41L2 and CDH4 is down-regulated in LUAD [illegible]ssues. [illegible] expressi[illegible]f circ_EPB41L2 and CDH4 was detected using qRT-PCR in LUAD tissues. **C**, The correl[illegible]n betwee[illegible]PB41L[illegible]nd CDH4 was analyzed using Spearman's correlation test. *p<0.05.

genesis, the expression of circ_EPB41L2 and CDH4 was detected in 32 pairs of LUAD tissues and adjacent normal tissues. qRT-PCR analysis showed that circ_EPB41L2 and CDH4 levels were markedly down-regulated in LUAD tissues (Figure 1A, B). Furthermore, a positive correlation between circ_EPB41L2 and CDH4 expression in LUAD tissues was observed (Figur[illegible]C). These results indicated that circ_EPB4[illegible] a[illegible] CDH4 possibly participated in tumorige[illegible] of LUAD.

The E[illegible]pression of c[illegible]EPB41L2 a[illegible]H4 Is Down-R[illegible]gulated In [illegible]AD Cell Lines

The expressi[illegible] of circ_EPB41L2 and CDH4 [illegible] also detecte[illegible] LUAD cell lines, and results s[illegible]d circ_EP[illegible]L2 and CDH4 levels were decrea[illegible] cell lines (Figure 2A-C). In addition, to [illegible]rm the existence of circ_EPB41L2, [illegible]al RNA from proliferating A549 and HCC827 [illegible]reated with RNase R exonuclease, and [illegible]en q[illegible]-PCR analysis exhibited that circ_EP-



[illegible]e 2. The expression of circ_EPB41L2 and CDH4 is down-regulated in LUAD cell lines. **A-C**, The expression of circ_E[illegible] and CDH4 was detected in LUAD cell lines using qRT-PCR or Western blot, respectively. **D, E**, qRT-PCR analysis of ci[illegible]PB41L2 and EPB41L2 mRNA after treatment with RNase R in A549 and HCC827 cells was performed. **F, G**, Circ_EPB41L2 in either the cytoplasm or the nucleus was analyzed using qRT-PCR analysis in A549 and HCC827 cells. *p<0.05.

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B41L2 existed in a circular form because of the resistance to digestion by RNase R (Figure 2D, E). Moreover, qRT-PCR analysis of nuclear and cytoplasmic RNAs in A549 and HCC827 cells indicated that circ_EPB41L2 was preferentially localized in the cytoplasm (Figure 2F, G). Thus, circ_EPB41L2 and CDH4 were down-regulated in LUAD cell lines.

Circ_EPB41L2 Acts As a Suppressor In the Proliferation, Invasion and Migration of LUAD Cells

To explore the role of circ_EPB41L2 in LUAD, we used two siRNA oligonucleotides to target the unique back splicing junction of circ_EPB41L2, and circ_EPB41L2 expression was expectedly decreased in A549 and HCC827 cells (Figure 3A, B). Besides, circ_EPB41L2-overexpressing plasmid was synthesized, and circ-AKT3 expression was significantly increased in A549 and HCC827 cells (Figure 3A, B). After that, functional experiments were conducted. Results showed overexpressed circ_EPB41L2 inhibited proliferation of A549 and HCC827 cells, reflected by the decreased proliferating cells (Figure 3C, D) an[illegible]67 protein level (Figure 3E). Afterwards, t[illegible] assay indicated that circ_EPB41L2 res[illegible]ion suppressed the migration and invasion of [illegible]9 and HCC827 cells (Figure 3F, G). Consiste[illegible] Western blot further demonstrated circ_EPB41[illegible] restoration inhibited cell mig[illegible] invasio[illegible] in LUAD by increasing th[illegible]vel o[illegible]adherin (Figure 3H). Conversely, [illegible]ckdown [illegible]circ_EP-B41L2 promoted cell pro[illegible]on ([illegible] migration and invasi[illegible] (Fig[illegible]) in LUAD. Taken together, ci[illegible]PB41L2 [illegible] a protective role by rep[illegible]g proliferati[illegible]gration and invasion i[illegible]lls.

CDH4 In[illegible]bits Cell Pr[illegible]ation, Invasion a[illegible] Migration In L[illegible]

To i[illegible]estigate the role of CDH4 in LUAD, the exp[illegible]on of C[illegible]H4 was increased or decreased by t[illegible]ti[illegible] with CDH4 or si-CDH4. As expected, [illegible] expre[illegible]n was up-regulated in [illegible]9 and [illegible]27 [illegible]s transfected with CDH4, b[illegible]s down[illegible]ated in A549 and HCC827 cell[illegible]ansfected with si-CDH4 (Figure 4A, B). Sub[illegible]ently MTT assay indicated CDH4 over-e[illegible]ppressed A549 and HCC827 cell [illegible]liferation (Figure 4C, D), and the inhibition [illegible] proliferation was also demonstrated by the [illegible]uction of Ki67 protein in A549 and HCC827 cells (Figure 4E). Meanwhile, CDH4 overexpression inhibited cell migration and invasion in LUAD, as illustrated by the de[illegible]d migrated and invaded A549 and HC[illegible] (Figure 4F, G), as well as the inc[illegible]sed E-cadherin level in A549 and HCC8[illegible] cells (Figure 4H). To the contrary, CDH4 k[illegible]wn showed the carcinogenic effects to indu[illegible] prolif-eration (Figure 4I-K), m[illegible]ation a[illegible]sion (Figure 4L-N) in LU[illegible]. Altogether, [illegible] functioned as a tum[illegible]ppressor to inhibit [illegible] tumorigenesis in L[illegible]

MiR-211-5p D[illegible]ctly In[illegible]ts With Circ_[illegible]41L2 and [illegible]

As circR[illegible]stly serve as [illegible]ges of miRNAs to [illegible]gulat[illegible]wnstream genes, we next investigated the abi[illegible]f circ_EPB41L2 to bind to m[illegible]s. Accordin[illegible]he prediction of the o[illegible]oinformatics d[illegible]abase (starBase3.0), [illegible] found miR-211-5p was a potential target of [illegible]c_EPB41L2 ([illegible]ure 5A). Then, Dual-Lucif-[illegible]e reporter as[illegible] showed a great reduction of L[illegible]rase activ[illegible] in A549 and HCC827 cells co-t[illegible]th circ_EPB41L2-wt and miR-211-5p, [illegible]ing the direct interaction between [illegible]EPB41L2 and miR-211-5p (Figure 5B, C). In [illegible]e also observed that the expression of [illegible]5p was increased by circ_EPB41L2 de[illegible]letion, but was decreased by circ_EPB41L2 overexpression in A549 and HCC827 cells (Figure 5D, E). Besides that, we also found miR-211-5p contained the binding sites of CDH4 (Figure 5F) through searching the Targetscan online bioinformatics database. Consistently, a significant reduction of Luciferase activity in A549 and HCC827 cells co-transfected with CDH4-wt and miR-211-5p confirmed the direct interaction between CDH4 and miR-211-5p (Figure 5G, H). Moreover, the level of CDH4 at mRNA and protein level was up-regulated by miR-211-5p inhibition but was down-regulated by miR-211-5p overexpression in A549 and HCC827 cells (Figure 5I-K). These results indicated miR-211-5p directly interacts with circ_EPB41L2 and CDH4.

Circ_EPB41L2 Indirectly Regulates CDH4 Expression By Binding to MiR-211-5p In LUAD Cells

Western blot assay was used to elaborate the regulatory relationship among miR-211-5p, circ_EPB41L2 and CDH4 in LUAD cells. Results showed circ_EPB41L2 overexpression increased the level of CDH4, while this increase was reversed by miR-211-5p up-regulation or CDH4

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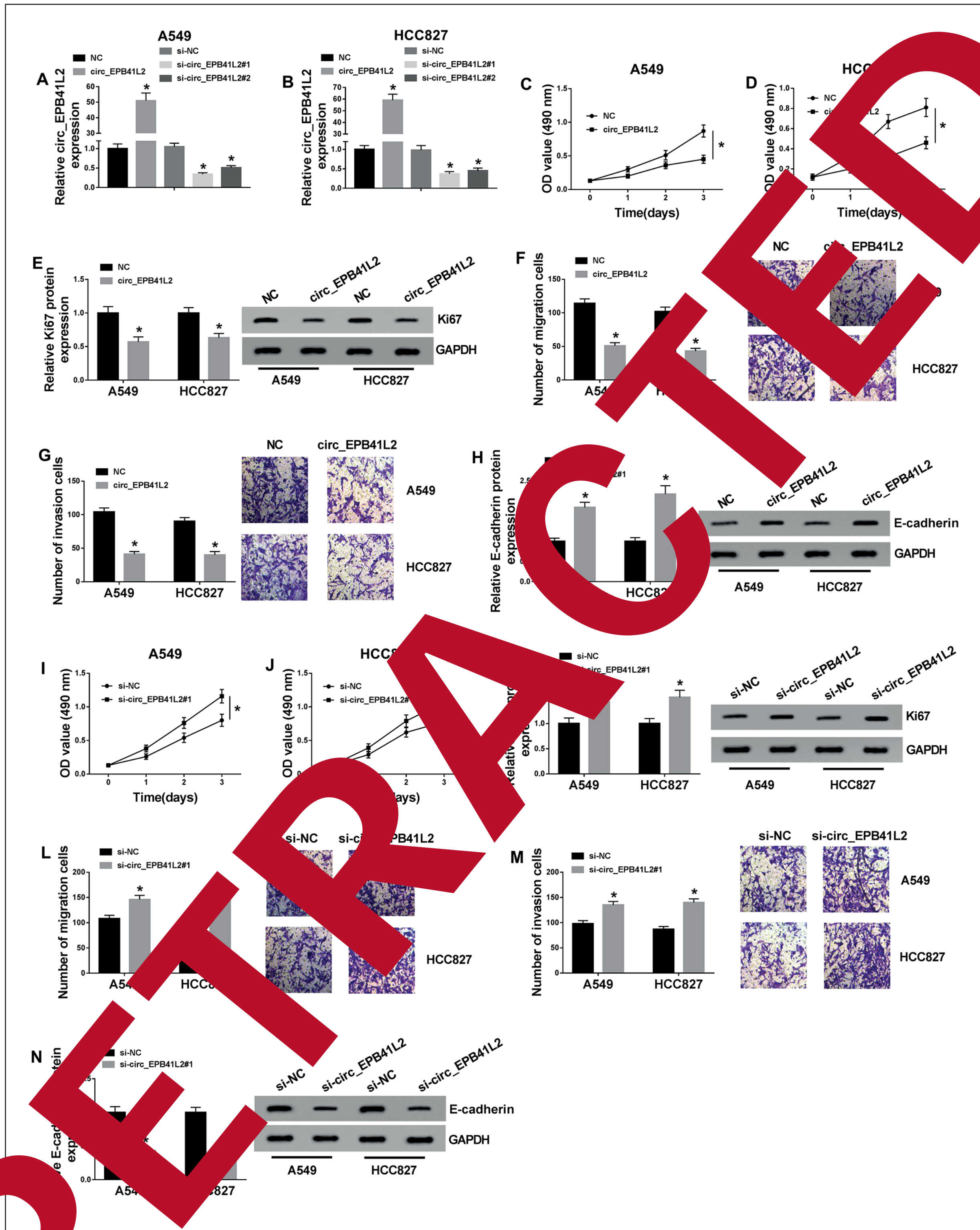

F[...] [...]_EPB41L2 acts as a suppressor in the proliferation, invasion and migration of LUAD cells. A549 and [...]C827 cells were transfected with NC, circ_EPB41L2, si-NC, or si-circ_EPB41L2. **A, B**, The transfection efficiencies [...]etected by qRT-PCR. **C, D, I, J**, Cell proliferation was analyzed using MTT assay. **E, H, K, N**, Western blot was ap[...] examine the expression of Ki67 and E-cadherin. **F, G, L, M**, Cell migration and invasion were determined by transw[...] assay (magnification 100×), $*p<0.05$.

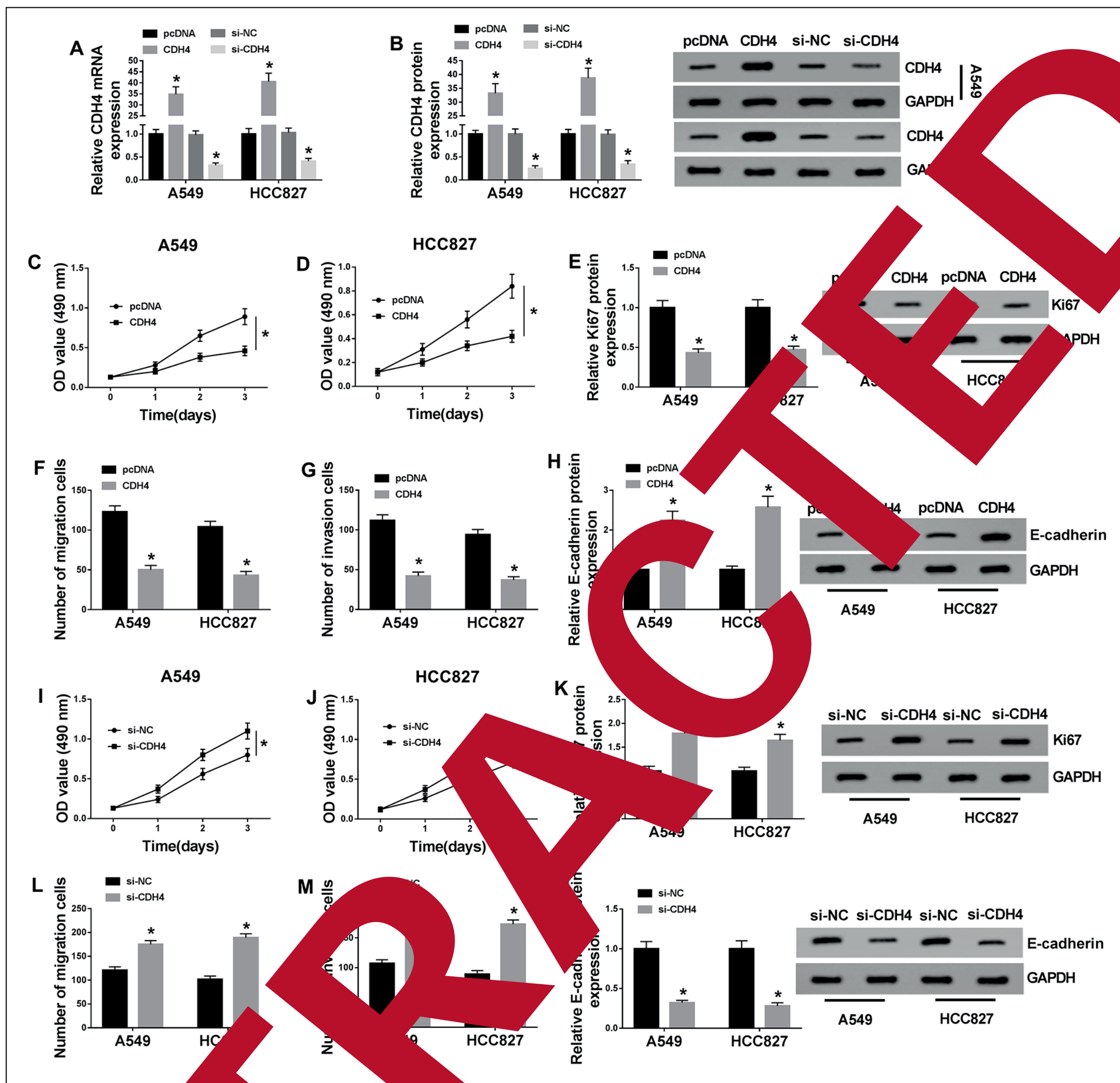

Figure 4. CDH[illegible] proliferation, [illegible]asion and migration in LUAD. A549 and HCC827 cells were transfected with pcDNA, C[illegible]4, si-N[illegible] CDH4. **A, B**, The transfection efficiencies were detected by Western blot. **C, D, I, J**, Cell proliferation [illegible]as analyzed usi[illegible] assay. **E, H, K, N**, The expression of Ki67 and E-cadherin was assessed using Western blot. **F, G, [illegible]**, Transwell assay [illegible]d to detect cell migration and invasion. *$p<0.05$.

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dow[illegible]regulatio[illegible] A549 and HCC827 cells (Figure [illegible]. T[illegible]s, a reg[illegible]atory network of circ_EPB41L[illegible]211-5p/[illegible]H4 axis was identified [illegible]UAD c[illegible]

Cir[illegible]PB41L2 [illegible]erforms Anti-Tumor Eff[illegible] By Regulating CDH4 and [illegible]n LUAD Cells

[illegible]ased on the circ_EPB41L2/ miR-211-5p/ [illegible] axis, we further explored the role of cir[illegible]B41L2/miR-211-5p/CDH4 axis in LUAD tumorigenesis. Rescue assay suggested the inhibition on proliferation (Figure 7A, B), migration and invasion (Figure 7D, E) induced by circ_EPB41L2 overexpression was markedly reversed by miR-211-5p up-regulation or CDH4 down-regulation in A549 and HCC827 cells, indicating circ_EPB41L2 suppressed cell tumorigenesis in LUAD by regulating CDH4 and miR-211-5p. Furthermore, Western blot analysis also exhibited that miR-211-5p up-regulation or CDH4 silence attenuated circ_EPB41L2 overexpression mediated suppression on Ki67 expression (Figure 7C) and promotion on E-cadherin level (Figure 7F)

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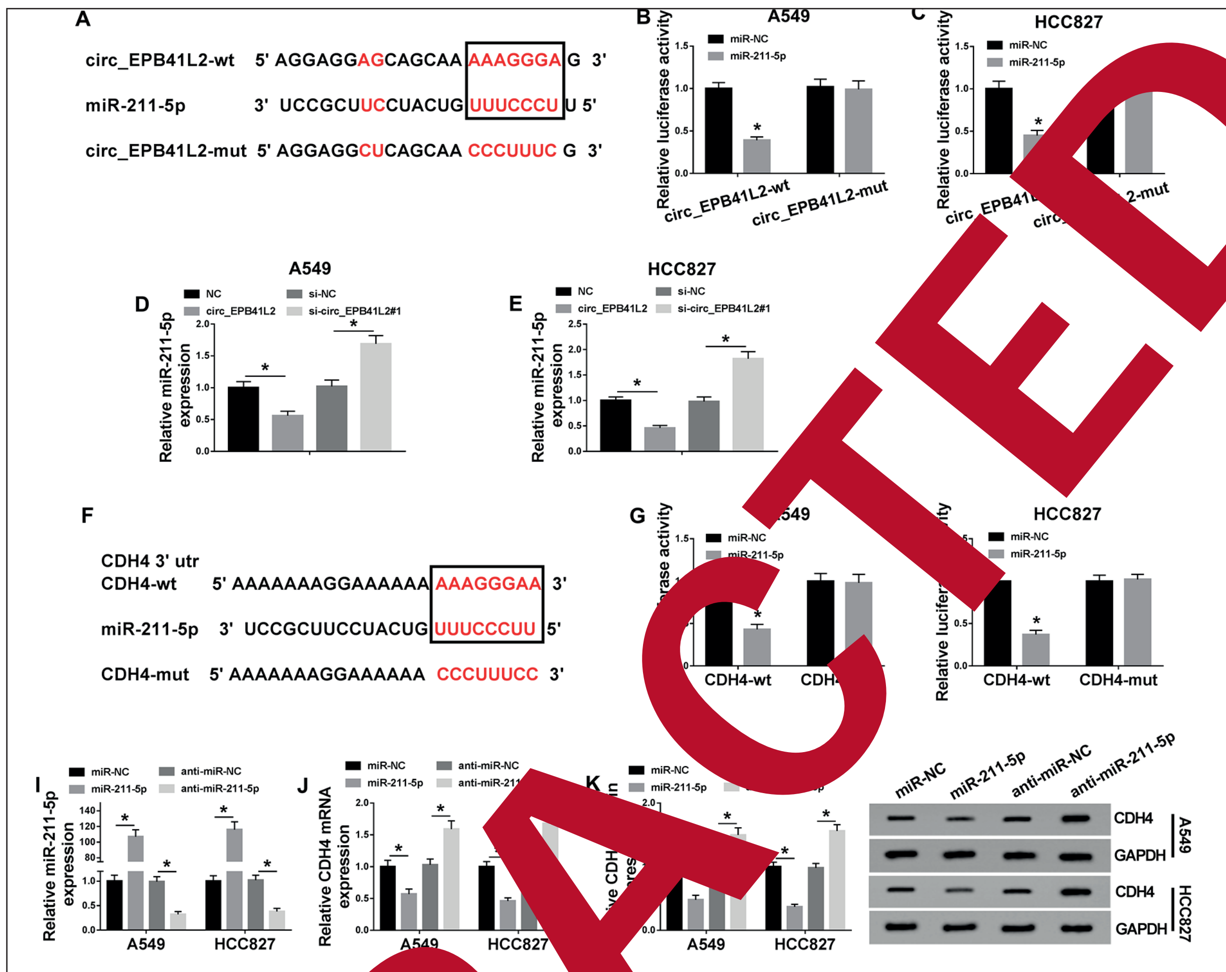
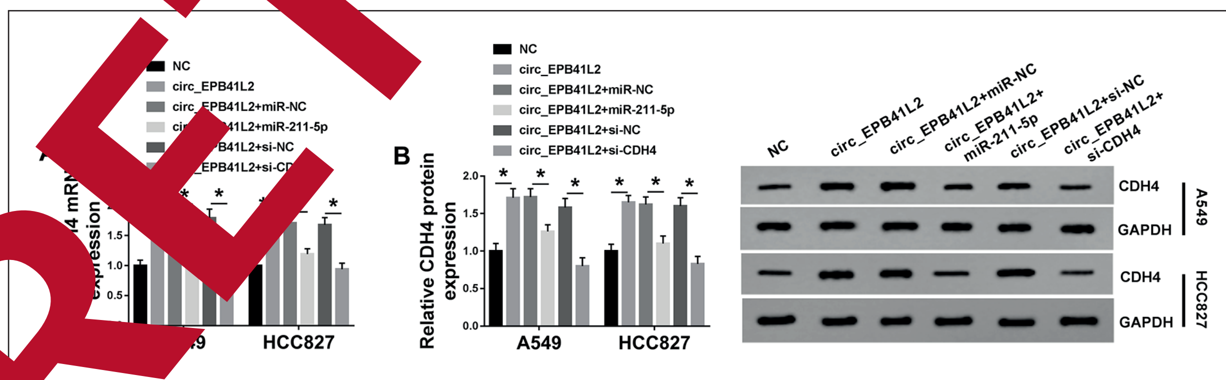


Figure 5. MiR-211-5p directly int[illegible]s with c[illegible]PB41L2 a[illegible]DH4. **A**, The putative binding sites between circ_EPB41L2 and miR-211-5p was presented. **B, C**[illegible]e interacti[illegible]etween circ_EPB41L2 and miR-211-5p was confirmed by the dual-luciferase reporter assay in A549 and H[illegible]ells, re[illegible] **D, E**, The expression of miR-211-5p was examined using qRT-PCR in cells transfected with NC, circ_E[illegible]si[illegible]EPB41L2. **F**, The putative binding sites between CDH4 and miR-211-5p were listed. **G, H**, The [illegible]raction [illegible] CDH4 and miR-211-5p was confirmed using dual-luciferase reporter assay in A549 and HCC827 cells, res[illegible]ively. **I-K**, Th[illegible]A and protein expression of CDH4 was examined in A549 and HCC827 cells transfected with mi[illegible]iR-211-5p, anti-[illegible]or anti-miR-211-5p using qRT-PCR and Western blot, respectively. *$p<0.05$.



F[illegible]. Circ_EPB41L2 indirectly regulates CDH4 expression by binding to miR-211-5p in LUAD cells. **A, B**, Western blot was [illegible] to detect the level of CDH4 in A549 and HCC827 cells transfected with NC, circ_EPB41L2, circ_EPB41L2 + miR-NC, circ_EPB41L2 + miR-211-5p, circ_EPB41L2 + si-NC, or circ_EPB41L2 + si-CDH4. *$p<0.05$.

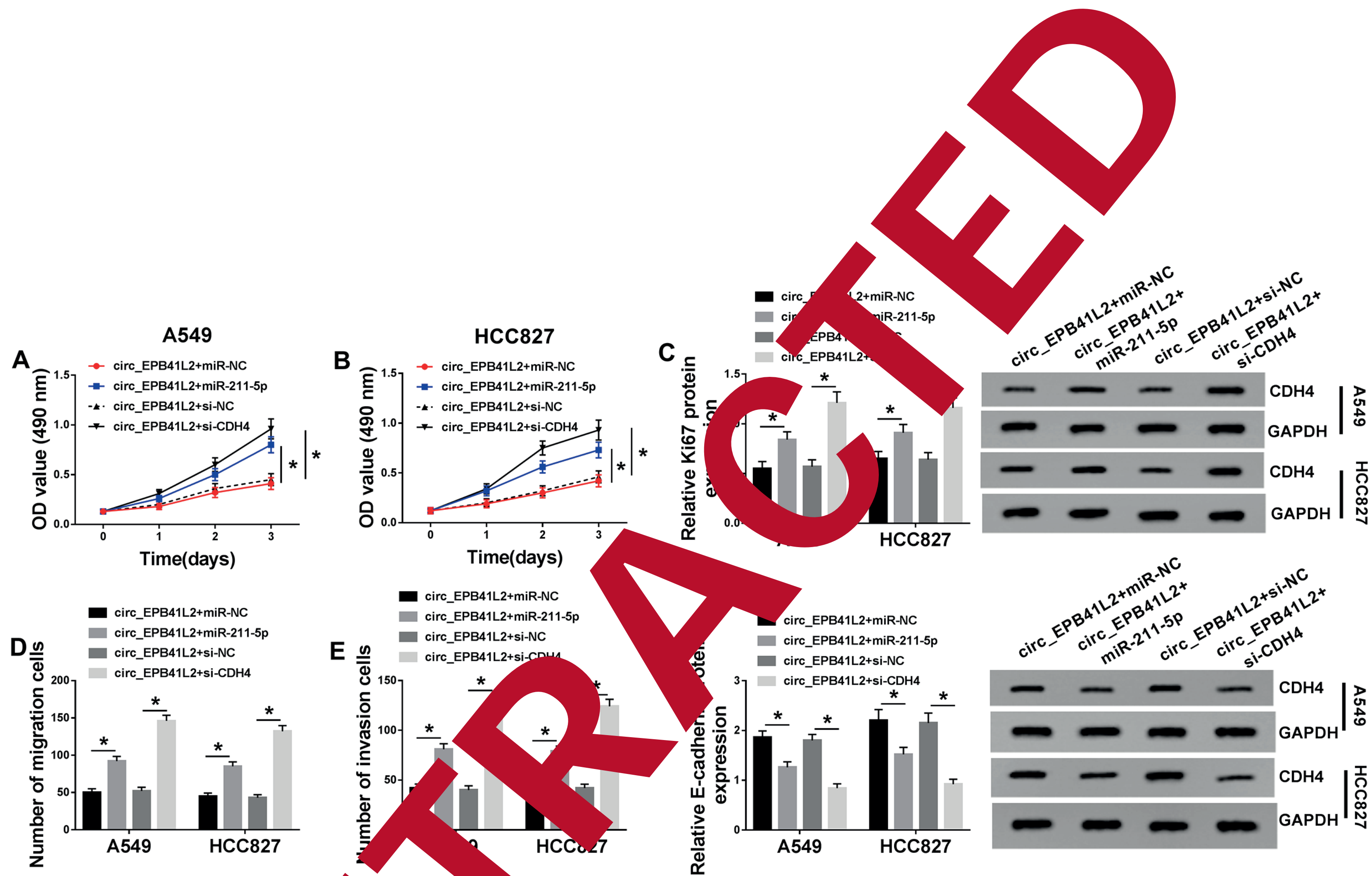


Figure 7. Circ_EPB41L2 performs anti-tumor effects by regulating CDH4 and miR-211-5p in LUAD cells. **A, B**, Cell proliferation was analyzed using MTT assay. **C, F**, The expression of Ki67 and E-cadherin was analyzed using Western blot. **D, E,** Transwell assay was used to detect cell migration and invasion. *$p<0.05$.

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in A549 and HCC827 cells. These data suggested that circ_EPB41L2 suppressed cell malignancy in LUAD by regulating miR-211-5p/CDH4 axis.

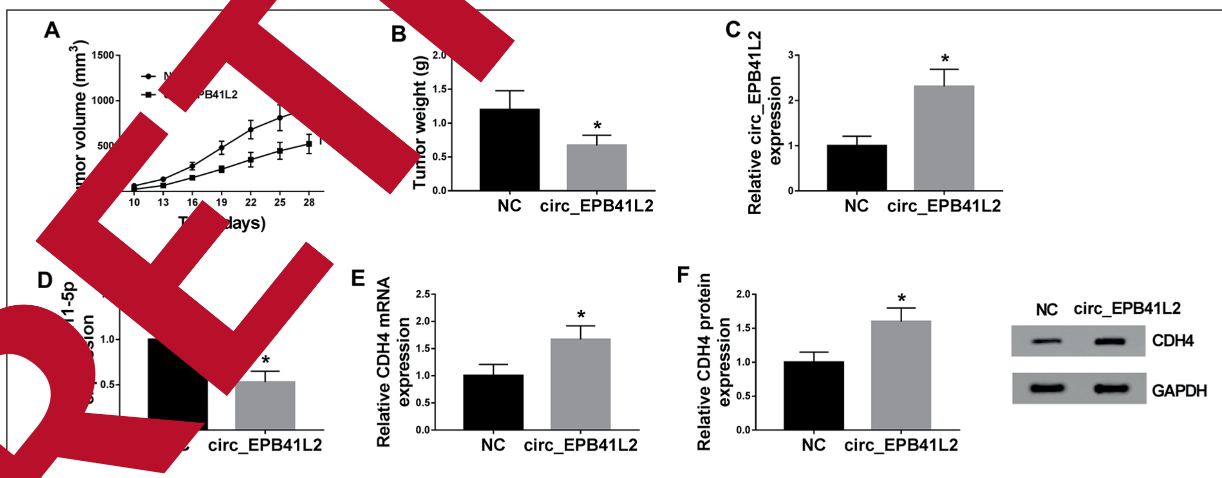
Circ_EPB41L2 Inhibits LUAD Tumor Growth In Vivo

The carcinogenesis roles of circ_EPB41L2 *in vivo* were further elucidated. We found circ_EPB41L2 re-expression inhibited NSCLC tumor growth, demonstrated by the inhibition of tumor volume and weight in circ_EPB41L2 overexpression group (Figure 8A, B). Additionally, molecular analysis showed circ_EPB41L2 re-expression increased the levels of circ_EPB41L2 (Figure 8C) and CDH4 (Figure 8E, F), but reduced the levels of miR-211-5p *in vivo* (Figure 8D). Collectively, these results revealed that circ_EPB41L2 hindered LUAD tumor growth *in vivo* via regulating miR-211-5p and CDH4 expression.

Discussion

The involvement of circRNA in cancers has been frequently reported, and its dysfu[illegible] has a significant impact on cancer c[illegible]origenesis, metastasis, and apoptosis[15] [illegible]. CircRNAs can make contributions to cell p[illegible]eration, migration and invasion in LUAD c[illegible]. Overexpressed circPRKCI drives cell proli[illegible]ation and tumorigenesis of [illegible] up-regulating E2F7 through spo[illegible]ng m[illegible]545 and miR-589[17]. CircCRIM1 [illegible]egulate[illegible]eukemia inhibitory factor recepto[illegible]indi[illegible] and miR-182 to repress cell invasion and metastasis in LUAD[18]. CircPUM1 contributed [illegible]ll proliferation, migration and invasi[illegible]duced apoptosis through binding [illegible]miR-326 in LUAD[19]. Therefore, it is of grea[illegible]nical significance to uncover the role of [illegible]PB41L2 in LUAD cell tumorigenesis. In the [illegible]t study, we found decreased cir[illegible]PB41L2 [illegible]UAD specimens and cell li[illegible], circ_EPB41[illegible]toration suppressed [illegible] prolife[illegible]ation, inva[illegible]n and migration of L[illegible] cell[illegible]while circ_EPB41L2 down-re[illegible]ulat[illegible]ed opp[illegible]e effects. Besides, [illegible]vivo exp[illegible]nts al[illegible]ndicated circ_EPB[illegible]2 overexpre[illegible] inhibited tumor gro[illegible]ough regulat[illegible]miR-211-5p and CD[illegible]. The[illegible] circ_EPB41L2 played a protective role in L[illegible].

Ci[illegible]NA can act as [illegible]ecular sponge of micr[illegible] (miRNAs) to in[illegible]lve in tumorigenesis [illegible] aggressiveness[20]. Herein, through Luciferase [illegible]porter assays, [illegible] confirmed circ_EPB41L2 was [illegible]ble of direc[illegible] interacting with miR-211-5p in [illegible]D cells. [illegible]R-211-5p has been revealed to func[illegible]or suppressor in renal cancer[21], breast ca[illegible], and hepatocellular carcinoma[23], [illegible]d has carcinogenic effects on NSCLC[24], sug[illegible]e cell- or disease-context-dependent [illegible]nct[illegible] of miR-211-5p. In this study, we found that the inhibition on proliferation, migration and invasion mediated by circ_EPB41L2 overexpression was markedly reversed by miR-211-5p up-regulation in LUAD cells. Thus, circ_EPB41L2 suppressed cell malignancy in LUAD by regulating miR-211-5p.



F[illegible]. Circ_EPB41L2 inhibits LUAD tumor growth *in vivo*. **A,** Tumor volumes were calculated every 3 days. **B**, Tumor mass[illegible]ere excised and weighed on 28 days. **C, D,** The expression of circ_EPB41L2 and miR-211-5p was determined using qRT-PCR. **E, F,** CDH4 expression was measured using qRT-PCR and Western blot. $*p<0.05$.

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Previous researches have reported that CDH4 participates in cell tumorigenesis, metastasis, differentiation and progression, and may perform anti-tumor functions in a variety of cancers, such as osteosarcoma, salivary adenoid cystic carcinoma, and gastric cancer[25-27]. This study investigated that CDH4 expression was down-regulated in LUAD, and overexpressed CDH4 performed anti-tumor effects in LUAD cells tumorigenesis, while decreased CDH4 promoted cell malignancy in LUAD. Besides that, we also demonstrated the upstream regulatory mechanism of CDH4 disorder in LUAD; circ_EPB41L2 bound to miR-211-5p, ultimately regulating CDH4 expression and suppressing LUAD cell tumorigenesis. Additionally, CDH4 inhibition could reverse circ_EPB41L2 re-expression mediated suppression on the carcinogenesis of LUAD. Thus, a circ_EPB41L2/miR-211-5p/CDH4 regulatory network was identified in the tumorigenicity.

Conclusions

We demonstrated that circ_EPB41L2 u[illegible]ulated CDH4 expression by interacti[illegible] miR-211-5p to repress LUAD cell proli[illegible]on, migration, and invasion. The biological m[illegible]lar therapies developing recently have prov[illegible] promising strategies for the treatment of dive[illegible] cancers[28,29]. Emerging evide[illegible]onfirmed that nonconding RNAs, inc[illegible]ng ci[illegible]NAs and miRNAs, are vital biom[illegible]ers and [illegible]rapeutic biological targets for dive[illegible]ease[illegible] flammatory diseases[illegible]tabo[illegible]ses and cancers[29,30]. This study [illegible]stly ident[illegible]e circ_EPB41L2/miR-211-[illegible]DH4 axis in [illegible] which provided a no[illegible]on the mecha[illegible]sms underlying LUA[illegible] tumor[illegible]is, and might be targeted for [illegible]lecular therap[illegible] benefits.

Con[illegible]rest

The Auth[illegible]re that th[illegible]ave no conflict of interests.

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