CircACAP2 promotes cell proliferation and migration in lung adenocarcinoma via LASP1-mediated TGF- β /Smad3 pathway

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Abstract

Lung adenocarcinoma (LUAD), a common malignant tumor, has led to a great number of deaths around the world. Circular RNAs (circRNAs) have been certified as essential players in the progression of diverse cancers. CircRNA ACAP2 (hsa_circ_0068568) is an oncogene in several cancers. However, the role of circACAP2 in LUAD remains unknown. This study revealed that the expression of circACAP2 was significantly elevated in LUAD tissues and cell lines, especially in the tissues of LUAD patients at advanced stage. Additionally, circACAP2 enhanced cell proliferation, migration, invasion abilities and epithelial-mesenchymal transition (EMT) process in LUAD. Moreover, miR-342-3p interacted with circACAP2 in LUAD cells. Importantly, we found that miR-342-3p targeted LIM and SH3 protein 1 (LASP1), and circACAP2 positively regulated LASP1 expression by competing for miR-342-3p in LUAD. Further, it was confirmed that circACAP2 promoted the malignant behaviors and stimulated the activation of TGF- β /Smad3 pathway in LUAD by modulating the miR-342-3p/LASP1 axis. To conclude, the molecular regulatory mechanism of circACAP2 in LUAD was under discussion in the current study. The findings revealed that circACAP2 facilitated malignant phenotypes in LUAD via the activation of the TGF- β /Smad3 pathway.

Introduction

Lung carcinoma, the most frequent serious malignancies characterized by high occurrence, poor prognosis and high mortality, is a great threat to global human health (Dong, Liu, Sun, & Ping, 2020; Ho & Leung, 2018). There are two types of lung cancer, non-small cell lung cancer accounting for about 80% and small cell lung cancer accounting for approximately 20% (Lemjabbar-Alaoui, Hassan, Yang, & Buchanan, 2015; Yuan, Liu, Qu, Liu, & Li, 2019). Lung adenocarcinoma (LUAD) is the most common type of non-small cell lung cancer (Inamura, 2018). Despite progress made in LUAD therapies, the five-year survival rate of LUAD patients is still low because of inapparent early symptoms as well as high recurrence and metastasis rates after advanced LUAD diagnosis and treatment (Behera et al., 2016; Denisenko, Budkevich, & Zhivotovsky, 2018). Accordingly, it is urgent to explore the molecular regulatory mechanism of LUAD tumorigenesis and growth.

Circular RNAs (circRNAs), non-coding RNA molecules with closed loop structures, play important roles in diverse human diseases, including cancers (Liu, Li, Luo, & Zhu, 2019; Wei & Liu, 2019). For instance, circDDX17 acts as a tumor suppressor by suppressing proliferation and accelerating apoptosis in colorectal cancer cells (Xiao et al., 2019). Besides, circRNA hsa_circ_0000263 is upregulated and played carcinogenic role through promoting cell cycle and tumor growth in cervical cancer (Cai et al., 2019). Further, circRNAs also function as key participators in LUAD. For example, circ-CAMK2A targets miR-615-5p/fibronectin 1 to promote LUAD metastasis (Du, Zhang, Qiu, Yu, & Yuan, 2019). CircRNA cMras inhibits LUAD development by downregulating PTPRG (Yu & Tian, 2019). CircRNA ACAP2 (hsa_circ_0068568) has been reported to exert carcinogenic influence on some tumors. In breast cancer, circACAP2 sponges miR-29a/b-3p to facilitate cell proliferation and metastasis by targeting COL5A1 (B. Zhao, Song, & Guan, 2020). In colon cancer, circACAP2/miR-21-5p/Tiam1 feedback loop drives cell proliferation, migration,

and invasion (J. H. He et al., 2018). Nevertheless, the specific function and molecular regulatory mechanism of circACAP2 in LUAD remain unclear.

We aimed to study the molecular mechanism and function of circACAP2 in LUAD. The influence of circACAP2 on proliferation, migration, invasion, and EMT of LUAD cells was under investigation, which may offer an innovative theoretical basis for LUAD.

Materials And Methods

Tissue specimens

Fifty paired tissues of LUAD and adjacent noncancerous tissues were obtained from LUAD patients at Harbin Medical University Cancer Hospital. The collected tissues were immediately frozen in liquid nitrogen and conserved at -80°C for experimental needs. No patients had received corresponding radiotherapy or chemotherapy before surgery, and all tissue specimens were confirmed pathologically. Written informed consents were got from every patient. The study was permitted by the Ethics Committee of Harbin Medical University Cancer Hospital.

Cell lines and reagent

Three LUAD cell lines (A549, H1975 and PC9) and one human bronchial epithelial cell line BEAS-2B were provided by the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences, Shanghai Institute of Cell Biology (Shanghai, China). All cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Thermo Fisher, Shanghai, China) consisting of 10% fetal bovine serum (FBS; Thermo Fisher) in humidified conditions with 5% CO₂ at 37°C. For blocking transcription, Actinomycin D (2 mg/ml; Sigma-Aldrich) was used with dimethylsulphoxide (Sigma-Aldrich) as a control. Besides, 3 U/μg of RNase R (Epicentre Technologies) was used for RNase R treatment.

Cell transfection

For knocking down circACAP2/LASP1, sh-circACAP2#1/2 and sh-LASP1 were constructed with sh-NC as control. Besides, pcDNA3.1/LASP1 was transfected into LUAD cells for the overexpression of LASP1, and empty pcDNA3.1 was seen as an internal control. In addition, miR-342-3p mimics and NC mimics (control) were also constructed to increasing miR-342-3p expression. All above-mentioned plasmids were provided by GenePharma (Shanghai, China). Then the cell transfection was conducted with Lipofectamine 3000 (Invitrogen, USA) for 48 hours.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was collected from LUAD tissues and cells by TRIzol reagent following the manufacturer's instructions. The extracted RNA was reverse transcribed into complementary DNA (cDNA) using a Reverse Transcription Kit (Takara, Dalian, China). Then, qRT-PCR was performed with Applied

Biosystems™ TaqMan and Taqman microRNA RTKit (ABI, USA). Relative expression of genes was calculated with the $2^{-\Delta\Delta C_t}$ method with GAPDH or U6 as the internal control.

Western blot analysis

Total protein in LUAD cells was separated by RIPA lysis buffer (Beyotime, Shanghai, China) and the protein concentration was detected with a Bio-Rad Protein Assay Kit (Bio-Rad, USA). Then proteins were separated with 10% SDS-PAGE and transferred to PVDF membranes (Millipore, USA). Blocked in 5% nonfat milk for 2 hours at room temperature, the membranes were incubated with primary antibodies (E-cadherin, N-cadherin, Vimentin, LASP1, TGF- β , Smad3 and p-Smad3, GAPDH) at 4°C overnight. Subsequently, after washing the membranes with TBST solution, secondary antibodies were added to coculture with the membranes at 37°C for another 2 hours. All the antibodies were obtained from Abcam (Shanghai, China). Finally, the blots were detected with an ECL Detection System (Thermo Fisher Scientific, USA).

MTT assay

The transfected LUAD cells were cultured in 96-well plates at 37°C. After 0, 24, 48 and 72 hours, each well was added with 10 μ l of MTT solution. After 4 hours, every well was supplemented with 150 μ l of DMSO to dissolve formazan crystal. Then, the optical density (OD) value at 490 nm was measured by a microplate reader (Bio-Rad).

Colony formation assay

The transfected LUAD cells (1000 per well) were placed into 6-well plates with RPMI 1640 medium comprising 10% FBS at 37°C. Two weeks later, the cells were washed twice with phosphate buffered saline (PBS, Thermo Fisher Scientific), fixed with 5% paraformaldehyde for 30 minutes and stained with 0.1% crystal violet (Beyotime) for another 30 minutes. Then, PBS was used to wash the cells until the solution was clear and cell colonies were counted.

Transwell assays

Transwell assays were used to examine the migration and invasion abilities of LUAD cells. For invasion assay, the transfected cells in serum-free RPMI 1640 medium were added to the upper chambers (Millipore) with Matrigel (BD Biosciences, USA). The lower chambers contained RPMI 1640 medium with 10% FBS. After 2 days, crystal violet was used to stain the invaded cells in the lower chamber and cell number was counted manually in five random fields with a fluorescence microscope (Olympus, Beijing, China). To detect cell migration ability, the procedures were the same as cell invasion assay except for the upper chambers precoated without Matrigel.

Wound healing assay

The transfected LUAD cells were seeded into 6-well plates, and then incubated in RPMI 1640 medium overnight. After a linear scratch wound was created with a sterile pipette tip, serum-free RPMI 1640 medium was used to culture the cells. At 0 h and 24 h, wound areas were imaged and assessed by ImageJ software (National Institutes of Health, USA).

RNA pull down assay

A549 and H1975 cells were transfected with circACAP2 and NC-circRNA labeled with biotin. Then, streptavidin magnetic beads were used to incubate with A549 and H1975 cell lysates for 4 h at 4 °C. Subsequently, the beads were rinsed in precooled lysis buffer and salt buffer. Later, levels of miRNAs interacting with circACAP2 were detected using qRT-PCR after the purification of pull-down RNAs.

Luciferase reporter assay

The pGL3-miR-342-3p-Wt/Mut and pmirGLO-LASP1-Wt/Mut vectors were obtained by separately cloning the wild type (WT) and mutant (Mut) circACAP2 or miR-342-3p binding sites to miR-342-3p sequence or LASP1 3'-UTR into pmirGLO (Promega) vectors. Then, the constructed vectors mentioned above were transfected with sh-circACAP2#1 or miR-342-3p mimics (NC mimics) into A549 or H1975 cells. After 2 days, Luciferase Reporter Assay System (Promega, USA) was used to evaluate the luciferase reporter activities.

RNA immunoprecipitation assay (RIP assay)

With the application of Magna RIP Kit (Millipore), RIP assay was carried out. A549 or H1975 cells were treated with RIPA lysis buffer, and the mixture was added with magnetic beads conjugated with Ago2 or IgG antibodies (Abcam). Afterwards, RNAs were purified and assessed by qRT-PCR analysis.

Fluorescence in situ hybridization assay (FISH assay)

FISH assay was conducted to determine the subcellular location of circACAP2 in LUAD cells. CircACAP2 probes were designed and synthesized by RiboBio (Guangzhou, China). The probe signals were detected with a FISH Kit (RiboBio). Briefly, LUAD cells were fastened in 4% paraformaldehyde for 15 minutes. After prehybridization in PBS, the cells were hybridized in hybridization solution (RiboBio) for 30 minutes and counterstained by DAPI (Beyotime), followed by visualization under an Olympus fluorescence microscope.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 7 Software (GraphPad Software, USA). Differences between groups were assessed by Student's *t* test (comparison evaluation between two groups) or one-way ANOVA (comparison evaluation over two groups). Spearman's correlation analysis displayed the expression correlation between genes. Data were shown as the mean $\pm$ standard deviation (SD) based on at least three experiments. $p < 0.05$ was regarded as statistical significance.

Results

CircACAP2 expression is significantly upregulated in LUAD and correlated with LUAD progression

To identify the association between circACAP2 and LUAD, qRT-PCR analysis showed that circACAP2 expression in LUAD tissues was higher than that in adjacent normal tissues (Figure 1A). Additionally, the circACAP2 was highly expressed in LUAD cell lines (A549, H1975 and PC9) relative to that in BEAS-2B cell line (Figure 1B). Further, it was suggested that circACAP2 expression in LUAD patients at advanced stage was upregulated in comparison with those at early stages (Figure 1C). Later, the stability and distribution of circACAP2 in A549 and H1975 cells were investigated. After treating with Actinomycin D, we observed that the half-life of circACAP2 was over 24 h, while that of ACAP2 mRNA was about 4 h in both A549 and H1975 cells (Figure 1D). In addition, we discovered the resistance of circACAP2 to RNase R digestion. Both data suggested the circular feature of circACAP2 (Figure 1E). Later, FISH assay confirmed that circACAP2 was mainly distributed in the cytoplasmic section of A549 and H1975 cells (Figure 1F), revealing the post-transcriptional regulation of circACAP2 in LUAD. To conclude, data above suggested that circACAP2 was a cytoplasmic RNA which harbored a loop structure and upregulated in LUAD tissues and cell lines.

CircACAP2 promotes the malignancy of LUAD

Further, the biological function of circACAP2 in LUAD was explored. In A549 and H1975 cells transfected with sh-circACAP2#1/2 vectors, the expression of circACAP2 was more decreased by sh-circACAP2#1 than by sh-circACAP2#2 (Figure 2A), so sh-circACAP2#1 vectors were used to conduct follow-up assays. MTT assay exhibited that cell viability was reduced by circACAP2 knockdown (Figure 2B). Consistently, the number of colonies was decreased after sh-circACAP2#1 transfection, indicating that circACAP2 downregulation weakened the proliferation capacity of LUAD cells (Figure 2C-D). Then, as presented by wound healing assay, cell migration suffered an evident reduction because of circACAP2 silencing (Figure 2E). The inhibition of circACAP2 knockdown on migration and invasion abilities in LUAD cells was further confirmed using transwell assay (Figure 2F). In Figure 2G, the expression of E-cadherin was raised while levels of N-cadherin and Vimentin were lessened in circACAP2-repressed A549 and H1975 cells, which indicated that EMT in LUAD was hindered by circACAP2 suppression. Taken together, circACAP2 promotes cell proliferation, migration, invasion and EMT in LUAD.

CircACAP2 interacts with miR-342-3p in LUAD

The molecular regulatory of circACAP2 in LUAD was elucidated. Based on that circACAP2 can regulate gene expression at post-transcriptional level, we hypothesized that circACAP2 exerted functions on LUAD by acting as a miRNA sponge. Afterwards, thirteen circACAP2-binding miRNAs were predicted by starBase database screened by CLIP Data (strict stringency) (Figure 3A). Then, qRT-PCR manifested three upregulated miRNAs (miR-9-5p, miR-29a-3p, miR-942-5p) and six downregulated miRNAs (miR-29b-3p, miR-766-5p, miR-342-3p, miR-653-5p, miR-450b-5p and miR-186-5p) in LUAD cells (Figure 3B). To narrow the selection, the six upregulated miRNAs were analyzed by RNA pull down assay. The results indicated

the significant enrichment of miR-342-3p in biotinylated circACAP2 probe compared with the rest five miRNAs (Figure 3C). Hence, miR-342-3p was selected for following experiments. Spearman's correlation analysis demonstrated that circACAP2 expression was negatively correlated with miR-342-3p expression in LUAD tissues (Figure 3D). Further, the binding sequence of miR-342-3p on circACAP2 was obtained from starBase (Figure 3E). According to luciferase reporter assay, the luciferase activities of wild-type miR-342-3p reporters were elevated by circACAP2 silencing, while mutant-type miR-342-3p reporters showed no evident change (Figure 3F). Further, enrichment of circACAP2 and miR-342-3p was found in Ago2 antibodies rather than in IgG antibodies (Figure 3G). Both findings validated that circACAP2 could bind with miR-342-3p. Overall, miR-342-3p interacted with circACAP2 in LUAD.

MiR-342-3p targets LASP1 in LUAD

On the basis of the above results, we further probed into the target gene of miR-342-3p in LUAD. From Venn diagram (microT, miRanda, miRmap and RNA22), seven mRNAs (ANKLE2, OAS3, LASP1, CDK12, FBN2, GPR63 and SHB) had putative binding sites with miR-342-3p (Figure 4A). After overexpressing miR-342-3p, LASP1 was significantly downregulated among the seven candidates (Figure 4B-C). Therefore, LASP1 was chosen for our assays. Besides, LASP1 was expressed at a high level in the LUAD tissues and cell lines (Figure 4D). In addition, the negative correlation between expression of miR-342-3p and LASP1 as well as the positive correlation between expression of circACAP2 and LASP1 in LUAD tissues was determined via Spearman's correlation analysis (Figure 4E). Later, the binding site between miR-342-3p and LASP1 was obtained from starBase, and the WT/Mut luciferase reporters were constructed (Figure 4F). As demonstrated in Figure 4G, miR-342-3p mimics exerted no influence on the luciferase activity of pmirGLO-LASP1-Mut reporters, but greatly inhibited that of pmirGLO-LASP1-Wt reporters. Additionally, enrichments of miR-342-3p and LASP1 in Ago2-coated pellets further uncovered that miR-342-3p could bind with LASP1 (Figure 4H). Moreover, LASP1 mRNA and protein expression were reduced upon miR-342-3p enhancement or circACAP2 deficiency (Figure 4H). To conclude, LASP1 is targeted by miR-342-3p and circACAP2 positively regulates LASP1 expression by competing for miR-342-3p in LUAD.

CircACAP2 facilitates LUAD malignancy by targeting miR-342-3p/LASP1 axis

To investigate whether circACAP2 facilitated cell proliferation, migration, invasion and EMT in LUAD via upregulating LASP1, we conducted rescue assays. At first, LASP1 expression was increased under pcDNA3.1/LASP1 transfection into A549 cells (Figure 5A). LASP1 elevation reversed the inhibitory impact of circACAP2 reduction on cell viability and proliferation in LUAD (Figure 5B and 5C). LASP1 overexpression reversed the circACAP2 repression caused by silenced circACAP2 on LUAD cell migration and invasion (Figure 5D-G). Furthermore, the effect of circACAP2 depletion on E-cadherin, N-cadherin and Vimentin was rescued via LASP1 overexpression in LUAD (Figure 5H). Therefore, LASP1 upregulation rescued circACAP2 silencing-induced inhibition on cell proliferation, migration, invasion, and EMT in LUAD. To conclude, circACAP2 promotes LUAD cellular process by upregulation of LASP1.

CircACAP2 activates the TGF- β /Smad3 pathway via modulating LASP1 in LUAD

It has been reported that the TGF- β /Smad3 pathway promotes the progression of LUAD (Jiang et al., 2019), and LASP1 can activate the TGF- β /Smad3 pathway in lung cancer (Shen, Yang, & Li, 2020). Here, we wondered whether LASP1 could affect the TGF- β /Smad3 pathway in LUAD. First, the expression of LASP1 was decreased by sh-LASP1 in A549 and H1975 cells (Figure 6A). Then, the effect of LASP1 on TGF- β /Smad3 pathway was investigated using western blot. The protein levels of phosphorylated Smad3 were reduced after LASP1 knockdown in A549 and H1975 cells, whereas the total protein expression of TGF- β and Smad3 were unchanged (Figure 6B). Further, we found that circACAP2 knockdown reduced p-Smad3 protein levels, but this effect was counteracted by overexpressing LASP1 (Figure 6C), indicating that circACAP2 could activate the TGF- β /Smad3 pathway by mediating LASP1. To further confirm this, TGF- β was used to carry out rescue assays in A549 and H1975 cells transfected with sh-circACAP2#1. The results revealed that the treatment of TGF- β reversed the suppressive effect caused by silenced circACAP2 on cell proliferation, migration and invasion (Figure 6D-F). In summary, circACAP2 promotes LUAD cell growth and migration via activating the TGF- β /Smad3 pathway.

Discussion

A mounting body of evidence has suggested that circACAP2 is a key regulator in malignant tumors, consisting of breast cancer and colon cancer (J. H. He et al., 2018; B. Zhao et al., 2020). However, the biological function and regulatory mechanism of circACAP2 in LUAD remain uncertain. Our study firstly unveiled that circACAP2 was upregulated in LUAD tissues and cell lines. Further, the circular feature and cytoplasmic localization of circACAP2 in LUAD were confirmed. Moreover, circACAP2 was validated to propel cell proliferation, migration, invasion and EMT in LUAD. These suggested the oncogenic role and post-transcription of circACAP2 in LUAD.

MicroRNAs (miRNAs) are endogenous non-coding single-stranded small RNAs with approximately 22 nucleotides (Matsuyama & Suzuki, 2019). They are implicated in various biological processes of cancers, such as breast cancer (Y. Li et al., 2020), gastric cancer (Wang et al., 2019), and lung cancer (Yang et al., 2017). CircRNAs, special endogenous RNAs, has been identified to implicate in malignancies of cancers through competing endogenous RNA (ceRNA) network that circRNAs release mRNA expression by acting as miRNA sponges (Cheng et al., 2019; Z. J. Zhao & Shen, 2017). As examples, circRNA ADAM9 contributes to cell proliferation and metastasis in pancreatic cancer by targeting miR-217/PRSS3 axis (Xing & Ye, 2019). CircRNA_001895 absorbs miRNA-296-5p to facilitate the development of clear cell renal cell carcinoma via upregulating SOX12 (Z. Chen et al., 2020). Here, through starBase database, we found that miR-342-3p harbored binding sites with circACAP2. As a tumor-suppressor, miR-342-3p inhibits cell proliferation, migration and invasion by targeting FOXM1 in cervical cancer (X. R. Li et al., 2014). Besides, miR-342-3p obstructs hepatocellular carcinoma cell proliferation by restraining IGF-1R-induced Warburg effect (W. Liu et al., 2018). In addition, miR-342-3p suppresses cell proliferation, migration, and invasion in osteosarcoma by combing with AEG-1 (Zhang et al., 2017). Then our further experiments illuminated that miR-342-3p was sponged by circACAP2 in LUAD and their expression levels were in a negative association in LUAD tissues.

According to ceRNA network, miRNAs acts as mRNA targets to regulate gene or protein expression in multiple processes of cancers (Andrés-León, Cases, Alonso, & Rojas, 2017; Urgese, Paciello, Acquaviva, & Ficarra, 2016). In current work, LASP1 was predicted as the underlying target of miR-342-3p. In view of previous literature, LASP1 accelerates cell proliferation and migration in glioma and is negatively regulated by miR-377-3p (Y. Liu et al., 2018). LASP1 propelled nasopharyngeal carcinoma progression through negatively modulating PTEN (Gao et al., 2018). Overexpression of LASP1 enhances cell growth in esophageal squamous cell carcinoma (B. He et al., 2013). In current study, the findings elucidated that LASP1 was targeted by miR-342-3p and circACAP2 positively regulated LASP1 expression by competing for miR-342-3p. Besides, circACAP2 promoted LUAD development by miR-342-3p/LASP1 axis.

It has been recognized that circRNAs can affect the progression of many diseases or malignancies by modulating the TGF- β /Smad3 pathway. For example, CircRNA circUCK2 overexpression reduces apoptosis of cerebral ischemia-reperfusion injury by targeting miR-125b-5p/GDF11 to regulate TGF- β /Smad3 signaling (Chen, Wang, Feng, & Chen, 2020). Circ_0003204 suppresses endothelial cell proliferation, migration and tube formation in atherosclerosis by targeting miR-370-3p/TGF β R2 and the activation of TGF- β /Smad3 pathway (Zhang et al., 2020). CircRNA-51217 can promote the prostate cancer cell invasion by targeting miRNA-646 and activating the TGF- β /Smad3 pathway (Xu et al., 2020). This paper demonstrated that circACAP2 stimulated the TGF- β /Smad3 pathway via regulating the miR-342-3p/LASP1 axis in LUAD.

In conclusion, circACAP2 activated the LASP1-mediated TGF- β /Smad3 pathway to accelerate proliferation, migration, invasion, and EMT of LUAD cells via sponging miR-342-3p. This discovery may provide a new clue to improve therapeutic methods of LUAD.

Declarations

Acknowledgement

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Competing interests

There are no competing interests.

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Ethics approval and consent to participate

Written informed consents were got from every patient. The study was permitted by the Ethics Committee of Harbin Medical University Cancer Hospital.

Consent for publication

Not applicable

Availability of data and material

The data underlying this article will be shared on reasonable request to the corresponding author.

Authors' contributions

Jianyu Xu conceived and designed the experiments. Jianyu Xu, Jianli Ma, Bixi Guan, Jian Li, Yan Wang, and Songliu Hu carried out the experiments. Jianyu Xu and Songliu Hu analyzed the data. Jianyu Xu and Songliu Hu drafted the manuscript. All authors agreed to be accountable for all aspects of the work. All authors have read and approved the final manuscript.

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Figures

Figure 1

CircACAP2 expression is significantly elevated in LUAD. (A-B) The expression of circACAP2 in LUAD tissues and adjacent normal tissues as well as in LUAD cell lines (A549, H1975 and PC9) and human

bronchial epithelial cell line BEAS-2B was examined by qRT-PCR analysis. (C) The expression of circACAP2 in different clinical stages was measured via qRT-PCR analysis. (D-E) CircACAP2 and ACAP2 expression in A549 and H1975 cells treated with actinomycin D or RNase R. (F) The distribution of circACAP2 in A549 and H1975 cells was determined via FISH assay. *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 2

CircACAP2 promotes the cell malignancy in LUAD. (A) The efficiency of circACAP2 knockdown in A549 and H1975 cells was evaluated via qRT-PCR. (B-D) A549 and H1975 cell viability and proliferation in sh-NC or sh-circACAP2#1 group were assessed via MTT and colony formation assays. (E-F) A549 and H1975 cell migration and invasion in sh-NC and sh-circACAP2#1 groups were probed by wound healing and transwell assays. (G) The E-cadherin, N-cadherin and Vimentin levels in A549 and H1975 cells posttransfection of sh-NC or sh-circACAP2#1 were estimated by western blot analysis. *p < 0.05, **p < 0.01.

Figure 3

CircACAP2 interacts with miR-342-3p in LUAD. (A) Thirteen potential circACAP2-binding miRNAs were found on the starBase database. (B) qRT-PCR was used to examine levels of predicted miRNAs in LUAD cell lines. (C) RNA pull down assay was conducted for selecting the potential miRNAs binding to circACAP2. (D) The expression correlation between circACAP2 and miR-342-3p was accessed using Spearman's correlation analysis. (E-F) The binding site and interaction between miR-342-3p and circACAP2 was separately examined using starBase and luciferase reporter assay. (G) RIP assay was used to explore the interaction between miR-342-3p and circACAP2. **p < 0.01, ***p < 0.001.

Figure 4

MiR-342-3p targets LASP1 in LUAD. (A) Seven target mRNAs of miR-342-3p were predicted from starBase. (B-C) The qRT-PCR analysis was utilized to test expressions of seven putative mRNAs in miR-342-3p mimics-transfected A549 and H1975 cells. (D) The LASP1 expression in LUAD tissues and cell lines was evaluated via qRT-PCR analysis. (E) Spearman's correlation analysis was conducted to evaluate the expression correlation between LASP1 and circACAP2 (miR-342-3p). (F-G) StarBase website and

luciferase reporter assay were respectively employed to verify the binding sequence and interaction between miR-342-3p and LASP1. (H) The binding of miR-342-3p to LASP1 was subjected to RIP assay. (I) The qRT-PCR and western blot analyses were performed to measure the mRNA and protein expressions of LASP1 after upregulating miR-342-3p or downregulating circACAP2. **p < 0.01, ***p < 0.001.

Figure 5

CircACAP2 accelerates LUAD malignancy through targeting miR-342-3p/LASP1 axis. (A) The qRT-PCR analysis was performed to examine the efficiency of LASP1 overexpression in A549 cells. (B-C) MTT and colony formation assays were performed to assess A549 cell viability and proliferation in each group. (D-G) Wound healing and transwell assays were used to detect migration and invasion of A549 cells

transfected with sh-NC, sh-circACAP2#1 or sh-circACAP2#1+pcDNA3.1/LASP1. (H) Western blot analysis was carried out to evaluate the expressions of E-cadherin, N-cadherin and Vimentin in A549 cells with indicated transfections. ** $p < 0.01$, *** $p < 0.001$.

Figure 6

CircACAP2 activates the TGF-β/Smad3 via the miR-342-3p/LASP1 axis in LUAD. (A) The efficiency of sh-LASP1 in A549 cells was examined via qRT-PCR. (B) Western blot analysis presented the protein expressions of TGF-β, Smad3 and p-Smad3 in A549 cells in sh-NC and sh-LASP1 groups. (C) Western blot analysis showed the protein levels of TGF-β, Smad3 and p-Smad3 in A549 cells transfected with appointed plasmids. (D-F) LUAD cell proliferation, migration and invasion in each group were assessed. ** $p < 0.01$, *** $p < 0.001$.