Original Article

Circular RNA *circZFR* promotes tumorigenic capacity of lung cancer via *CCND1*

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Background: To explore the role of circular RNA (circRNA) *circZFR* in tumorigenic capacity of lung cancer (LC).

Methods: Thirty primary LC tissues were used to detect circRNAs expression. *CircZFR* was silenced in two LC cell lines using lentivirus-mediated short hairpins RNAs. Quantitative real time PCR (qRT-PCR), northern blot and in situ hybridization (ISH) assay were used to measure the expression of circRNA.

Results: CircRNA *circZFR* was highly expressed in LC tumors. *CircZFR* deficiency significantly abrogated clone formation. *CircZFR* depletion substantially decreased tumor growth compared to WT control cells. *CircZFR* overexpression was dramatically increased cell growth in LC cell lines. Consequently, *circZFR* overexpression substantially promoted tumor propagation. Consistently, circZFR deficiency significantly reduced the expression of *CCND1* and major cell cycle genes in LC cell lines. In contrast, *circZFR* depletion did not alter the expression of ZFR. Consequently, *circZFR* deficiency dramatically decreased H3K4me3 levels on the *CCND1* promoter at –1,100 to –900 bp segment of *CCND1* promoter.

Conclusions: *CircZFR* was related with LC growth *in vitro* and *in vivo* and tumorigenic capacity of LC. The possible mechanism was to regulating expression of *CCND1*, indicating the *circZFR/CCND1* signaling might be a promising therapeutic target for LC treatment.

Keywords: Circular RNA (circRNA); *CircZFR*; lung cancer (LC); *CCND1*

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Introduction

Lung cancer (LC) is the third human malignant tumor and the leading cause of cancer-associated mortality around the world (1,2). Surprisingly, survival from a diagnosis of LC is longer for those who experienced a previous cancer than for those without previous cancer (3). LC is a serious cancer which can be cured if it is diagnosed at early stages, but its early diagnosis is not good (4). Thus, it is very critical to understand the molecular mechanism of LC progression and to discover novel therapeutic targets. Circular RNAs (circRNAs), which are discovered as a class of endogenous non-coding RNAs, have recently shown huge capabilities to regulate gene expression including viruses, plants, and animals (5). As a novel class of noncoding RNAs, circRNAs was reported to play important roles in various biological processes (6). CircRNAs have the potential in application of

Table 1 Clinical characteristics of lung cancer patients

Characteristics	Lung cancer (n=30)*
Sex	
Male	18 [60]
Female	12 [40]
Age (yr)	55.2±10.2
≤55	16 [53]
>55	14 [47]
Tumor size (cm)	
≤5	17 [57]
>5	13 [43]
Differentiation	
Low	12 [40]
Medium	15 [50]
High	3 [10]

*, data are shown as means ± standard deviation (SD) or numbers [%].

individualized cancer medicine (7). Microarray analysis of circRNAs identifies hsa_circ_0014130 as a new biomarker in non-small cell lung cancer (NSCLC) (8). Many studies showed that circRNAs were potential biomarkers for tumor diagnosis (9-11). In this study, circRNA *circZFR* (circBase ID: hsa_circ_0072088) located at chromosome 5p13.3. *CircZFR* was highly expressed in LC tissues compared with adjacent normal tissues. The deficiency and overexpression of circRNA *circZFR* in LC cell lines were performed to study its effects on tumor propagation, and its potential mechanism was explored.

Methods

Patients and sample collection

Thirty LC patients were recruited from Department of Respiratory Medicine, Beijing Aerospace General Hospital (Beijing, China) in this study. Pathological diagnosis was made according to the histology of tumor specimens or biopsy and examined by experienced pathologists. All tissue samples were obtained from consenting patients and this study was approved by Ethics committee of Beijing Aerospace General Hospital (Ethical approval number: KS-2016-01). The clinical data for the above patients were summarized in *Table 1*.

Cell lines and reagents

LC cell lines were from ATCC and maintained in DMEM supplemented with 10% fetal bovine serum (FBS) (Invitrogen, NY, USA), 100 μg/mL penicillin G, and 100 U/Ml streptomycin (Invitrogen, NY, USA).

ShRNA-mediated interference

ShRNAs against circRNA were designed with MIT's siRNA designer (http://sirna.wi.mit.edu/home.php). At least four quadruplexs were designed for each gene, and the most effective shRNAs were used for subsequent studies. The sequences of the effective shRNAs were provided as follows: Sh 1#: 5'-TACCTCGAGTGTAGCTACG-3', 2# 5'-CGCATAACTCGCATCGACC-3'. shRNAs and control hairpins were cloned into pSICO R vector. Production of lentiviral particles and transduction of cells was performed according to protocols from the RNAi consortium (http://www.broadinstitute.org/rnai/trc). Cells were transfected with lentiviral constructs expressing shRNA or shCtrl as described above for 24 hours. Positive cells were selected with puromycin (MCE, NJ, USA) for 5 days. Then, cells were collected for protein and RNA analysis.

CircRNA overexpression

Full-length *circular* cDNA was cloned into pCDNA3.1 vector, and transfected cells were generated as described above. Stable clones were obtained by selection with G418 (Thermo Fisher Scientific, USA). All constructs were confirmed by DNA sequencing.

Immunoblotting assay

Cells were lysed in RIPA lysis buffer supplemented with cocktail protease inhibitor (Roche, UK). Cytoplasamic or nuclear proteins were separated by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membrane (GE Healthcare, USA). The PVDF membranes were incubated with primary antibodies, followed by incubation with secondary antibodies coupled to horseradish peroxidase (R&D systems, USA).

Quantitative real time PCR (qRT-PCR) assay

Total RNA was isolated using TRIzol (Invitrogen, USA) and an RNeasy kit (QIAGEN, USA) with DNase I digestion

according to the manufacturers' instructions. cDNA was synthesized from total RNA using M-MLV reverse transcriptase (Promega) and random primers (Promega, USA). qRT-PCR was performed on an ABI7300 Real-Time PCR System (ABI 7300, Applied Biosystems). Data were normalized to 18S rRNA or β-actin or to control samples. Primers sequences for the detected genes were as follows: Circ-F: 5'-TTATCCCTACCTACTGCTGTGCC-3'; Circ -R: 5'-CCTTACTCTCCTGTTGTTGC-3'; *CCND1*-F: 5'-CAATCACTCCGCTCATGCTC-3', *CCND1*-R: 5'-CATGTGCAGCCTACCGGTT-3'; CCND2-F: 5'-TTACTCCTACTGCACTGCATC-3', CCND2-R: 5'-ATTCGGCATACATGAATG-3'; 18S-F: 5'-AATCCGTTGAACCCCATT-3', 18S-R: 5'-CCATCCAATCGGTAGTAGCG-3'; ACTB-F: 5'-TCCCAACAGCTT-3', ACTB-R: 5'-ATGACGTCTGGGCCTGTCTA-3'.

In situ hybridization (ISH) assay

Digoxigenin-conjugated circRNA probes (targeting junction sequence of circRNA, #1: 5'-ATTCCATAGTTCTCGGCTCG-3', #2: 5'-AGCTTGCAATTATCGAGAGC-3', #3: 5'-GAACTTGTGCGTTCCAAT-3') were designed according to protocols of Biosearch Technologies (https://www.biosearchtech.com/). Indicated samples treated in non-denaturing conditions were hybridized with probe sets overnight, then incubated with HRP-conjugated secondary antibodies. After rinsing with RNA-free PBS, the sections were incubated with DAB, counterstained with hematoxylin, dehydrated and mounted. All experiments were performed according to manuals of Biosearch Technologies.

Northern blot

Total RNA was extracted from indicated samples using Trizol, then subjected to electrophoresis on a formaldehyde denaturing agarose gel for 1.5 h. Samples were transferred to positively charged NC membranes (GE Healthcare, USA) with 20× SSC (Invitrogen, USA) buffer for 12–16 hours. After UV cross-linking and prehybridization, the with secondary antibodies coupled to horseradish peroxidase (R&D systems, USA). Membrane was incubated with biotin-labeled probes at 65 ℃ for 16–20 hours. After washed with washing buffer, biotin signals were detected with Chemiluminescent Nucleic Acid Detection Module according to the manufacturer's instructions. For detecting circRNAs only, junction sequences were used for probes. For detecting both circRNAs and linear RNAs, exon sequences were used for probes.

Cell proliferation assay

About 2,000–5,000 indicated cells were planted to a well of a 6-well plate and incubated for 2–3 weeks until growing to 80% in a CO_2 incubator at 37 ℃ with 5% CO_2. After washing three times with PBS, the cells were fixed with 4% PFA (Sigma, USA) for 10 min and incubated with 0.1% crystal violet for 30 min at room temperature. Plates were washed gently with distilled water until the background is clear, then subsequent to air-dry. After photographed, 33% acetic acid was added to each well to decolorize, then subsequent to measuring their absorbance value at 570 nm after sufficient shaking.

RNA fluorescence in situ hybridization (FISH)

Fluorescence-conjugated *CircRNA* probes were used for RNA FISH. RNA FISH was performed as previously described. Hybridization was carried out using DNA probe sets (Biosearch Technologies) according to the protocol of Biosearch Technologies. Oncosphere and control cells were observed with a FV1000 confocal laser microscopy (Olympus, Japan).

Xenograft growth in nude mice

For subcutaneous injection models, different dilutions of control and treated cells were implanted into mice (male BALB/c nude mice), aged 4 to 6 weeks, with a matrigel scaffold (BD matrigel matrix, BD biosciences) into two sides of the same nude mouse at the posterior dorsal flank region (n=4 to 6 per group). Tumors were measured every other day. Mouse experiments were approved by the Institutional Animal Care and Use Committees at Peking Union Medical College Hospital. The mice were maintained under standard conditions according to the institutional guidelines for animal care.

Statistical analysis

Statistical analysis was performed with SPSS 17.0 software (IBM, Chicago, IL). Data were shown as mean ± SD. The

significance of the differences was determined by Student's *t*-test. P<0.05 was considered significant.

Results

CircZFR is highly expressed in LC tumor tissues

Based on circRNA sequencing and circBase (http://www.circbase.org/) analysis, *circZFR* transcript was transcribed from the *ZFR* gene due to variable cyclizations (*Figure 1A*). *CircZFR* was an exon circRNA consisting of 13th to 17th exons of *ZFR* gene (*Figure 1A*). The circRNA was further validated by RNase R digestion (*Figure 1B*) and actinomycin D treatment (*Figure 1C*). In addition, *circZFR* was highly expressed in tumor tissues than peri-tumor tissues (*Figure 1D*). These observations were further validated by qRT-PCR analysis (*Figure 1E*). Furthermore, *circZFR* was mainly distributed in the nucleus of LC cells by RNA FISH (*Figure 1F*). Collectively, a conserved circRNA *circZFR* was highly expressed in LC tumors.

circZFR deficiency inhibits LC tumor propagation

To further test the role of *circZFR* in LC, we depleted *circZFR* in LC cells and examined its ability of tumor growth *in vitro* and *in vivo*. Given that intronic complementary sequences flanking exons are required for formation of exonic circRNAs, the exonic circRNAs do not generate if either of intronic complementary sequences is missing. The *circZFR* was silenced in two LC cell lines using lentivirus-mediated short hairpins RNAs (shRNAs) (*Figure 2A*). *CircZFR* deficiency significantly abrogated clone formation (*Figure 2B*). The *circZFR* deficient or wild type (WT) cells were subcutaneously injected into BALB/c nude mice. We observed that *circZFR* depletion substantially decreased tumor propagation compared to WT control cells (*Figure 2C,D*). Collectively, *circZFR* depletion impaired the lung tumor growth *in vitro* and *in vivo*.

circZFR overexpression promotes LC tumorigenic capacity

CircZFR was overexpressed in LC cell lines (*Figure 3A*). Results indicated that *circZFR* overexpression did not affect the expression of its parental gene *ZFR* (data not shown). *CircZFR* overexpression was dramatically increased the capacity of cell growth in LC cell lines (*Figure 3B*). Consequently, *circZFR* overexpression substantially promoted tumor propagation (*Figure 3C*). Taken together, *circZFR* overexpression enhances tumorigenic capacity of LC.

CircZFR promotes tumor propagation via CCND1 mediated cell cycle activation

To further determine target genes of *circZFR*, transcriptome microarray analysis was performed for *circZFR* deficiency and WT control LC cells. We noticed that the *circZFR* was positively correlated with cell cycle signaling (*Figure 4A*). These observations were validated in LC cell lines. Consistently, *circZFR* deficiency reduced the expression of *CCND1* and other cell cycle genes in LC cell lines (*Figure 4B,C*). In contrast, *circZFR* depletion did not alter the expression of *ZFR*. Consequently, *circZFR* deficiency dramatically decreased H3K4me3 levels on the *CCND1* promoter at −1,100 to −900 bp segment of *CCND1* promoter (*Figure 4D,E*). Taken together, *circZFR*-mediated *CCND1* transcription activates cell cycle signaling in LC.

Discussion

CircRNA, a class of non-coding RNAs, is a new group of RNAs that are related to tumorigenesis, and the role of circRNAs in various diseases has been already highlighted (12). CircRNAs shape a covalently closed continuous loop which have no 5'-3' polarity and contain no polyA tail (13). There is increasing evidence that circRNAs are involved in cancer development (14-16). Our results that compared with adjacent normal tissues, *circZFR* was highly expressed in LC tissues. *CircZFR* depletion impaired the lung tumor growth *in vitro* and in vivo, while its overexpression enhanced tumorigenic capacity of LC. CircRNA *circZFR* (circBase ID: hsa_circ_0072088) was first reported by Wei *et al.* (17). They found that circRNA *circZFR* was significantly upregulated in papillary thyroid carcinoma tissues compared with adjacent normal tissues, and *circZFR* expression level was negatively correlated with clinical severity, demonstrating that circRNA *circZFR* exerted oncogenic roles via regulating miR-1261/C8orf4 axis in papillary thyroid carcinoma. In another study, *circZFR* knockdown significantly suppressed cell proliferation and epithelial-mesenchymal transition in hepatocellular carcinoma, and may play carcinogenic role in hepatocellular carcinoma through regulating miR-3619–5p/CTNNB1 axis and activating Wnt/β-catenin pathway (18). *CircZFR* could significantly distinguish the cancer samples, with an AUC of 0.7069 to distinguish live cancer cases and normal controls

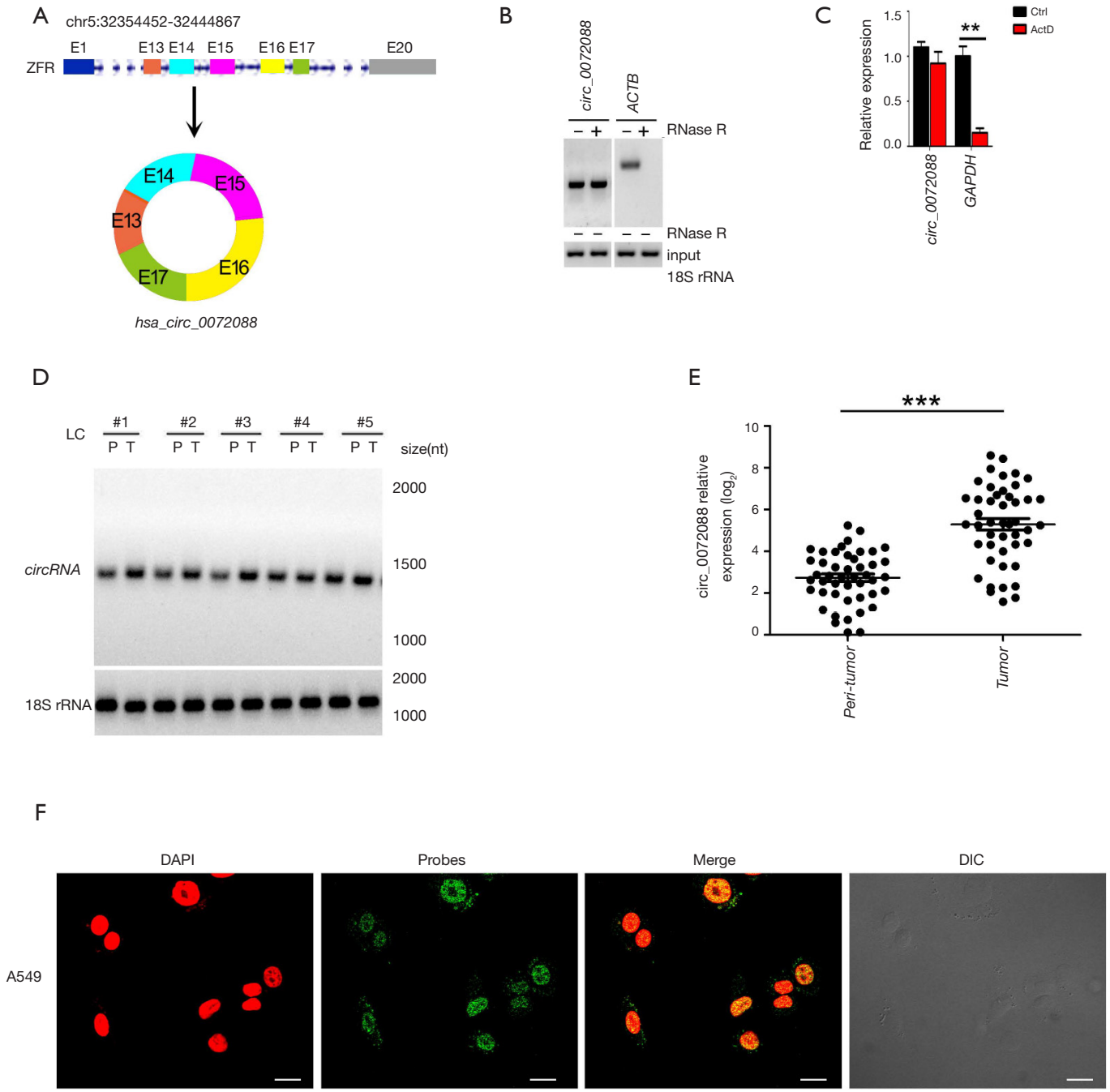

Figure 1 CircZFR is highly expressed in lung cancer tumor tissues. (A) Schematic representation of human circZFR; (B) total RNAs were digested with or without 2 Unit/g RNase R for 1 hour (h) at 37 ℃, followed by RNA extraction and analysis. For Northern blot, RNA was extracted from LC cells. 18S rRNA was used as a loading control; (C) LC cells were treated with 2 g/mL Act D for 16 h, and corresponding RNAs were extracted for qRT-PCR analysis; (D,E) expression levels of circZFR in LC primary tumors and peri-tumors by Northern blot (D) and qRT-PCR analysis (E); (F) human circZFR expression was tested in LC primary samples by RNA FISH. Representative images are shown. Scale bar, 10 μm. **, P<0.01; ***, P<0.001 by two-tailed Student's *t* test. Data are representative of at least three independent experiments. E, exon; LC, lung cancer; Act D, actinomycin D; qRT-PCR, quantitative real-time PCR; Ctrl, control; nt, nucleotide; DAPI, 4',6-diamidino-2-phenylindole; DIC, differential interference contrast.

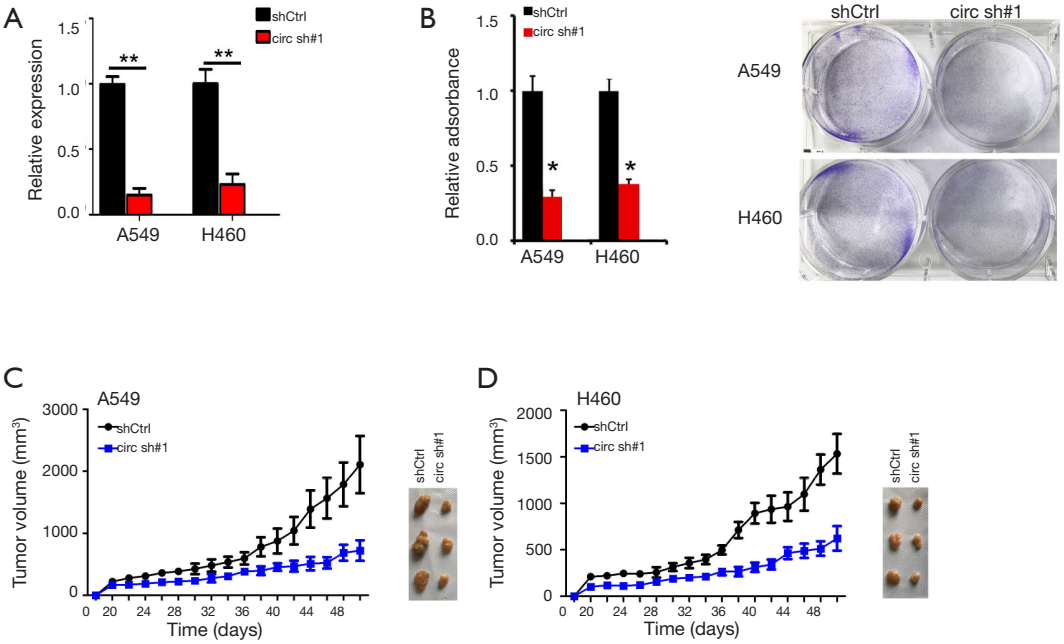

Figure 2 *CircZFR* deficiency impairs LC tumor propagation. (A) CircZFR was deleted in LC cell lines by short hairpin RNA (circ sh#1) compared with control (shCtrl) cells. 18S rRNA served as a positive control; (B) clone formation capacities were tested in circ-depleted and control LC cell lines. Representative pictures are shown (right panel), and statistical results are shown as means ± SD (left panel); 1×106 circZFR deficient or shCtrl A549 (C) and H460 (D) cells were subcutaneously injected into BALB/c nude mice for tumor growth measurement. Representative tumor images (right panel) and statistical results are shown as means ± SD (left panel). n=5 for each group. *, P<0.05; **, P<0.01.

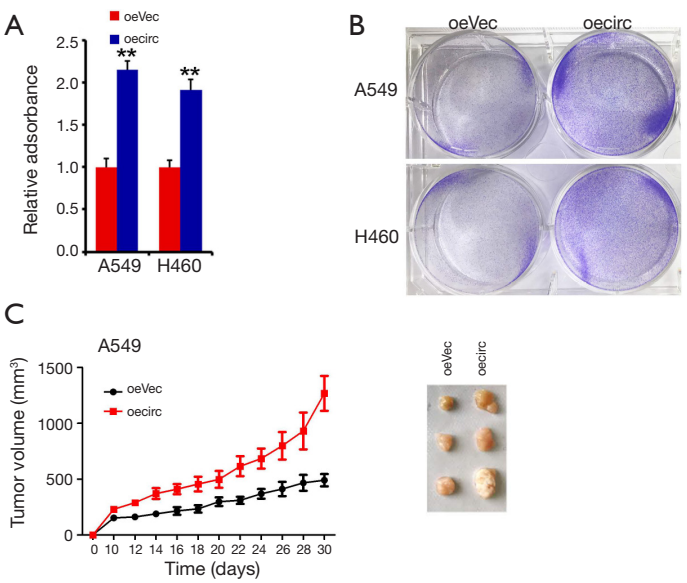

Figure 3 CircZFR overexpression enhances LC tumorigenic capacity. (A) CircZFR-overexpressing LC cell lines were established; (B) Overexpression of circZFR enhanced the capacity of clone formation. Representative images and statistical results are shown; (C) 1×10^6 circ-overexpressing or oeVec LC cells were subcutaneously injected into BALB/c nude mice for tumor growth measurement. Representative tumor images (right panel) and statistical results are shown as means ± SD (left panel). n=5 for each group. oeVec, overexpression of empty vector; oecirc, overexpression of circZFR. **, P<0.01.

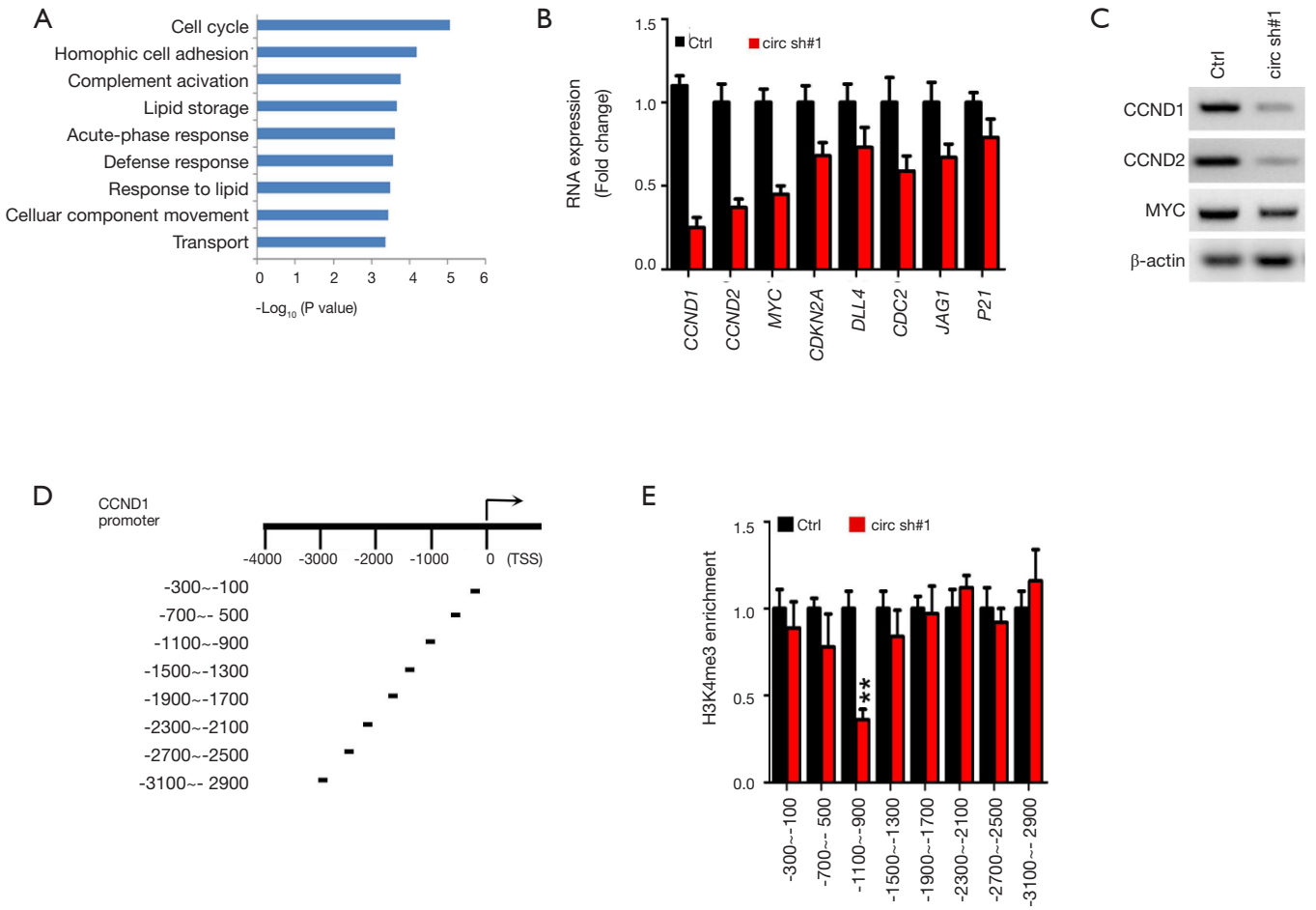

Figure 4 CircZFR depletion suppresses tumor propagation via CCND1. (A) GO analysis of downregulated genes in *circZFR* deficiency *vs.* control cells. (B,C) quantitative measurement of cell cycle-related genes in circZFR-depleted and Ctrl LC cells by qRT-PCR (B) and western blot (C); (D,E) ChIP-qPCR analysis of H3K4me3 enrichment on the *CCND1* promoter in Circ-depleted cells. Results are shown as means ± SD. **, P<0.01, by two-tailed Student's *t* test. Data are representative of at least three independent experiments. GO, gene ontology; qRT-PCR, quantitative real-time PCR; ChIP, chromatin immunoprecipitation.

(19). However, there are still few studies to explore circRNA *circZFR* in cancer research. Through many articles have showed the relationship of circRNAs with LC (8,11,20), to our knowledge, our study firstly reported that circRNA *circZFR* exerted carcinogenic role on LC. And possible mechanism is that *circZFR*-mediated *CCND1* transcription activates cell cycle signaling in LC. *CCND1* gene encodes Cyclin Dl, a cyclin involved in cell cycle regulation at the Gi-S transition (21). *CCND1* G870A polymorphism may be a risk factor for LC (22). Study found that miR-326 played a pivotal role on NSCLC through inhibiting cell proliferation, migration, invasion, and promoting apoptosis by targeting oncogenic *CCND1* (23). The results were consistent our results, circRNA *circZFR* deficiency dramatically decreased H3K4me3 levels on the *CCND1* promoter at −2,700 to −2,500 bp segment of *CTNNB1* promoter and suppressed chromatin accessibility of the *CTNNB1* locus, indicating that *circZFR* played important role in cell cycle signaling of LC by targeting *CCND1*. In conclusion, *circZFR* as a new circRNA was highly expressed in LC tumors, its depletion impaired the LC growth *in vitro* and *in vivo* and overexpression enhanced tumorigenic capacity of LC, and possible mechanism was related with expression of *CCND1*. Our results suggested the *circZFR/*

CCND1 signaling might be a promising therapeutic target for LC treatment.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2020.04.24). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All tissue samples were obtained from consenting patients and this study was approved by Ethics committee of Beijing Aerospace General Hospital (Ethical approval number: KS-2016-01).

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