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CircZSWIM4 facilitates tumor development in lung adenocarcinoma by targeting miR-370-3p and miR-873-5p to regulate the axis of FOXM1/β-catenin

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ABSTRACT

Circular RNA (circRNA) is a kind of RNA generated by a covalently closed loop and possesses sophisticated capacities of gene regulation in tumorigenesis and development. However, the role of circZSWIM4 on lung adenocarcinoma (LUAD) remains largely unclear. In the present study, we used reverse transcription–qPCR (RT-qPCR) to examine whether circZSWIM4 was significantly overexpressed in LUAD cells. The impacts of circZSWIM4 on the properties of proliferation, apoptosis and migration were assessed by loss-of and gain-of-function assays, such as CCK-8 experiments, flow cytometry analysis and wound healing experiments. Moreover, TOP/FOP flash experiments and FISH experiments were carried out to prove that circZSWIM4 stimulated the Wnt/β-catenin pathway. Downstream targets of circZSWIM4 were forecasted by bioinformatics tools and validated by RNA immunoprecipitation (RIP), RNA pulls down as well as luciferase reporter experiments. Forkhead box M1 (FOXM1) was confirmed to be the corporate targets of miR-370-3p and miR-873-5p. Through co-IP assay, we verified the combination between FOXM1 and β-catenin. Totally, circZSWIM4 activated the Wnt/β-catenin pathway by targeting miR-370-3p and miR-873-5p to regulate FOXM1 and β-catenin and facilitated the progression of LUAD.

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Introduction

Lung cancer is an often kind of malignancy and is the main cause of cancer-linked mortality around the globe (1). Among this, NSCLC is geared to a form of lung malignancy and accounts for 84% (2). More, lung adenocarcinoma (LUAD) belongs to the most frequent variety of NSCLC and exhibits a high incidence rate and death rate (3). Despite the great efforts for the improvement of LUAD treatments, LUAD patients are still confronted with poor survival rates (4). Thus, the exploration of novel treatment strategies is the primary task for LUAD.

CircRNAs are a type of special long noncoding RNA that forms a covalently shut consecutive loop featuring no 5'-3' polarity along with a polyA tail (5). A large scale of evidences has proved that circRNAs are tightly linked to human cancers, and may function as better potential markers for their enrichment and stability (6). In parallel, a large member of circRNAs has been proven to play significant roles in the occurrence and processes of LUAD. CircPUM1 elevates the malignant behavior of LUAD via targeting miR-326 (7). CircPRKCI is important for tumorigenesis and functions as a possible target for LUAD treatment (8). As a fresh circRNA, circZSWIM4 has not been documented in LUAD. Accordingly, the biological behaviors and potential mechanisms of circZSWIM4 remain to be further investigated.

Mechanistically, abnormal activation of signaling pathways plays key roles in the development of cancers, encompassing the Wnt/β-catenin pathway (9,10). In this pathway, β-catenin can aggregate in the cytoplasmic part, followed by being transmitted to the nuclear part. In the nucleus, β-catenin combines with TCF/LEFs, leading to the activation of proto-oncogenes containing cyclin D1 and c-Myc, and thus affecting the development of malignancy (11). Increasingly studies have disclosed that circRNAs can irritate this pathway to involve in cancerous development (12). CircRNA_100290 plays a promoting role in colorectal cancer development by targeting miR-516b and FZD4 to promote the pathway (13). Cir-ITCH hampers lung cancer cell proliferation through the inactivation of the pathway (14). In our study, we also probed into the underlying mechanism between circZSWIM4 and this pathway in the LUAD.

Moreover, circRNAs can act as a competing endogenous RNA (ceRNA) to promote mRNAs via targeting miRNAs (15). As reported previously, Circular RNA cMras negatively affects LUAD progression by targeting the miR-567/PTPRG axis (16). Circ_0003998 accelerates chemoresistance via the regulation of miR-326 in LUAD cells (17). However, whether circZSWIM4 acted as a ceRNA to regulate miRNA needs to be further specified in our study.

In a word, our study emphasized exploring the functional role as well as the underlying mechanism of circZSWIM4 in LUAD. First, we found that circZSWIM4 expressed a high level in LUAD cells. Knockdown or overexpression of circZSWIM4 inhibited or promoted the proliferative and migratory capacities of LUAD cells. Moreover, circZSWIM4 activated the pathway by targe-

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ting miR-370-3p and miR-873-5p. Conclusively, our study might be conducive to identifying new methods for LUAD treatments.

Materials and Methods

Cell culture

LUAD cells (A-549, NCI-H1975, NCI-H1299 and NCI-H460) and human normal lung epithelial cells (BEAS-2B) were commercially attained from ATCC (Manassas, VA). In addition, PC-9 cells were obtained from COBIOER Company Ltd (Nanjing, China). PC-9, NCI-H1975, NCI-H1299 and NCI-H460 cells were placed in RPMI-1640 medium, replenished with 10% FBS in a humidified environment at 37 °C with 5% CO_2. Also, A-549 was cultivated in the F-12K medium (Gibco) and BEAS-2B was cultivated in the BEGM medium (Gibco) under the same culture condition. For RNA digestion, RNase R was treated in cells for incubation at 37 °C for 60 min and next subjected to RT-qPCR analysis.

Reverse transcription–qPCR (RT-qPCR)

As per the supplier's requirements, total RNA from LUAD cells was isolated utilizing TRIzol reagent, and cDNA was gotten through reverse transcription. RT-qPCR was then implemented. After that, the expression levels of genes were acquired utilizing the $2^{-\Delta\Delta Ct}$ approach. GAPDH was selected for circRNA and mRNA reference, while U6 was used as an internal control for miRNA.

Actinomycin D experiment

A-549 and NCI-H1975 cells were inoculated in 24-well plates (5×10^4/well) treated with Actinomycin D (2 μg/ml, Abcam) for 0 h, 2 h, 4 h, 6 h, and 8 h after 24 h. Then, the relative circZSWIM4 and linear ZSWIM4 expression levels were severally probed by RT-qPCR.

Cell transfection

To knock downcircZSWIM4 expression, three shRNAs targeting circZSWIM4 (sh/circZSWIM4#1/2/3) and sh/NC were designed and synthesized. After that, pcDNA3.1 (+) CircRNA Mini Vector as well as circZSWIM4, miR-1286 mimics, miR-370-3p mimics, miR-873-5p mimics, mimics control (miR-NC), shRNAs targeting FOXM1 (sh/FOXM1#1/2) and sh/NC, vector and FOXM1 as well we CTNNB1 were also commercially attained from GenePharma. Cell transfection used Lipofectamine 3000 (Invitrogen) on the basis of the provider's instructions for 48 h. The experiment was in triplicate.

Cell counting kit-8 (CCK-8)

The viability of LUAD cells was assessed by CCK-8 reagent (Dojindo Molecular Technologies, Japan). Transfected cells were added into a 96-well plate (10^4/well), followed by incubation of 24 h, 48 h and 72 h. Next,10 μl CCK-8 solutions were treated into every well and hatched for another 4 h. At 450 nm, the absorbance was examined through the application of a microplate reader.

Colony formation

About 500 cells were placed in 6-well plates for 10 days. Subsequently, cells were severally fixed and stained with 4% paraformaldehyde and 0.5% crystal violet for 10 minutes. Finally, we observed and counted the number of colonies manually.

Flow cytometry analysis

Subsequent to the transfection, cells were gathered and double stained by Annexin V-FITC/PI kit. After that, cells were then washed, followed by detection via flow cytometry (BD Biosciences). The experiment was conducted three times.

TUNEL

LUAD cell apoptosis was assessed via TUNEL Apoptosis Kit (Invitrogen) according to the provider's suggestions. DAPI was used to stain the cell nucleus and then observed by fluorescence microscopy (Olympus). The experiment was in triplicate.

Wound healing

Wound healing experiments were performed to evaluate LUAD cells migration. Transfected cells (1×10^6) were seeded into 24-well plates and then a sterile 200μl pipette tip was used to create a scratch in the cell monolayer. After that, the wound closure at 0 h and 24 h were photographed via microscopy.

Transwell

In the serum-free medium, transfected cells were added into the upper Transwell chamber (Corning, New York, USA). In the bottom chamber, a medium with 10% FBS was joined. Twenty-four hours later, cells on the upper chamber were removed and then the migrated cells were subjected to fixationin4% paraformaldehyde and dyed with 0.5% crystal violet in the lower chamber. Lastly, in five random visual fields, migrated cell number was counted using a microscope (Olympus).

Luciferase reporter assay

LUAD cells were planted in 96-well plates, followed by transfection with a variety of luciferase reporters constructs, in order to examine activities of Notch, p53, MAPK/ERK, MAPK/JNK, TGF-β, pRb-E2F, Wnt, Myc/Max, Hypoxia and NF-κB pathways, respectively. Moreover, the circZSWIM4-WT, circZSWIM4-MUT, CTNNB1-WT and CTNNB1-MUT reporters were next subjected to 48 h of co-transfection with miR-1286 mimics, miR-370-3p mimics, miR-873-5p mimics or NC mimics in LUAD cells, respectively. Luciferase activities were assessed.

TOP/FOP flash assay

The assay was carried out according to the manufacturer's requirements. LUAD cells (2×10^4 cells/well) were planted in 24-well plates, followed by co-transfection with Top-flash plasmid and sh/circZSWIM4#1/2 or sh/NC or circZSWIM4 or vector or miRNA mimics or miR-NC. Similarly, cells were co-transfection with Fop-flash plasmid and sh/circZSWIM4#1/2 or sh/NC or circZSWIM4 or vector or miRNA mimics or miR-NC. The luciferase activity was examined.

FISH

The RNA FISH Kit was acquired from RiboBio (Guangzhou, China). Cells laid on the slides were fixed and then permeated. Next, the slides were incubated with a prehybridization buffer at 37 °C overnight. After washing, the nucleus was counterstained with DAPI. Finally, the

slides were fixed and captured.

Immunofluorescence

Transfected cells were fixed and permeated. Nucleus staining was used with DAPI. After blocking, cells were hatched with β-catenin antibody and then hatched with the appropriate secondary antibody for 60 min. After that, the cell nucleus was dyed utilizing DAPI and the images were visualized using a fluorescence microscope.

Western blot

Cells were lysed, followed by quantification of protein concentration. After that, proteins were isolated by SDS-PAGE and shifted to PVDF membranes. After blocking with 5% defatted milk, proteins were incubated with the following primary antibodies: GAPDH, β-catenin, c-MYC, FOXM1, Lamin B1(ab16048, Abcam, USA) and cyclin D1(ab16663, Abcam, USA). Later, the membranes with proteins were grown. Lastly, the protein detections were using ECL Western Blotting Substrate (Invitrogen, Carlsbad, CA, USA).

Subcellular fractionation

LUAD cells were separated using a PARIS kit based on the manufacturer's advice. U6 and GAPDH were utilized to be internal references. The percentage of total RNA was determined.

RNA pull down assay

Biotin-labeledcircZSWIM4 probes, biotin-labeled wild-type and mutant-type of miR-370-3p/miR-873-5p probes were transcribed in vitro for 48 h, respectively. Then cells were cross-linked, followed by lysis in cell lysis buffer. Next, DNase I and streptomyces avidin magnetic beads were added to digest the gene in the cell lysis buffer and capture the RNA complex. Subsequent to washing, the protease K was utilized to relieve the cross-link and followed by RT-qPCR analysis.

RNA immunoprecipitation (RIP) assay

LUAD cells were split by buffer, followed by incubation with magnetic beads coated with antibodies against Ago2 or IgG. Then, RNA was purified and analyzed by RT-qPCR.

Statistical analysis

GraphPad Prism 5.0 software was adopted for data analyses. Student's t-test and one-way ANOVA were utilized for the assessment of the significance of the difference. Data were verified as mean±SD. P-value lower than 0.05 was considered to be the threshold for statistical significance. Each experiment was conducted three times.

Results

CircZSWIM4 expresses a high level in LUAD cells

To identify the expression profile of circZSWIM4 in LUAD, we adopted RT-qPCR to analyze circZSWIM4 expression level in LUAD cells (A-549, PC-9, NCI-H1975, NCI-H1299 and NCI-H460), and BEAS-2B was used as the control. As exhibited in Figure 1A, circZSWIM4 expression level displayed a noticeably high level in LUAD cells, and was the highest in A-549 cells as well as the lowest in NCI-H1975 cells. Thus both cells were selected for the follow-up experiments. Besides, the schematic diagram illustrated the genomic distribution and splicing model of circZSWIM4 (Figure 1B). Besides, to verify that circZSWIM4 was circRNA rather than the products of trans-splicing or genomic rearrangements, RNA primers were designed. Divergent primers were designed to magnify the circZSWIM4 while convergent primers were devised to magnify the linear ZSWIM4. The data from agarose gel electrophoresis disclosed that circZSWIM4 was amplified from complementary DNA (cDNA) by divergent primers not from genomic DNA (gDNA) (Figure 1C). Simultaneously, we treated Actinomycin D into A-549 and NCI-H1975 cells for 8 h, and used RT-qPCR to analyze the half-life of circZSWIM4. It was found that circZSWIM4 was more stable than ZSWIM4 mRNA (Figure 1D). Additionally, the fragment of ZSWIM4 mRNA was digested by RNase R, and circZSWIM4 had no change by RNase R treatment (Figure 1E). Taken together, circZSWIM4 was highly expressed in LUAD cells.

CircZSWIM4 promotes LUAD cells proliferation and migration

To test the role of circZSWIM4 in LUAD cells, we first stably down-regulated or overexpressed the expression of circZSWIM4 in A-549 or NCI-H1975 cells transfected with shRNAs targeting circZSWIM4or pcDNA3.1 targeting circZSWIM4. RT-qPCR analysis proved the effectiveness of down- or up-regulated circZSWIM4 expression (Figure 2A-2B). Then several cell function assays were carried on. In CCK-8 assays, circZSWIM4 deficiency or overexpression significantly inhibited or elevated the cell viability in LUAD cells (Figure 2C). Simultaneously, a colony formation assay was carried out. Consistent with the results of the CCK-8 assay, silenced or promoted circZSWIM4 expression suppressed or increased the number of colonies (Figure 2D). The abovementioned data disclosed the effect of circZSWIM4 on LUAD cell proliferation. After that, we uncovered that the cell apoptosis rate was increased by circZSWIM4 knockdown, while decreased by circZSWIM4 up-regulation (Figure 2E-2F). Moreover, we also assessed the effect of circZSWIM4 on migratory property in LUAD cells. As exhibited by the wound healing experiment, the

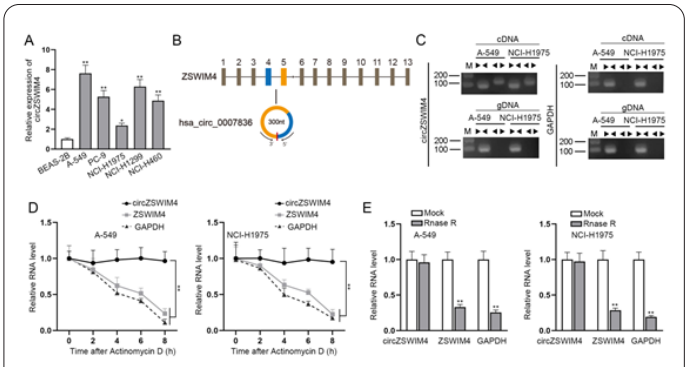

Figure 1. CircZSWIM4 is overexpressed in LUAD cells. A. Expression of circZSWIM4 in LUAD cells and normal lung epithelial cells was unclosed by RT-qPCR. B. Sanger sequencing disclosed a back-spliced junction of circZSWIM4. C. Agarose gel electrophoresis of circZSWIM4 PCR products amplified by divergent primers in gDNA or cDNA. D. Actinomycin D treatment was applied to detect the half-life of circZSWIM4 and ZSWIM4 mRNA. E. circZSWIM4 and ZSWIM4 mRNA expression were detected by RT-qPCR subsequent to RNase R treatment. $^{*}P<0.05$, $^{**}P<0.01$.

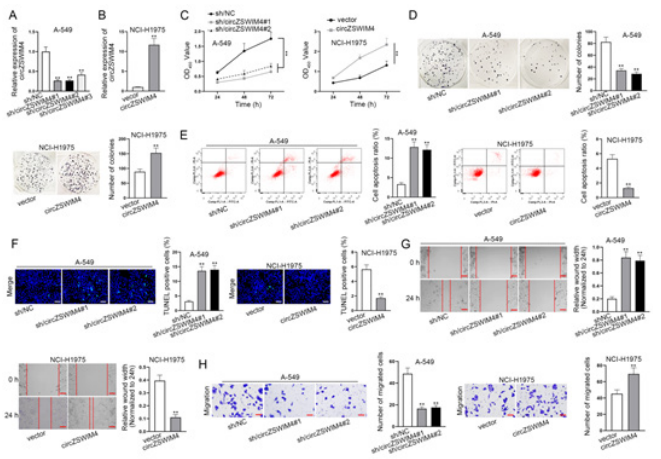

Figure 2. CircZSWIM4 promotes the progression of LUAD. A-B. Expression of circZSWIM4 was analyzed in LUAD cells transfected with shRNAs targeting circZSWIM4 or pcDNA3.1 targeting circZSWIM4. C. CCK-8 assay detected the cell viability in LUAD cells transfected with shRNAs targeting circZSWIM4 or pcDNA3.1 targeting circZSWIM4. D. Colony formation experiments in LUAD cells subsequent to transfection with shRNAs targeting circZSWIM4 or pcDNA3.1 targeting circZSWIM4. E. Flow cytometry analysis in LUAD cells after transfection with shRNAs targeting circZSWIM4 or pcDNA3.1 targeting circZSWIM4. F. TUNEL (bar value = 100 μm) assay in LUAD cells subsequent to transfection with shRNAs targeting circZSWIM4 or pcDNA3.1 targeting circZSWIM4. G. Wound healing (bar value = 80 μm) assay in LUAD cells transfected with shRNAs targeting circZSWIM4 or pcDNA3.1 targeting circZSWIM4. H. Transwell (bar value = 40 μm) assay measured the migratory ability in LUAD cells after transfection with shRNAs targeting circZSWIM4 or pcDNA3.1 targeting circZSWIM4. $^{**}P<0.01$.

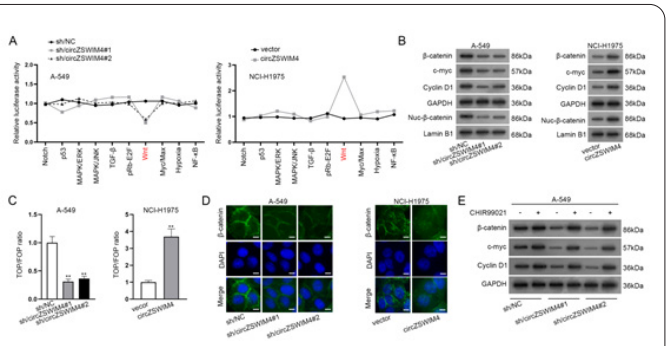

Figure 3. CircZSWIM4 enhances the Wnt/β-catenin pathway for EMT induction in LUAD cells. A. The relative luciferase activity of various signaling pathways was detected in LUAD cells subsequent to transfection with shRNAs targeting circZSWIM4 or pcDNA3.1 targeting circZSWIM4. B. Western blot uncoveredβ-catenin,c-MYC, Cyclin D1 and Nuc-β-catenin levels in LUAD cells transfected with shRNAs targeting circZSWIM4 or pcDNA3.1 targeting circZSWIM4. C.TOP/FOP flash experiment in LUAD cells after transfection with shRNAs targeting circZSWIM4 or pcDNA3.1 targeting circZSWIM4. D. FISH (bar value = 15 μm) assay analyzed the cellular location of β-catenin in LUAD cells subsequent to transfection with shRNAs targeting circZSWIM4 or pcDNA3.1 targeting circZSWIM4. E. Western blot analyzedβ-catenin,c-MYC, and Cyclin D1 levels in LUAD cells transfected with shRNAs targeting circZSWIM4 or pcDNA3.1 targeting circZSWIM4 upon the treatment of agonist (CHIR99021). $^{**}P < 0.01$.

wound width was augmented by circZSWIM4 silencing and could be lessened after the deletion of circZSWIM4 (Figure 2G). In the transwell experiments, migrated cell number was impeded or enhanced by down- or up-regulated circZSWIM4 (Figure 2H). All of the above data revealed that circZSWIM4 was implicated in the progression of LUAD cells.

CircZSWIM4 enhances the Wnt/β-catenin pathway for induction of the EMT in LUAD cells

It is well known that this pathway is tightly related to diverse cancer development, encompassing LUAD (18). Here we suspected whether circZSWIM4 modulated this pathway in LUAD. To verify our assumption, luciferase reporter assays were implemented to examine the luciferase intensity of multiple common signaling pathways, respectively. As shown in Figure 3A, the activity of this pathway was inhibited or promoted when circZSWIM4 was knockdown or overexpression, whereas no obvious difference of that in other signaling pathways. To further validate that circZSWIM4 was responsible for this pathway activation, the following experiments were carried on. First, western blot unclosed β-catenin, c-MYC, Cyclin D1 and Nuc-β-catenin levels, which were the relative genes of the Wnt pathway. Notably, c-MYC and Cyclin D1 serve as the downstream genes of this pathway can induce the EMT process (19). The data showed that circZSWIM4 deficiency declined the protein levels of these genes, while circZSWIM4 overexpression could increase the protein levels of these genes, which hinted that circZSWIM4 could affect the EMT process via the modulation of the pathway (Figure 3B). Next, the TOP/FOP flash assay was exploited for the examination of the influence of circZSWIM4 on transcriptional activity depending on β-catenin/TCF (20). As expected, the activity of TOP/FOP flash was also inhibited or promoted by silenced or overexpressed circZSWIM4 (Figure 3C). Moreover, we discovered that the cytoplasm level of β-catenin was weakened after circZSWIM4 deletion, and circZSWIM4 promotion could translocate the level of β-catenin from the cytoplasmic part to the nuclear part, implying that circZSWIM4 might induce this pathway activation via increasing the nuclear translocation of β-catenin (Figure 3D). Additionally, we added the agonist (CHIR99021) into A-549 cells for activation of the pathway and examined the impact of circZSWIM4 on the protein levels of β-catenin, c-MYC and Cyclin D1. As indicated in Figure 3E, the protein levels of these genes were decreased by circZSWIM4 knockdown while restored after the treatment of CHIR99021 (Figure 3E), implying that circZSWIM4 could induce the EMT process through pathway activation. Totally, circZSWIM4 activated the pathway to induce the EMT process in LUAD cells.

CircZSWIM4 acts as a ceRNA to modulate miR-1286/miR-370-3p/miR-873-5p in LUAD cells

To further determine the regulatory relationship between circZSWIM4 and this pathway, we detected the subcellular localization of circZSWIM4. Based on the relevant assays, we found that circZSWIM4 was mainly in the cytoplasm (Figure 4A-4B), suggesting that circZSWIM4 exerted functions in post-transcriptional regulation. As we know, the ceRNA pattern belongs to a common post-transcriptional regulation and is widely researched in the progression of various cancers, encompassing LUAD (21). Here, we conjectured whether circZSWIM4

acted as a ceRNA to modulate the pathway in LUAD cells. First, circinteractome was applied to observe 11 potential miRNAs (miR-1205, miR-1286, miR-1289, miR-370-3p, miR-486-3p, miR-558, miR-580, miR-615-5p, miR-637, miR-767-3p and miR-873-5p) combined with circZSWIM4. Then, we used a probe to label the biotinylated circZSWIM4 and performed RNA pull-down experiments to confirm which miRNA is directly bound to circZSWIM4 in LUAD cells. The data manifested that miR-1286, miR-370-3p and miR-873-5p were significantly enriched in Bio-circZSWIM4-WT groups while other miRNAs had no changes, which relative to Bio-circZSWIM4-MUT groups and Bio-NC groups (Figure 4C). For all we know, RNA-induced silencing complexes (RISCs) are shaped by miRNA ribonucleoprotein complexes (miRNPs) that existed in anti-Ago2 immunoprecipitates (22). Hence we used RIP assays to verify the correlation between RNAs and Ago2 for the confirmation of the ceRNA mechanism. The results presented that the enrichments of circZSWIM4, miR-1286, miR-370-3p and miR-873-5p were all promoted in Ago2 groups (Figure 4D), which meant that circZSWIM4 functioned as a ceRNA to regulate these three miRNAs. Furthermore, we cloned the vector of circZSWIM4-WT and circZSWIM4-MUT, followed by co-transfection with the mimics of these miRNAs, respectively, to probe the luciferase activity of LUAD cells. It was found that the luciferase intensity of circZSWIM4-WT was obviously weakened when miR-1286 or miR-370-3p or miR-873-5p was overexpressed, whereas no statistical significance of that in circZSWIM4-MUT groups (Figure 4E). Totally, circZSWIM4 acted as a ceRNA for modulation of miR-1286/miR-370-3p/miR-873-5p in LUAD cells.

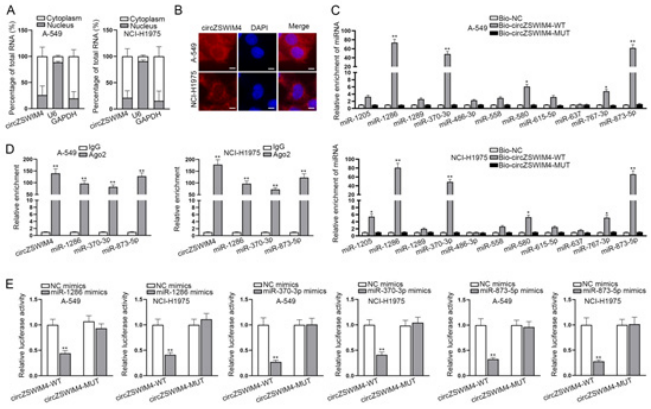

Figure 4. CircZSWIM4 functions as a ceRNA to regulate miR-1286/miR-370-3p/miR-873-5p in LUAD cells. A-B. Subcellular fractionation and FISH (bar value = 15 μm) assays examined the cellular distribution of circZSWIM4. C. RNA pull-down assay unclosed the abundance of 11 miRNAs in Bio-circZSWIM4-WT or Bio-circZSWIM4-MUT groups. D. RIP assays detected the association between RNAs and Ago2. E. The relative luciferase intensity of circZSWIM4-WT or circZSWIM4-MUT was unearthed. $^{*}P<0.05$, $^{**}P<0.01$.

CircZSWIM4 combines with miR-370-3p and miR-873-5p to regulate FOXM1 in LUAD cells

According to bioinformatics analysis, we found that FOXM1 was the target of miR-1286, miR-370-3p and miR-873-5p. Then we evaluated FOXM1 expression level in LUAD cells and uncovered that its expression profile was similar to circZSWIM4 (Figure 5A). Next, we found FOXM1 was down-regulated by circZSWIM4 knockdown or up-regulated by circZSWIM4 overexpression, which was positively correlated with circZSWIM4 expression (Figure 5B). We knocked down or overexpressed the expression of FOXM1 to perform the FISH assay and validated that the cytoplasm level of β-catenin was lessened by down-regulated FOXM1. Intriguingly, up-regulated FOXM1 translocated β-catenin from the cytoplasmic part to the nuclear part (Figure 5C). To screen which miRNA could regulate FOXM1 to affect the pathway, we probed the expression of these three miRNAs in LUAD cells. RT-qPCR disclosed that miR-370-3p and miR-873-5p expression were obviously reduced in LUAD cells relative to BEAS-2B cells, while miR-1286 displayed no notable significance in LUAD cells and BEAS-2B cells (Figure 5D). Then we conducted TOP/FOP flash assays to attest that enhanced miR-370-3p and miR-873-5p expression levels evidently suppressed the activity of TOP/FOP flash, while up-regulation of miR-1286 had no effects on it (Figure 5E). To further prove the relation among circZSWIM4, miR-370-3p, miR-873-5p and FOXM1, the relevant assays were followed. It was found that circZSWIM4, miR-370-3p, miR-873-5p and FOXM1 were highly abundant in Anti-Ago2 groups, indicating they were co-existed in the RISC (Figure 5F). In luciferase reporter assays, overexpression of miR-370-3p or miR-873-5p hindered pmirGLO/3'UTR-WT activity, while co-transfected with circZSWIM4 could reverse this effect (Figure 5G).

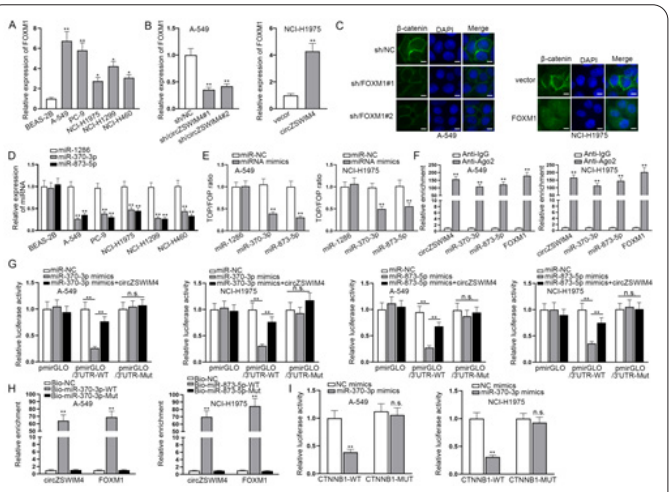

Figure 5. CircZSWIM4 combines with miR-370-3p and miR-873-5p to regulate FOXM1 in LUAD cells. A. Expression of FOXM1 in LUAD cells and normal lung epithelial cells. B. Relative expression of FOXM1 was detected in LUAD cells transfected with shRNAs targeting circZSWIM4 or pcDNA3.1 targeting circZSWIM4. C. FISH (bar value = 15 μm) assay uncovered the cellular location of β-catenin in LUAD cells subsequent to transfection with shRNAs targeting FOXM1 or pcDNA3.1 targeting FOXM1. D. Expression of miR-1286 or miR-370-3p or miR-873-5p in LUAD cells and normal lung epithelial cells. E. TOP/FOP flash assay in LUAD cells after transfection with miR-1286 mimics or miR-370-3p mimics or miR-873-5p mimics. F. RIP assays disclosed the association between RNAs and Ago2. G. Luciferase reporter assays examined pmirGLO/3'UTR-WT or pmirGLO/3'UTR-MuT activity in LUAD cells subsequent to transfection with miR-NC or miR-370-3p mimics or miR-873-5p mimics or miR-370-3p mimics+circZSWIM4 or miR-873-5p mimics+circZSWIM4. H. RNA pull-down assay showed the abundance of circZSWIM4 and FOXM1 in different groups. I. Relative luciferase intensity of CTNNB1-WT and CTNNB1-MUT was unclosed in LUAD cells after transfection with miR-370-3p mimics. $^{*}P<0.05$, $^{**}P<0.01$, n.s.: no significance.

Meanwhile, we found that both circZSWIM4 and FOXM1 were abundant in the Bio-miR-370-3p-WT or Bio-miR-873-5p-WT groups, further confirming the combination among them (Figure 5H). Finally, we also constructed the wild and mutant luciferase reporter plasmids of CTNNB1, and co-transfected with mimics to confirm whether miR-370-3p could combine with CTNNB1 to activate this pathway. The data exhibited that miR-370-3p mimics declined CTNNB1-WT activity, and had no alteration of that in CTNNB1-WT groups (Figure 5I). In short, circZSWIM4 combined with miR-370-3p and miR-873-5p to regulate FOXM1 in LUAD cells.

CircZSWIM4 promotes LUAD progression via targeting miR-370-3p and miR-873-5p

To investigate whether circZSWIM4 targeted miR-370-3p and miR-873-5p to affect the progression in LUAD, a succession of rescue experiments were conducted. In the experiments, cell proliferation was hampered by circZSWIM4 deficiency, while knockdown of miR-370-3p or miR-873-5p could restore this effect caused by circZSWIM4 deficiency (Figure 6A-6B). Moreover, the apoptosis rate induced by circZSWIM4 deficiency could be abolished by co-transfection of miR-370-3p mimics or miR-873-5p mimics (Figure 6C-6D). Additionally, the inhibition of cell migration mediated by circZSWIM4 deletion was rescued after co-transfection with miR-370-3p mimics or miR-873-5p mimics (Figure 6E-6F). Totally, circZSWIM4 affected LUAD cells proliferation, apoptosis and migration via targeting miR-370-3p and miR-873-5p.

CircZSWIM4 up-regulates FOXM1 expression to activate the Wnt/β-catenin signaling pathway and facilitates the progression of LUAD

To further determine whether circZSWIM4 targeted FOXM1 to affect this pathway and the progression in LUAD, rescue experiments were performed. First, through co-IP assay, we found that FOXM1 could combine with β-catenin, and silenced circZSWIM4 or overexpressed circZSWIM4 could decrease or increase the combination between FOXM1 and β-catenin (Figure 7A). Then we found that the luciferase intensity of TOP/FOP flash was inhibited by circZSWIM4 silencing and co-transfection with FOXM1 could partly rescue this effect, while co-transfection with FOXM1 and CTNNB1 could completely enhance this effect (Figure 7B). Similarly, circZSWIM4 silencing declined the cytoplasm level of β-catenin and co-transfection with FOXM1 translocated the expression of β-catenin from the cytoplasmic part to the nuclear part, while co-transfection with FOXM1 and CTNNB1 promoted the nucleus level of β-catenin (Figure 7C). Besides, we found that the cell proliferation impaired by circZSWIM4 depletion could be partially countervailed by FOXM1co-transfection, and was fully reversed by co-transfection with FOXM1 and CTNNB1 (Figure 7D-7E). Moreover, circZSWIM4 knockdown induced the apoptosis rate in LUAD cells could be partly lessened by co-transfection with FOXM1, while was totally abolished by overexpression with FOXM1 and CTNNB1 (Figure 7F-7G). In addition, the reduced migratory ability caused by

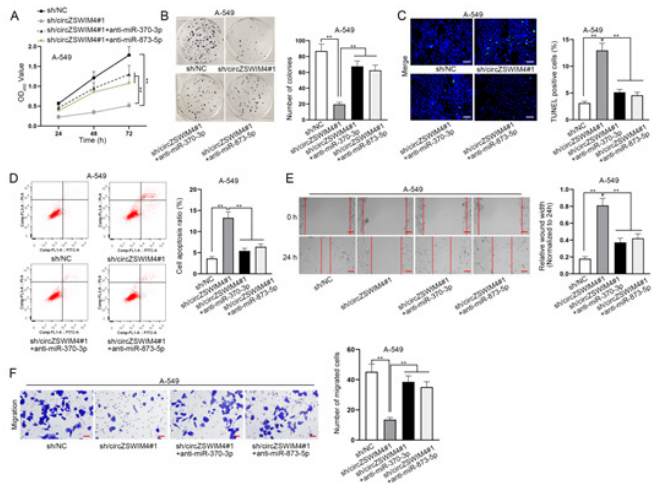

Figure 6. CircZSWIM4 promotes the progression of LUAD by targeting miR-370-3p and miR-873-5p. Rescue experiments were conducted in A-549 cells subsequent to transfection with sh/NC or sh/circZSWIM4#1 or sh/circZSWIM4#1+anti-miR-370-3p or sh/circZSWIM4#1+anti-miR-873-5p. A. CCK-8 experiment was implemented to assess cell proliferation. B. Colony formation experiment further test cell proliferation. C. TUNEL (bar value = 100 μm) assay was conducted to measure the influence on cell apoptosis. D. Flow cytometry analysis further tested cell apoptosis in different groups. E. Wound healing (bar value = 80 μm) assay was utilized to assess cell migration. F. Transwell (bar value = 40 μm) assay was utilized to estimate cell migration. $^{**}P < 0.01$.

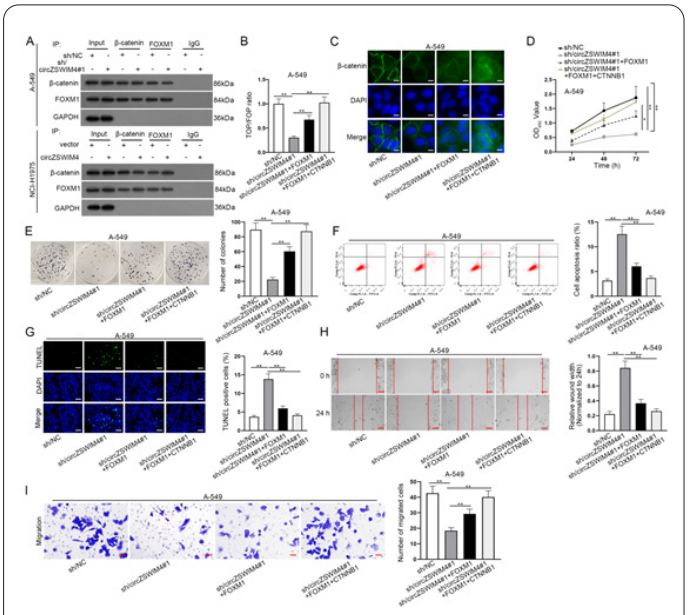

Figure 7. CircZSWIM4 up-regulates FOXM1 expression to irritate the Wnt/β-catenin pathway and promotes LUAD progression. A. Co-IP assay examined the combination between FOXM1 and β-catenin after the knockdown of circZSWIM4 or overexpressed circZSWIM4. B-I. Rescue assays were performed in A-549 cells subsequent to transfection with sh/NC, sh/circZSWIM4#1, sh/circZSWIM4#1+FOXM1 and sh/circZSWIM4#1+FOXM1+CTNNB1. B. TOP/FOP flash experiment was conducted in A-549 cells of different groups for detecting the influence on the intensity of the pathway. C. FISH (bar value = 15 μm) assay was utilized to detect the location of β-catenin in different groups. D. CCK-8 assay evaluated cell proliferation in various groups. E. Colony formation assay measured cell proliferation in different groups. F. Flow cytometry analysis detected cell apoptosis in multiple groups. G. TUNEL (bar value = 100 μm) assay was carried out for verifying cell apoptosis in a variety of groups. H. Wound healing (bar value = 80 μm) assay detected cell migration capability. I. Transwell (bar value = 40 μm) assay further uncloses cell migration ability. $^{*}P<0.05$, $^{**}P<0.01$.

circZSWIM4 knockdown could be partly overturned by co-transfection with FOXM1, while was thoroughly elevated by co-transfection with FOXM1 and CTNNB1 (Figure 7H-7I). As shown in the graphical abstract, all these data suggested that circZSWIM4 up-regulated FOXM1 expression for pathway activation and promoted LUAD progression.

Discussion

Although the rapid advances in the therapeutic strategies of LUAD, it is still the major cause of death-related cancer worldwide (23). In recent years, emerging evidences have suggested that the aberrant circRNA expression is a crucial regulator in LUAD progression. Hsa_circ_0025036 is markedly overexpressed in LUAD tissues, and hsa_circ_0025036 silencing impedes cell proliferation and increases cell apoptosis in LUAD (24). CircRNA-ENO1 is identified to be significantly expressed in LUAD cells, and repression of circRNA-ENO1 suppresses tumor progression in LUAD (25). Similarly, our study unclosed that circZSWIM4 expression was obviously elevated in LUAD cells. Through loss-of and gain-of-function assays, we found that knockdown or overexpression of circZSWIM4 evidently inhibited or promoted LUAD cell proliferation and migration. As a result, circZSWIM4 facilitated LUAD development, which might be a promising target for LUAD treatment.

Mechanistically, circRNAs have been registered to influence LUAD development via Wnt/β-catenin pathway modulation (26). Circ_0006427 impedes LUAD processes through miR-6783-3p to modulate DKK1 and inactivate this pathway (27). Unlike with this reference, our study discovered that circZSWIM4 could enhance this pathway in LUAD cells, which was in line with the study provided by Yao et al. (28). They propose that circ_0001946 positively affects the cell growth in LUAD via the regulation of miR-135a-5p and SIRT1, and Wnt/β-catenin pathway activation, which further supports the promoting role of circZSWIM4 in the progression of LUAD. In addition, c-MYC and Cyclin D1 belong to the downstream genes of the Wnt pathway which have been reported that can induce the EMT process of cancers (29). Consistently, we found that circZSWIM4 promoted EMT by the activation of the pathway in LUAD cells.

Moreover, our present research discovered that circZSWIM4 combined with miR-1286 and miR-370-3p and miR-873-5p. It has been reported that circAGFG1 affects cervical cancer processes by regulating miR-370-3p and RAF1 (30). Besides, circRNA_NEK6 targets miR-370-3p to influence the Wnt pathway, affecting the proliferation and invasion of the thyroid (31). These studies further verified the ceRNA hypothesis. Notably, our study first pointed to that circZSWIM4 as a ceRNA could target three miRNAs in LUAD cells. As we know, miRNAs are small RNAs that consists 18–25 nucleotides and without protein-coding capacity and exert function by targeting their mRNAs to regulate mRNAs expression in the biological process (32,33). Here, our study found the common target mRNA of these three miRNAs, FOXM1. It has been reported that Merlin hinders pancreatic cancer pathogenesis by hampering the FOXM1-mediated Wnt/β-Catenin signaling (34). Moreover, in cervical cancer, Pin1 regulates cisplatin resistance by elevating FOXM1 and this pathway (35). In our research, we found that circZSWIM4 could target miR-370-3p and miR-873-5p to regulate FOXM1 expression, and thereupon induce the pathway activation in LUAD cells. Simultaneously, circZSWIM4 could also target miR-370-3p to affect CTNNB1 expression to affect this pathway. Additionally, we confirmed that circZSWIM4 increased LUAD cell processes by targeting miR-370-3p and miR-873-5p.

Finally, our study performed a battery of rescue experiments to confirm that FOXM1 could combine with β-catenin, and pathway activation could completely restore the inhibition of the progression mediated by circZSWIM4, which is in accordance with the study discovered by Gao N et al (36). They present that circ-SOX4 influences LUAD via the miR-1270/PLAGL2 axis to elevate this pathway. Together, all these findings contributed to the tentative target of circZSWIM4 in LUAD treatment (37,38).

In conclusion, our study suggested that circZSWIM4 facilitated tumor development in LUAD through miR-370-3p and miR-873-5p to regulate the pathway, which may contribute to identifying a new biomarker for LUAD.

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Conflict of interest

None

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