Original Article

Has_circ_0002360 promotes the progression of lung adenocarcinoma by activating miR-762 and regulating PODXL expression

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Background: Circular RNAs (circRNAs) have been found to be linked to cancer progression and metastasis, but there is not much known about their connection to lung adenocarcinoma (LAC). In the previous study reported by our group, has_circ_0002360 was highly expressed in LAC tissues. The goal of this study was to investigate the potential impact of has_circ_0002360 in LAC.

Methods: Bioinformatics software, TargetScan, and miRanda were used to study the interactions of RNAs. Luciferase reporter assays further confirmed their relationship. The relative expression of has_circ_0002360 in 122 patients and four cell lines of the lung were obtained using real-time qualitative polymerase chain reaction (qRT-PCR). The target gene podocalyxin-like (PODXL) expression was confirmed by immunohistochemistry (IHC) in ten pairs of clinical samples. Then, cell counting kit-8 (CCK8), wound healing, and transwell experiments were applied to examine cell growth, migration, and infection-induced cell invasion. LAC cell lines were infected, and the process was monitored by examination of the related epithelial-mesenchymal transition (EMT) proteins.

Results: The resulting data indicated that has_circ_0002360 and PODXL were overexpressed in LAC tissues, whereas miR-762 expression was repressed. The reduction of has_circ_0002360 or upregulation of miR-762 mitigated the proliferation, migration, invasion of LAC cells. Mechanistically, has_circ_0002360 upregulated PODXL expressions by targeting miR-762 to promote LAC progression.

Conclusions: In general, the has_circ_0002360/miR-762/PODXL axis affected the progress of LAC. The results of our study identified has_circ_0002360 as a novel oncogenic RNA in LAC.

Keywords: Lung adenocarcinoma (LAC); has_circ_0002360; miR-762; podocalyxin-like (PODXL); epithelial-mesenchymal transition (EMT)

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Introduction

Circular RNAs (circRNAs) are endogenous non-coding RNAs, which may regulate gene expression. Compared to linear RNA, circRNAs have shown good expression, conservation, and cytoplasm stability (1). Their expression in mammalian cells is tissue- and developmental stage-specific (2). With the development of bioinformatics, more and more RNAs have been discovered by researchers, and their functions have been explored. Meanwhile, various biological functions of circRNAs have been found, i.e., their action as microRNA (miRNA) sponge to prevent messenger RNA (mRNA) translation, binding to RNA related proteins, and affecting gene expression (3-5). Mao *et al.* discovered that the silencing of hsa_circ_0068871 mitigated bladder cancer progression that might occur along the signal transducer and activator of transcription 3 (STAT3) signaling pathway (6). Chen *et al.* showed that circPVT1 was a proliferative factor and also an important gene for gastric cancer by targeting miR-125 (7). Using a dynamic Boolean network, Shantanu Gupta *et al.* discovered new positive circuits that contribute to treatment resistance in non-small cell lung cancer (NSCLC). In these circuits, MiR-145 appears as a key regulator, highlighting its critical function in NSCLC treatment resistance (8). In addition, hsa_circ_0012673 had miR-22 sponge-like properties and enhanced the development of lung adenocarcinoma (LAC) (9). In other words, circRNAs affect the progress of different tumor cells in various ways.

Lung cancer is one of the primary causes of death, ranking among the top in morbidity and mortality (10,11), which emphasizes the relevance of current research with a focus on the mechanisms of lung cancer progression and metastasis. LAC is the most common subtype of NSCLC, with increasing numbers reported over the last years, especially among women, non-smokers, and young people (12,13). Most patients are diagnosed at a late stage with severe local infiltration or distant metastasis. This group of patients have lost the opportunities to choose surgical eradication, and the majority of them extend the survival period through drug chemotherapy. However, there is a big problem of drug resistance in chemotherapy. It is crucial to discover effective diagnostic markers and therapeutic targets for early-stage LAC to ensure timely treatment, improved diagnosis, and survival rate.

We had screened has_circ_0002360 for differences in LAC and adjacent tissues by whole genome sequencing in our previous studies. We identified differences between LAC and adjacent tissues in 46 pairs of LAC tissues (14). The bioinformatics analysis results suggested that target binding exists between has_circ_0002360, miR-762, and podocalyxin-like (PODXL). Thus, we hypothesized that the development of LAC might be driven by the has_circ_0002360/miR-762/PODXL axis.

In this study, we investigated the connection between the level of has_circ_0002360 and the pathological characteristics in tumor patients with a special focus on the mechanisms that influence tumor development. We present this article in accordance with the MDAR reporting checklist (available at https://tcr.amegroups.com/article/view/10.21037/tcr-24-279/rc).

Highlight box

Key findings

- According to this study, lung adenocarcinoma (LAC) tissue has a higher quantity of the gene Has_circ_0002360 than does normal lung tissue. In addition to improving lung cancer cells' capacity for invasion and migration, the gene Has_circ_0002360 is linked to lymph node metastasis and cell differentiation in LAC patients. Additionally, it was discovered that this gene might use the miR762-podocalyxin-like (PODXL) axis to accomplish the biological function indicated before.

What is known and what is new?

- It has been discovered by researchers that the PODXL gene influences the biological activity of tumor cells.
- This work explores the upstream genes in the gene pathway of the lung cancer gene PODXL in addition to clarifying the significance of this gene in the biological activity of lung cancer cells.

What is the implication, and what should change now?

- This study offers a theoretical foundation for the creation of targeted medications for the treatment of lung cancer and biomarkers for the early detection of LAC. Tumor modeling is necessary for *in vivo* experimental research and eventually even clinical medication trials, although this work is currently in the *in vitro* experimental stage.

Methods

Clinical samples

One hundred and twenty-two pairs of samples from LAC tissue and adjacent non-tumor tissue were used in this study. The samples were taken from patients who had surgery at the Affiliated People's Hospital of Jiangsu University between January 2018 and June 2019. These patients had

Table 1 The primer sequences used

Gene	Primer sequences
has_circ_0002360	CTCAGAGTCAGATGCAGGG
	TGATGGCTCTGTGGTAGG
miR-762	GGGGCTGGGGCCGGGG
	AGTGCAGGGTCCGAGGTATT
PODXL	AGATACAGCCCAGCAGAGCA
	GCCACGGTAGTGTTGACTGG
GAPDH	CCCACTTCTCTCTAAGGAGAAT
	CACGAAAGCAATGCTATCAC

not been given chemotherapy or radiation before surgery but had been pathologically diagnosed with LAC. After surgery, tissue samples were kept at –80 ℃ for future utilization and testing. All participants and/or their legal guardian(s) gave their informed consent in writing, and this study was approved by the Medical Ethics Committee of the University of Hong Kong-Shenzhen Hospital (No. [2024]001). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Specificity verification for has_circ_0002360

The polymerase chain reaction (PCR) products were amplified by primers. Then they were separated with 1% agarose gel to confirm the specificity of the has_circ_0002360. The product of PCR was determined to be specific, with the result that one single band presented in the gel. Sanger sequencing was used as supporting information to validate the sequence of has_circ_0002360.

CeRNA network construction

The miRanda database was consulted for predicting an existing relationship between circRNA and miRNA. The MicroRNA Target Prediction Database (MiRDB) was used for the prediction of the miRNA and mRNA binding relationship. R language was used to assess the matching relationship. The competing endogenous RNA (ceRNA) network was built using Cytoscape (version 3.7.1).

RNA extraction and reverse transcription

Total RNA was extracted from LAC tissues and paired non-tumor tissues with the TRIzol™ Reagent (Life Technologies, Scotland, UK). The RNA integrity test was carried out using denatured agarose gel electrophoresis. RNA quantification and quality were confirmed spectrophotometrically using the absorbance at 260 and 280 nm.

Real-time qualitative polymerase chain reaction (qRT-PCR)

The PCR was completed on a CFX-96 system (Bio-Rad, USA), equipped with an SYBR Green PCR Kit (cat. #RR042A; Takara, Japan) according to the manufacturer's guidelines. Using random primers and the Prime Script RT Master Mix, 500 ng of total RNA was reverse transcribed into a total volume of 20 µL cDNA with random primers, using standard conditions and the Prime Script RT Master Mix (cat. #RR037A; Takara). The levels of has_circ_0002360, miR-762, and PODXL expressions were normalized to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), beta actin, and U6 snRNA, with each PCR analyzed in triplicate. Relative gene expression is reported as the fold change ($2^{-\Delta\Delta CT}$). *Table 1* shows the primer sequences used.

Cell culture and transfection

The bronchial epithelium cell line (BEAS-2B), as well as three LAC cell lines (A549, H1975, H1299), were obtained from Cell center, Chinese Academy of Sciences (Shanghai, China). Each cell line was cultured in RPMI-1640 medium (HyClone, USA) after adding 10% fetal bovine serum (FBS) (Gibco, California, USA). The cell lines were stored in a humidified incubator containing 5% CO_2 at 37 ℃. Short interfering RNA against circ_0002360 (Si-circ_0002360), the control oligos (Si-NC), the human miR-762 inhibitor, and the miR-762-mimics were transfected into LAC cells with the Lipofectamine 2000 Reagent (Life Technologies, New York, USA).

Cell proliferation and colony formation assays

H1975 and A549 cells were cultured on 96-well plates, at a concentration of approximately 1×10^4 cells per well, to determine the proliferation rate. Various cell treatment conditions were employed during cell culture, which was followed by incubation of the cells, and cell counting with the cell counting kit-8 (CCK8) solution. Counts were determined spectrophotometrically by measuring the

absorbance at 450 nm. Five hundred transfected cells were inoculated into a 6-well plate for colony formation test. After 14 days of culture, colonies were observed in the well. A crystal violet solution was used to sustain the colonies. The number of colonies was obtained by counting from the photos taken.

Transwell assay

Cell migration was examined in a filtered Transwell chamber with 8 µm pore polycarbonate membrane (Corning, MA, USA). A 10% FBS medium was put to the bottom chamber as a chemo-attractant. Transfected cells (5×10^4 cells/well) suspended in the serum-free RPMI-1640 medium were seeded into the upper chamber and then incubated in a humidified incubator containing 5% CO_2 at 37 ℃. After 24 hours of culture, the cells on the membrane were gently wiped with cotton swabs, and the other side of the filter was fixed with 4% paraformaldehyde for 30 min. Next, they were stained for 10 min with 0.1% crystal violet. Microscope images were taken for counting the migrated cells.

Wound-healing assay

The cells grew to 90% confluence overnight with the original density of 5×10^5 cells/well on a 6-well plate. A linear scratch was drawn into the single-cell layer utilizing a 10 µL pipette tip. Then the cells were rinsed using phosphate-buffered saline (PBS) solution three times. After a 48 h serum-free RPMI-1640 incubation, microscope images of the migrating cells were taken.

Western blotting analysis

Total proteins of the LAC cell lines were extracted by centrifugation in a lysis buffer. The concentration was obtained using a Bio-Rad Protein Assay Kit (Bio-Rad, CA, USA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins of different molecular sizes. The isolated proteins were imprinted on PVDF membrane and incubated with primary antibody overnight at 4 ℃ (Boster, Wuhan, China), including anti-PODXL, anti-E-cadherin, anti-N-cadherin, anti-Vimentin antibody and GAPDH. Subsequently, the membrane was incubated with the second antibody (Boster, Wuhan, China) at room temperature for 2 h. After incubation, the polyvinylidene fluoride (PVDF) membrane is completely soaked in an appropriate amount of Tris-buffered saline with Tween-20 (TBST), the rocaker slowly shake and wash (avoid friction influence protein), about 10 min after a new TBST wash, wash 3 times. The proteins were analyzed by using the chemiluminescence detection method (ECL Plus Substrate, Thermo Fisher Scientific, Massachusetts, USA).

Dual-luciferase reporter assays

The LAC cells were co-transfected with wild-type or mutated has_circ_0002360 reporter plasmids, and with miR-762 mimics, or mimics-negative controls (mimics-NC), utilizing Lipofectamine 2000 (Life Technologies, New York, USA). The miRNA mimics were acquired from Sangon Biotech. Firefly and Renilla luciferase activities were examined 24 h after the transfection with a Dual-Luciferase Reporter System (Promega, Madison, USA). The effect of miRNA on the luciferase assay with has_circ_0002360 or PODXL was normalized to that of the luciferase reporter with the mutated. The fold change between each miRNA was calculated compared to the negative control (NC).

Immunohistochemistry (IHC)

LAC tissues from different clinical stages were collected and fixed in 4% paraformaldehyde for 6 h, rinsed with ethanol solution, and the rinsed tissues were dehydrated and transparent with high concentrations of alcohol and xylene, and embedded in paraffin. Paraffin-embedded tissue was divided into 4 m sections, placed in a baking dish, and immersed in xylene three times for 5 min, rehydrated, repaired by microwave, and added with TBST containing 5% goat serum to seal for 1 hour at room temperature. Sections were incubated with a 1:400 ratio-diluted PODXL antibody at 4 ℃ overnight, then sections were washed with PBS (three times for 5 min each), incubated with HRP Polymer (enzyme-conjugated secondary antibody) for 30 min at room temperature, and washed with PBS (three times for 5 min each). After coloring, restaining, and transparent sealing sheet, PODXL expression was determined. Microscopic images at the appropriate magnification were observed and preserved.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism 5 and SPSS 22.0. The Student's *t*-test was used to compare data from two groups only. One-way analysis of variance

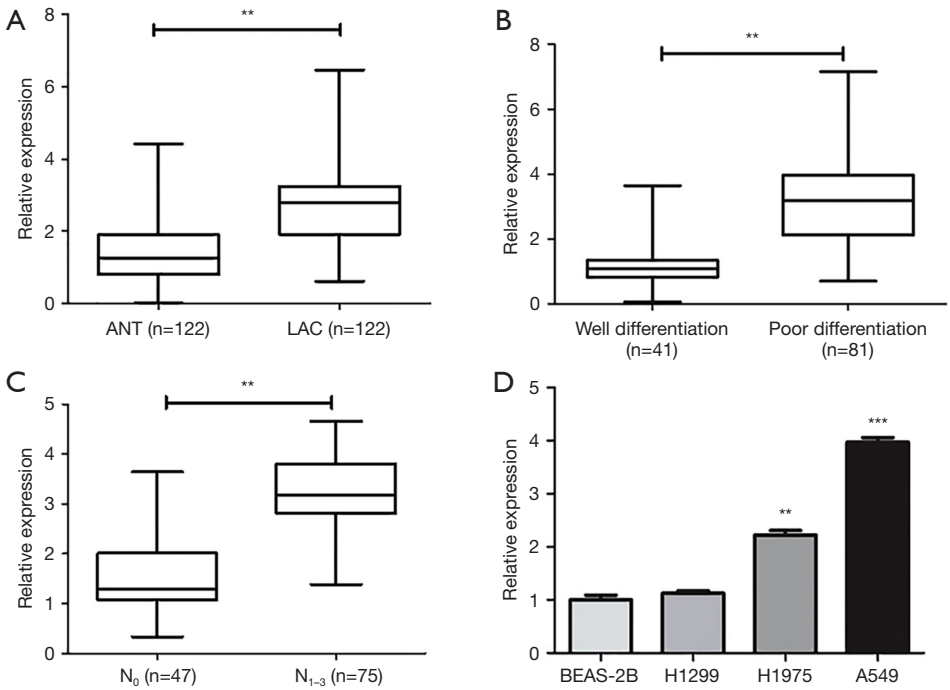

Figure 1 Detection of has_circ_0002360 in tissues and cell lines by qRT-PCR. (A-C) Relative expression of has_circ_0002360 in LAC tissues and their paired noncancerous tissues. (A) Relative expression of has_circ_0002360 in ANT lung tissues and LAC tissues (P=0.003). (B) Relative expression of has_circ_0002360 in well-differentiated LAC and poorly differentiated LAC (P=0.003). (C) Relative expression of has_circ_0002360 in patients with lymph nodes at N_0 and N_{1-3} stages (P=0.002). (D) Relative expression of has_circ_0002360 in LAC cell lines (H1299, H1975, and A549) and bronchial epithelial cell line (BEAS-2B) (P=0.01, P<0.001). **, P<0.01; ***, P<0.001. qRT-PCR, real-time qualitative polymerase chain reaction; LAC, lung adenocarcinoma; ANT, adjacent non-tumor.

(one-way ANOVA) was used for multiple comparisons, where a P value of P<0.05 indicated a statistically significant difference. The experiment was repeated more than 3 times.

Results

Has_circ_0002360 expression in LAC

We tested the expression of has_circ_0002360 in LAC and corresponding para-cancer tissues of 122 patients with LAC, to determine whether has_circ_0002360 was expressed in LAC tissue. The qRT-PCR results revealed a significantly higher expression of has_circ_0002360 in LAC tissues as compared to para-carcinoma non-tumor tissues (*Figure 1A-1C*). We tried to examine whether a high level of has_circ_0002360 in patients was related to clinicopathological parameters. *Table 2* shows that the has_circ_0002360 expression was not significantly different between groups of different sexes (P=0.49), ages (P=0.51), smoking/non-smoking (P=0.47), or tumor sizes (P=0.55). Tumor differentiation stages were associated with the has_circ_0002360 expression (P=0.02). Besides, the has_circ_0002360 level was correlated with lymphatic metastasis (P=0.004). Subsequently, the expression level of has_circ_0002360 in LAC cell lines and bronchial epithelial cell lines was assessed by qRT-PCR. The has_circ_0002360 expression in LAC cell lines (A549 and H1975) increased relative to that of BEAS-2B (*Figure 1D*).

Identification of has_circ_0002360

We designed convergent and divergent primers, specifically amplifying the normal or reverse splicing primers, to verify the ring structure of has_circ_0002360 (*Figure 2A*). Next, Sanger sequencing was applied to confirm the has_circ_0002360 splice junction (*Figure 2B*). Products containing the divergent primers were resistant to digestion by RNase-R after reverse transcription. In contrast, the convergent primers initially enhanced the linear RNA, and disappeared after RNase-R digestion (*Figure 2C,2D*). None

Table 2 Correlation between has_circ_0002360 expression and clinical characteristics

Characteristics	Total patients	Mean ± SD	P value
Age (years)			0.51
≤60	55	2.76±1.03	
>60	67	2.94±1.10	
Gender			0.49
Male	52	2.77±0.86	
Female	70	2.95±1.21	
Smoking			0.47
Yes	43	2.58±0.85	
No	79	2.42±0.81	
Lymph node metastasis			0.004
N_0	47	2.28±0.80	
N_{1-3}	75	2.91±0.74	
Tumor size (cm)			0.55
<1	86	2.49±0.83	
≥1	36	2.41±0.74	
Tumor differentiation			0.02
Low	81	2.62±0.88	
High	41	2.16±0.64	

SD, standard deviation.

of these different primers were able to amplify any of the genomic DNA products (gDNA).

Silencing of has_circ_0002360 and inhibition of LAC cell progression

We constructed small interfering RNA (Si-RNA) to silence circRNAs and then used PCR to test for transfection efficiency and study the action mechanisms of has_circ_0002360 in LAC. A549 and H1975 cells were treated with Si-circ_0002360 (siRNA1, siRNA2, and siRNA3) for 24 h and detected transfection by qRT-PCR. The results indicated that siRNA oligos were able to eliminate the expression of circ_0002360 (*Figure 3A*,*3B*). siRNA1 appeared to be the most effective siRNA for subsequent experiments (*Figure 3A*,*3B*). A CCK8 assay tested whether has_circ_0002360 was connected to the proliferation of LAC cells (*Figure 3C*,*3D*). The test results revealed that siRNA1 had a significant inhibitory effect on LAC cells, while siRNA in the control group did not show any binding activity of has_circ_0002360. It did not affect cell proliferation and the knockdown of has_circ_0002360 blocked the proliferation of LAC cells. Considering that the expression of has_circ_0002360 in LAC tissue was associated with lymph node metastasis, we performed transwell and wound-healing assays on LAC cells transfected with Si-circ_0002360 and Si-NC. Cell invasion and migration were inhibited after silencing has_circ_0002360 in A549 and H1975 cells, as shown in *Figure 3E-3J*. In general, these results demonstrated that the silencing of has_circ_0002360 inhibited LAC cell proliferation, migration, and invasion.

Has_circ_0002360 sponge for miR-762

The network related to has_circ_0002360 was extracted from all ceRNA analyses, and nine miRNAs with the potential to bind to has_circ_0002360 were recorded (*Figure 4A*). The MiRDB database was further used to predict microRNA target genes, suggesting that nine microRNA matched mRNAs. Functional analysis of mRNAs gave a P value of less than 0.05, signifying apparent differences. Finally, according to the expression trends and function of has_circ_0002360, the related microRNA and mRNA, has_circ_0002360, miR-762, and PODXL were selected as follow-up research targets. We also tested whether has_circ_0002360 functioned as an oncogene by acting as a sponge of miR-762. First, the luciferase reporter assay confirmed that has_circ_0002360 interacted with miR-762 (*Figure 4B*). PCR further showed that the expression of miR-762 was down-regulated in LAC tissues, and was negatively correlated with the expression of has_circ_0002360 (R^2=0.3212, P<0.05; *Figure 4C*). At the same time, the miR-762 expression increased after has_circ_0002360 was silenced in LAC cell lines (*Figure 4D*,*4E*).

Dysregulation of miR-762 on the function of has_circ_0002360

Experiments were conducted to confirm whether the biological function of has_circ_0002360 depended on the regulation of miR-762. LAC cells were transfected separately with Si-circ_0002360 + miR-NC, Si-circ_0002360 + miR-762 inhibitor, and Si-NC + miR-NC. Transfection activity was detected by qRT-PCR. The results from CCK-8, transwell, and wound-healing assays indicated

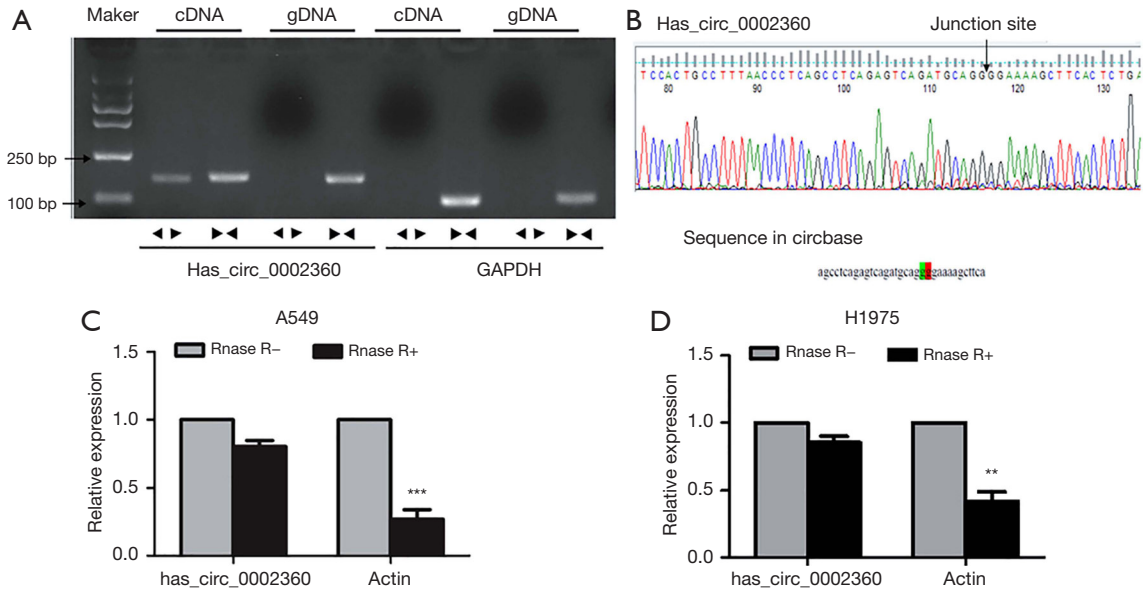

Figure 2 Identification of has_circ_0002360. (A) qRT-PCR assay with divergent or convergent primers revealing a ring structure of has_circ_0002360. (B) Sanger sequencing of has_circ_0002360 showing the splice junction. (C,D) Relative expression of has_circ_0002360 in (C) A549 and (D) H1975 showed that has_circ_0002360 was resistant to digestion by RNase-R (C: P<0.001; D: P=0.004). **, P<0.01; ***, P<0.001. qRT-PCR, real-time qualitative polymerase chain reaction.

an inverse correlation between the miR-762 expression and has_circ_0002360 in Si-circ_0002360 + miR-NC groups. In fact, miR-762 reversed the cell proliferation, invasion, and migration mechanisms, triggered by changes of has_circ_0002360 in LAC (*Figure 4B*, *Figure 5A-5H*).

Oncogene PODXL as the target gene of miR-762

Meng *et al.* demonstrated that PODXL has important pathogenic functions, and its dysregulation promotes the development of the LAC cell lines (15). Our bioinformatics studies showed that PODXL was the target gene of miR-762 in LAC cell lines (*Figure 4A*). We also found that PODXL was upregulated in LAC tissues, as shown in *Figure 6A,6B*, and the level of PDDXL was correlated with the has_circ_0002360 expression (R^2=0.4487, P<0.01; *Figure 6C*). The level of miR-762 was negatively correlated with the PODXL expression (R^2=0.4306, P<0.01; *Figure 6D*). Transfection of miR-762 mimics inhibited PODXL expression in LAC cell lines (A549, H1975) and transfection of the miR-762 inhibitor promoted PODXL expression in two cell lines (*Figure 6E*). The Luciferase reporter assays' results confirmed that the miR-762 mimics substantially reduced luciferase activity of the wild-type reporter, while no significant reduction of luciferase activity of a mutant reporter was observed. The results also revealed that PODXL was the target gene of type miR-762 in LAC cells (*Figure 6F*).

Has_circ_0002360 as the facilitator of cell EMT through miR-762/PODXL axis

The regulatory action of has_circ_0002360 on cell-related proteins was examined using Western blotting. Western blot analysis demonstrated that ectopic miR-762 changed the level of PODXL caused by silencing only has_circ_0002360 in LAC cells (*Figure 7A-7E*). These results confirmed that has_circ_0002360 worked as a sponge for miR-762 and upregulated the PODXL protein. Expression levels of PODXL, Vimentin, Snaill, and N-cadherin were clearly down-regulated while E-cadherin was significantly upregulated in Si-circ_0002360 groups. Further investigations revealed that PODXL had a close relationship with cell EMT (*Figure 7F-7H*), and that has_circ_0002360 was the main factor in the EMT of LAC cells via the miR-762/PODXL axis.

Discussion

CircRNAs belong to non-coding RNAs, which are widely

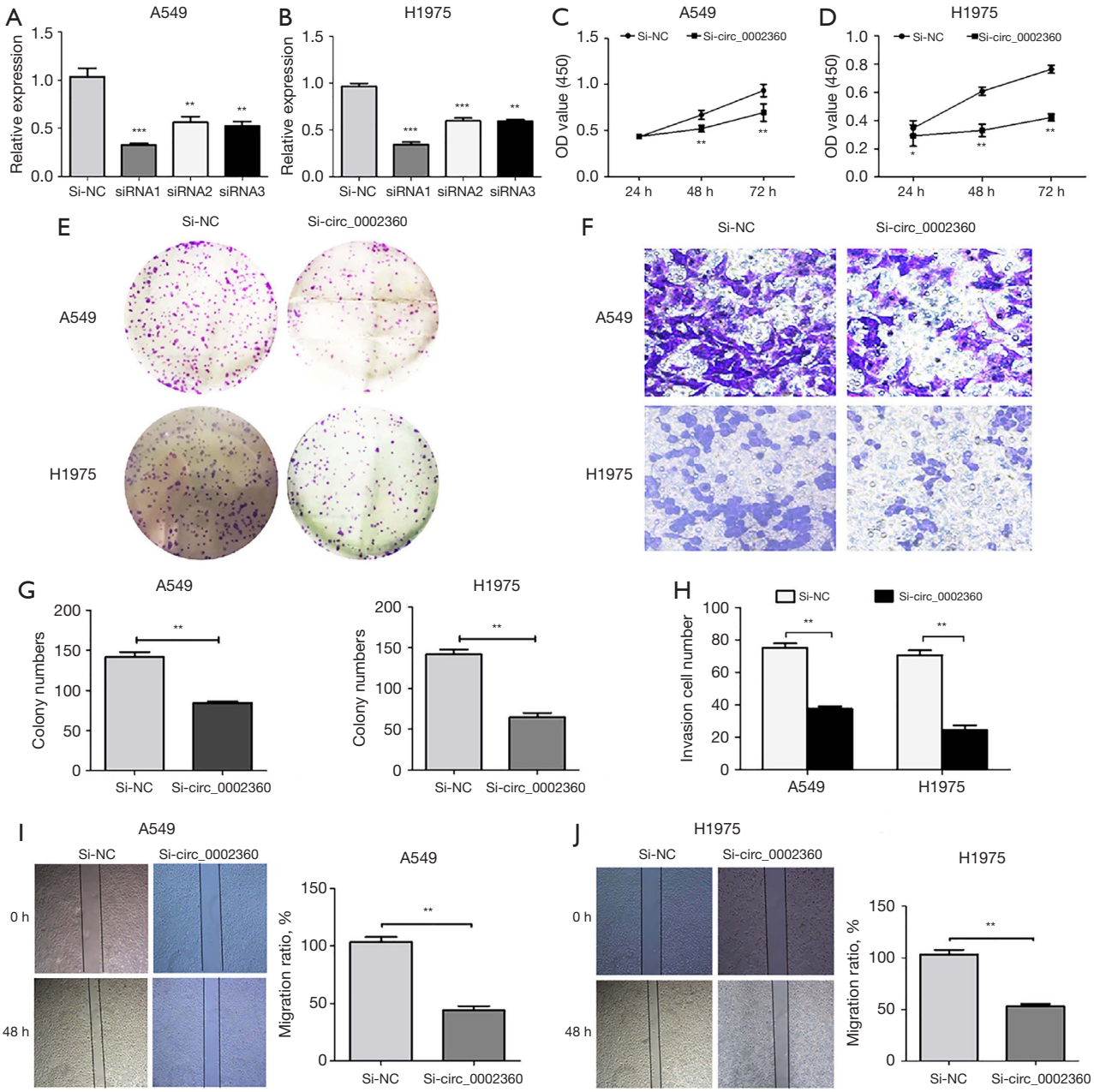

Figure 3 Effects of has_circ_0002360 on proliferation, migration, and invasion of LAC cells. (A,B) Relative expression results in small interfering RNA, Si-circ_0002360 (siRNA1, siRNA2, siRNA3). Treated (A) A549 and (B) H1975 cells show that has_circ_0002360 was inhibited by three types of siRNAs, siRNA1, siRNA2, and siRNA3, in LAC cells (A: P<0.001, P=0.004, P=0.005; B: P<0.001, P<0.001, P=0.009). (C,D) LAC cell viability detected by CCK-8 assays. The figures show the absorbance (OD, measured at 450 nm) of the solutions of (C) A549 and (D) H1975 cells treated with Si-NC and Si-circ_0002360 with different treating time, 24, 48, and 72 h (C: P=0.008, P=0.008; D: P=0.04, P=0.005, P=0.005). (E,G) Effects of has_circ_0002360 on colony formation of LAC cells (0.1% crystal violet) (P=0.003, P=0.002). (F,H) Effects of has_circ_0002360 on cell invasion capacities detected using Transwell assays (0.1% crystal violet, 400× microscope images) (P=0.005, P=0.003). (I,J) Effect of has_circ_0002360 on cell migration capacities detected using wound-healing assays (100× microscope images) (P=0.002, P=0.002). *, P<0.05; **, P<0.01; ***, P<0.001 (Si-NC *vs.* Si-circ_0002360). NC, negative control; OD, optical density; LAC, lung adenocarcinoma; CCK-8, cell counting kit-8.

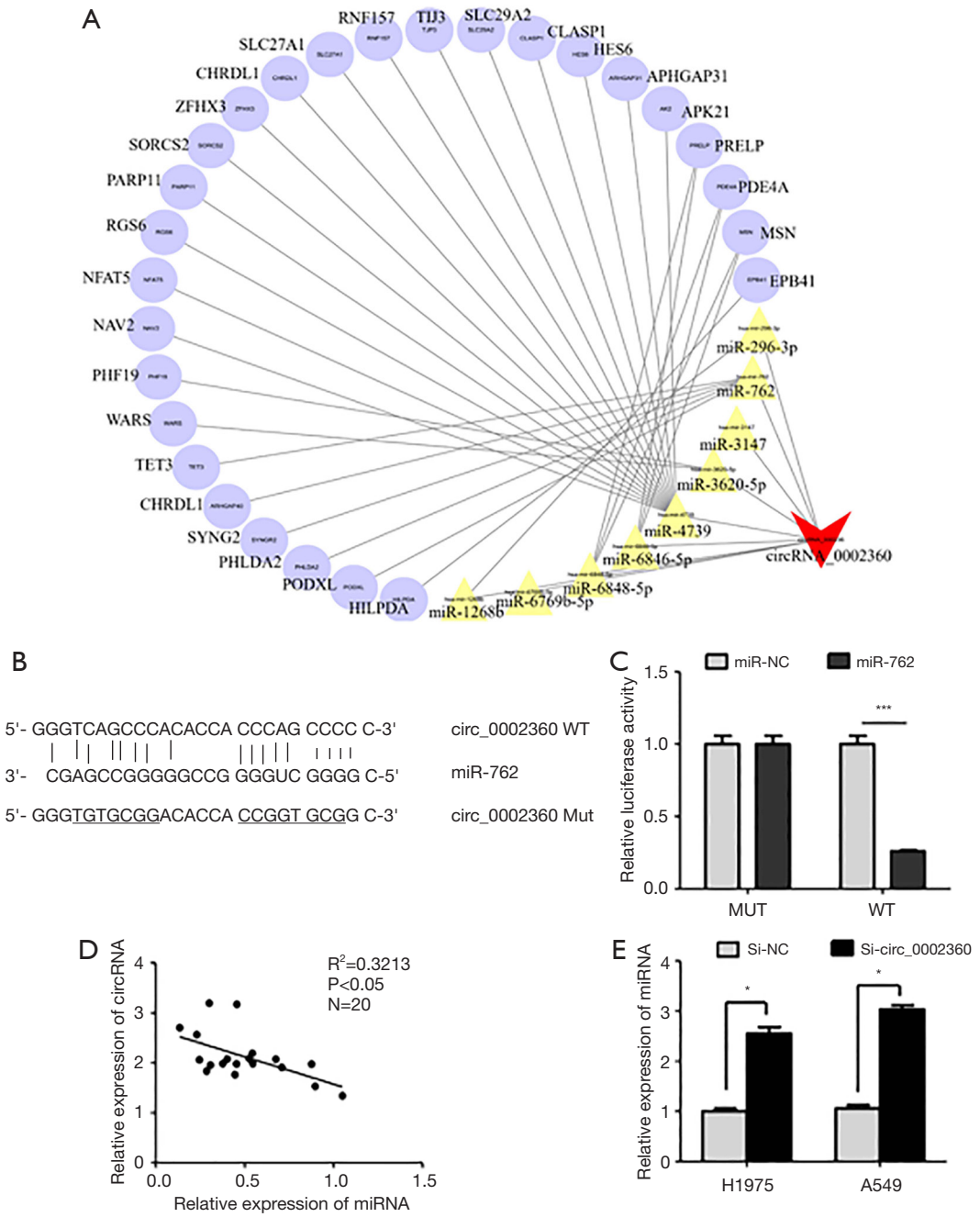

Figure 4 The targeted correlation between miR-762 and has_circ_0002360. (A) Has_circ_0002360 network (Red represents circRNA; yellow represents miRNA; purple represents mRNA). (B) The seed sequences of circ_0002360, miR-762, and circ_0002360 mutant. (C) Results of dual-luciferase reporter assays conducted to examine the binding ability between miR-762 and has_circ_0002360 in 293T cells. (D) Results of correlation analysis of miR-762 and has_circ_0002360 obtained in LAC tissues. (E) Effect of has_circ_0002360 on miR-762 expression (P=0.001, P=0.001). *, P<0.05; ***, P<0.001. MUT, mutation; WT, wild-type; NC, negative control; LAC, lung adenocarcinoma.

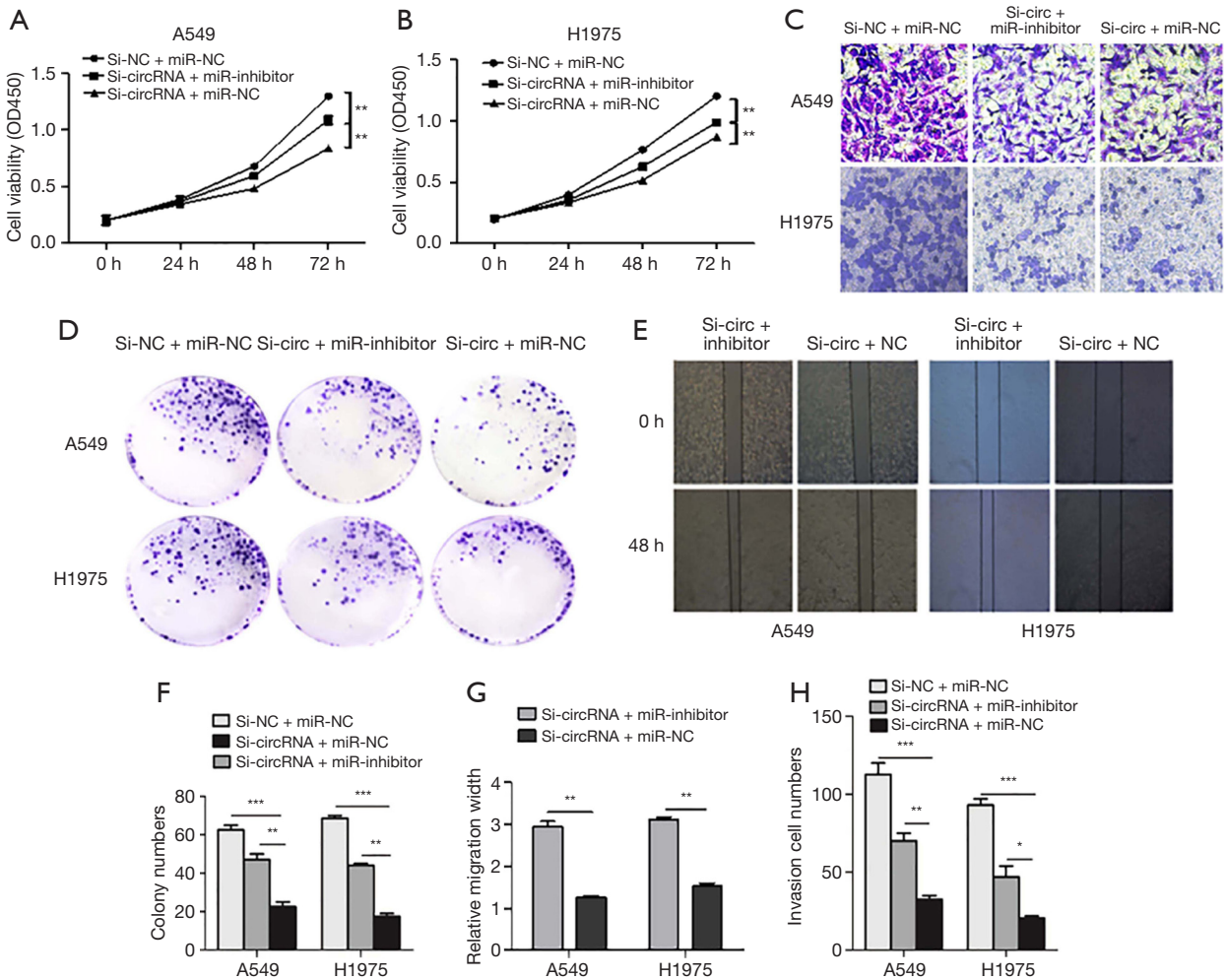

Figure 5 The dysregulation of miR-762 affected the biological function of has_circ_0002360. (A,B) Cell proliferation was determined in LAC cell lines, (A) A549 and (B) H1975, after transfection (A: P<0.001, P<0.001; B: P<0.001, P<0.001). (D,F) Effects of has_circ_0002360 on colony formation of LAC cells (A549 and H1975) (0.1% crystal violet) (P<0.001, P=0.007, P<0.001, P=0.008). (E,G) Migration of LAC cells assessed by wound-healing assays by wound-healing assays (100× microscope images) (P=0.005, P=0.006). (C,H) Invasion of LAC cells assessed by Transwell assays (0.1% crystal violet, 400× microscope images) (P<0.001, P=0.003, P<0.001, P=0.03). *, P<0.05; **, P<0.01; ***, P<0.001 (Si-circRNA + miR-NC *vs.* Si-circRNA + miR-inhibitor, Si-NC + miR-NC *vs.* Si-circRNA + miR-NC). NC, negative control; OD, optical density; LAC, lung adenocarcinoma.

expressed in mammals, and are important contributors to cell growth, differentiation, and tumorigenesis (16,17). Currently, the most frequently reported functional mode of circRNAs is the formation of the miRNA-mRNA axis and the sponge of miRNA (18,19). For example, Yang *et al.* revealed that circ-ITCH inhibited bladder cancer progression through the sponge effect of miR-17/miR-224 and the regulation of p21 or PTEN expression (20). Through targeting the miR-558/heparin enzyme axis, Li group observed that overexpression of circHIPK3 reduced the potential invasion of bladder cancer cells and inhibited metastasis (21). The increasing incidence rate and mortality rate of lung cancer, especially LAC, have attracted much attention in the field of respiratory diseases. Investigating circRNA has become a prospective research direction due to its unique function in the field of respiratory diseases. Several circRNAs are known to be related to lung cancer progression (22,23) although the functions of most

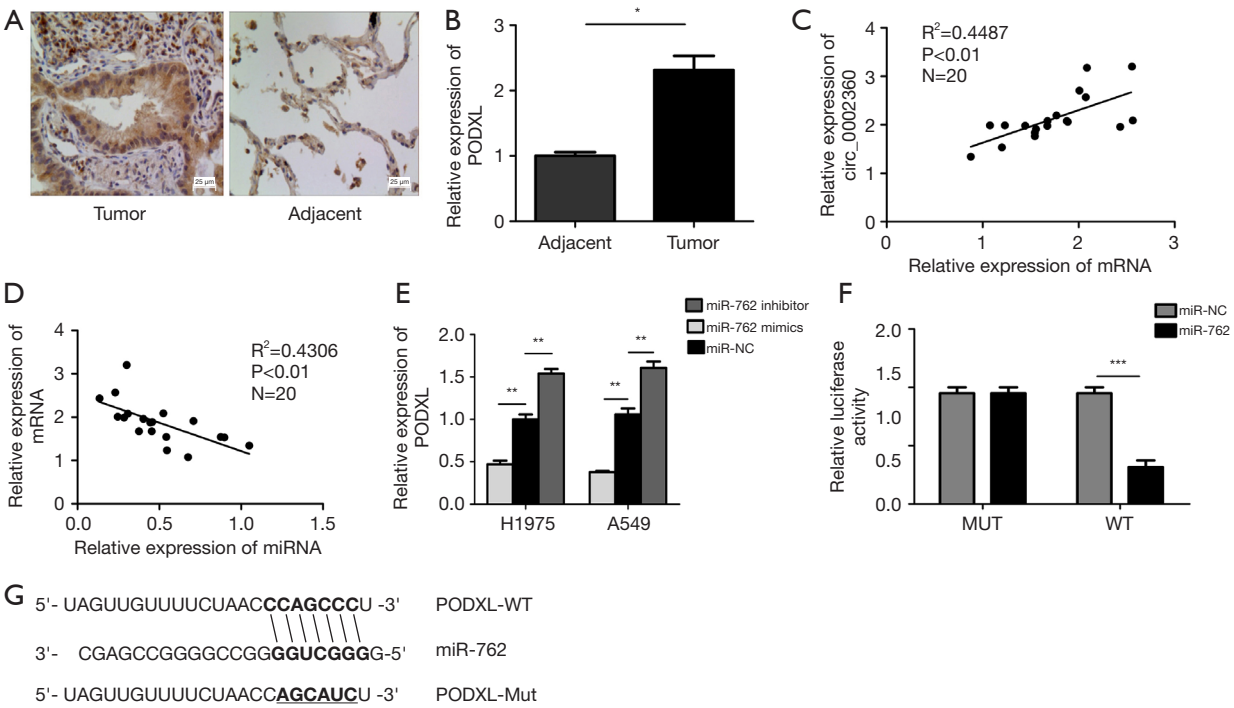

Figure 6 Oncogene PODXL as a target gene of miR-762. (A,B) IHC analyses (hematoxylin staining, 400×) of the tumor and adjacent non-tumor tissues show the PODXL levels in the 10 LAC tissues and the control group (P=0.001). (C,D) Correlation analysis of has_circ_0002360, PODXL, and miR-762 expressions in 20 LAC tissues. (E) Relative mRNA expression obtained by qRT-PCR after transfecting (miR-762 inhibitor *vs.* miR-NC, and miR-762 mimics *vs.* miR-NC) (P=0.002, P=0.002, P=0.001, P=0.003). (F) Dual-luciferase reporter assays for obtaining the correlation between miR-762 and PODXL 3'-UTR in 293T cell. (G) The seed sequences of PODXL 3'-UTR, miR-762, and PODXL 3'-UTR mutant (P<0.001). *, P<0.05; **, P<0.01; ***, P<0.001. PODXL, podocalyxin-like; NC, negative control; MUT, mutation; WT, wild-type; IHC, immunohistochemistry; LAC, lung adenocarcinoma; qRT-PCR, real-time qualitative polymerase chain reaction.

circRNAs require further investigation. In addition, more information is needed on the molecular mechanisms of circRNA in LAC.

In the early stage, our group screened out has_circ_0002360 as the research subject using the next generation sequencing (NGS) (14). In this study, we proposed that has_circ_0002360 might function as a sponge of miR-762, identified PODXL as a potential target of miR-762, and constructed the corresponding network. At the same time, the dual-luciferase reporter assays indicated that miR-762 targeted both has_circ_0002360 and PODXL, providing strong evidence that has_circ_0002360 acts as a sponge of miR-762 to modulate PODXL expression. PCR assays with divergent or convergent primers and Sanger sequencing data supported the existence of has_circ_0002360 in the LAC cell lines.

Our results revealed that the expression levels of has_circ_0002360 and PODXL in LAC tissues were substantially greater than that in normal tissues. We also observed that their levels were correlated with the differentiation degree of tumor cells and lymph node metastasis. The expression of miR-762 was relatively low in LAC tissues. Our results showed that the expression trend of circRNA was the same as that of target gene PODXL, but it was not directly proportional to the expression of miR-762. Therefore, we speculated whether there is a connection between the three genes. In view of the spongy mechanism of circRNA, we inferred that the has_circ_0002360/miR-762/PODXL pathway might play a critical role in LAC progression. We designed experiments to test our hypothesis and found that the Si-circ_0002360 + miR-762 and the miR-762 inhibitor groups led to an increase in PODXL expression. Both promoted cell invasion, migration, and growth as compared to the Si-NC + miR-NC groups. In the miR-762 mimic group, PODXL expression decreased, and the biological functions of the cells were inhibited. In other

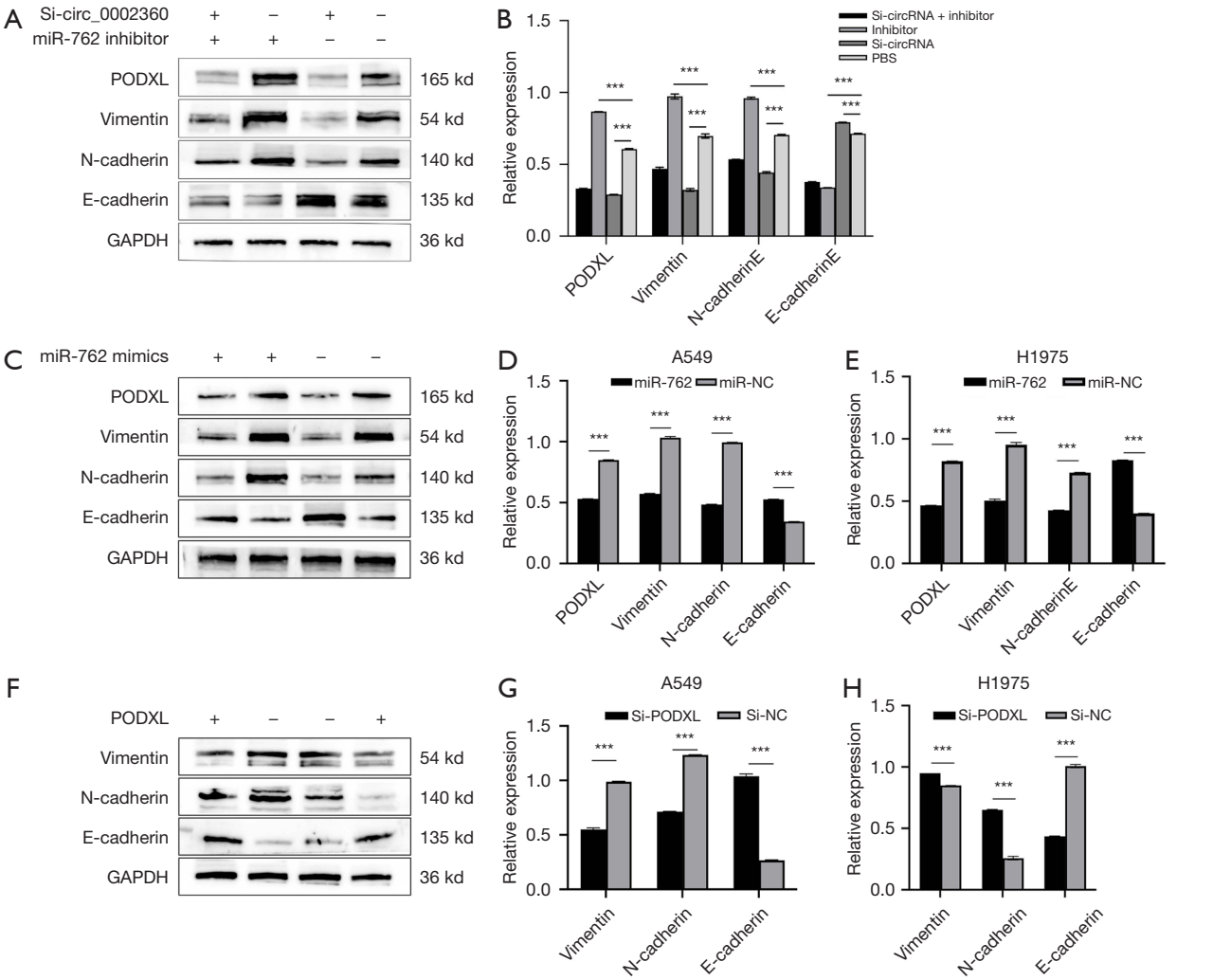

Figure 7 Has_circ_0002360 regulates cell EMT through the miR-762/PODXL axis. (A,B) The levels of PODXL and EMT-related proteins (Vimentin, E-cadherin, N-cadherin) obtained by Western blot after transfection (P<0.001, P<0.001, P<0.001, P<0.001, P<0.001, P<0.001, P<0.001, P<0.001) (Si-circ_0002360 *vs.* PBS, miR-762 inhibitor *vs.* PBS, and Si-circ_0002360 + miR-762 inhibitor *vs.* Si-circ_0002360). (C-E) The levels of PODXL and EMT-related proteins (Vimentin, N-cadherin, and E-cadherin) measured after transfection with miR-762 mimics or the control, miR-NC, in A549 and H1975 cell lines (D: P<0.001, P<0.001, P<0.001, P<0.001; E: P<0.001, P<0.001, P<0.001, P<0.001). (F-H) The levels of EMT-related proteins (Vimentin, N-cadherin, and E-cadherin) measured in A549 and H1975 cell lines with Si-PODXL or the control, Si-NC (G: P<0.001, P<0.001, P<0.001; H: P<0.001, P<0.001, P<0.001). ***, P<0.001. PODXL, podocalyxin-like; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PBS, phosphate-buffered saline; NC, negative control; EMT, epithelial-mesenchymal transition.

words, the cytological function experiments suggested that has_circ_0002360 and miR-762 had reverse effects on cell proliferation, invasion, and migration in LAC, while both has_circ_0002360 and PODXL promoted the progression of LAC. In addition, the results of the dual-luciferase experiment revealed that there were binding targets among the three genes. Therefore, we confirmed that has_circ_0002360 promotes the progression of LAC via competing mechanisms with endogenous RNAs.

PODXL is classified as a type I transmembrane glycoprotein and belongs to the CD34 family. It operates as a pro-adhesive molecule in lymphocytes, enhancing

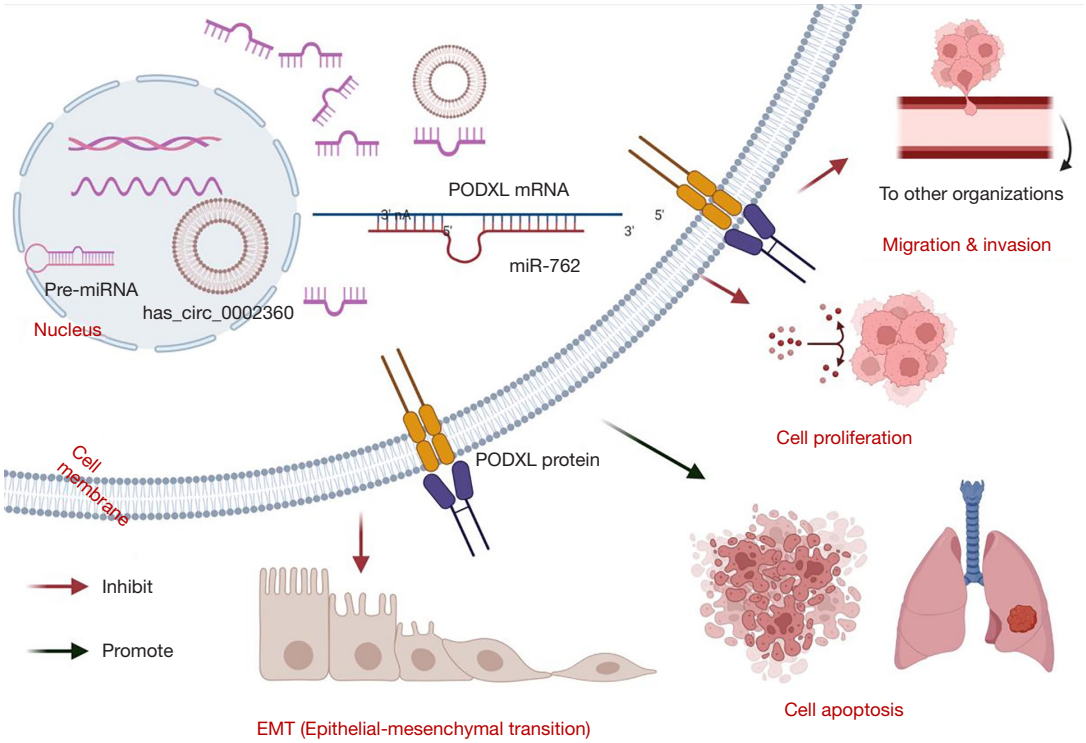

Figure 8 Schematic diagram of the role of has_circ_0002360 in LAC. PODXL, podocalyxin-like; LAC, lung adenocarcinoma.

their adhesion to immobilized L-selectin (24-28). Reports indicate that the expression of PODXL is related to tumor aggression and adverse prognosis in several cancers (29-33). Meng *et al.* found that PODXL expression leads to molecular transformations (e.g., Vimentin, E-cadherin) related to TGF-β induced EMT of human LAC (15). They demonstrated the crucial role of PODXL in the EMT and metastasis of cancers. EMT has been reported to have a significant influence on cancer progression (34), manifested by upregulation of mesenchymal markers such as vimentin and downregulation of epithelial related genes such as E-cadherin (35). After EMT, tumor cells acquire a higher potential for metastasis and invasion, analogous to embryonic mesenchymal cells, with increasing ability to invade adjacent stroma and form new tumor lesions (36,37). Our study revealed that the expression of EMT-related proteins also changed in the Si-circ_0002360 group, miR-762 inhibitor group, miR-762 mimics group, and Si-PODXL. These findings support the assumption that the silencing of has_circ_0002360 and PODXL inhibits EMT in LAC cells. We also found that has_circ_0002360 and PODXL had consistent effects on cell phenotypes.

Conclusions

In summary, our results suggest that the expression of has_circ_0002360 in LAC is correlated with the differentiation degree of tumor cells and lymphatic metastasis, has_circ_0002360 upregulated PODXL expressions by targeting miR-762 to promote LAC progression (*Figure 8*). CircRNA is closely related to the progress of lung cancer and plays a key regulatory role in the occurrence and development of lung cancer and other respiratory diseases by interacting with relevant miRNAs. Our study reveals that has_circ_0002360 is promising to be a prognostic tool and therapeutic target for LAC.

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Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tcr.amegroups.com/article/view/10.21037/tcr-24-279/coif). 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. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). All experimental protocols were approved by the University of Hong Kong-Shenzhen Hospital (No. [2024]001). Informed consent was obtained from all subjects and/or their legal guardian(s).

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