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CircTADA2A Up-regulates MAPK8 by targeting MiR-214-3p and recruiting EIF4A3 to promote the invasion and migration of non-small cell lung cancer cells

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Summary. Background. Non-small cell lung cancer (NSCLC) occupies 87% of all lung cancer cases. Due to delayed diagnosis, the prognosis of NSCLC is unfavorable. To improve the survival of patients with NSCLC, more effective therapeutic targets urgently need to be identified. Recently, circular RNAs (circRNAs) have been revealed to play a crucial role in NSCLC progression.

Purpose. This research focused on the influence of circTADA2A on the malignant phenotype of NSCLC cells and its in-depth regulatory mechanisms.

Methods. RT-qPCR and western blot assays were done to examine the level of gene/protein of interest. Wound healing and transwell assays were conducted to monitor the migration and invasion of NSCLC cells. Bioinformatics tools and mechanistic assays were utilized to delve into the underlying mechanism of circTADA2A in NSCLC cells.

Results. The results demonstrated that circTADA2A presented a high expression in NSCLC. CircTADA2A knockdown was revealed to hamper migration and invasion of NSCLC cells. Mechanistically, circTADA2A elevated MAPK8 expression through sequestering miR-214-3p and recruiting EIF4A3.

Conclusion. CircTADA2A enhances MAPK8 expression by serving as a miR-214-3p sponge and EIF4A3 decoy, consequently promoting invasion and migration of NSCLC cells.

Key words: Non-small cell lung cancer, circTADA2A, miR-214-3p, EIF4A3, MAPK8

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Introduction

Lung cancer represents a predominant cause of cancer-associated death (Nagano et al., 2019). Non-small cell lung cancer (NSCLC) takes up 87% of lung cancer cases with 16% of patients diagnosed in stage I.

Abbreviations. NSCLC, Non-small cell lung cancer; circRNAs, Circular RNAs; HMGB3, High mobility group box 3; ceRNA, Competing endogenous RNA; mRNAs, Messenger RNAs; miRNAs, MicroRNAs; MAPK1, Mitogen-activated protein kinase 1; RBP, RNA binding protein; FXR1, Fragile X-Related 1; PRKCI, Protein kinase C, iota; ETFA, Electron transfer flavoprotein alpha subunit; VEGFA, Vascular endothelial growth factor A; ATCC, American Type Culture Collection; RPMI , Roswell Park Memorial Institute 1640; FBS, Fetal bovine serum; si-RNAs, Small interference RNAs; TADA2A, Transcriptional adaptor 2A; EIF4A3, Eukaryotic translation initiation factor 4A3; NCs, Negative controls; MAPK8, Mitogen-activated protein kinase 8; RT-qPCR, Quantitative reverse transcription polymerase chain reaction; cDNA, Complementary DNA; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; FISH, Fluorescence in situ hybridization; PFA, Paraformaldehyde; DAPI, 4',6-diamidino-2-phenylindole; 3'UTR, 3'untranslated region; Wt, Wild-type; Mut, Mutant; Bio, Biotinylated; ECL, Enhanced chemiluminescence; RIP, RNA binding protein immunoprecipitation; AGO2, Argonaute 2; DGCR8, DiGeorge syndrome critical region gene 8; U2AF65, U2 small nuclear ribonucleoprotein auxiliary factor 65; IgG, Immunoglobulin G; SD, Standard deviation; ANOVA, Analysis of variance; GEO, GENE EXPRESSION OMNIBUS; ZFR, Zinc finger RNA-binding protein; NOL10, Nucleolar protein 10; gDNA, Genomic DNA; MMP2 , Matrix metallopeptidase 2; MMP9, Matrix metallopeptidase 9; LUSC, Lung squamous cell carcinoma; TMEM248, Transmembrane protein 248; CTNNB1, Catenin beta1; FGFR1, Fibroblast growth factor receptor 1; ALPK2, Alpha protein kinase 2; CNIH1, Cornichon Family AMPA Receptor Auxiliary Protein 1; NUFIP2, Nuclear fragile X mental retardation-interacting protein 2; GNAL, G protein subunit alpha L; RUBCN, Rubicon autophagy regulator; BCL2L11, BCL-2-like protein 11; Bax, BCL-2 associated X; TWF1, Twinfilin actin binding protein 1; CMPK1, Cytidine/uridine monophosphate kinase 1; DCUN1D4, Defective in cullin neddylation 1 domain containing 4; HuR, Human antigen R; TXNIP, Thioredoxin-interacting protein; SEMA5A, Semaphorin 5A.

Currently, surgery is a suitable choice for treating patients with the operable stage I NSCLC. Nevertheless, around 25% of NSCLC patients at early stage are not suitable for lobectomy due to severe medical comorbidities (Herbst et al., 2018; Tandberg et al., 2018; He et al., 2020). As for the treatment of stage III NSCLC, the combination of chemotherapy, radiation and surgical resection is traditionally involved. However, only a fraction of those patients can have complete resection and the long-term prognosis remains poor (Myall and Das, 2020).

In recent years, molecular targeted therapy has greatly developed within the field of NSCLC treatment and several targetable genetic alterations have been identified in NSCLC (Nagano et al., 2019; Sankar et al., 2020). Circular RNAs (circRNAs), identified as a type of RNAs with loop structure, are attracting increasing attention, and mounting evidence has supported that circRNAs highly engage in the development of different cancers, including NSCLC (Chen et al., 2019a; Sun et al., 2020b; Li et al., 2021). For instance, circRNA_010763 has been revealed to accelerate the growth and invasive process of NSCLC cells (Zhang et al., 2020b). CircRNA_102179 has been reported to promote NSCLC cell proliferative, migratory and invasive processes by modulating the miR-330-5p/HMGB3 axis (Zhou et al., 2020). CircTADA2A has been validated to facilitate osteosarcoma progression and metastasis (Wu et al., 2019) as well as promoting NSCLC cell proliferative and migratory processes (Zhang et al., 2021). Considering the vital roles of circRNAs in NSCLC, we decided to research the relationship of circRNA functions and NSCLC cell malignant behaviors.

Existing research work points out that the competing endogenous RNA (ceRNA) model involves the function of messenger RNAs (mRNAs) and non-coding RNAs, including microRNAs (miRNAs) and circRNAs (Qi et al., 2015). The regulation model of ceRNA manifests that circRNA serves as the sponge of miRNA to indirectly modulate downstream target genes (Han et al., 2020). For example, circRNA_101237 has been found to up-regulate MAPK1 through sponging miR-490-3p, which contributes to NSCLC cell growth (Zhang et al., 2020c). Recent studies have demonstrated that MAPK8 exerts influence on the progression of several cancers, including glioblastoma and NSCLC (Xu et al., 2018; Zhang et al., 2016). Moreover, MAPK8 has been confirmed to be involved in the circMED13L_012-mediated ceRNA model and play a vital role in NSCLC pathogenesis (Chen et al., 2021). Apart from that, circRNA has also been identified to act as RNA binding protein (RBP) decoys to participate in human diseases (Wang et al., 2019). As Chen et al. suggest, circ_0000079 decoys FXR1 to interfere in the formation of the FXR1/PRCKI complex, which consequently suppresses NSCLC cell invasion and drug resistance (Chen et al., 2020). Reportedly, EIF4A3 has been identified to play an essential part in the onset and development of several cancers (Zhu et al., 2021). Furthermore, its role as an RBP has also been studied in cancers. For example, EIF4A3 recruited by circETFA stabilizes CCL5 to facilitate cell proliferation and migration in hepatocellular carcinoma (Lu et al., 2021). Hence, the ceRNA and RBP mechanisms attracted our attention.

This study attempted to deeply investigate the role of circTADA2A in affecting the migration and invasion of NSCLC cells, and tried to discuss the circTADA2A-mediated ceRNA and RBP mechanisms in NSCLC cells. Since circTADA2A has been validated to work as an oncogene in NSCLC (Jin et al., 2022), we hypothesized that circTADA2A might serve as a miRNA sponge and RBP decoy to exert its oncogenic role in NSCLC cells. We hope this study can reveal more potential mechanisms of circTADA2A in NSCLC cells and provide promising novel biomarkers for NSCLC diagnosis.

Materials and methods

Cell culture

Human lung epithelial cell line (BEAS-2B) and human NSCLC cell lines (A549, NCI-H1299 and PC9) were all bought from American Type Culture Collection (ATCC, Manassas, VA, USA). They were cultivated in 24-well plates with RPMI 1640 medium mixed with 20% fetal bovine serum (FBS) in a humid incubator which was placed in an environment with 5% CO_2 at 37°C. Upon reaching approximately 90% confluence, the cells were collected for subsequent assays.

Cell transfection

All small interference RNAs (si-RNAs) against circTADA2A and EIF4A3 as well as their negative controls (si-NCs) were prepared. The pcDNA3.1-MAPK8 was utilized to overexpress MAPK8. Empty pcDNA3.1 functioned as NC. MiR-214-3p mimics, mimics NC, miR-214-3p inhibitor, and inhibitor NC were also procured. The aforementioned plasmids were then transfected into NSCLC cells. After 48-hour incubation, successfully transfected cells were harvested and used in the subsequent assays.

Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

Total RNA from indicated cells was isolated with the use of Trizol and then reversely transcribed to complementary DNA (cDNA). After that, qPCR analysis was conducted, and the $2^{-\Delta\Delta Ct}$ method was employed to calculate the final consequences. GAPDH and U6 served as the internal controls. Three bio-repeats were run in three technical replicates.

Wound healing assay

Cells of indicated groups were seeded onto 6-well plates. When the cell confluence reached 80%, the

surface of every well was scratched vertically using a sterile pipette. After being scratched, wounded cells were washed away and the pictures of wound width were captured immediately with a microscope. After cells were incubated for another 24 hours with 5% CO_2 at 37°C, a microscope was used again to take pictures for analyzing. Three bio-repeats were run in three technical replicates.

Transwell assay

The transwell invasion assay was conducted using Matrigel-coated chambers. Cells were collected and put in the top compartment of 24-well plates containing non-serum medium. The lower compartment medium was mixed with 500 μL FBS. Twenty-four hours later, the cells successfully traversing to the lower compartment were fixed in methanol and dyed by crystal violet. The amount of invaded cells in five randomly chosen regions was counted. The procedures of migration assay were the same as the invasion assay except the top compartment was not covered with Matrigel. Three bio-repeats were run in three technical replicates.

Fluorescence in situ hybridization (FISH) assay

The FISH probe targeting circTADA2A was used in FISH assay. Cells were fixed with the help of 4% paraformaldehyde (PFA) at room temperature. Subsequently, 300 μL TritonX-100 was added in 24-well plates for permeabilizing the cells at 4°C. Afterwards, cells were incubated with circTADA2A FISH probe in the hybridization buffer overnight. Cell nuclei were stained with DAPI and the fluorescence of circTADA2A was visualized via a laser scanning confocal microscope. Three bio-repeats were run in three technical replicates.

Luciferase reporter assay

CircTADA2A/MAPK8 3'UTR sequences covering wild-type (Wt) or mutant (Mut) binding sites on miR-214-3p were sub-cloned into pmirGLO reporter vectors to construct pmirGLO-circTADA2A-Wt/Mut and pmirGLO-MAPK8 3'UTR-Wt/Mut. The aforementioned vectors along with the empty pmirGLO were then co-transfected with miR-214-3p mimics or mimics NC into NSCLC cells. Following 48-hour transfection, the luciferase activities were measured with the application of a dual luciferase reporter assay kit, and the activity was then normalized to renilla luciferase. Three bio-repeats were run in three technical replicates.

RNA pull-down assay

Biotinylated (Bio)-circTADA2A probes, Bio-miR-214-3p-Wt/Mut and Bio-NC probes as well as biotin-labeled MAPK8 3'UTR sense/Anti-sense were designed and produced in advance. Cells were lysed and then cultivated with probes and magnetic beads for a whole night. After that, the RNAs or proteins precipitating with biotin-labeled probes were purified and subjected to RT-qPCR or western blot detection. As for western blot assays, purified proteins were incubated with primary antibody (Anti-EIF4A3) at 4°C for a whole night and then secondary antibody was supplemented for incubation. Finally, the proteins were analyzed with an enhanced chemiluminescence (ECL) detection system. Three bio-repeats were run in three technical replicates.

RNA binding protein immunoprecipitation (RIP) assay

RIP assay was done to assess the binding relationship of RNAs and proteins. The cell lysates were cultured with human Anti-AGO2, Anti-DGCR8, Anti-EIF4A3, Anti-U2AF65 or NC Anti-IgG linked to magnetic beads. The immunoprecipitated RNAs were isolated and quantified by RT-qPCR to detect the enrichment of target genes. Three bio-repeats were run in three technical replicates.

Statistical analyses

Statistical data gathered from at least three independent experiments were processed utilizing Graphpad Prism 6 software, and displayed as mean ± standard deviation (SD). Data difference between two groups was analyzed by Student's t-test. ANOVA test model was constructed to compare data differences among multiple groups. When one or two variables were involved, one-way or two-way analysis of variance (ANOVA) was applied, respectively. Tukey and Dunnett tests were done as post hoc tests. P value less than 0.05 was regarded as statistically significant.

Results

In this study, we concentrated on the function and mechanism of circTADA2A in NSCLC cells. Firstly, we revealed the expression of circTADA2A in NSCLC and tested its circular structure. Next, functional assays were implemented to detect the impact of circTADA2A deficiency on NSCLC cell migration and invasion. Bioinformatics tools and mechanistic assays were utilized to determine the underlying molecular pathways (ceRNA and RBP mechanisms) regulated by circTADA2A in NSCLC cells. Finally, functional assays in a rescue manner were performed to evaluate the influence of circTADA2A-mediated pathways on NSCLC cell migration and invasion.

CircTADA2A displays the stable loop structure and high expression in NSCLC

At the very beginning, we searched GEO dataset (ID: GSE101586) to analyze the remarkably up-regulated circRNAs in NSCLC tissues, relative to the tumor-free lung tissues. As hsa_circRNA_102049, with the most differential expression, has been well-explored

in a previous study (Cui et al., 2019), we excluded it and took the remaining top three candidates (hsa_circRNA_102051, hsa_circRNA_103809 and hsa_circRNA_102619) into consideration. According to the host gene of these candidates, we named them as circTADA2A, circZFR and circNOL10 respectively (Fig. 1A). Later, the expression of three candidate circRNAs was examined in human bronchial epithelial cells (BEAS-2B) and human NSCLC cells (A549, NCI-H1299 and PC9), and the RT-qPCR outcome illustrated that\ circTADA2A was the most significantly expressed in NSCLC cells, especially in A549 and PC9 cells (Fig. 1B). Hence, circTADA2A was determined to be the research object of this study. To figure out the structure of circTADA2A, PCR-agarose gel electrophoresis (AGE) was conducted. We noticed that cDNA of circTADA2A was amplified by divergent and convergent primers, while the gDNA was only amplified by the convergent primer (Fig. 1C). Further, in RNase R-treated A549 and PC9 cells, the expression of TADA2A mRNA was greatly diminished, but that of circTADA2A was hardly affected (Fig. 1D). These findings indicated that circTADA2A had the stable circular structure. In summary, circTADA2A presents a high expression in NSCLC and has the closed loop structure.

CircTADA2A promotes migration and invasion of NSCLC cells

Prior to investigating the influence of circTADA2A on NSCLC cell phenotype, we examined the efficacy of circTADA2A knockdown through RT-qPCR, and si-circTADA2A-1/2 was involved in the subsequent experiments for the relatively higher efficiency (Fig. 2A). Moreover, we observed that migratory and invasive abilities of NSCLC cells were hampered on account of circTADA2A depletion (Fig. 2B,C). The mRNA levels of invasion-linked genes, including MMP2 and MMP9,

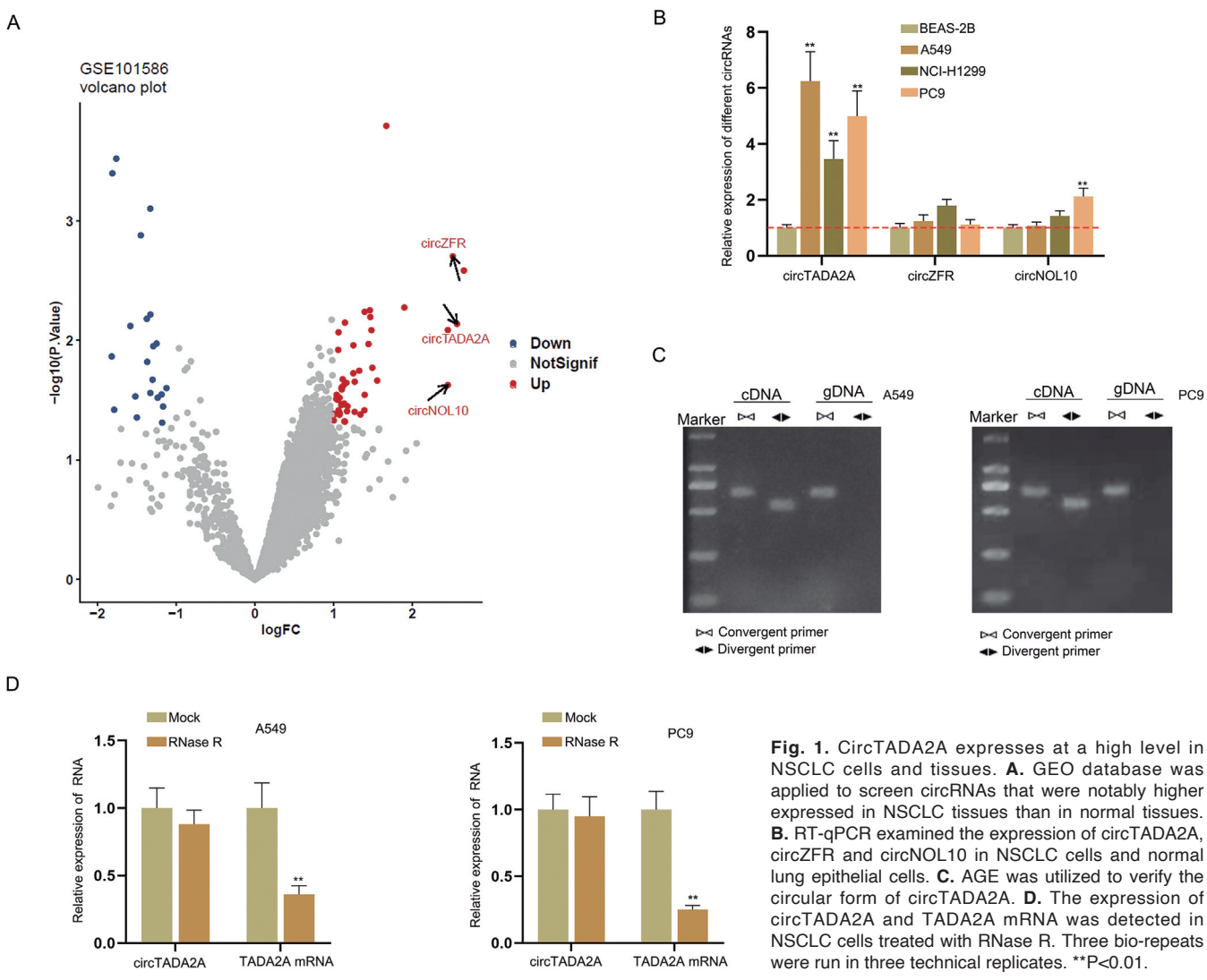

Fig. 1. CircTADA2A expresses at a high level in NSCLC cells and tissues. **A.** GEO database was applied to screen circRNAs that were notably higher expressed in NSCLC tissues than in normal tissues. **B.** RT-qPCR examined the expression of circTADA2A, circZFR and circNOL10 in NSCLC cells and normal lung epithelial cells. **C.** AGE was utilized to verify the circular form of circTADA2A. **D.** The expression of circTADA2A and TADA2A mRNA was detected in NSCLC cells treated with RNase R. Three bio-repeats were run in three technical replicates. **P<0.01.

was also decreased, attributing to circTADA2A down-regulation (Fig. 2D). To conclude, circTADA2A facilitates the migration and invasion of NSCLC cells.

CircTADA2A sponges miR-214-3p in NSCLC cells

In this part, we continued to delve into the underlying mechanisms of circTADA2A in NSCLC cells. Above all, the main distribution of circTADA2A in A549 and PC9 cells was ascertained. It turned out that circTADA2A primarily amassed in NSCLC cell cytoplasm (Fig. 3A). Next, circTADA2A was found to be overtly enriched in Anti-AGO2 (Fig. 3B). As reported, cytoplasmic circRNA able to bind with AGO2 can act as a ceRNA of miRNA (Shi et al., 2020), so we speculated circTADA2A might also sponge a certain miRNA in a ceRNA manner. To verify our speculation, we used circBank (http://www.circbank.cn/) to project

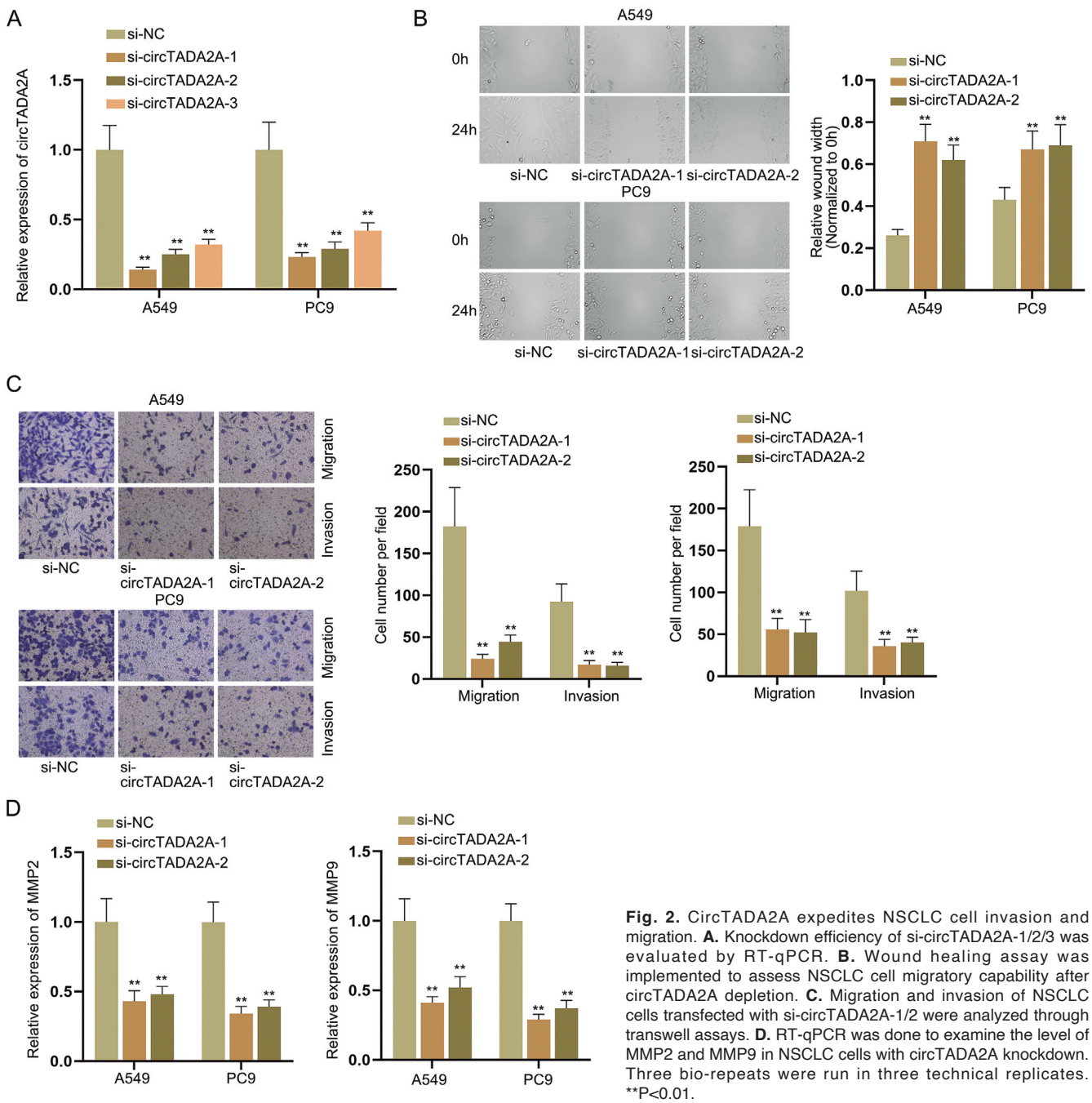

Fig. 2. CircTADA2A expedites NSCLC cell invasion and migration. **A.** Knockdown efficiency of si-circTADA2A-1/2/3 was evaluated by RT-qPCR. **B.** Wound healing assay was implemented to assess NSCLC cell migratory capability after circTADA2A depletion. **C.** Migration and invasion of NSCLC cells transfected with si-circTADA2A-1/2 were analyzed through transwell assays. **D.** RT-qPCR was done to examine the level of MMP2 and MMP9 in NSCLC cells with circTADA2A knockdown. Three bio-repeats were run in three technical replicates. **P<0.01.

miRNAs potential to bind with circTADA2A. The search results were listed in a descending order based on targetscan binding site (positions) (Fig. 3C). The correlation between circTADA2A and the top one miRNA (miR-203a-3p) has been studied in other research (Xu et al., 2019), so we excluded it. The remaining top 3 miRNAs (miR-214-3p, miR-761 and miR-129-5p) were chosen. Subsequently, RNA pull-down assay revealed that miR-214-3p was the most enriched in Bio-circTADA2A among the three candidate miRNAs (Fig. 3D). Hence, miR-214-3p was involved in the following assays. Noteworthily, circTADA2A could be highly enriched in Bio-miR-214-3p-Wt, which further confirmed the binding affinity of circTADA2A to miR-214-3p (Fig. 3E). Prior to luciferase reporter assays, the miR-214-3p mimics were proved to be efficiently up-regulate miR-214-3p (Fig. 3F). Then, the luciferase activity of pmirGLO-circTADA2A-Wt was observed to be significantly weakened in response to miR-214-3p up-regulation, verifying the strong binding of circTADA2A and miR-214-3p (Fig. 3G). Further, RT-qPCR analysis uncovered that miR-214-3p expression was hardly changed after circTADA2A reduction (Fig. 3H). To conclude, circTADA2A play a role of a ceRNA to sequester miR-214-3p in NSCLC cells.

CircTADA2A competitively binds with miR-214-3p to up-regulate MAPK8

CircRNAs have been proved to regulate mRNA expression through acting as a miRNA sponge (Yang et al., 2019). Therefore, we utilized bioinformatics tools to screen potential target mRNAs for miR-214-3p. Utilizing search results on miRDB (http://mirdb.org/)

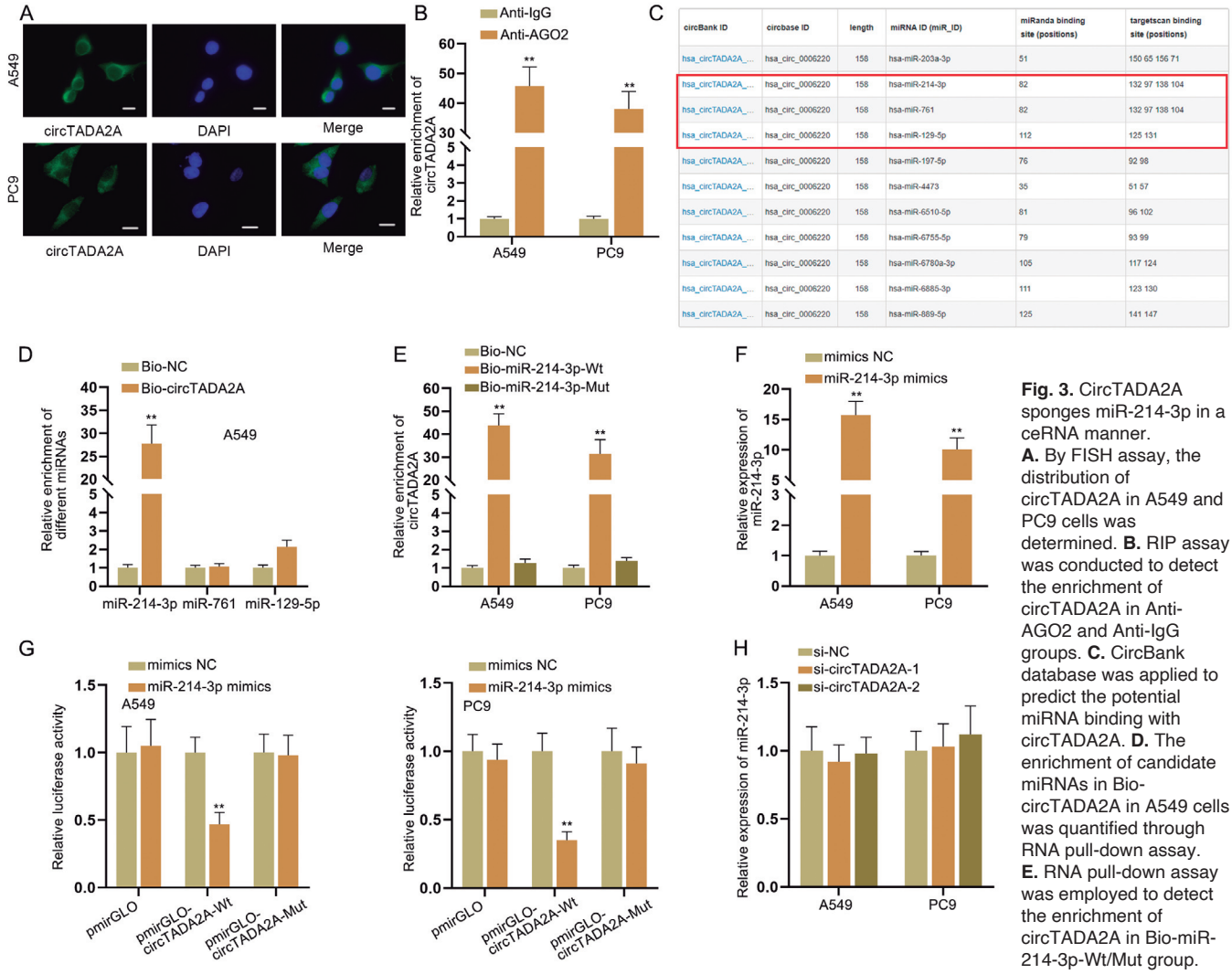

Fig. 3. CircTADA2A sponges miR-214-3p in a ceRNA manner. **A.** By FISH assay, the distribution of circTADA2A in A549 and PC9 cells was determined. **B.** RIP assay was conducted to detect the enrichment of circTADA2A in Anti-AGO2 and Anti-IgG groups. **C.** CircBank database was applied to predict the potential miRNA binding with circTADA2A. **D.** The enrichment of candidate miRNAs in Bio-circTADA2A in A549 cells was quantified through RNA pull-down assay. **E.** RNA pull-down assay was employed to detect the enrichment of circTADA2A in Bio-miR-214-3p-Wt/Mut group. **F.** The overexpression efficacy of miR-214-3p mimics was tested by RT-qPCR. **G.** The binding relationship between miR-214-3p and circTADA2A-Wt/Mut was assessed by luciferase reporter assay. **H.** The expression of miR-214-3p was detected through RT-qPCR in A549 and PC9 cells upon circTADA2A knockdown. Three bio-repeats were run in three technical replicates. **P<0.01.

and miRWalk (http://mirwalk.umm.uni-heidelberg.de/), a venn diagram was generated. As displayed in Fig. 4A, 12 mRNAs were predicted. UALCAN (http://ualcan.path.uab.edu/index.html) was then used to find online information about the expression of 12 mRNAs in lung squamous cell carcinoma (LUSC) tissues and normal tissues. The results indicated that TMEM248, CTNNB1 and FGFR1 expression in LUSC tissues was not significantly different to that in the normal tissues. ALPK2, CNIH1, NUFIP2, GNAL, RUBCN, BCL2L11, Bax, MAPK8 and TWF1 all displayed a conspicuously higher expression in LUSC tissues compared to normal tissues. As a result, TMEM248, CTNNB1 and FGFR1 were excluded. Meanwhile, previous literature proposed that the relationship of ALPK2 and miR-214-3p as well as their impacts on NSCLC cells has been investigated (Salim et al., 2013); MAPK8 has been pointed out to exert a pivotal role in NSCLC development (Chen et al., 2021); TWF1 overexpression has been revealed to be linked to poor prognosis of lung adenocarcinoma (the most common subtype of NSCLC) (Kaishang et al., 2018). As no sufficient evidence supported the research

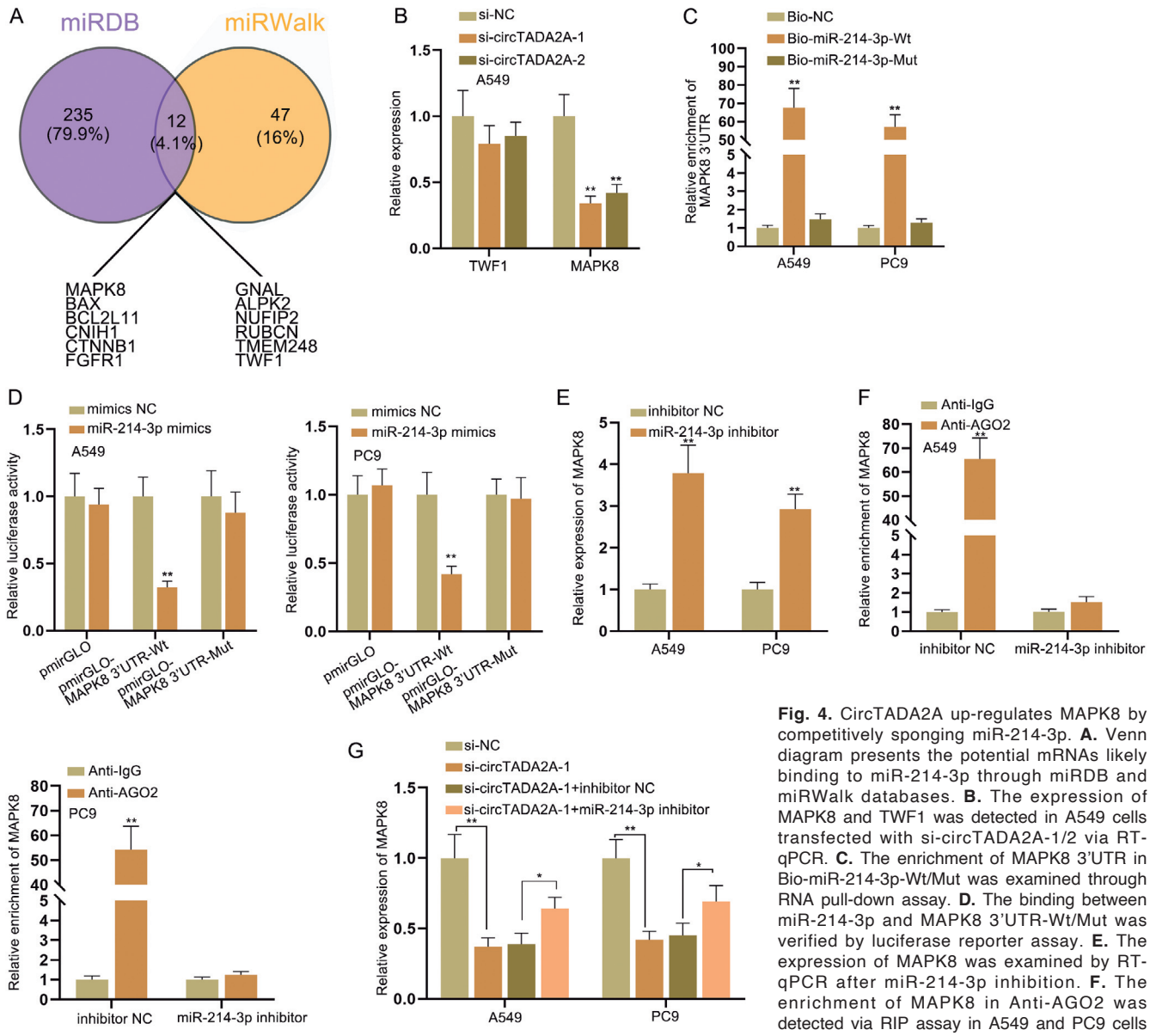

Fig. 4. CircTADA2A up-regulates MAPK8 by competitively sponging miR-214-3p. **A.** Venn diagram presents the potential mRNAs likely binding to miR-214-3p through miRDB and miRWalk databases. **B.** The expression of MAPK8 and TWF1 was detected in A549 cells transfected with si-circTADA2A-1/2 via RT-qPCR. **C.** The enrichment of MAPK8 3'UTR in Bio-miR-214-3p-Wt/Mut was examined through RNA pull-down assay. **D.** The binding between miR-214-3p and MAPK8 3'UTR-Wt/Mut was verified by luciferase reporter assay. **E.** The expression of MAPK8 was examined by RT-qPCR after miR-214-3p inhibition. **F.** The enrichment of MAPK8 in Anti-AGO2 was detected via RIP assay in A549 and PC9 cells upon miR-214-3p inhibition. **G.** RT-qPCR was conducted to detect the expression of MAPK8 in the indicated NSCLC cells under different conditions (si-NC, si-circTADA2A-1, si-circTADA2A-1+inhibitor-NC and si-circTADA2A-1+miR-214-3p inhibitor). Three bio-repeats were run in three technical replicates. *P<0.05, **P<0.01.

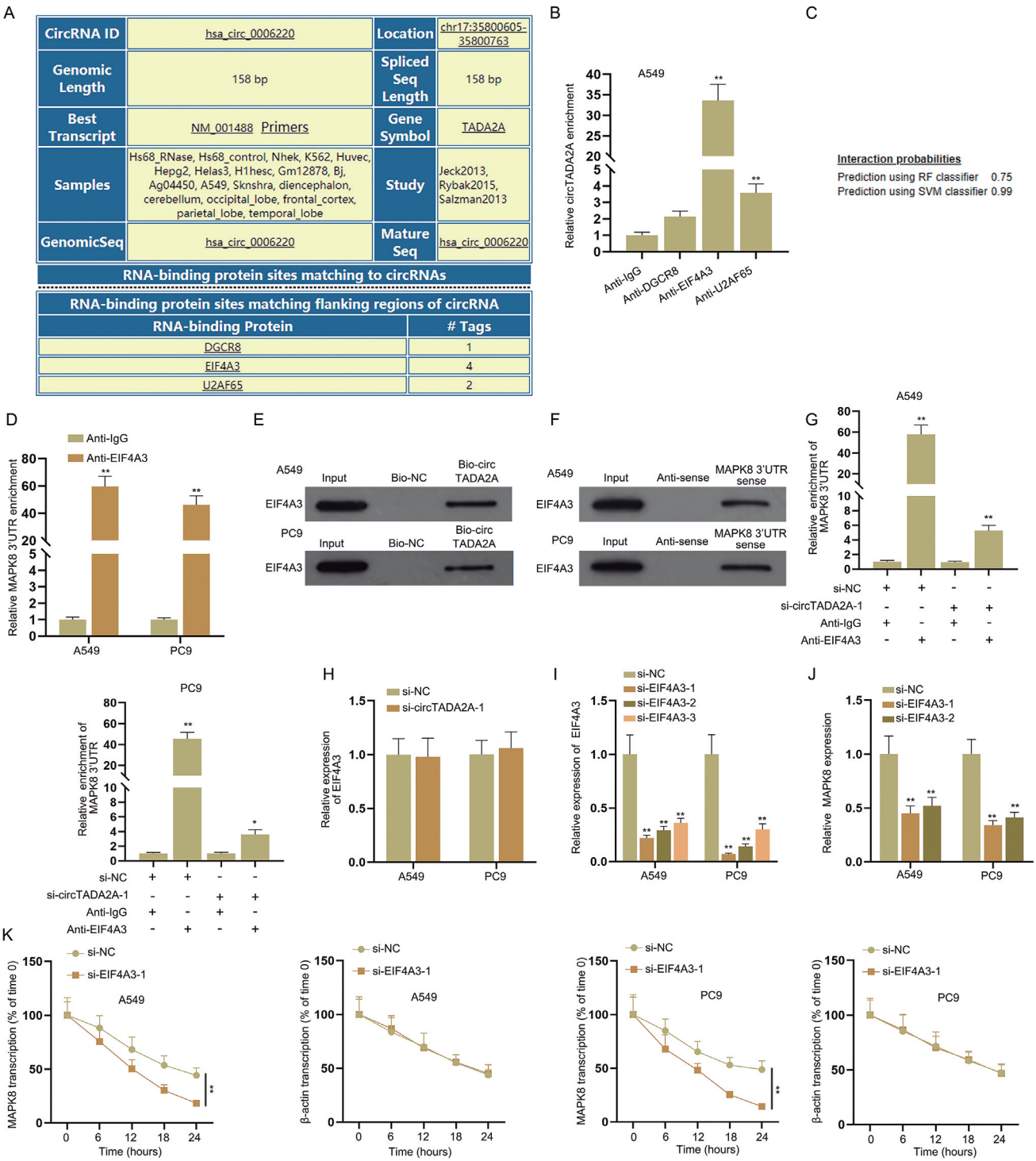

Fig. 5. CircTADA2A recruits EIF4A3 to stabilize MAPK8 mRNA. **A.** RBPs possible to bind with circTADA2A were predicted on Circinteractome. **B.** The binding relationship of circTADA2A and DGCR8/EIF4A3/U2AF65 was assessed by RIP assay. **C.** Pridb website was utilized to project the interaction possibility of EIF4A3 and MAPK8 3'UTR. **D.** The enrichment of MAPK8 3'UTR in Anti-EIF4A3 was examined with the application of RIP assay. **E, F.** RNA pull-down assays were conducted to analyze the binding affinity of EIF4A3 and MAPK8 3'UTR/circTADA2A. **G.** The expression of MAPK8 3'UTR precipitated with Anti-EIF4A3 was evaluated by RIP assay under the condition of circTADA2A depletion. **H.** The expression of EIF4A3 was measured by RT-qPCR after NSCLC cells were transfected with si-circTADA2A-1. **I.** The knockdown efficacy of si-EIF4A3-1/2/3 was detected via RT-qPCR. **J.** RT-qPCR was done to examine the expression of MAPK8 after si-EIF4A3-1/2 was transfected into NSCLC cells. **K.** RT-qPCR was applied to detect the levels of MAPK8 mRNA and β-actin in NSCLC cells treated with α-amanitin and si-EIF4A3-1. Three bio-repeats were run in three technical replicates. *P<0.05, **P<0.01.

basis and value of other candidate mRNAs in NSCLC, we only chose MAPK8 and TWF1 to do the subsequent assays. RT-qPCR analysis revealed circTADA2A depletion only noticeably diminished the expression of MAPK8, which made MAPK8 our optimal choice (Fig. 4B). Next, we noticed that MAPK8 3'UTR was abundantly enriched in Bio-miR-214-3p-Wt (Fig. 4C) and the luciferase activity of MAPK8 3'UTR was overtly decreased in response to miR-214-3p overexpression (Fig. 4D), which verified the strong affinity between MAPK8 and miR-214-3p. MAPK8 was also found to be conspicuously up-regulated, which was attributed to miR-214-3p inhibition (Fig. 4E). Moreover, RIP assay reflected that miR-214-3p inhibition greatly reduced MAPK8 mRNA expression enriched in Anti-AGO2 (Fig. 4F). Later, RT-qPCR data represented that miR-214-3p inhibition could only partially recover the suppressed expression of MAPK8 caused by circTADA2A down-regulation (Fig. 4G). Taken together, circTADA2A can up-regulate MAPK8 via sponging miR-214-3p.

CircTADA2A recruits EIF4A3 to stabilize MAPK8 mRNA

Given the above findings, we inferred that circTADA2A might regulate the expression of MAPK8 through other pathways. Published cancer research has revealed that circRNA can recruit RBP to regulate downstream target mRNA (Chen et al., 2019a; Liu et al., 2020). Hence, we inferred that circTADA2A might regulate MAPK8 expression through recruiting a certain RBP. In the first place, Circinteractome (https://circinteractome.nia.nih.gov/) was employed to project the potential RBPs, possibly combining with circTADA2A. DGCR8, EIF4A3 and U2AF65 were found (Fig. 5A). The following results of RIP assays unveiled that circTADA2A was largely enriched in Anti-EIF4A3 (Fig. 5B). Existing literature has pointed out that EIF4A3 can bind with the 3'UTR region of mRNA and further stabilize that mRNA (Song et al., 2020), which was in line with our speculation. Considering the prediction from RPISeq database (http://pridb.gdcb.iastate.edu/RPISeq/), the interaction possibility of EIF4A3 and MAPK8 mRNA 3'UTR was very high (Fig. 5C). Additionally, the findings of RIP assay indicated that MAPK8 3'UTR was greatly enriched in Anti-EIF4A3 (Fig. 5D). The subsequent RNA pull-down assays confirmed that circTADA2A and MAPK8 3'UTR were able to bind with EIF4A3 (Fig. 5E,F). Then, we observed that the enrichment of MAPK8 3'UTR in Anti-EIF4A3 was lessened in A549 and PC9 cells with circTADA2A knockdown (Fig. 5G). Meanwhile, it was also found that circTADA2A deficiency made no difference to EIF4A3 expression (Fig. 5H). Following

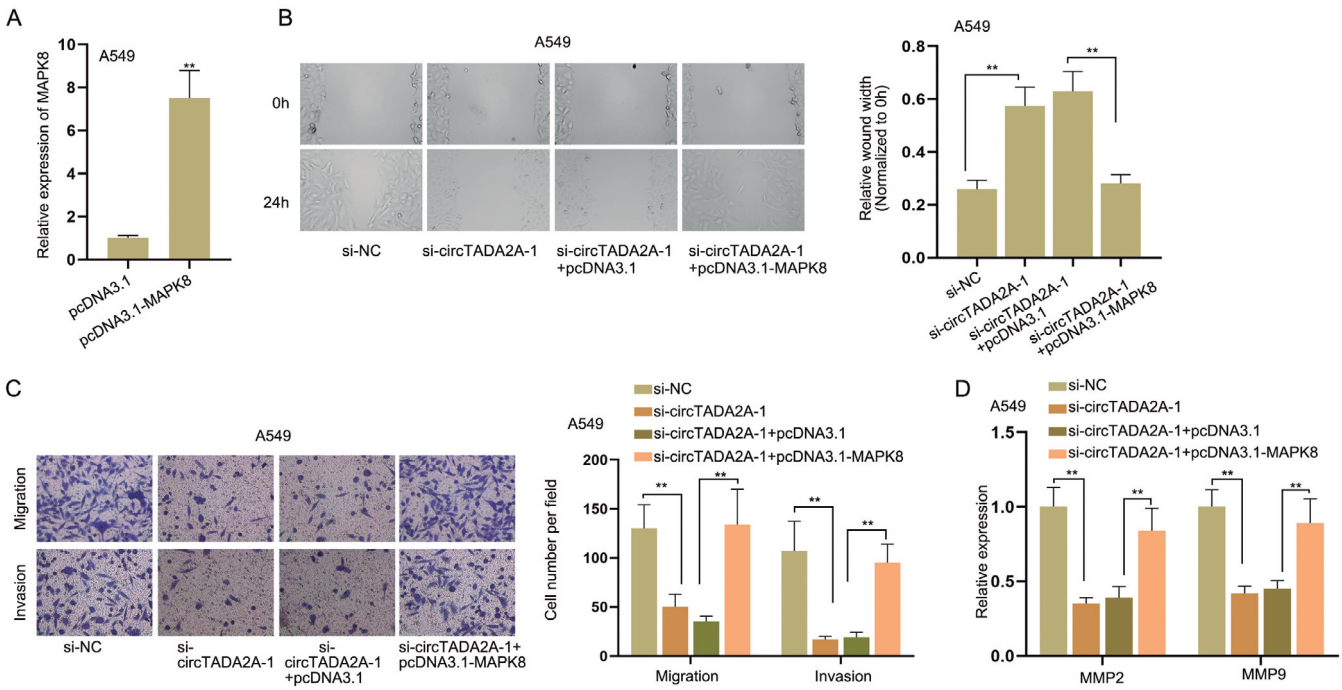

Fig. 6. CircTADA2A regulates invasion and migration of NSCLC cells through modulating MAPK8. **A.** The efficiency of MAPK8 overexpression was detected by RT-qPCR. A549 cells were transfected with different plasmids, including si-NC, si-circTADA2A-1, si-circTADA2A-1+pcDNA3.1 and si-circTADA2A-1+pcDNA3.1-MAPK8. **B.** Wound healing assay was performed to evaluate the migration of NSCLC cells under different conditions. **C.** Transwell assay was conducted to assess the migration and invasion of NSCLC cells with different treatments. **D.** RT-qPCR was conducted to detect the influence of different treatments on MMP2 and MMP9 expression. Three bio-repeats were run in three technical replicates. **P<0.01.

the verification of high EIF4A3 knockdown efficacy (Fig. 5I), we carried out RT-qPCR, finding that MAPK8 expression was decreased upon EIF4A3 depletion (Fig. 5J). Moreover, After A549 and PC9 cells were treated with 50 mM α-amanitin to block RNA transcription, we noticed that MAPK8 expression was significantly reduced in response to EIF4A3 knockdown (Fig. 5K). To sum up, circTADA2A recruits EIF4A3 to improve MAPK8 mRNA stability in NSCLC cells.

CircTADA2A contributes to NSCLC cell migration and invasion by elevating MAPK8 expression

To figure out the influence of circTADA2A/MAPK8 pathway on NSCLC cell malignant behaviors, we carried out the following rescue assays. Firstly, we proved MAPK8 could be successfully up-regulated by pcDNA3.1-MAPK8 (Fig. 6A). Subsequently, wound healing assays demonstrated that the suppression of NSCLC cell migration resulting from circTADA2A deficiency was fully recovered by pcDNA3.1-MAPK8 (Fig. 6B). Transwell assays reflected that the inhibitory effect of circTADA2A knockdown on NSCLC cell migration and invasion was entirely abrogated, accounting for MAPK8 overexpression (Fig. 6C). Moreover, RT-qPCR assays implied that circTADA2A depletion led to a reduction in the expression of MMP2 and MMP9, but MAPK8 elevation restored their expression completely (Fig. 6D). To sum up, circTADA2A up-regulates MAPK8 to promote NSCLC cell migration and invasion.

Discussion

Lung cancer is the most diagnosed malignant cancer with growing fatality rate worldwide, and NSCLC is a main subtype of lung cancer (Brown et al., 2019; Thakur et al., 2020). The research of new targeted therapies for NSCLC patients largely relies on a bettwe understanding of the molecular mechanism of tumorigenesis and the construction of molecular biology platforms able to identify new potential biomarkers (Basse et al., 2021). CircRNAs have been unveiled to serve as potential biomarkers for NSCLC. For example, Sun et al. have demonstrated that circ_0000376 is an oncogenic circRNA in NSCLC and might be potentially used as a diagnostic biomarker (Sun et al., 2020a). In the current study, circTADA2A was uncovered to be highly expressed in NSCLC cells and tissues. CircTADA2A down-regulation was corroborated to hamper migratory and invasive processes of NSCLC cells. Reportedly, circRNAs are novel molecular markers which have a close relationship with tumor invasion and migration (Xu et al., 2020). Herein, our study proved that circTADA2A was able to affect NSCLC cell migration and invasion, so circTADA2A might serve as a novel potential biomarker.

Recent literature has demonstrated that circRNAs work as miRNA sponges to modulate mRNA expression (Zhang et al., 2018), and this ceRNA model is linked to the development of multiple cancers, including NSCLC (Cui et al., 2020). For instance, circ-CMPK1 facilitates NSCLC cell proliferation through competitively binding to miR-302e and elevating cyclin D1 (Cui et al., 2020). This study uncovered that circTADA2A acted as a miR-214-3p sponge to elevate MAPK8 expression. Moreover, circTADA2A strengthened migratory and invasive abilities of NSCLC cells by up-regulating MAPK8. A former study has reported that circMED13L_012 exacerbates the malignant behaviors of NSCLC cells through binding with miR-433-3p and elevating MAPK8 expression (Chen et al., 2021). In agreement with the previous study, our study found that circTADA2A sponged miR-214-3p to up-regulate MAPK8 to further improve the migration and invasion abilities of NSCLC cells.

Currently, circRNAs can exert regulatory effects on diverse biological processes as RBP decoys, signifying their huge potential as biomarkers or therapeutic targets of various cancers (Zhang et al., 2020a). In addition, existing evidence has suggested that circRNAs can stabilize mRNA expression by recruiting RBPs in cancers. For instance, circDCUN1D4 has been revealed to restrain tumor metastasis and glycolysis in lung adenocarcinoma through interacting with HuR and enhancing TXNIP expression (Liang et al., 2021). This research verified that circTADA2A decoyed EIF4A3 to strengthen the stability of MAPK8 mRNA, which was similar to the finding of a recent study that circSEMA5A recruits EIF4A3 to stabilize SEMA5A mRNA (Wang et al., 2020).

In conclusion, circTADA2A is verified to sponge miR-214-3p and recruit EIF4A3 to up-regulate MAPK8, thereby improving the migratory and invasive abilities of NSCLC cells. Since the present findings are limited in in-vitro assays, we will make an effort to complete in-vivo assays and clinical samples collection in the future. This study revealed the circTADA2A/miR-214-3p/EIF4A3/MAPK8 axis and we hope our findings might contribute to the development of novel promising biomarkers for NSCLC early diagnosis.

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