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ORIGINAL RESEARCH

lncRNA *MALAT1* potentiates the progression of tongue squamous cell carcinoma through regulating *miR-140-5p*-PAK1 pathway

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Background: Tongue squamous cell carcinoma (TSCC) is the second most common malignancy in oral carcinoma. lncRNA metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*) was regarded as an oncogenic factor in various carcinomas. However, its underlying molecular mechanisms in the development and progression of TSCC have not been well featured till now.
Methods: The expressions of *MALAT1*, *miR-140-5p* and p21 (RAC1)-activated kinase 1 (PAK1) mRNA were measured by RT-qPCR assay. The protein level of PAK1 was determined by western blot analysis. Cell viability was detected by Cell Counting Kit-8 assay. Transwell chamber was used to detect cell migratory and invasive capability. Luciferase reporter assay, RNA-binding protein immunoprecipitation (RIP) assay and biotin pull-down assay were applied to evaluate the relationship between *MALAT1*, *miR-140-5p* and PAK1. Xenograft experiments were performed to assess the effect and mechanism of *MALAT1* in TSCC tumor growth.
Results: The expression of *MALAT1* and p21 (RAC1)-activated kinase 1 (PAK1) was upregulated and microRNA-140-5p (*miR-140-5p*) expression was downregulated in TSCC tissues and cells. *MALAT1* knockdown induced *miR-140-5p* expression by direct interaction. Moreover, *MALAT1* knockdown inhibited proliferation, migration, and invasion by upregulating *miR-140-5p* expression in TSCC cells. Additionally, PAK1 was identified as a direct target of *miR-140-5p*. Also, *MALAT1* knockdown inhibited PAK1 expression by upregulating *miR-140-5p* in TSCC cells. Furthermore, *miR-140-5p* overexpression curbed the proliferation, migration, and invasion of TSCC cells by targeting PAK1. Finally, *MALAT1* knockdown inhibited tumor growth by upregulating *miR-140-5p* and downregulating PAK1 in mouse xenograft models of TSCC.
Conclusion: *MALAT1* contributed to TSCC progression via *miR-140-5p*-PAK1 regulatory axis, highlighting a potential target for TSCC management.
Keywords: tongue squamous cell carcinoma, lncRNA, *MALAT1*, miRNA-140-5p, PAK1

Introduction

Tongue squamous cell carcinoma (TSCC), the second most common oral malignancy, was responsible for ~30% of all newly diagnosed oral carcinomas in the USA in 2018.[1] In China, the incidence rate of oral carcinoma is ~4.81% and the mortality rate is about 2.21% in all carcinomas.[2] Moreover, the 5-year survival rate of TSCC patients has shown no obvious improvement over the past decades, while the incidence is gradually increasing in young people.[3] Despite the great advances in diagnosis and therapy of TSCC,[4,5] it is still necessary to clarify the molecular mechanisms underlying TSCC pathogenesis in order to identify more effective interventions.

lncRNAs, a group of transcripts longer than 200 nucleotides (nt) without protein-coding potential, have been well documented as critical players in the development and

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progression of carcinomas including TSCC.[6,7] Metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*), an lncRNA located at chromosome 11q13, was first identified as a prognostic marker in non-small-cell lung carcinoma.[8] Recently, *MALAT1* has been found to be implicated in tumorigenesis and progression of a great variety of carcinomas.[9,10] Moreover, some researchers have provided evidence of *MALAT1* as a biomarker and prognostic indicator in human carcinomas.[11,12] For instance, *MALAT1* facilitated cell epithelial–mesenchymal transition (EMT) and suppressed apoptosis by regulating Wnt/β-catenin signaling in TSCC.[13] Knockdown of *MALAT1* resulted in the upregulation of small proline-rich proteins, thereby impairing the proliferative and migratory capacities of TSCC cells.[14] *MALAT1* promoted EMT-mediated metastasis in oral squamous cell carcinoma (OSCC) through activating β-catenin and NF-κB pathways.[15] However, the molecular mechanisms of *MALAT1* in the development and progression of TSCC have not been thoroughly elucidated.

miRNAs, a class of endogenous small non-coding RNAs about 22 nt long, can regulate the translation and stability of mRNAs at post-transcriptional levels.[16] Emerging evidence shows that miRNAs can act as potential oncogenic factors or tumor suppressors in human carcinomas by regulating the processes associated with tumorigenesis, such as inflammation, cell cycle, stress response, differentiation, apoptosis, and invasion.[17] *miR-140-5p* has been reported as an antitumor factor in multiple carcinomas, such as gastric carcinoma,[18] hepatocellular carcinoma,[19] lung carcinoma,[20] and ovarian carcinoma.[21] Moreover, Kai et al pointed out that *miR-140-5p* could suppress cell migration and invasion in TSCC.[22]

In the present study, it is demonstrated that *MALAT1* expression was upregulated and *miR-140-5p* expression was downregulated in TSCC tissues and cells. Further functional and mechanism analysis demonstrated that *MALAT1* promoted the development of TSCC by *miR-140-5p*/p21 (RAC1)-activated kinase 1 (PAK1) regulatory pathway in vitro and in vivo.

Materials and methods

Tissue samples

TSCC tissues and adjacent normal tissues were obtained from 40 patients diagnosed with TSCC between January 2014 and December 2016 at our hospital. All tissues were immediately frozen in liquid nitrogen during the surgical procedure. The study was approved by the Ethics Committee of Changhai Hospital, Second Military Medical University. All patients signed the written informed consents prior to enrolling in this study. This study was conducted in accordance with the Declaration of Helsinki.

Cell culture

Primary normal human oral keratinocytes (NHOKs) were isolated from keratinized oral epithelial tissues of patients who suffered from flap operation to remove impacted wisdom teeth. This experiment was carried out with the written informed consents from the patients and the approval of Ethics Committee of Changhai Hospital. NHOKs were cultured in Defined Keratinocyte-serum-free medium (Thermo Fisher Scientific, Waltham, MA, USA). Human TSCC cell lines (Tca8113, SCC-2, SCC-4, SCC-9, and Cal-27) were purchased from BeNa Culture Collection (Beijing, China) and incubated in Roswell Park Memorial Institute (RPMI) 1640 medium (Thermo Fisher Scientific) supplemented with 10% FBS (Thermo Fisher Scientific). All cells were grown in a humidified incubator containing 5% CO_2 at 37°C.

Cell transfection

siRNA targeting *MALAT1* (si-*MALAT1*), scrambled control siRNA (si-NC), *miR-140-5p* mimics, miRNA control (miR-NC), *miR-140-5p* inhibitor (anti-*miR-140-5p*) and its control anti-miR-NC were synthesized by GenePharma Co., Ltd (Shanghai, China). To construct *MALAT1* or PAK1 overexpression plasmid, the full-length sequences of *MALAT1* or PAK1 were amplified by PCR and then subcloned into pcDNA3.1 vector (Thermo Fisher Scientific), named as pcDNA3.1-*MALAT1* (*MALAT1*) and pcDNA3.1-PAK1 (PAK1). All these plasmids and oligonucleotides were transfected into TSCC cells by Lipofectamine 2000 reagent (Thermo Fisher Scientific) according to the manufacturer's instructions.

Quantitative reverse transcriptase PCR (RT-qPCR) assay

Total RNA was extracted from TSCC tissues and cells by TRIzol reagent (Thermo Fisher Scientific) following the manufacturer's protocols. For the expression analysis of *MALAT1*, an equal amount of RNA (1 μg) was reverse transcribed into cDNA first strand using M-MLV reverse transcriptase (Promega, Madison, WI, USA), followed by quantitative analysis using FastStart Universal SYBR Green Master (Roche Diagnostics, Mannheim, Germany) with GAPDH as the internal reference. The expression analysis of *miR-140-5p* was performed by the S-Poly(T) method. Briefly, RNA was first polyadenylated using Poly(A) Polymerase Tailing Kit (Epicenter, Madison, WI, USA), followed by the reverse transcription with M-MLV High-Performance Reverse Transcriptase (Epicenter) via *miR-140-5p*-RT primer. At last, the expression level of *miR-140-5p* was detected by *miR-140-5p* primers (forward and reverse) and Taqman probe with small nucleolar RNA SNORD47 as an endogenous control.

Cell Counting Kit-8 (CCK-8) assay

Cell viability was detected by CCK-8 (MedChemExpress, Monmouth Junction, NJ, USA), referring to the manufacturer's instructions. Briefly, Tca8113 and SCC-9 cells (10^4–10^5 cells/well) were inoculated into 96-well plates and transfected with corresponding oligonucleotides or plasmids. Then, CCK-8 solution (10 μL) was added into each well of 96-well plates at the indicated time points (24, 48, 72, and 96 hours) after transfection and then incubated for another 3 hours. At last, the absorbance was measured by a microplate reader at the wavelength of 450 nm.

Cell migration and invasion assay

Cell migration assay was performed using the Transwell chamber (8 μm pore size; BD Biosciences, Franklin Lakes, NJ, USA) to detect cell migratory capability. Briefly, Tca8113 and SCC-9 cells (5×10^4) in serum-free RPMI 1640 medium were inoculated into the upper chambers, while medium with 10% FBS was added to the lower chambers. After 48 hours of incubation at 37°C, cells on the upper side of the membranes were removed using a cotton swab. Cells adhering to the lower surface were photographed and counted after fixing with 100% pre-cold methanol and staining using 0.1% crystal violet solution. For cell invasion assay, the same experimental procedures were performed, except that the Transwell chambers were precoated with Matrigel (BD Biosciences).

Western blot assay

Total protein was obtained from TSCC Cells using pre-cold RIPA lysis buffer (Sigma-Aldrich, St Louis, MO, USA) containing protease inhibitor cocktail (Roche Diagnostics). An equal amount of protein (40 μg per lane) was separated by 10% SDS-PAGE gel and transferred to nitrocellulose membrane (EMD Millipore, Billerica, MA, USA). After blocking with 5% skimmed milk for 1 hour at room temperature, the membranes were incubated with primary antibody against PAK1 or β-actin (Abcam, Cambridge, UK) overnight at 4°C. Subsequently, the membranes were further probed with appropriate HRP-conjugated secondary antibody for 1 hour at room temperature. Finally, the protein signals were measured by BeyoECL Plus (Beyotime, Shanghai, China) and quantified with ImageJ software. The experiment was repeated three times.

Bioinformatics analysis

The potential miRNAs that have a chance to interact with *MALAT1* were predicted by miRcode database (http://www.mircode.org/). The candidate target mRNAs of *miR-140-5p* were predicted using the miRcode (http://www.mircode.org/) and miRwalk (http://mirwalk.umm.uni-heidelberg.de/) databases.

Luciferase reporter assay

The fragments of *MALAT1* and PAK1-3′UTR region containing putative *miR-140-5p* binding sites were amplified by PCR and constructed into pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega), named as *MALAT1*-WT and PAK1-3′UTR-WT reporter, respectively. QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) was used to generate *MALAT1*-MUT and PAK1-3′UTR-MUT reporters containing mutant *miR-140-5p* binding sites. Then, the constructed luciferase reporters were, respectively, co-transfected with miR-NC or *miR-140-5p* mimic into TSCC cells. At 48 hours after transfection, luciferase activity was detected with Dual-Luciferase Reporter Assay System (Promega) referring to the manufacturer's instructions.

RNA-binding protein immunoprecipitation (RIP) assay

The RIP assay was performed using the EZ-Magna RIP Kit (EMD Millipore), referring to the instructions of the manufacturer. Briefly, TSCC cells were lysed by RIP lysis buffer supplemented with cocktail (Roche Diagnostics). Then, cell supernatants were incubated overnight at 4°C with primary antibody against Ago2 or mouse IgG (EMD Millipore) and protein A/G magnetic beads. Following this, RNase-free DNase I (Promega) and Proteinase K were used to digest extra DNAs and proteins. At last, purified RNA was analyzed by the RT-qPCR assay to measure the expression of *MALAT1* and *miR-140-5p*.

Biotin pull-down assay

The pull-down assay was performed according to a previous report.[23] Briefly, Tca8113 cells were transfected with biotinylated *MALAT1* probe (Bio-*MALAT1*-probe) or a negative control probe (Bio-NC-probe). At 48 hours after transfection, cells were harvested for biotin-based pull-down assay, followed by RT-qPCR assay to determine *miR-140-5p* level.

Animal experiments

The animal experiments were performed with the approval of the Ethics Committee of Changhai Hospital, Second Military Medical University. Animal experiments were carried out according to the National Institutes of Health guidelines to the Care and Use of Laboratory Animals. Female Nu/Nu nude mice (6–8 weeks old, n=12) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd (Shanghai, China). Lentiviruses carrying *MALAT1* shRNA (sh-*MALAT1*) or control shRNA (sh-NC) were purchased from Genomeditech Co., Ltd (Shanghai, China) and then added into Tca8113 cells. Next, Tca8113 cells (5×10^6)

infected with lentiviruses carrying sh-NC or sh-*MALAT1* were injected subcutaneously into the right flanks of sh-NC or sh-MALAT group mice with six mice in each group. Tumor volume was monitored using a caliper every week with the formula: volume = (long diameter × short diameter^2)/2. Tumors were resected, photographed, and weighed at 5 weeks after injection. The levels of *MALAT1* and *miR-140-5p* were measured by RT-qPCR assay in resected xenograft tumors at the end of experiments. PAK1 protein level in xenograft tumors was determined by Western blot assay at the end of experiments.

Statistical analysis

Results were presented as mean ± SD from at least three independent experiments. Student's *t*-test/Wilcoxon test (for two-group data) or one-way ANOVA together with Tukey's post hoc test (for more than two-group data) was used for the analysis of differences. The correlation between *miR-140-5p* and *MALAT1* or PAK1 was evaluated by Pearson correlation analyses. $P<0.05$ represented the difference was statistically significant.

Results

MALAT1 expression was upregulated and *miR-140-5p* expression was downregulated in TSCC tissues and cells

First, the expression patterns of *MALAT1* and *miR-140-5p* were investigated in TSCC tissue specimens and cell lines by RT-qPCR assay. Results showed that *MALAT1* expression was markedly increased (Figure 1A) and *miR-140-5p* expression was strikingly decreased (Figure 1B) in TSCC tissues compared with adjacent non-carcinomatous tissues. Moreover, *MALAT1* expression was found to be associated with tumor size, lymph node metastasis (LNM), and TNM stage in TSCC (Table 1). Correlation coefficient analysis also showed that *miR-140-5p* level was inversely associated with *MALAT1* level in TSCC tissues (Figure 1C). Moreover, RT-qPCR assay showed that *MALAT1* expression was remarkably upregulated (Figure 1D) and *miR-140-5p* expression was notably downregulated (Figure 1E) in TSCC cell lines (Tca8113, SCC-2, SCC-4, SCC-9, and Cal-27) compared with NHOK cell line. In summary, these data indicated

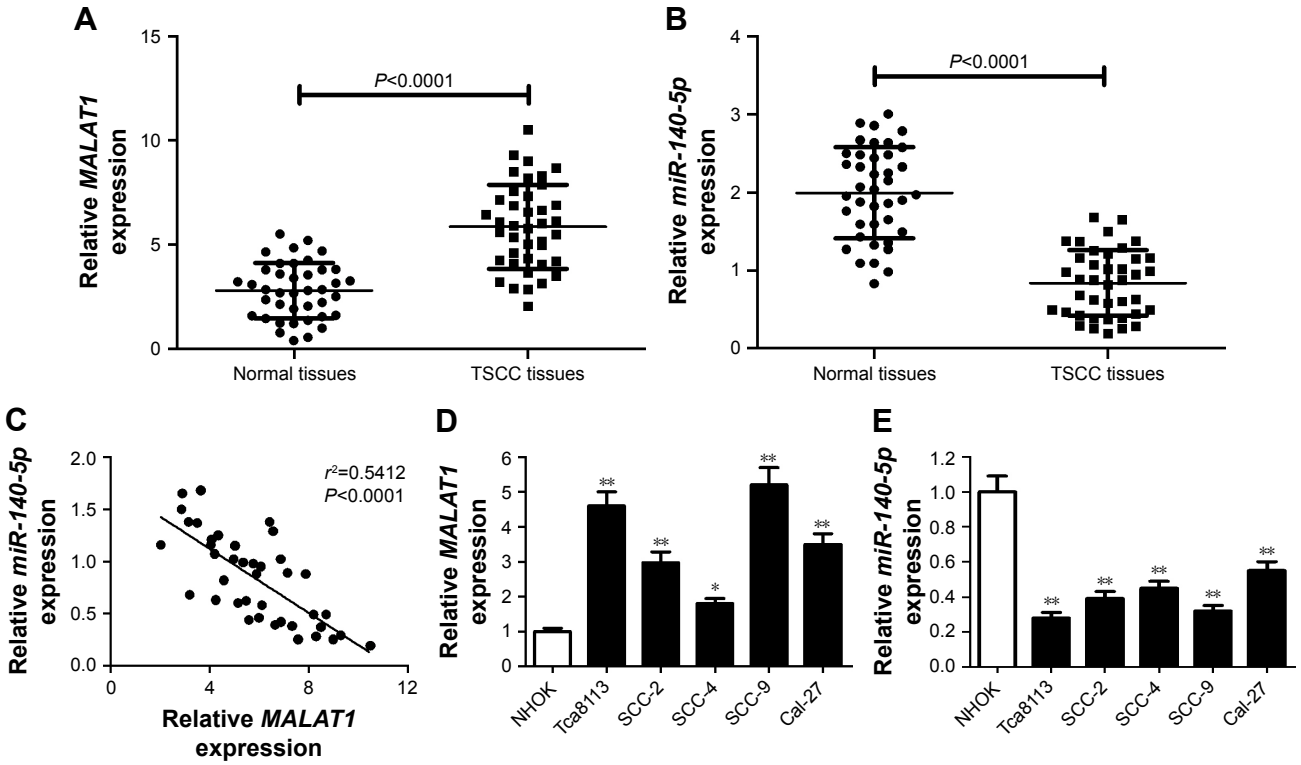

Figure 1 *MALAT1* expression was increased and *miR-140-5p* expression was decreased in TSCC tissues and cells.
Notes: (**A** and **B**) RT-qPCR analysis of *MALAT1* and *miR-140-5p* expression in 40 pairs of TSCC tumor tissues and adjacent normal tissues. (**C**) The correlation analysis of *MALAT1* and *miR-140-5p* expression levels in 40 cases of TSCC tumor tissues. (**D** and **E**) RT-qPCR analysis of *MALAT1* and *miR-140-5p* expression in NHOK and TSCC cell lines (Tca8113, SCC-2, SCC-4, SCC-9, Cal-27). *$P<0.05$, **$P<0.01$.
Abbreviations: NHOK, normal human oral keratinocyte; RT-qPCR, quantitative reverse transcriptase PCR; TSCC, tongue squamous cell carcinoma.

Table 1 Correlation between *MALAT1* level and clinical characteristics in TSCC patients

Clinicopathologic feature	*MALAT1* level		*P*-value
	High, n (%)	Low, n (%)	
Age (years)			0.536
<55	12 (57.1)	9 (42.9)	
≥55	9 (47.4)	10 (52.6)	
Gender			0.775
Female	9 (50)	9 (50)	
Male	12 (54.5)	10 (45.5)	
Tumor size (cm)			0.027
>2	15 (68.2)	7 (31.8)	
≤2	6 (33.3)	12 (67.7)	
Lymph node metastasis			0.028
Yes	16 (66.7)	8 (33.3)	
No	5 (31.3)	11 (68.7)	
TNM stage			0.024
I–II	8 (36.4)	14 (63.6)	
III–IV	13 (72.2)	5 (27.8)	

Abbreviations: *MALAT1*, metastasis-associated lung adenocarcinoma transcript 1; TSCC, tongue squamous cell carcinoma.

that *MALAT1* and *miR-140-5p* might be implicated in the development of TSCC.

MALAT1 inhibited *miR-140-5p* expression by direct interaction

Accumulating evidence reveals that lncRNAs can serve as miRNA sponges or decoys to modulate endogenous miRNAs, thus leading to derepression of target mRNAs.[24] Thus, whether *MALAT1* could act as a *miR-140-5p* sponge to exert its oncogenic effects was further explored in TSCC. Bioinformatics analysis by miRcode website showed that there were several complementary sites between *MALAT1* and *miR-140-5p* (Figure 2A). Dual-luciferase reporter assay proved that the overexpression of *miR-140-5p* markedly attenuated luciferase activity of *MALAT1*-WT reporter, but not that of *MALAT1*-MUT reporter in Tca8113 cells (Figure 2B). RIP assay further revealed that both *MALAT1* and *miR-140-5p* were remarkably enriched by Ago2 antibody in Tca8113 cells compared with anti-IgG negative control group (Figure 2C). Subsequent pull-down assay demonstrated that biotin-labeled *MALAT1* probe (Bio-*MALAT1*-probe) could substantially enrich *miR-140-5p* in Tca8113 cells compared with the control group (Figure 2D). Moreover, *MALAT1* expression was reduced (Figure 2E) and *miR-140-5p* expression was enhanced (Figure 2F) in Tca8113 and SCC-9 cells transfected with si-*MALAT1*. That is to say, *MALAT1* knockdown promoted *miR-140-5p* expression via direct interaction in TSCC cells.

MALAT1 knockdown suppressed the proliferation, migration, and invasion by regulating *miR-140-5p* in TSCC cells

First, the transfection efficiency of *miR-140-5p* inhibitor in TSCC cells was confirmed. As presented in Figure 3A and B, a notable decrease in *miR-140-5p* expression was observed in Tca8113 and SCC-9 cells compared with untransfected group or anti-miR-NC group. Then, the effects of *MALAT1* on the proliferation, migration, and invasion of TSCC cells were detected by CCK-8, Transwell migration, and invasion assay. The results showed that downregulation of *MALAT1* by si-*MALAT1* significantly suppressed the proliferation (Figure 3C and D), migration (Figure 3E), and invasion (Figure 3F) in both Tca8113 and SCC-9 cells. To further investigate whether the effects of *MALAT1* in TSCC progression were mediated by *miR-140-5p*, *miR-140-5p* inhibitor was introduced into si-MALATA–transfected TSCC cells. The results showed that si-MALATA–induced inhibition of proliferation (Figure 3C and D), migration (Figure 3E), and invasion (Figure 3F) was greatly attenuated by knockdown of *miR-140-5p*. Taken together, these data indicate that *MALAT1* exerted its oncogenic effects partially through suppressing *miR-140-5p* expression.

MALAT1 knockdown suppressed PAK1 expression by upregulating *miR-140-5p*

It is well known that miRNAs exert their functions by modulating the expression of target genes. By using miRcode website, the complementary sequences between *miR-140-5p* and PAK1 3′UTR were identified (Figure 4A). Subsequent dual-luciferase reporter assay showed that *miR-140-5p* overexpression decreased the luciferase activity of PAK1-WT reporter, while little change was observed in the luciferase activity of PAK1-MUT reporter between miR-NC and *miR-140-5p* groups (Figure 4B). To explore the effect of *miR-140-5p* on PAK1 expression, *miR-140-5p* mimic was transfected into TSCC cells. As expected, transfection of *miR-140-5p* mimic resulted in an obvious elevation of *miR-140-5p* level in Tca8113 and SCC-9 cells (Figure 4C). Western blot analysis revealed that the ectopic expression of *miR-140-5p* dramatically suppressed PAK1 protein expression in Tca8113 and SCC-9 cells (Figure 4D). PAK1 expression was found to be markedly upregulated in six randomly selected TSCC tissues compared with corresponding normal tissues (Figure 4E). Moreover, a notable upregulation of PAK1 mRNA level was observed in 40 cases of TSCC tissues compared with normal tissues (Figure 4F). Additionally, PAK1 mRNA level was

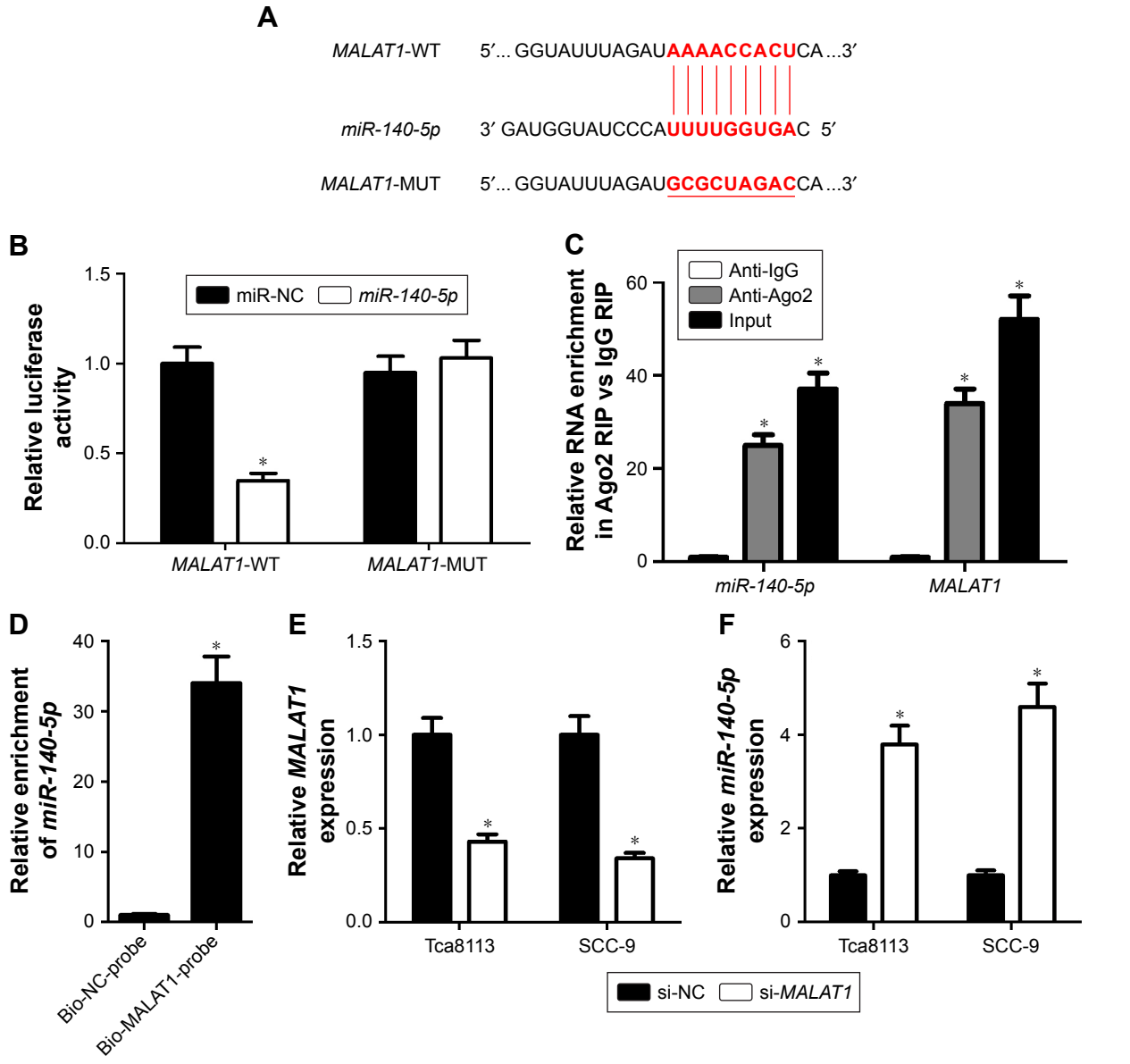

Figure 2 *MALAT1* suppressed *miR-140-5p* expression by direct interaction.

Notes: (**A**) The putative binding sites of *MALAT1* and *miR-140-5p* and mutant sites in MALAT-MUT reporter. (**B**) Effect of *miR-140-5p* overexpression on the luciferase activity of *MALAT1*-WT or *MALAT1*-MUT reporter was detected by luciferase assay in Tca8113 cells. (**C**) RIP assay was performed in Tca8113 cells using anti-Ago2 or anti-IgG, followed by RT-qPCR analysis of *MALAT1* and *miR-140-5p* levels in the IgG or Ago2 immunoprecipitation complex. (**D**) RNA pull-down assay was conducted to test the binding capacity of *MALAT1* and *miR-140-5p*. (**E** and **F**) RT-qPCR analysis of *MALAT1* and *miR-140-5p* expression in Tca8113 and SCC-9 cells transfected with si-*MALAT1* or si-NC. *$P<0.05$.

Abbreviations: RIP, RNA-binding protein immunoprecipitation; RT-qPCR, quantitative reverse transcriptase PCR; si-*MALAT1*, siRNA targeting *MALAT1*; si-NC, scrambled control siRNA.

markedly increased in several TSCC cells (Tca8113, SCC-2, SCC-4, SCC-9, and Cal-27) compared with NHOK control cells (Figure 4G). Subsequent correlation analysis revealed that PAK1 mRNA level was negatively associated with PAK1 level in TSCC tissues (Figure 4H). Furthermore, *MALAT1* knockdown resulted in a prominent decline of PAK1 protein expression in both Tca8113 and SCC-9 cells, while this effect was markedly weakened by *miR-140-5p* inhibitor (Figure 4I). Overall, these data indicate that *MALAT1* could act as a molecular sponge of *miR-140-5p* to sequester *miR-140-5p* from its target gene PAK1, resulting in the upregulation of PAK1 level in TSCC cells.

miR-140-5p repressed the proliferation, migration, and invasion through targeting PAK1 in TSCC cells

To further explore whether *miR-140-5p* affected TSCC development by regulating PAK1, the transfection efficiency of PAK1 overexpression plasmid (pcDNA3.1-PAK1) was first investigated in TSCC cells. As displayed in Figure 5A and B,

PAK1 protein level was markedly enhanced in Tca8113 and SCC-9 cells following transfection with pcDNA3.1-PAK1 (Figure 5A and B), suggesting its applicable values in subsequent gain-of-function experiments. Then, TSCC cells were co-transfected with *miR-140-5p* mimics and pcDNA3.1-PAK1. Results showed that *miR-140-5p* overexpression led to a significant decrease in proliferation, migration, and invasion of Tca8113 and SCC-9 cells, while these effects were substantially abated following increase of PAK1 expression (Figure 5C–F). All these data suggest that *miR-140-5p* suppressed the proliferation, migration, and invasion via downregulating PAK1 in TSCC cells.

MALAT1 knockdown inhibited the growth of TSCC xenograft tumors through upregulating *miR-140-5p* and downregulating PAK1 in vivo

First, *MALAT1* expression level was measured in Tca8113 cells infected with sh-NC or sh-*MALAT1* lentiviruses.

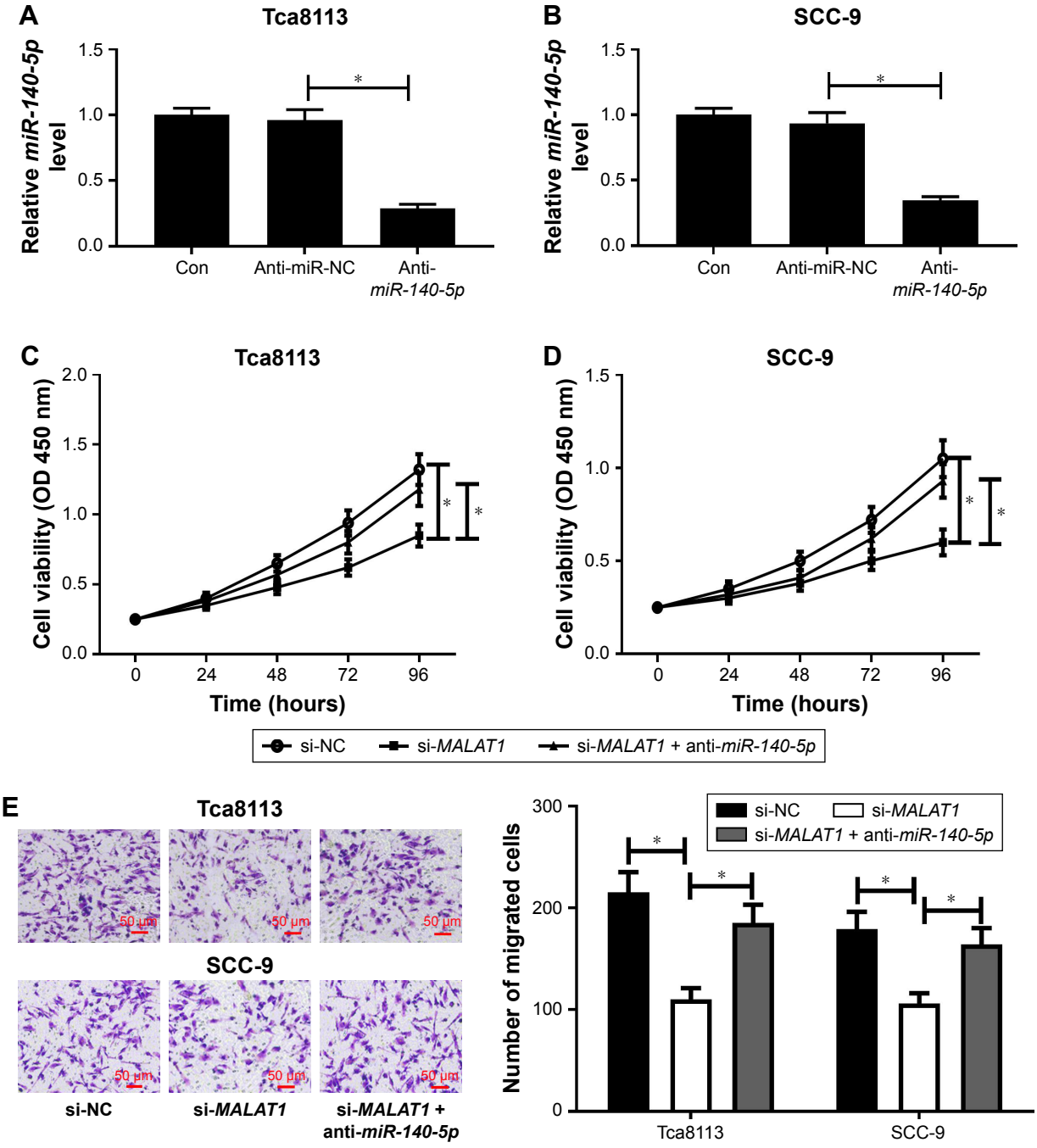

Figure 3 (*Continued*)

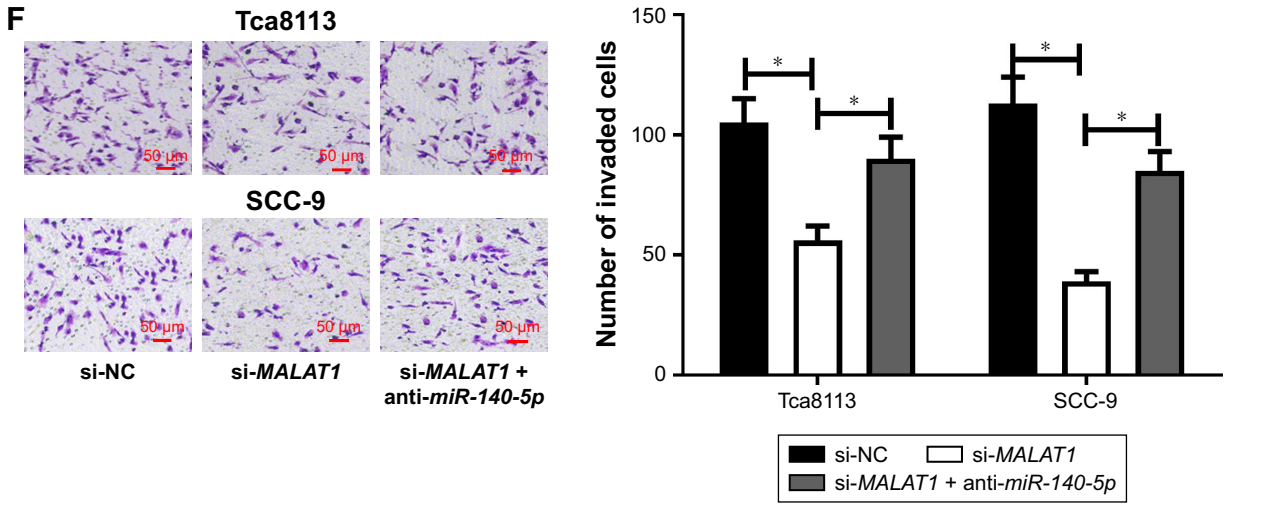

Figure 3 *MALAT1* downregulation repressed the proliferation, migration, and invasion by regulating *miR-140-5p* in TSCC cells.
Notes: (**A** and **B**) RT-qPCR assay was used to measure the expression of *miR-140-5p* in Tca8113 and SCC-9 cells after transfection with anti-miR-NC or anti-*miR-140-5p*. Untransfected cells served as the negative control group. (**C**–**F**) Tca8113 and SCC-9 cells were transfected with si-*MALAT1*, or together with anti-*miR-140-5p*, followed by CCK-8 analysis of cell proliferation (**C** and **D**), as well as Transwell assay of cell migration (**E**) and invasion (**F**). *$P<0.05$.
Abbreviations: CCK-8, Cell Counting Kit-8; RT-qPCR, quantitative reverse transcriptase PCR; si-*MALAT1*, siRNA targeting *MALAT1*; si-NC, scrambled control siRNA; TSCC, tongue squamous cell carcinoma; Con, negative control.

Results showed that MALAT expression was strikingly reduced in Tca8113 cells infected with sh-*MALAT1* lentiviruses compared with sh-NC group (Figure 6A), indicating that sh-*MALAT1*–infected Tca8113 cells could be used for the following in vivo loss-of-function experiments. Then, the roles and molecular mechanisms of *MALAT1* on the growth of TSCC xenograft tumors were further tested. Results displayed that *MALAT1* knockdown inhibited tumor growth in xenograft models of TSCC, as evidenced by the reduction of tumor volume (Figure 6B) and tumor weight (Figure 6C) in MALAT-depleted mice. Moreover, downregulated *MALAT1* level (Figure 6D), increased *miR-140-5p* expression (Figure 6E), and decreased PAK1 expression (Figure 6F) were observed in *MALAT1*-silenced xenograft tumors of TSCC. These data verified that *MALAT1* contributed to tumor growth via *miR-140-5p*/PAK1 regulatory axis in vivo.

Discussion

Accumulating evidence indicates that abnormal expression of lncRNAs and miRNAs is closely associated with the development and progression of human carcinomas.[25] Moreover, some reports pointed out that lncRNAs could act as potential tumor suppressors or oncogenic factors in TSCC. For instance, lncRNA AFAP1 antisense RNA 1 (AFAP1-AS1) facilitated proliferation, migration, and invasion via activating Wnt/β-catenin pathway in TSCC cells.[26] NKILA impaired cell migratory and invasive capacities by inhibiting EMT via regulation of NF-κB activity in TSCC.[27] Moreover, the carcinogenic effects of *MALAT1* on TSCC have been highlighted in several reports.[14,28] Nevertheless, the underlying molecular mechanisms of *MALAT1* in TSCC progression are far from being elucidated.

A growing body of literature points out that lncRNAs can serve as miRNA decoys or sponges to affect their expression.[29] *miR-140-5p* has been found to inhibit cell migration and invasion in TSCC.[22] Moreover, previous studies showed that *MALAT1* promoted proliferation and invasion of uveal melanoma cells through silencing miR-140,[30] and *MALAT1* knockdown enhanced the blood–tumor barrier permeability by increasing miR-140 expression.[31]

In the present study, it is found that *MALAT1* expression was markedly upregulated in TSCC tissues and cells. Moreover, *MALAT1* expression was associated with tumor size, LNM, and TNM stage in TSCC. Consistently, Fang et al pointed out that TSCC patients (n=59) with LNM have higher *MALAT1* expression compared with patients without LNM (n=68).[14] Zhou et al disclosed that OSCC patients (n=19) with higher *MALAT1* expression have a shorter survival time compared with patients with lower *MALAT1* expression (n=35).[15] Also, *miR-140-5p* expression was notably downregulated in TSCC tissues and cells. Moreover, there existed a significant inverse correlation between *MALAT1* and *miR-140-5p* expression in TSCC tissues. Subsequent bioinformatics analysis showed that *MALAT1* contained binding sequences of *miR-140-5p*. Luciferase reporter experiments, RIP, and pull-down assay further validated the interplay between *MALAT1* and *miR-140-5p*. Furthermore, *MALAT1*

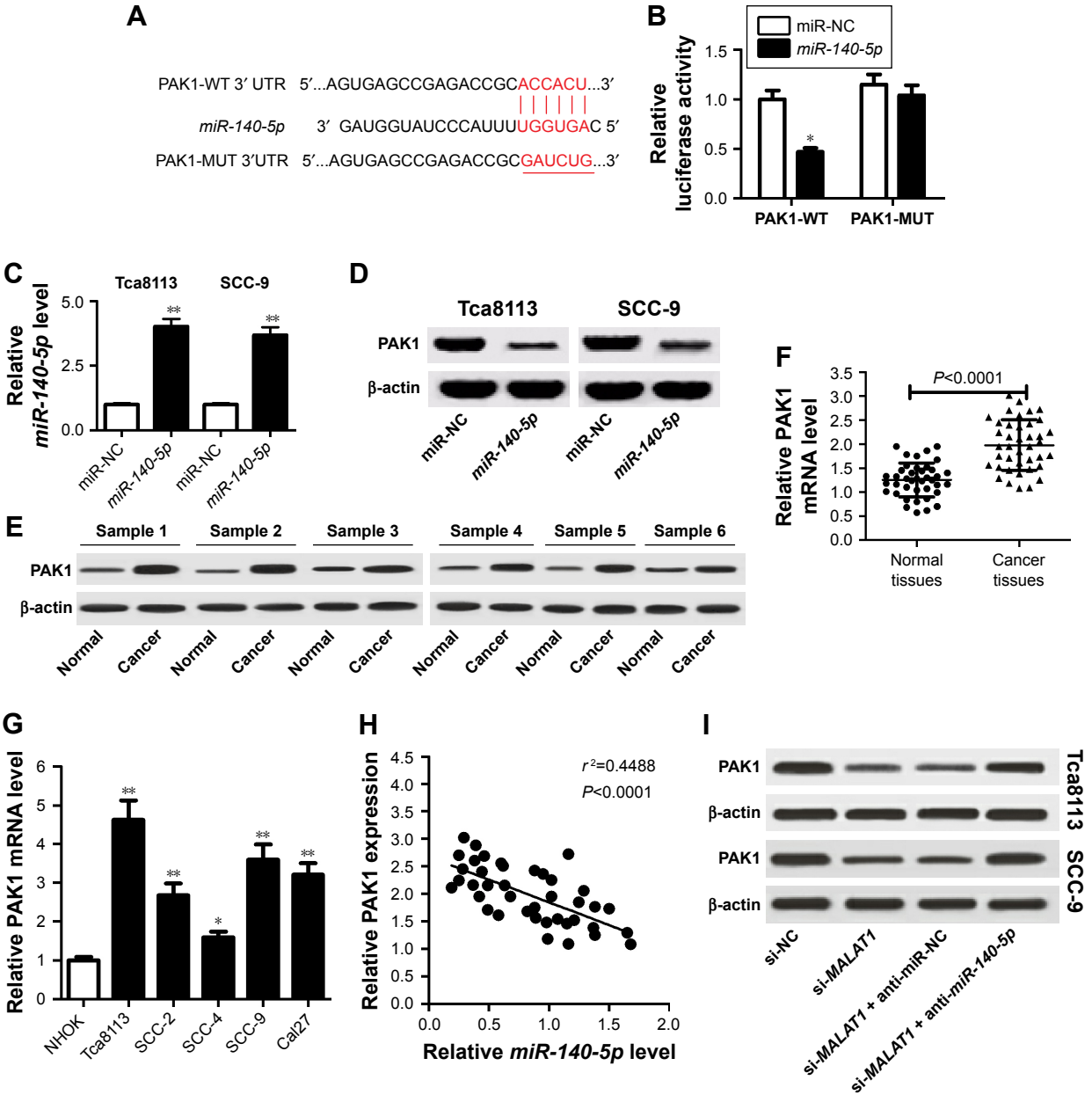

Figure 4 *miR-140-5p* downregulated PAK1 expression by targeting its 3′UTR region.

Notes: (**A**) Predictive binding sites between PAK1 3′UTR and *miR-140-5p* and mutant sites in PAK1-MUT reporter. (**B**) Dual-luciferase reporter assay was carried out to detect the effect of *miR-140-5p* overexpression on the luciferase activity of PAK1-WT or PAK1-MUT reporter in Tca8113 cells. (**C**) Tca8113 and SCC-9 cells were transfected with *miR-140-5p* mimic or miR-NC, followed by the measurement of *miR-140-5p* expression via RT-qPCR assay at 48 hours after transfection. (**D**) Western blot assay of PAK1 protein expression in Tca8113 and SCC-9 cells transfected with *miR-140-5p* or miR-NC at 48 hours upon transfection. (**E**) PAK1 protein analysis in six randomly selected tumor tissues and corresponding non-carcinomatous tissues. (**F**) RT-qPCR analysis of PAK1 mRNA expression in 40 pairs of TSCC tissues and corresponding non-carcinomatous tissues. (**G**) PAK1 mRNA level detection in NHOK and TSCC cell lines (Tca8113, SCC-2, SCC-4, SCC-9, and Cal27). (**H**) Correlation analysis between PAK1 mRNA level and *miR-140-5p* level in 40 cases of TSCC tissues. (**I**) Western blot assay of PAK1 protein expression in Tca8113 and SCC-9 cells transfected with si-NC, si-*MALAT1*, si-*MALAT1* + anti-miR-NC, or si-*MALAT1* + anti-*miR-140-5p*. *$P<0.05$; **$P<0.01$.

Abbreviations: NHOK, normal human oral keratinocyte; RT-qPCR, quantitative reverse transcriptase PCR; si-*MALAT1*, siRNA targeting *MALAT1*; si-NC, scrambled control siRNA; TSCC, tongue squamous cell carcinoma.

knockdown strikingly facilitated *miR-140-5p* expression in TSCC cells. All these data indicate that *MALAT1* reduced *miR-140-5p* expression by direct interaction. Then, functional analysis showed that downregulation of *MALAT1* hampered the proliferation, migration, and invasion of TSCC cells, while these effects were obviously abrogated by inhibition of *miR-140-5p*. Overall, *MALAT1* exerted its oncogenic effects through silencing *miR-140-5p* expression in TSCC cells.

Then, web-based tools demonstrated that PAK1-3′UTR possessed possible binding sites of *miR-140-5p*. Moreover, luciferase reporter and Western blot assays confirmed that

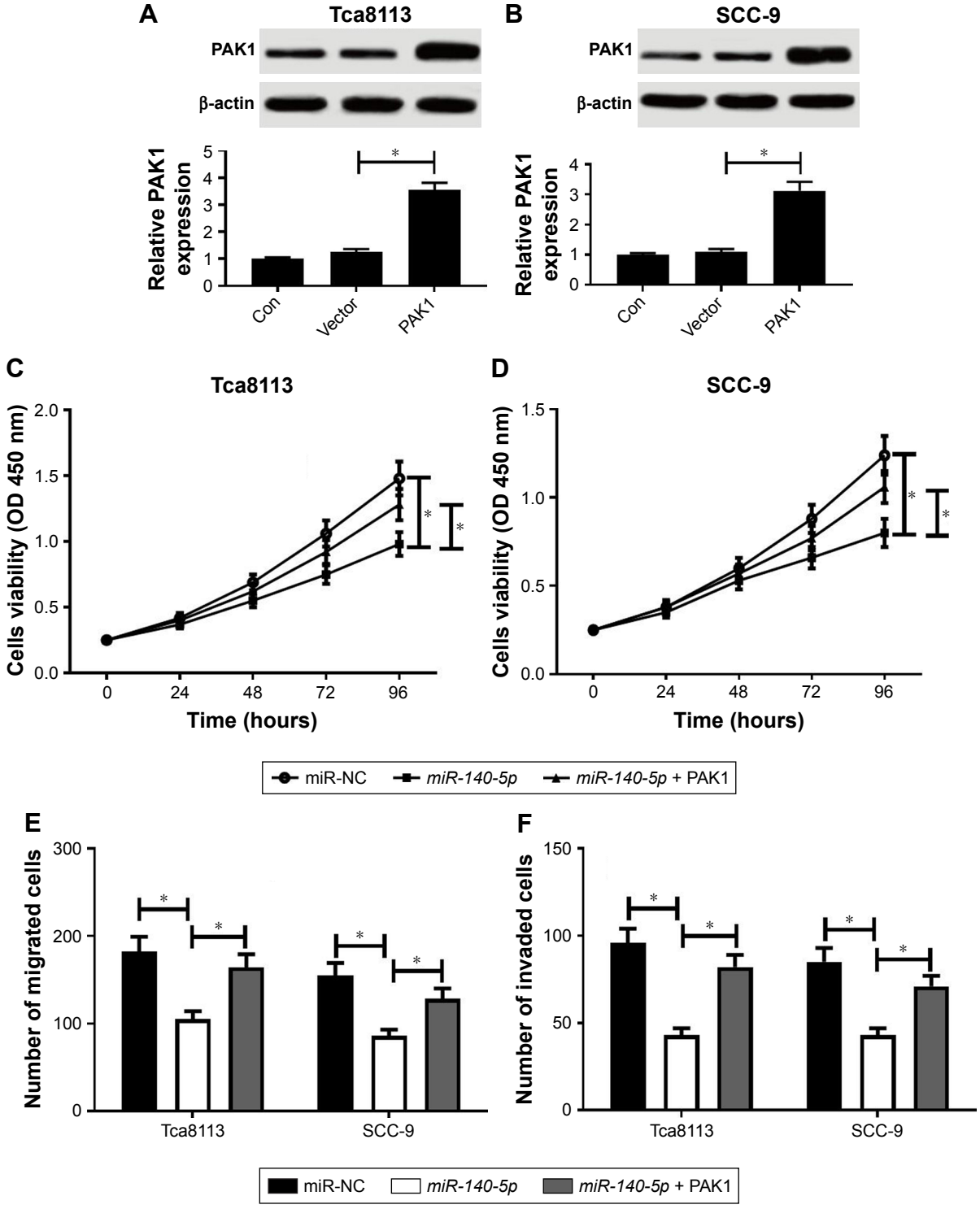

Figure 5 *miR-140-5p* overexpression inhibited proliferation, migration, and invasion by modulating PAK1 in TSCC cells.
Notes: (**A** and **B**) Tca8113 and SCC-9 cells were transfected with pcDNA3.1 or pcDNA3.1-PAK1 plasmid, followed by detection of PAK1 protein level at 48 hours. Untransfected cells served as the negative control group. (**C–F**) Tca8113 and SCC-9 cells were transfected with miR-NC, *miR-140-5p* mimics, or together with pcDNA-PAK1, followed by CCK-8 analysis of cell proliferation (**C** and **D**), as well as Transwell assay of migration (**E**) and invasion (**F**). *$P<0.05$.
Abbreviations: CCK-8, Cell Counting Kit-8; TSCC, tongue squamous cell carcinoma; Con, negative control.

PAK1 was a direct target of *miR-140-5p*. Furthermore, PAK1 expression was markedly increased in TSCC tissues and cells and negatively correlated with *miR-140-5p* expression in TSCC tissues. Additionally, *MALAT1* knockdown inhibited PAK1 expression by upregulating *miR-140-5p* in TSCC cells.

Subsequent functional analysis clarified that *miR-140-5p* repressed the proliferation, migration, and invasion in TSCC

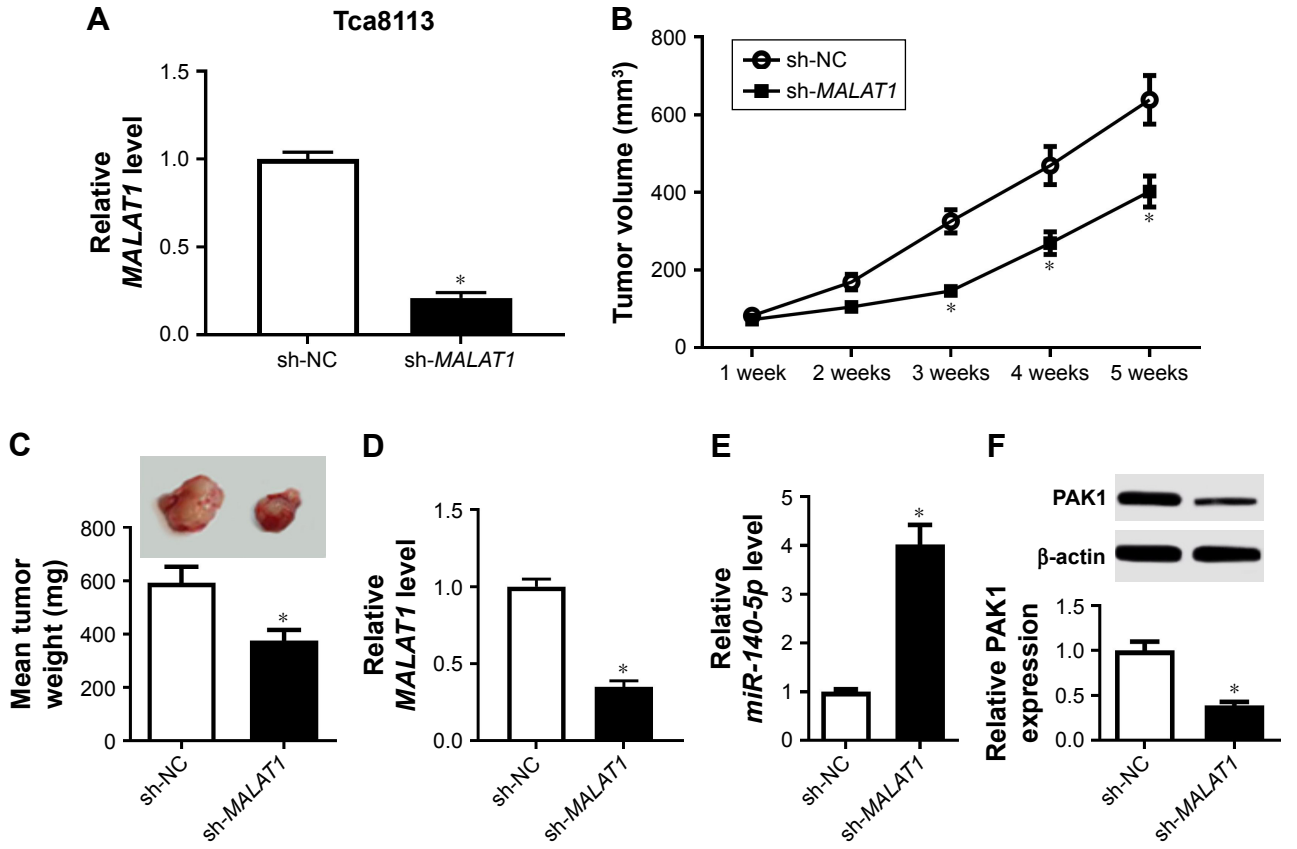

Figure 6 *MALAT1* knockdown inhibited the growth of TSCC xenograft tumors through upregulating *miR-140-5p* and downregulating PAK1 in vivo.
Notes: (**A**) Tca8113 cells were infected with sh-NC or sh-*MALAT1* lentiviruses. At 72 hours post-infection, *MALAT1* level was determined by RT-qPCR assay. (**B**) Tumor volume was monitored using a caliper every week for a total of 5 weeks after injection. (**C**) Tumors were resected, photographed, and weighed at 5 weeks after injection. (**D** and **E**) The levels of *MALAT1* and *miR-140-5p* were measured by RT-qPCR assay in TSCC xenograft tumors at the end of experiments. (**F**) PAK1 protein level was determined by Western blot assay in TSCC xenograft tumors at the end of experiments. *$P<0.05$.
Abbreviations: RT-qPCR, quantitative reverse transcriptase PCR; sh-*MALAT1*, *MALAT1* shRNA; sh-NC, control shRNA; TSCC, tongue squamous cell carcinoma.

cells, whereas these effects were dramatically reversed after overexpressing PAK1, suggesting that the tumor-suppressive roles of *miR-140-5p* were mediated by PAK1. PAK1, a prototype of group I PAK family, has been elucidated as the cause for several oncogenic processes.[32,33] Several documents verified that PAK1 was a vital target of miRNA signal pathways. For example, miR-494 repressed the proliferation, migration, and invasion of breast carcinoma cells by targeting PAK1.[34] miR-98 directly targeted the 3′UTR of PAK1 to downregulate its expression, thus inhibiting cell proliferation and invasion in non-small-cell lung carcinoma.[35] In this study, it is further demonstrated that *MALAT1* knockdown inhibited the growth of TSCC xenograft tumors via upregulating *miR-140-5p* and downregulating PAK1 in vivo.

Previous studies showed that *MALAT1* facilitated OSCC development by regulating miR-125b/signal transducer and activator of transcription 3 (STAT3) axis.[36] *MALAT1* could interact with miR-320d by SRSF1, and miR-320d knockdown induced CCR7 expression in OSCC.[37] Moreover, Zhang et al pointed out that *MALAT1* knockdown inhibited the tumorigenesis and progression of tongue cancer in vitro and in vivo by miR-124/jagged1 (JAG1) pathway.[28] *MALAT1* could interact with miR-30a in human head and neck squamous cell carcinoma.[38] Moreover, some studies have shown that miR-124 could exert its antitumor effect by targeting STAT3 in hepatocellular carcinoma,[39] and that *miR-140-5p* suppressed glioma cell proliferation and invasion by targeting JAG1.[40] ADAM10, LAMC1, HDAC7, and PAX6 have been reported as the targets of *miR-140-5p* in tongue cancer.[22] Furthermore, Lee et al pointed out that PAK1 suppressed ADAM17 association and lipid raft transfer by phosphorylating paxillin, and HIV-1 and cancer cells could exploit a paxillin/integrin-controlled mechanism to release ADAM17/ADAM10-containing vesicles, hinting the link of PAK1 and ADAM10 (a target of *miR-140-5p*).[41] These data indicate that some downstream signaling pathways of *MALAT1* were mutually relevant.

Moreover, bioinformatics analysis by miRcode website suggests that *MALAT1* has a chance to interact with multiple miRNAs such as miR-503, miR-96, and miR-17-5p.

Additionally, prediction results from miRwalk show that ADAM2, WNT7B, PAQR7, and P2RY2 are the potential targets of *miR-140-5p*.

Taken together, this study demonstrated that *MALAT1* promoted the development of TSCC partly via regulating *miR-140-5p*/PAK1 pathway in vitro and in vivo. This study provides a novel insight into the involvement of *MALAT1* in TSCC development and elucidates a promising therapeutic candidate for TSCC treatment.

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Disclosure

The authors report no conflicts of interest in this work.

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