Circ_0005232 Promotes the Progression of Oral Squamous Cell Carcinoma by Sponging miR-1299 to Regulate CDK6

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Abstract

Objectives: CircRNA may play essential roles and act as biomarkers in tumor development due to their special stable structure. However, the mechanism by which circRNAs affect OSCC progression is still unclear.

Methods: qRT-PCR was performed to detect circ_0005232 expression level in oral squamous cell carcinoma (OSCC) tissues and cell lines. Colony formation assays, cell migration and invasion assays, and wound healing assays were performed to verify the effects of overexpression or knockdown of circ_0005232 on the biological function of OSCC cell lines. Western blot was performed to determine the effects of circ_0005232 on epithelial-to-mesenchymal transition (EMT) and expression of MMP2 and MMP9 in OSCC cell lines. Dual luciferase reporter assays, rescue assays, RNA immunoprecipitation assays, and EDU incorporation assays were performed to explore interactions among circ_0005232, miR-1299, and CDK6.

Results: qRT-PCR results confirmed that circ_0005232 was expressed significantly higher in OSCC tissue and cell lines. Functional experiments indicated that overexpression of circ_0005232 promoted OSCC cell lines proliferation、migration and invasion ability, while inhibition of circ_0005232 caused opposite results. MiR-1299 knockdown could rescue the changes in cell function caused by circ_0005232 knockdown. The dual luciferase reporter assay verified that circ_0005232 could bind with miR-1299 to affect the proliferation、migration and invasion ability of OSCC cell lines. RNA immunoprecipitation assays indicated that circ_0005232 could increase CDK6 expression by sponging miR-1299.

Conclusions: Our results demonstrate that circ_0005232 exerts its tumor-promoting effects by sponging miR-1299 which then affects function of CDK6. Therefore, circ_0005232 may represent a novel potential prognostic biomarker and therapeutic target in OSCC.

Background

Oral squamous cell carcinoma (OSCC) accounts for over 90% of oral malignant tumors, and it is the sixth most common cancer worldwide[1]. Due to the risk factor exposure, low cure rate, and high mortality rate, OSCC has become a global public health problem, with 5-year survival rates of about 80% for early OSCC (Stage I and II) but only 20% for advanced OSCC[2, 3]. Despite years of research, the pathogenesis of OSCC remains unclear.

The progression of OSCC are accompanied by a series of dysregulated non-coding RNAs (ncRNAs) in cancer cells, which may be potential diagnostic markers for OSCC treatment. NcRNAs are a type of RNA without coding function which can regulate the expression and function of various genes at the transcriptional and post-transcriptional levels. At present, reported ncRNAs mainly consist of microRNA (miRNA), long non-coding RNA (lncRNA) and circular RNA (circRNA)[4]. Recent studies have shown that circRNAs can be used as diagnostic markers for tumor progression[5–8]. With the development of research

and sequencing technology, circRNAs have become better understood. Emerging evidence show that circRNAs are endogenous ncRNAs with conserved sequences, stable states, and high abundance[9, 10]. Since there are many miRNA binding sites on circRNAs, they can regulate mRNA expression by competing miRNAs away from mRNA, leading circRNAs to be considered miRNA sponges. Due to the tissue specificity and stability of circRNAs, they have become potential markers for some diseases, especially tumors[11, 12]. CDK6, a member of cyclin-dependent kinase (CDK) family, is a classical cell kinase. Abnormal activation of this kinase can lead to uncontrolled cell proliferation and cancer. Therefore, the importance of CDK6 in promoting tumorigenesis and progression makes it an attractive target for drug inhibition[13].

In the current study, we use microarray to analyze expression of circRNAs in 3 pairs of OSCC tissues. Based on the microarray and PCR results, circ_0005232 was selected as our research target. Our findings confirm that circ_0005232 exerts its tumor-promoting effects through the miR-1299/CDK6 axis. Therefore, circ_0005232 may be a potential biomarker of OSCC.

Materials And Methods

Collection of clinical specimens and basic information

Our study collected specimens from patients who were diagnosed with OSCC and who received maxillofacial surgery at the Stomatological Hospital Affiliated to China Medical University. The specimens were stored at -80℃ for long-term preservation. Patients were diagnosed with OSCC by the pathology department, and OSCC tissues and corresponding adjacent healthy tissues (AHT) were taken at least 3 cm apart. This research was authorized by the ethics committee of the Stomatological Hospital Affiliated to China Medical University, and all patients signed informed consent. The relevant clinical information and pathological characteristic data were collected and collated for statistical analysis.

Cell culture

Human oral keratinocyte (HOK) cells and two human oral squamous cell lines (CAL27 and SCC9) were purchased from ATCC (American Type Culture Collection). The HOK cells were cultured in oral keratinocyte growth medium (ScienCell, Carlsbad, CA, USA), CAL27 were cultured in Dulbecco's modified Eagle's medium (DMEM), SCC9 cells were cultured in DMEM/F12 medium, all with 10% fetal bovine serum (FBS: Hyclone, Israel) and 100 U/ml penicillin and streptomycin (Invitrogen, Camarillo, CA, USA). Cells were incubated at 37°C with 5% CO_2. Cells were not contaminated with mycoplasma.

Total RNA extraction and quantitative real-time PCR

Total RNA was isolated from OSCC tissues and cell lines using Trizol reagent and cDNA was synthesized using the PrimeScriptTMRT reagent kit with random primers. Reverse transcription real-time fluorescent quantitative PCR was performed using SYBR® Premix Ex Taq real-time fluorescent quantitative PCR

reaction, with annealing temperatures adjusted for each primer, and GAPDH was used as the internal reference. All experiments were performed in triplicate.

Cell transfection

Experimental cells were plated (5×10^5) in 6-well plates. After 24 h, when the cell concentration had grown to 70% and the cells were in the logarithmic growth phase, the cells were transfected with si-circ_0005232 using Lipofectamine 3000. Cells were collected at 48h for functional experiments.

Colony formation assay

In this assay, 500–1000 squamous cells were added to every new well of 6-well cell culture plate, cultured for 96h, then medium changed. Cells were cultured for 12–15 days depending on the growth rate and of each cell, after which 1ml methanol was added to each well and cells were fixed for 10min. After fixing, methanol was discarded and 1ml crystal violet was added to each well to stain for 10min. Wells were then washed twice with 1ml PBS (phosphate belanced solution) until the colony was clearly visible. A light microscope was used to capture images. All experiments were performed in triplicate.

EdU incorporation assay

An EdU cell proliferation kit (RiboBio, Guangzhou, China) was used to test DNA synthesis and cell proliferation. Cells were incubated in 96-well plates at the required density. After growing for 36 h, 50 μM EdU was added to cells and incubated for 3 h, fixed with 4% paraformaldehyde, then stained. Images were observed under a fluorescence microscope, with EdU positive (red) and Hoechst 33342 positive (blue) cells counted in 5 randomly captured fields. The cell proliferation rate was calculated as EdU-positive cells /Hoechst33342-positive cells. All experiments were performed in triplicate.

Cell migration and invasion assays

Transwell assays were used to detect the effect of circ_0005232 on the migratory ability of cancer cells. For cell migration assays, tumor cells were evenly spread in double-free medium in the upper chamber of a transwell chamber, and complete medium containing 10% serum was added to the lower chamber. For cell invasion assays, cells were seeded in upper chambers pre-coated with 100% Matrigel (BD Biosciences) then treated the same as in the migration assay. Cells passing through the transwell were obtained after 24 h culture, at which point they were fixed with methanol for 1 minute, stained, dried and sliced. Images were collected under microscopy and cells were counted with Image J. All experiments were performed in triplicate.

Wound healing assay

Transfected cells were uniformly plated into a 12-well plate and cultured 24 h until 90% confluency. A 10-μL sterile tip held perpendicular to the 12-well plate was used for scratching to ensure a consistent scratch width. After scratching, the cells were washed two times with PBS and culturing continued. Images were obtained at 0 h, 6 h, 12 h, 24 h and 36 h. All experiments were performed in triplicate.

Western blot

Total protein was extracted from squamous cells. Cells were lysed (Beyotime, ShangHai, China) and total protein prepared following the manufacturer's instructions. The absorbance of each well was measured at 560nm with a microplate analyzer, then the known protein content and absorbance were used to draw a standard curve to calculate the sample concentration. Equal amounts of protein were then separated via electrophoresis before being transferred to PVDF membranes (Millipore, Darmstadt, Germany). PVDF membranes were blocked with 5% defatted dry milk for 2h, incubated with primary antibody overnight at 4°C. Next, PVDF membranes were incubated with secondary antibodys for 2h to complete a color reaction. Actin was used as the internal protein loading control. All experiments were performed in triplicate.

Dual luciferase reporter assay

The Dual Luciferase Reporter Assay System Kit (Promega, WI, USA) was used to detect fluorescence activity between firefly and Rinilla luciferase. In order to create luciferase reporter vectors, the sequences of circ_0005232 and its corresponding mutant version without miR-1299 binding sites were synthesized and subcloned into pmir-GLO vector (Promega, WI, USA), termed 0005232-1299-WT, circ_0005232-1299-MuT, respectively. SCC9 cells were seeded in 12-well plates for 24 h before transfection. Next, miR-1299 mimics □or miR-1299 inhibitor□and luciferase reporter vectors were cotransfected into the SCC9 cells. All experiments were performed in triplicate.

RNA immunoprecipitation

RNA immunoprecipitation (RIP) assay was performed using the EZMagna RIP Kit (Millipore, Billerica, MA, USA) according to the instructions. RNA-protein compounds were precipitated using antibodies against the target protein CDK6, then the RNA bound on the compounds were isolated and purified. Finally, the target RNA was analyzed via qRT-PCR. After co-transfection, cell lysate was prepared and mixed with magnetic beads, immunoprecipitation buffer was added, and the RNA binding protein immunoprecipitation reaction was performed. Retrieved RNA were then purified and analyzed with reverse transcription and qRT-PCR. All experiments were performed in triplicate.

Image processing and statistical analysis

GraphPad Prism8 and SPSS 17.0 were used to calculate experimental results. Each experiment was repeated three times.

Results

Characterization and expression of circ_0005232 in OSCC

To test whether circ_0005232 is abnormally expressed in OSCC, we used qRT-PCR to probe circ_0005232 expression levels in 53 pairs OSCC tissue and matched healthy tissues and found significantly higher levels in the cancer tissues. The results were evaluated as follows ratio = ΔCt (normal tissue)/ ΔCT (cancer tissue), with a higher ratio representing higher expression (Fig. 1a). In our experiment, a ratio > 1 is

considered high expression; otherwise, it is considered low expression. Clinicopathological parameters of the 53 patients diagnosed with OSCC are shown in Table 1, and circ_0005232 was found to relate to TNM stage and lymphatic metastasis. We then measured the expression level of circ_0005232 in two OSCC cell lines, CAL27 and SCC9, and in the noncancerous HOK cell line, and found higher circ_0005232 in the OSCC cell lines (Fig. 1b).

Decreased expression of circ_0005232 inhibits OSCC cell proliferation, migration, and invasion ability

To evaluate the influence of circ_0005232 on OSCC cell function, we conducted functional experiments by transfecting a vector encoding si-circ_0005232 into OSCC cells to decrease circ_0005232 expression. Using qRT-PCR, we found that circ_0005232 levels were significantly lower in cancer cells transfected with si-circ_0005232 (Fig. 2a). To determine the function of si-circ_0005232 in regulating cell proliferation, migration, and invasion abilities, we conducted colony formation, migration, invasion, and wound healing assays to evaluate differences in biological function between the two groups (Fig. 2b-e). We found that decreased circ_0005232 expression inhibited proliferation (Figs. 2b) and migration and invasion ability in both cell lines (Figs. 2c). Furthermore, the wound healing assay showed that decreased circ_0005232 also inhibited cell migration (Fig. 2d, e).

Overexpression of circ_0005232 promotes OSCC cell proliferation, migration, and invasion ability

We next transfected Circ_0005232 into CAL27 and SCC9 cells to create circ_0005232 overexpression (circ_0005232) and control (NC) groups. We found significantly higher expression of circ_0005232 in CAL27 and SCC9 cells after transfection than in the NC group (Fig. 3a). To determine the function of circ_0005232 overexpression in regulating cell proliferation, migration, and invasion abilities, we conducted colony formation, migration, invasion, and wound healing assays to evaluate differences in biological function between the two groups (Fig. 3b-e). Overexpression of circ_0005232 enhanced the proliferation, migration, and invasion abilities of CAL27 and SCC9 cell lines (Fig. 3b, c). The wound healing assay further demonstrated that overexpression of circ_0005232 could enhance migration in both cell lines (Fig. 3d, e).

Effect of circ_0005232 on EMT, MMP2, and MMP9 in OSCC cell lines

To further explore the role of circ_0005232 on epithelial-to-mesenchymal transition (EMT) and MMP family proteins, we transfected si-circ_0005232 into SCC9 cells then measured RNA and protein expression using qRT-PCR and Western blot. The qRT-PCR results showed significantly increased expression of E-cadherin and lower expression of MMP2 and MMP9 in the si-circ_0005232 group. Additionally, expression of N-cadherin, vimentin, and β-catenin were relatively down-regulated in the si-circ_0005232 group, but there was no statistically significant difference(Fig. 4a). Western blot showed that expression of E-cadherin was increased and expression of N-cadherin, β-catenin, vimentin, MMP2,

and MMP9 were significantly decreased in the si-circ_0005232 group (Fig. 4b). The qRT-PCR results suggest that si-circ_0005232 partly inhibits the EMT process and expression of MMP2 and MMP9 in SCC9 cells. The Western blot results suggest that si-circ_0005232 inhibits the protein expression level of the EMT process and expression of MMP2 and MMP9 in SCC9 cells.

Circ-0005232 negatively regulated miR-1299

To explore the mechanism of circ_0005232 in tumorigenesis, we explored predicted target miRNAs of circ_0005232. Potential binding miRNAs for circ_0005232 were predicted by Circ-interactom and the Circ-bank database. Overlapping predicted targets from the two databases were harvested, and the index with the higher context score is shown (Fig. 5a). We extracted RNA from SCC9 cells after transfection with circ_0005232 and probed expression levels of three miRNAs (miR-1299, miR-502-5p, and miR-545) by qRT-PCR. We found that circ_0005232 negatively regulated expression of miR-1299 (Fig. 5b). To determine whether circ_0005232 acts as a sponge for miR-1299, we constructed a dual luciferase reporter plasmid containing the WT or MUT circ_0005232 sequence (Fig. 5C). Luciferase activity significantly decreased in the circ_0005232-1299-WT group when miR-1299 was overexpressed in SCC9 cells, while luciferase activity in the circ_0005232-1299-MUT group showed no significant difference (Fig. 5c). Similarly, miR-1299 inhibitors caused significantly increased luciferase activity in the circ_0005232-1299-WT group, with no significant change in circ_0005232-1299-MUT (Fig. 5c). These results indicate the presence of a highly efficient interaction between circ_0005232 and miR-1299 via the implicated binding sites and provide direct evidence that miR-1299 is sponged by circ_0005232.

Circ_0005232 affects biological functions of OSCC cell lines by sponging miR-1299

To verify whether circ_0005232 regulates OSCC cell proliferation, migration, and invasion through miR-1299, we performed rescue experiments. We verified that miR-1299 knockdown could rescue the changes in these cell functions caused by circ_0005232 knockdown through functional experiments. SCC9 cell lines were used to perform rescue experiments with four different transfection groups: si-NC, si-circ_0005232, si-circ_0005232 + inh-miR-1299, and si-circ_0005232 + inh-NC. We found that si-circ_0005232 + inh-miR-1299 could restore si-circ_0005232-induced proliferation, migration, and invasion ability (Fig. 5d, e). Additionally, qRT-PCR and western blotting showed that inh-miR-1299 could restore expression of EMT-related markers and invasion-related MMP family markers (Fig. 5f). Overall, these results indicate that circ_0005232 promotes cell proliferation, migration, invasion, EMT, and MMP family expression in OSCC cells at least in part by competitively binding to miR-1299.

We next predicted target genes of miR-1299 using TargetScan and selected CDK6 for further study based on references (Fig. 6a). RIP testing indicated that miR-1299 complexes with CDK6 (Fig. 6b). Our results thus far suggest that circ_0005232 plays a role in OSCC through sponging miR-1299, so we tested whether circ_0005232 regulates expression of CDK6, a known target of miR-1299. QRT-PCR and Western blot indicated that CDK6 was significantly decreased and increased after circ_0005232 knockdown and overexpression, respectively (Fig. 6c). Rescue experiment results suggested that inh-miR-1299 restored

the effects of si-circ_0005232 on CDK6, while miR-1299 mimics reversed the effects of circ_0005232 on CDK6 (Fig. 6d). EdU rescue experiments were performed with si-circ_0005232 and CDK6 mimics, and CDK6 mimics were found to restore the effects of si-circ_0005232 on proliferation changes (Fig. 6e). These results support the hypothesis that circ_0005232 affects CDK6 to promote proliferation, migration, and invasion at least partially by sponging miR-1299.

Discussion

Extensive research on circRNAs has raised many new insights into cancer development. Researchers now use advanced sequencing and micro-array technology to better investigate circRNAs and verify their function and molecular mechanism[14–19]. CircRNA are considered potential biomarkers and therapeutic targets for cancer because of their special stable structure[20, 21]. However, the mechanisms by which circRNAs affect OSCC progression are still unclear. Our study was based on a previous study[22] in which 407 differentially expressed circRNAs were detected between OSCC and AHT using high-throughput sequencing analysis, with 134 highly expressed and 273 lowly expressed in OSCC. Circ_0005232 was an implicated highly expressed circRNA. Following preliminary qRT-PCR experiments, we chose circ_0005232 as the focus of this study. The relationship between circ_0005232 and OSCC was confirmed through bioinformatic analysis and functional experiments. Circ_0005232 is a newly discovered circRNA which has not yet been reported in any other cancers. QRT-PCR results from 53 pairs of OSCC tissue confirmed that circ_0005232 has significantly higher expression in OSCC. Correlation analysis of clinical pathological characteristics indicated that high circ_0005232 is related to lymphatic metastasis. To better define the relationship between circ_0005232 and OSCC, we knocked down circ_0005232 in OSCC cell lines by transfecting with si-circ_0005232 then performed colony formation, transwell migration and invasion, and wound healing assays. We found that circ_0005232 silencing inhibited proliferation, migration, and invasion in OSCC cells, suggesting that circ_0005232 plays an important role in malignant OSCC development. To determine functional effects of circ_0005232 in OSCC cells, we overexpressed circ_0005232 then repeated the functional experiments, finding that overexpression of circ_0005232 significantly improved proliferation, migration, and invasion ability. These results suggest that circ_0005232 may serve as a cancer-promoting gene that affects biological functions of CAL27 and SCC9 in OSCC, consistent with the results of clinical and pathological data analysis.

Epithelial-to-mesenchymal transformation (EMT) refers to the process by which cells transform from normal epithelial cells to abnormal mesenchymal cells[23, 24]. EMT is a typical event in the development of malignant tumors including OSCC[25]. MMP2 and MMP9 mainly play roles in cell invasion and migration progression[26]. Western blot and qRT-PCR for EMT markers, MMP2, and MMP9 after circ_0005232 knockdown indicated that si-circ_0005232 inhibits EMT progression and MMP family expression, thus inhibiting OSCC progression.

We also examined the TCGA database, but circ_0005232 was not included in the database, so our results could not be confirmed by a larger data set. We used 53 clinical samples to test the expression of

circ_0005232 in OSCC vs AHT. While this is a sizeable sampling, it is insufficient to generalize the expression of circ_0005232 in all of OSCC. Therefore, we hope that future studies can expand the sample size for a more detailed overview of the effects of circ_0005232 on OSCC.

A large amount of evidence has shown that circRNAs can adsorb miRNAs by acting as sponges to affect miRNA activity and regulate expression of their target genes, ultimately promoting or suppressing cancer. This process of regulation is a competitive mechanism[27, 28]. Potential miRNAs targets of circ_0005232 were predicted using Circ-interactom and Circ-bank software. We choose to overlay the results of the two databases then combine it with experimental results. Through dual luciferase assays, we confirmed that circ_0005232 negatively regulates miR-1299. Presently reported studies implicate miR-1299 in non-small cell lung cancer (NSCLC)[29], prostate cancer[30], ovarian cancer[31], liver cancer[32], breast cancer[33], osteosarcoma[34], esophageal squamous cell cancer (ESCC)[35], colon cancer[36], bile duct cancer[37], gastric cancer[38], and renal cell cancer[39]. For example, circ_0006528 could sponge miR-1299 in breast cancer to regulate CDK8 expression[33]. In NSCLC and ESCC, miR-1299 acted as a sponge of EGFR (epidermal growth factor receptor) and negatively regulated the expression of EGFR[29, 35].

Our study is the first to verify that circ_0005232 acts as a sponge of miR-1299 in OSCC. To further verify the relationship between circ_0005232 and miR-1299 in OSCC, we conducted rescue experiments which showed that inh-miR-1299 can restore the influence of si-circ_0005232 on cell biological functions. In addition, protein and RNA expression results were consistent with functional results, verifying that circ_0005232 exerts its biological function in OSCC cells by sponging miR-1299.

We predicted target proteins of miR-1299 by TargetScan and selected CDK6 for further study. CDK6 is encoded by the CDK6 gene, which is a cell division protein kinase regulated by cyclins[40]. Reports in breast cancer[41], liver cancer[42] and osteosarcoma[43] show that miR-1299 acts on CDK6 through sponging to play a role in tumor progression. RIP verified that miR-1299 binds to CDK6 in squamous cell carcinoma cell lines, so we speculated that circ_0005232 might regulate the miR-1299/CDK6 axis in OSCC. Mechanistic experiment results verified that miR-1299 could restore the effects of circ_0005232 on CDK6 RNA and protein expression and that CDK6 could restore the effects of circ_0005232 on proliferation in SCC9 cells. Taken together, our results suggest that circ_0005232 regulates expression of CDK6 and affects the malignant function of OSCC cells through sponging miR-1299.

Conclusions

In conclusion, the results of this study indicate that circ_0005232 is a cancer-promoting gene in OSCC. Circ_0005232 affects biological functions of OSCC through the miR-1299/CDK6 axis, and as such it may be a potential diagnostic marker of OSCC.

Abbreviations

OSCC: oral squamous cell carcinoma; EMT: epithelial-to-mesenchymal transition; CDK: cyclin-dependent kinase; AHT: adjacent healthy tissues; HOK: Human oral keratinocyte; ATCC: American Type Culture Collection; DMEM: Dulbecco's modified Eagle's medium; FBS: fetal bovine serum; RIP: RNA immunoprecipitation; NSCLC: non-small cell lung cancer; ESCC: esophageal squamous cell cancer; EGFR: epidermal growth factor receptor; PBS: phosphate belanced solution; MMP: matrix metallopeptidase; EdU: 5-Ethynyl-2'-deoxyuridine.

Declarations

Ethical Approval and Consent to participate

Not applicable.

Consent for publication

All authors have given approval to the publication of the article.

Availability of data and materials

The data and materials used in the current study are all available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

Founding

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Authors' contributions

Xue Zhang: performed the experiment and wrote the manuscript; Guang-Yu Guo: performed the data analyses；Zhen-Hua Wang: significant contributions to analysis and manuscript review; Zhong-Ti Zhang: significant contributions to the whole article review.

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Table

Table 1. Correlation between clinicopathological factors and circ_0005232 expression levels (ΔCt) in OSCC patients. * means $P < 0.05$.

Characteristics	Cases (n)	Mean ± SD	*p* value
Gender			0.823
Male	41	0.54 ± 0.505	
Female	12	0.5 ± 0.522	
Age			0.167
≤60	20	0.65± 0.489	
>60	33	0.45 ± 0.506	
Smoking			0.614
Yes	43	0.51 ± 0.506	
No	10	0.6 ± 0.516	
Drinking			0.284
Yes	21	0.62± 0.498	
No	32	0.47 ± 0.507	
muscular Invasion			0.728
Yes	31	0.55 ± 0.506	
No	22	0.5 ± 0.512	
TNMstage			**0.003 ***
I-II	31	0.35 ±0.486	
III-IV	22	0.77 ± 0.429	
Lympho node metastasis			**0.037 ***
Positive	28	0.68 ± 0.476	
Negative	25	0.39 ± 0.497	

Figures

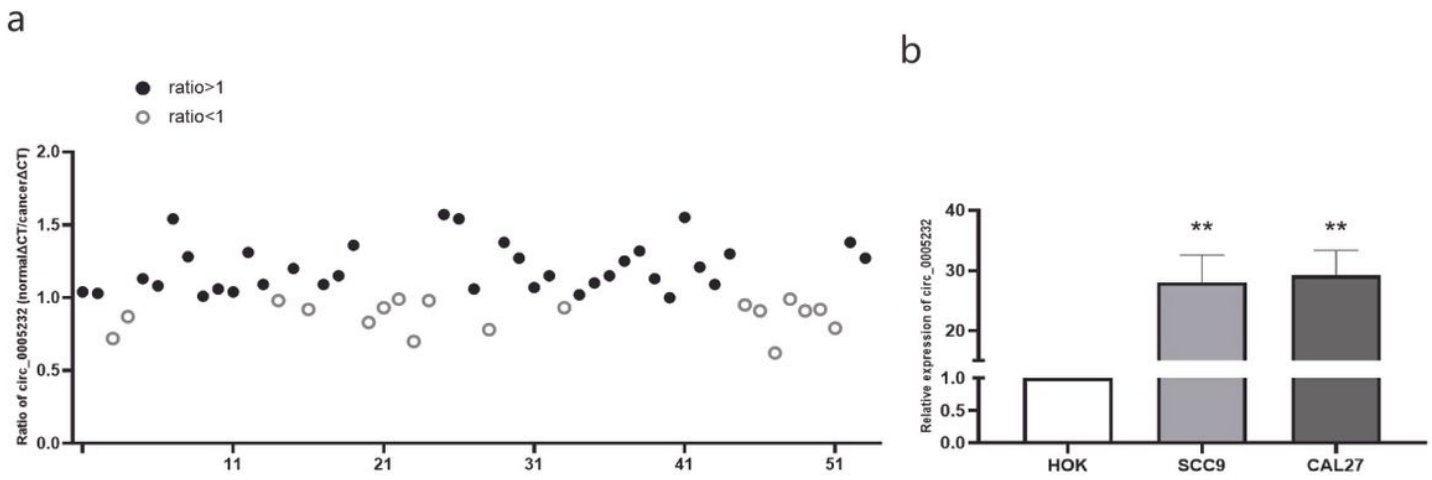

Figure 1

Expression level of circ_0005232 in OSCC tissues and cell lines. a Expression level of circ_0005232 in 53 pairs of OSCC tissues detected by qRT-PCR. b Expression level of circ_0005232 in OSCC cell lines detected by qRT-PCR (n = 3). ** means P < 0.01.

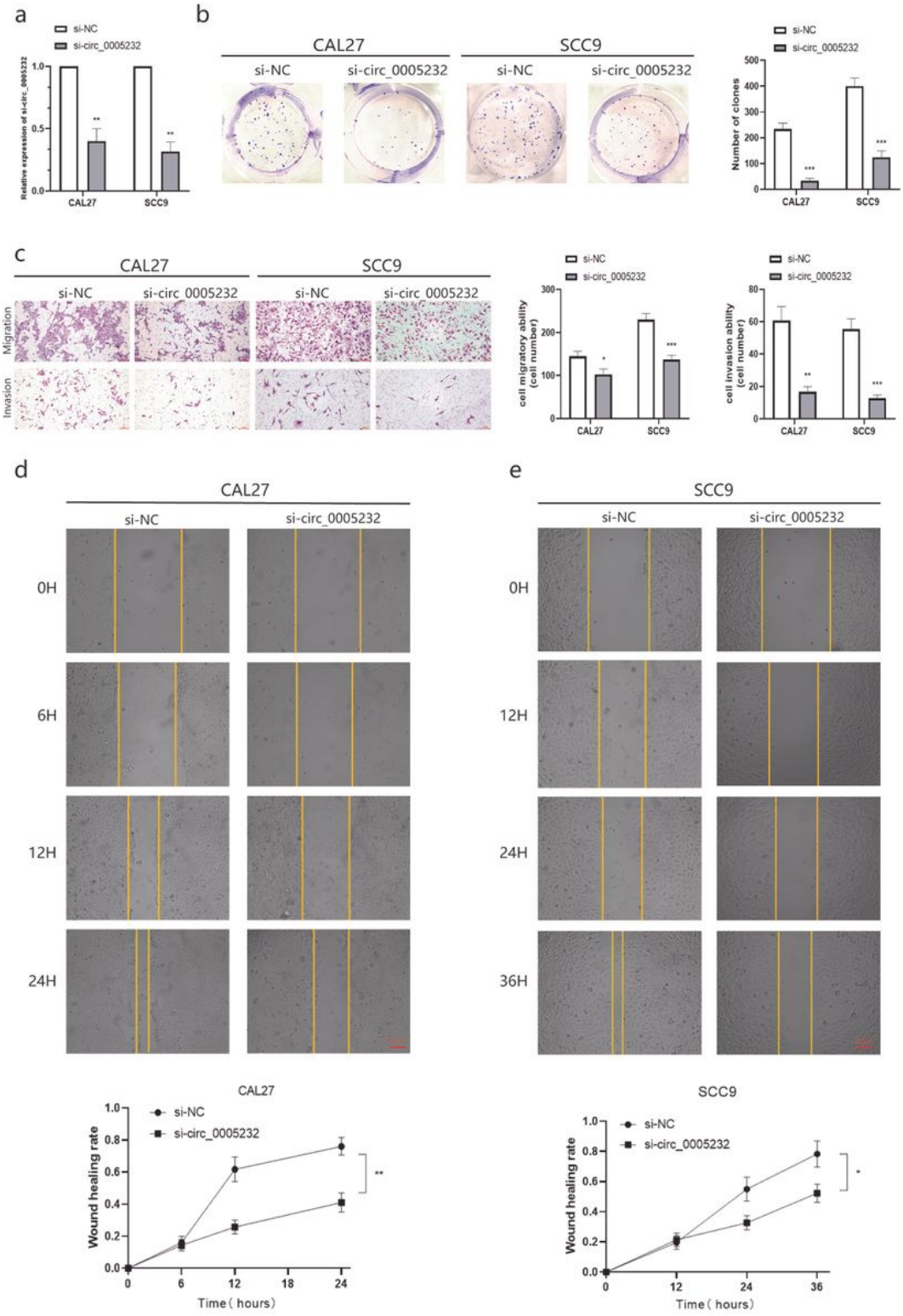

Figure 2

Changes in biological function of squamous cell carcinoma cells after circ_0005232 knockdown. a The knockdown efficiency of circ_0005232 in CAL27 and SCC9 cell lines (n = 3). b Proliferation changes of CAL27 and SCC9 cell lines transfected with si-circ_0005232 as detected by colony formation assay (n = 3). c Migration and invasion changes of CAL27 and SCC9 cell lines transfected with si-circ_0005232 as detected by transwell migration and invasion assay (n = 3). d Scratch healing images and rate at 0h, 6h,

12h, 24h in CAL27 cell lines (n = 3). e Scratch healing images and rate at 0h, 12h, 24h, 36h in SCC9 cell lines (n = 3). * means P < 0.05, ** means P < 0.01, *** means P < 0.001.

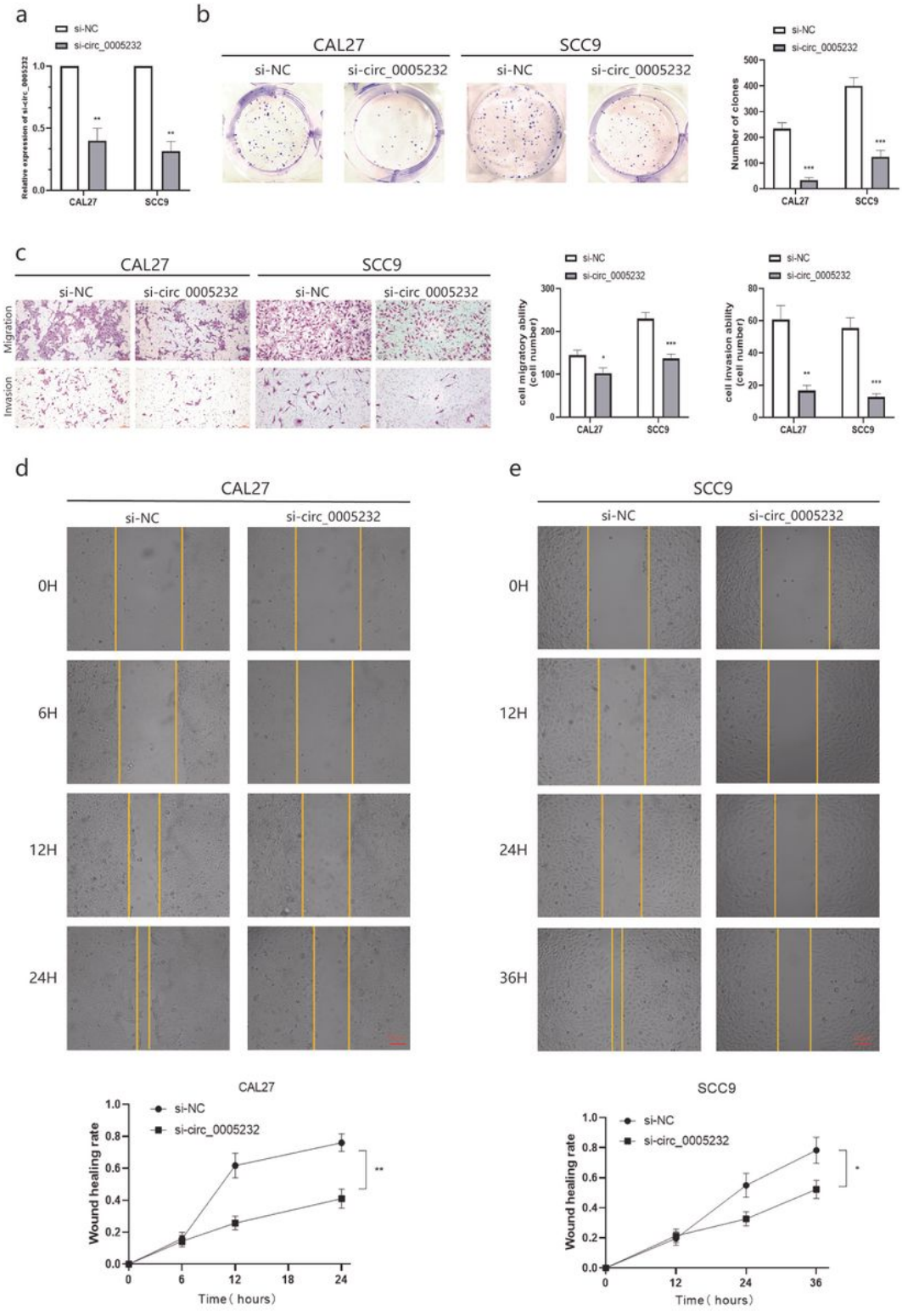

Figure 3

Changes in biological function of squamous cell carcinoma cells after circ_0005232 overexpression. a The overexpression efficiency of circ_0005232 in CAL27 and SCC9 cell lines (n = 3). b Proliferation changes of CAL27 and SCC9 cell lines transfected with circ_0005232 as detected by colony formation

assay (n = 3). c Migration and invasion changes of CAL27 and SCC9 cell lines transfected with circ_0005232 as detected by transwell migration and invasion assay (n = 3). d Scratch healing images and rate at 0h, 6h, 12h, 24h in CAL27 cell lines (n = 3). e Scratch healing images and rate at 0h, 12h, 24h, 36h in SCC9 cell lines (n = 3). ** means P < 0.01, *** means P < 0.001.

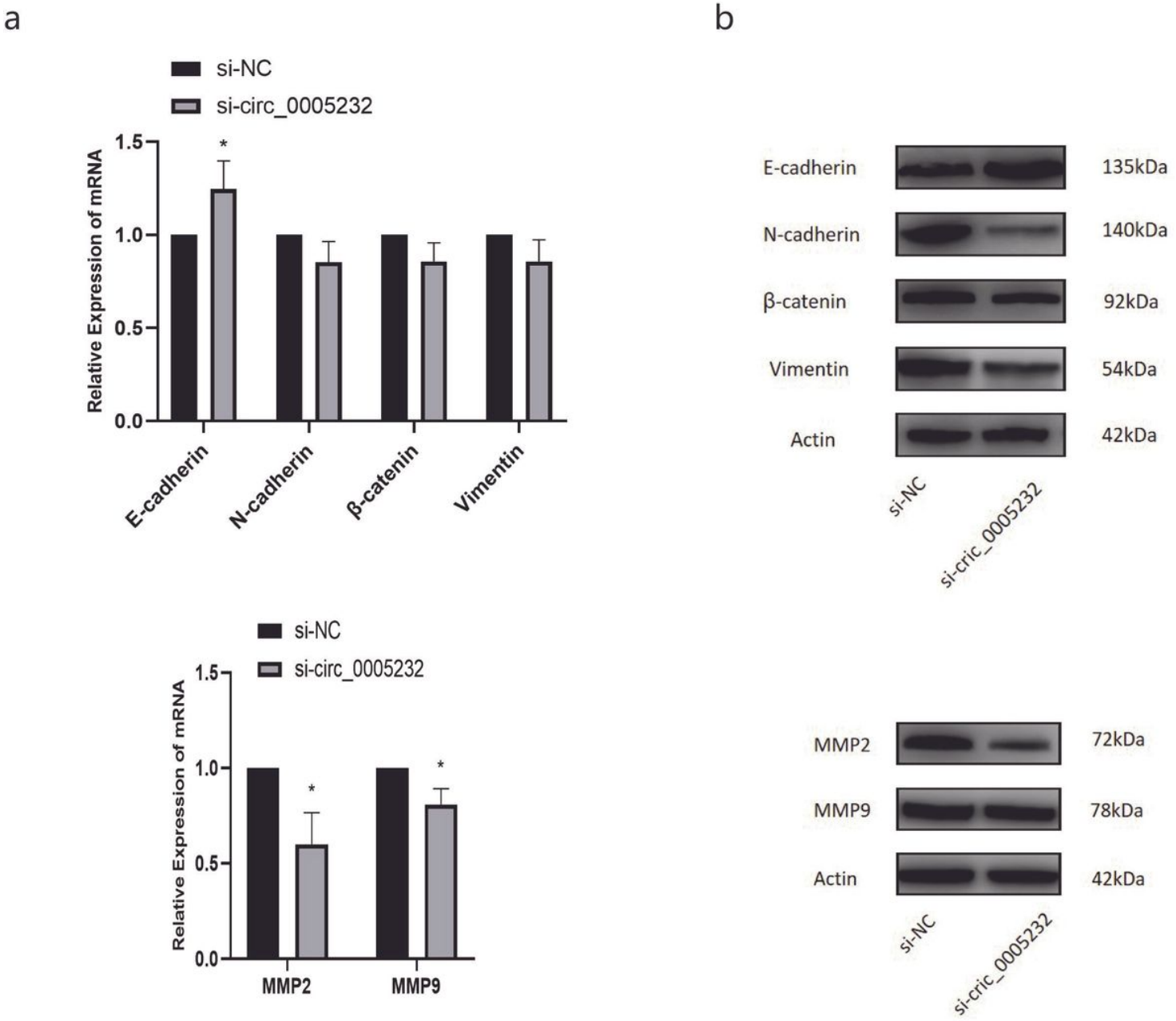

Figure 4

Effect of circ_0005232 on EMT, MMP2, and MMP9 of squamous cell carcinoma cells. a qRT-PCR was used to detect the effect of circ_0005232 knockdown on RNA expression levels of EMT-related markers and MMP family markers in SCC9 cell lines (n = 3). b Western blot was used to detect the effect of circ_0005232 knockdown on protein expression levels of EMT-related markers and MMP family markers in SCC9 cell lines (n = 3). * means P < 0.05.

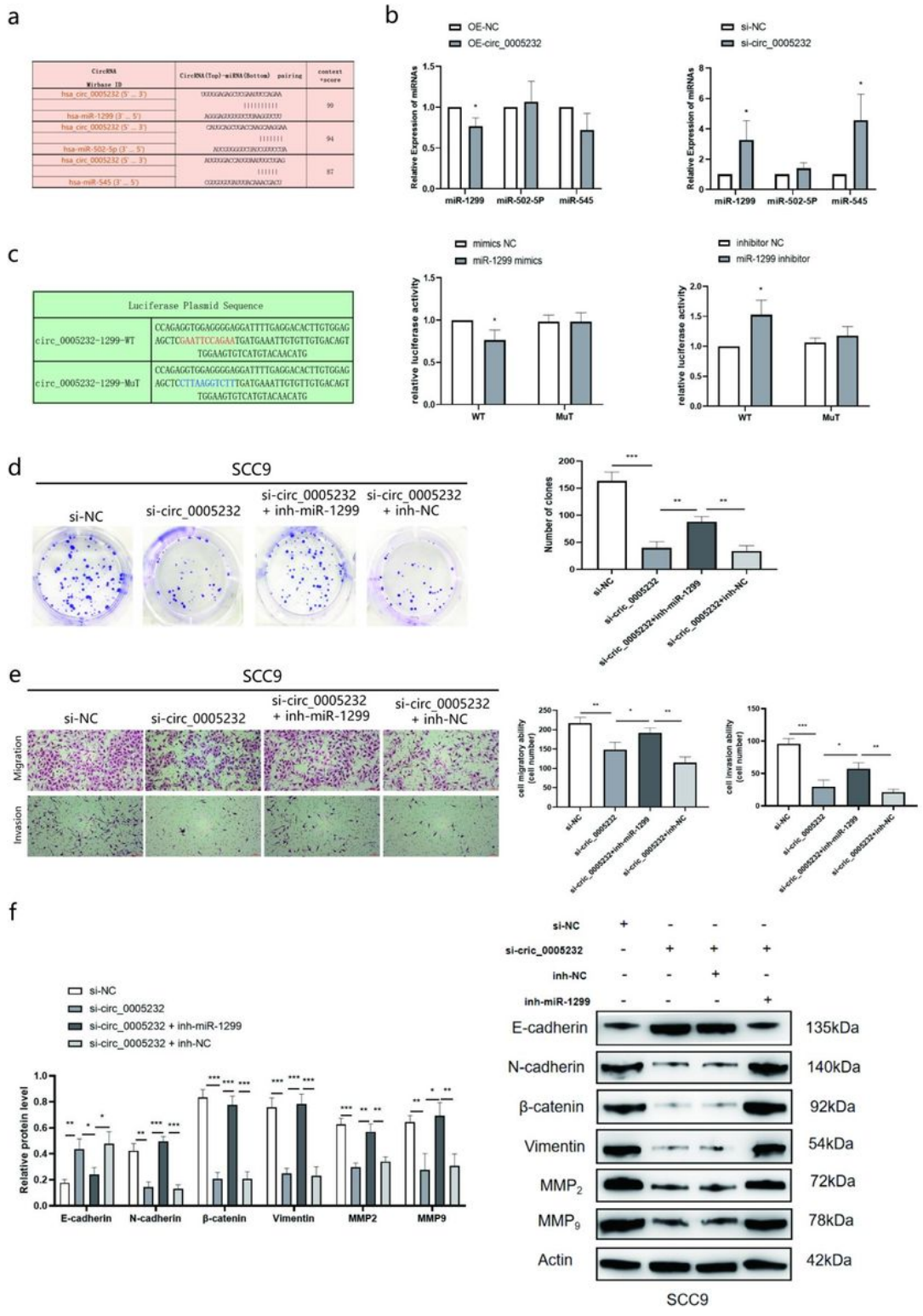

Figure 5

Circ_0005232 affects biological functions of OSCC cell lines by sponging miR-1299. a Potential miRNA binding targets of circ_0005232 predicted by Circ-interactom and Circ-bank. b qRT-PCR to detect differential miRNA expression in SCC9 cell lines after circ_0005232 knockdown or overexpression (n = 3). c A dual-luciferase reporter assay measuring the interaction between circ_0005232 and miR-1299 (n = 3). d Proliferation changes of SCC9 cell lines transfected with si-circ_0005232 and inh-miR-1299 as detected

by colony formation assay (n = 3). e Migration and invasion changes of SCC9 cell lines transfected with si-circ_0005232 and inh-miR-1299 as detected by transwell migration and invasion assay (n = 3). f Changes in the RNA and protein expression levels of EMT-related markers and MMP family markers by transfection with si-circ_0005232 and inh-miR-1299 as detected by Western blot (n = 3). * means P < 0.05, ** means P < 0.01, *** means P < 0.001.

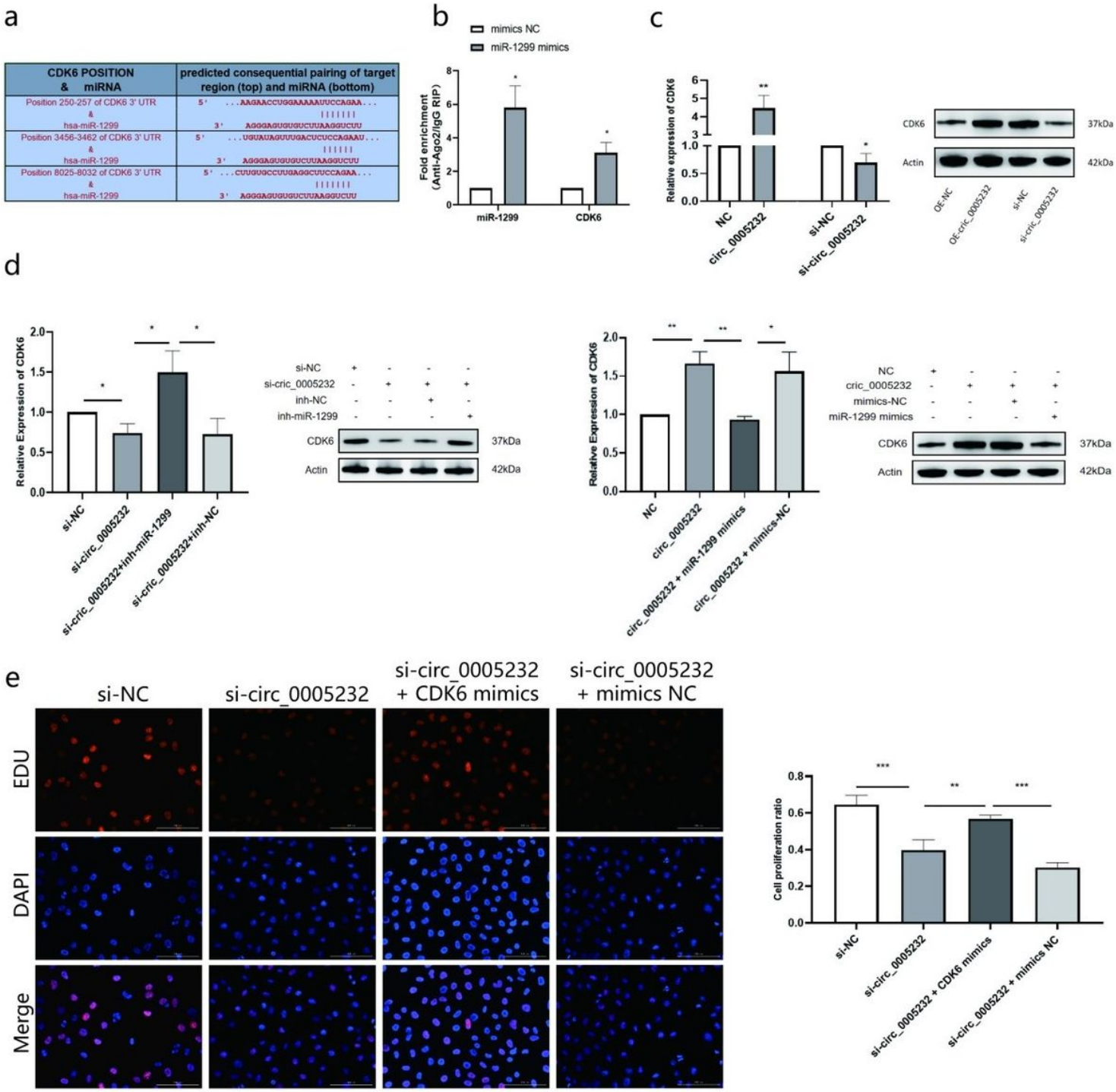

Figure 6

Circ_0005232 affects progression of OSCC cell lines through the miR-1299/CDK6 axis. a Potential binding sites between miR-1299 and CDK6 predicted via the TargetScan database. b qRT-PCR to test the

RIP results of direct binding between circ_0005232 and miR-1299 (n = 3). c qRT-PCR and Western blot results show that CDK6 is negatively regulated by circ_0005232 (n = 3). d miR-1299 restored the effects of circ_0005232 on CDK6 RNA and protein expression (n = 3). e CDK6 restored the effects of circ_0005232 on proliferation of squamous cell carcinoma SCC9 cell lines (n = 3). * means $P < 0.05$, ** means $P < 0.01$, *** means $P < 0.001$.