LncRNA HOXA-AS2 promotes tumor progression via suppressing miR- 567 expression in Oral Squamous Cell Carcinoma

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LncRNA HOXA-AS2 promotes tumor progression via suppressing miR-567 expression in Oral Squamous Cell Carcinoma

Running title: LncRNA HOXA-AS2 promotes tumor progression in OSCC

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Abstract:

Growing evidence shows that long non-coding RNAs (lncRNAs), such as lncRNA HOXA-AS2, have emerged as critical regulators in human cancer. However, the biological function and detail regulating mechanisms that how lncRNA HOXA-AS2 played in oral squamous cell carcinoma (OSCC) remains unexplored. In this study, high expression of lncRNA HOXA-AS2 was determined in OSCC cell lines and clinical tissues, which positively correlated with advanced TNM stage and poor survival of OSCC patients. In mechanism, we found that lncRNA HOXA-AS2 negatively regulated miR-567 expression by direct interaction. Additionally, we also found that the expression of miR-567 inversely decreased in OSCC tissues along with the up-regulation of lncRNA HOXA-AS2. Functionally, overexpression of lncRNA HOXA-AS2 significantly promoted OSCC cell proliferation, while knockdown of lncRNA HOXA-AS2 significantly inhibited it. Additionally, we determined that miR-567 targeted to CDK8 directly at the 3' UTR. In conclusion, lncRNA HOXA-AS2 was up-rugulated in OSCC, correlated with poor clinical outcomes, and promoted OSCC cell proliferation via sponging miR-567 to promote CDK8 expression. Therefore, the potential prognostic value of lncRNA HOXA-AS2 could be explored further.

Keywords: LncRNA HOXA-AS2, MiR-567, CDK8, Oral Squamous Cell Carcinoma, tumor progression

Introduction:

Oral squamous cell carcinoma (OSCC), a most prevalent type of head and neck cancer, is an aggressive malignant cancer, which often leads to tumor invasion and distance metastasis [1]. The 5-year survival rate of OSCC patients remains at about 60% and has not been improved obviously in recent decades[2]. Effective molecule changes are helpful to tumor prognosis and targeted therapy. Therefore, to identify more molecule changes in OSCC and explore their regulated mechanism is an urgent need for further understanding the tumor progress and metastasis.

With the rapid development of second generation RNA sequencing, more and more non-coding RNAs were recognized to play significant roles in tumor biogenesis and progress. Long non-coding RNA (lncRNA) are a kind of RNA non-coding RNA, whose length are more function than 200 nucleotides, and participated in cell all kinds of process through multiple mechanisms including direct gene regulation and functioning as competing endogenous RNA through interacting with miRNAs[3]. MiRNAs are another type of non-coding RNA, whose length is often 19-22 nucleotides, down-regulating gene expression at post-transcriptionally level via binding to the 3'-untranslated region (UTR) of targeted genes with seed region perfect match[4]. Recently, both dysregulated miRNAs and lncRNAs were reported to join in gene regulating network, such an lncRNA-miRNA-mRNA axis, and closely related with tumor growth and development[5, 6].

LncRNA HOXA-AS2 had been found to be up-regulated and promoted tumor progress in cancers such as non-small cell lung cancer, prostate cancer, and so on. [7, 8]. LncRNA HOXA-AS2 was reported to be regulating gene expression via lncRNA-miRNA-mRNA axis. TJ Cui et al [9] discovered that lncRNA HOXA-AS2 promoted non-small cell lung cancer cell growth and metastasis via sponging miR-520a-3p. What's more, lncRNA HOXA-AS2 was also reported to interact with several other miRNAs, such as miR-520c-3p,miR-124-3p, and miR-145-3p [10-12]. Despite of these achieved advances, how does lncRNA HOXA-AS2 participate in OSCC remained unclear？

In this study, we first determined that lncRNA HOXA-AS2 was high expressed in OSCC and its high expression was associated with OSCC tumor progression in clinical. In mechanism, we found that lncRNA HOXA-AS2 promtoted tumr progress via lncRNA HOXA-AS2-miR-567 -CDK8 axis.

Materials and methods

Cell culture and chenmicals

The OSCC cell lines, such as NHOK, TSCCA, Cal-27, Tca8113, SCC-9, and HEK293 were purchased from the American Type Culture Collection in China (ATCC). All cells were maintained as instruction. Without special notes, the cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS, Gemini Bio-Products), containing penicillin and streptomycin, as described before[13-15]. The chemicals were purchased from Sigma-Aldrich or Thermo Fisher Scientific. All of the DNA oligonucleotides were synthesized from BGI Genomics in China. The oligolists were listed in the **Supplement Data.1**.

Clinical samples

The ethical consent on clinical patients' participation was approved by the Committee for Ethical Review of Research Involving Human Subjects of The First Affiliated Hospital of Chongqing Medical University according to the International Ethical Guidelines for Biomedical Research Involving Human Subjects (CIOMS). The written informed consent was provided by all patients participated. Human OSCC samples and adjacent tissues were collected by surgical doctors from the Department of Oral and Maxillofacial Surgery at The First Affiliated Hospital of Chongqing Medical University. Pathological diagnostics for OSCC were determined by three pathologists. The clinical characteristics in 46 OSCC patients are presented in the **Table 1**.

Total RNA extraction from cells and tissues

The cultured cell total RNA was extracted with NucleoZOL RNA Isolation kits (Takara Bio USA, Mountain View, CA) according to the manufacturer's instructions as described[16-18].

The tissues were grinded in the autoclaved grinding bowl with liquid nitrogen. The tissues were then treated as the cultured cells, following the cell total RNA extraction protocol. Briefly, 20 mg of clinical tissues was grinded with the liquid nitrogen and lysed with 500 μL NucleoZOL for 60 mins at 4°C. 200 μL RNase free ddH2O was added to the lysate and vortexed vigorously. Then, 500 μL of the supernatant was pipetted and precipitated with 500 μL isopropanol. After washing with 75% ethanol twice, the total RNA was dissolved in 30 μL RNase free ddH2O.

Reverse transcription and Touchdown-qPCR (TqRCR)

For the interested genes or lncRNA quantification, reverse transcription reactions for the total RNA and TqPCR were performed as described before[14, 19]. All the qPCR primers were designed and optimized by software Primer3 Plus with the same parameters. The SYBR Green mixture (Biomake, Houston, TX) was used for the TqPCR. The reference gene was GAPDH, and the results were normalized to GAPDH using the $2^{-\Delta\Delta Ct}$ method. To assess the miR expression levels, the reverse transcription reactions were carried out by using miR-specific reverse primers with six nucleotides that complemented to 3'end region, preceded with a 44-nt artificial stem-loop sequence[20]. TqPCR was also performed to quantitatively assess the miR expression levels. The results for miRs were normalized to 5s RNA by using the $2^{-\Delta\Delta Ct}$ method. The SYBR Green mixture (Bimake, Houston, TX) was used for the TqPCR according to manufacturer's instructions.

WST-1 cell proliferation assay

WST-1 assay was performed to detect the cell proliferation ability following the instructed protocol. 3000 cells were pre-seeded in the 96-well plate. The absorbance was detected following the WST-1 Reagent manufacturer's protocol (Sigma-Aldrich) at 0h, 24h, 48h, and 72h. The absorbance valve was used for the cell proliferation curve.

Plasmid construction

The whole sequence of LncRNA HOXA-AS2 was amplified from HEK-293 genomic DNA by High-Fidelity PCR. After digested by the restricted enzyme HindIII and EcoRI, the fragments

were cloned for pSEB-LncHOXA-AS2. The pSEB vector was the control (pSEB-Ctrl). To get the gene silencing expression construct, three siRNA cassettes designed by siDirect Version 2.0 were inserted into the vector pSEB-361-BSG using cassette cloning as described before[8], resulting in pSEB-si- LncHOXA-AS2. The LncHOXA-AS2 wild type (WT) and mutated LncHOXA-AS2 (M1, M2) were cloned into the pSEB-Gluc vector resulting in pSEB-LncHOXA-AS2-WT/M1/M2 by HindIII and EcoRI. The mutated LncHOXA-AS2 (M1, M2) fragments were amplified from WT LncHOXA-AS2 using the site-directed mutagenesis PCR with the mutation sites in the primer. In brief, the spiked primer with a defined mutation was amplified for both side products. These products were purified and mixed at 1:1 (molecular number) for the 2nd step. In the second step, the whole LncHOXA-AS2 was amplified to get the mutated LncHOXA-AS2 (M1, M2) fragments. By the similar step, we constructed pSEB-CDK8-WT and pSEB-CDK8-MUT.

Gene overexpression or silencing

The stable cell line for LncRNA HOXA-AS2 overexpression or silencing expression was established by retrovirus packaging system as described before[8, 15]. Briefly, The plasmid for gene overexpression (such as pSEB-LncHOXA-AS2 or Control) or gene silencing (pSEB-si-LncHOXA-AS2 or control) were packaged with pSEB-Ampho were cotransfected in 293-PA cells. And the cultured medium was collected every 12 hours at Day 2, and then the medium were mixed and filtered for targeting cell infection.

Luciferase activity assay

pNRGluc-LncRNA HOXA-AS2-WT or pNRGluc-LncRNA HOXA-AS2-Mut1/2 was used to package into retroviruses using our retrovirus packaging system as described before[15]. After gained the stable cell line, we transiently transfected the cells with 1 μg pSEB-miR-567. After 48 hours, the cell culture medium was refreshed. Another 12 hours later, 100 μL cell culture medium was taken out for Gaussia luciferase assay. BioLux® Gaussia Luciferase Flex Assay Kit was used to detect Gaussia luciferase activity. At the same time, cells were collected for total protein qualification by the Pierce™ BCA Protein Assay Kit, which was used for data normalization. Each experiment was performed in triplicate.

Crystal staining for cell proliferation

$1.0*10^5$ OSCC cells (TSCCA and SCC-9) with LncRNA HOXA-AS2 overexpression or silencing expression were cultured in the 35 mm dishes for five days. For colony forming assay, 1000 OSCC cells grew in the 35 mm dishes for 21 days. To determine cell viability or cloning forming ability, the cells were stained with crystal violet staining solution (containing 4% formalin) in the same way as before[21].

In vivo tumor growth assay

The animal use and care was approved by the Committee for Ethical Review of Research of The First Affiliated Hospital of Chongqing Medical University and done according to the guidelines for the ethical review of laboratory animal welfare National Standard GB/T 35892-2018 issued by People's Republic of China[22]. Meanwhile, the in-vivo study was carried out in compliance with the ARRIVE guidelines 2.0[23]. The TSCCA or SCC-9 cells with LncRNA HOXA-AS2 silencing expression were harvested and re-suspended in sterile PBS (60 μL each injection), and injected subcutaneously into the flanks of athymic BALB/c nude mice (n = 3/group, female, 4-5 week old; 2 × 107 cells per injection site). After four weeks, the mice were sacrificed. Next, the tumor masses were extracted out, and their weight, length and width were measured. Tumor size was calculated with the formula, volume = length × width2/2.

Statistical analysis

All quantitative studies were carried out in triplicate. The Pearson correlation was analyzed by GraphPad Prism 6.0. A *P*-value < 0.05 was defined as statistically significant.

Results

The expression of LncRNA HOXA-AS2 and miR-567 in OSCC Clinical Tissue Samples and Cell Lines.

First, we detected LncRNA HOXA-AS2 expression in 46 pairs of OSCC and their adjacent normal tissues by qRT-PCR. The LncRNA HOXA-AS2 was highly expressed in OSCC tissues

compared to the adjacent normal tissues ($P < 0.01$, **Figure 1A**). To further analyze the correlation between LncRNA HOXA-AS2 expression and clinical characteristics of OSCC, we found that the high expression status of LncRNA HOXA-AS2 was positively related with TNM stage and Lymph node metastasis ($P < 0.05$, **Table 1**). Interestingly, we found that miR-567 was down-regulated in above 46 paired OSCC tissues. ($P < 0.05$**; Figure 1B**) To further analyze their expression correlation, we found that LncRNA HOXA-AS2 was negatively correlated with miR-567 with a Pearson correlation of r=-0.3164 ($P < 0.05$, **Figure 1C**). Further, the expression pattern of LncRNA HOXA-AS2 and miR-567 in OSCC cells was examined and the results showed that LncRNA HOXA-AS2 expression in OSCC cell lines, like TSCCA, Cal-27, Tca8113,and SCC-9 cells, was higher than that in normal human oral keratinocyte cell line (NHOK) cell lines (**Figure 1(D)**); while the miR-567 was down regulated in these cell lines (**Figure 1(E)**). These results suggested that lncRNA HOXA-AS2 was elevated in OSCC, which might be involved in OSCC development via miR-567.

LncRNA HOXA-AS2 negatively regulated miR-567 expression by direct targeting.

Through bioinformatics analysis, we found that lncRNA HOXA-AS2 contained the potential binding site of miR-567 in the seed region (**Figure 2A**). To validate this prediction, we built two constructs with the miR-567 seed region binding sites mutated into the Gaussia Luciferase Reporter (**Figure 2B**). The luciferase reporter assay results showed that the relative luciferase activities in lncRNA HOXA-AS2-WT and miR-567 co-transfected OSCC cells were greatly inhibited compared to lncRNA HOXA-AS2-WT and miR-NC co-transfected group, while the luciferase activities were not affected in lncRNA HOXA-AS2-MUT1/lncRNA HOXA-AS2-MUT2 and miR-567/miR-NC co-transfected OSCC cells (**Figure 2C**). Further, with lncRNA HOXA-AS2-WT overexpressed, the expression of miR-567 was down-regulated, however, the lncRNA HOXA-AS2-MUT1/lncRNA HOXA-AS2-MUT2 did not change the expression level of miR-567(**Figure 2D**).These results indicated that lncRNA HOXA-AS2 could regulate the expression of miR-567 via direct targeting.

Cell proliferation was promoted in lncRNA HOXA-AS2 overexpressed OSCC cell lines.

To validate above hypothesis, we constructed lncRNA HOXA-AS2 stably expressed cell lines in TSCCA and SCC-9 cells through overexpressing pSEB-lncRNA-HOXA-AS2. Compared with

the vector control, miR-567 was down regulated in both TSCCA and SCC-9 cells with lncRNA HOXA-AS2 overexpressed (**Figure 3A; Figure 3B**). To better observe the proliferation of cells, we planted 10^5 OSCC cells (TSCCA and SCC-9) with or without lncRNA HOXA-AS2 overexpressed in the 35 mm dishes. Five days later, the cells were stained with crystal violet staining buffer. The results showed that the cells were growing faster in the cells with lncRNA HOXA-AS2 overexpressed compared to the control group (**Figure 3C**). The WST-1 results were consistent with the cell crystal staining (**Figure 3D**). These data indicated that overexpression of lncRNA HOXA-AS2 promoted OSCC proliferation via negatively regulating miR-567.

Cell proliferation was suppressed in si-lncRNA HOXA-AS2 transfected OSCC cell lines.

To further confirm the functional roles of lncRNA HOXA-AS2 in OSCC development, we silenced lncRNA HOXA-AS2 expression using siRNA expressed by our siRNA retrovirus expression system that was described in methods. Finally, we got the lncRNA HOXA-AS2 silenced stable cell lines TSCCA-si-LncRNA HOXA-AS2 and SCC-9-si-LncRNA HOXA-AS2. In these lncRNA HOXA-AS2 silenced cells, lncRNA HOXA-AS2 was silenced, while miR-567 was up-regulated (**Figure 4A; Figure 4B**). Cell crystal staining experiments and WST-1 proliferation assay both showed that silencing of LncRNA HOXA-AS2 could inhibit cell viability(**Figure 4C; Figure 4D**). These data indicated that silencing LncRNA HOXA-AS2 inhibited OSCC proliferation via regulating miR-567 negatively.

CDK8 was a downstream target of miR-567 in the regulation of OSCC Cell proliferation.

By bioinformatics analysis, we found that miR-567 contained the putative binding sites of CDK8 in the 3' UTR (**Figure 5A**). And luciferase reporter assay was performed to verify this prediction, the data showed that the luciferase activities in CDK8-WT and miR-567 co-transfected SCC-9 and TSCCA cells were grea tly inhibited compared to CDK8-WT and miR-NC co-transfected group, while the luciferase activities we re not affected in CDK8-MUT and miR-567/miR-NC co-transfected SCC-9 and TSCCA cells (**Figure 5B and 5C**). Subsequently, we transfected mimic miR-567, mimic control or blank control into SCC-9 and TSCCA cells to detect the expression of CDK8 in the mRNA level and protein level. The results showed that mimic miR-567 significantly suppressed CDK8 and its protein expression (**Figure 5D and 5E**). The crystal staining experiments and WST-1 proliferation assay showed miR-567 inhibitor significantly i

ncreased CDK8 expression, while miR-567-mimic suppressed the expression of CDK8, compared to t he corresponding controls (**Figure 5F and 5G**). These data revealed that CDK8 was a downstream t arget of miR-567 in OSCC.

Silencing lncRNA HOXA-AS2 inhibited OSCC tumor growth by releasing miR-567 to suppress CDK8 expression.

Previously results showed overexpression or silencing expression of lncRNA HOXA-AS2 increased or decreased OSCC cells proliferation in vitro. Therefore, we examined whether lncRNA HOXA-AS2 influenced OSCC cell tumor growth in vivo. We subcutaneously injected TSCCA-si-LncHOXA-AS2 or SCC-9-si-LncHOXA-AS2 cells into nude mice with the vector control cells and monitored tumor growth daily. After mice sacrificed, and tumor masses were extracted to measure their weight and volume. The results showed that tumors grew slower in si-LncHOXA-AS2 group than in si-ctrl group (**Figure 6A; Figure 6B; Figure 6C**). Additionally, it was observed that miR-567 was up-regulated in the si-LncHOXA-AS2 group (**Figure 6D**), while the expression level of CDK8 was down-regulated in the si-LncHOXA-AS2 group (**Figure 6E**). These in vivo results strongly supported our hypothesis that si-LncHOXA-AS2 inhibited OSCC tumor growth via releasing miR-567 to suppress CDK8 expression.

Discussion

Growing evidence showed that lncRNAs play pivotal roles in gene transcriptional regulation, however, the mechanisms are complex and remain unclear[10-12, 24]. Recently, there are some studies indicated that lncRNA HOXA11-AS was up-regulated in OSCC[25], but the detail function and regulation mechanism of lncRNA HOXA-AS2 in OSCC is unclear. In the present study, we investigated the involvement of lncRNA HOXA-AS2 in OSCC and showed that lncRNA HOXA-AS2 promoted OSCC cell proliferation via sponging miR-567/CDK8. We demonstrated lncRNA HOXA-AS2-miR-567-CDK8-cell proliferation regulation network, which contribute greatly to OSCC tumorigenesis and development.

Tested on several OSCC cell lines, our data showed that lncRNA HOXA-AS2 was high expressed in OSCC cell lines, and high expression of LncRNA HOXA-AS2 positively correlated with cell proliferation. In our clinical data, lncRNA HOXA-AS2 was high expressed in 46 OSCC patients while miR-567 was expressed lowly, which the Pearson correlation r is -0.3164 (P <0.05). Based on

these clues, we proposed our hypothesis that lncRNA HOXA-AS2 promoted OSCC cell proliferation via binding to miR-567 as a miRNA sponge. To test this, we first overexpressed lncRNA HOXA-AS2 in TSCCA and SCC-9 using the retrovirus packaging system. We found that overexpression of lncRNA HOXA-AS2 decreased miR-567 and increased its target gene CDK8; meanwhile, the cell proliferation ability was promoted. When we silenced lncRNA HOXA-AS2, the opposite in vitro results were observed. In addition, to explore the regulation mechanism, bioinformatics ananlysis predicted that there was one potential miR-567 seed region binding sites on lncRNA HOXA-AS2. To validate this prediction, the predicted miR-567 binding sites were mutated and constructed into the Gaussia Luciferase Reporter expression system. In accordance to our prediction, with binding sites mutated, lncRNA HOXA-AS2 failed the ability to inhibit miR-567 expression and promote cell proliferation. Finally, after attained these in vitro indications, we did the xenograft tumor formation assay by injecting the nude mice with OSCC cells subcutaneously. Our in vivo results showed that tumors grew slower in the LncRNA HOXA-AS2 silenced group, which was in accordance to our in vitro data.

In summary, our current findings first demonstrated that LncRNA HOXA-AS2 was high expressed in OSCC, correlated with poor clinical outcomes, and promoted OSCC cell proliferation via sponging miR-567/CDK8. Therefore, LncRNA HOXA-AS2 participates in OSCC tumorigenesis and development, and can be served as a prognostic biomarker.

Declarations

Ethics approval and consent to participate

The animal use and care was approved by the Committee for Ethical Review of Research of The First Affiliated Hospital of Chongqing Medical University and done according to the guidelines for the ethical review of laboratory animal welfare National Standard GB/T 35892-2018 issued by People's Republic of China. The in-vivo animal study was carried out in compliance with the ARRIVE guidelines 2.0, as well. The ethical consent on clinical patients' participation was approved by the Committee for Ethical Review of Research Involving Human Subjects of The First Affiliated Hospital of Chongqing Medical University according to the International Ethical Guidelines for Biomedical Research Involving Human Subjects (CIOMS). The written informed consent was provided by all patients participated.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study available from the corresponding author (Zongyue Zeng) on reasonable request.

Competing interests

The authors declare that they do not have any competing conflicts of interest.

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Authors' Contributions

Rui Chen contributed to writing – original draft, investigation, and formal analysis. Xi Wang contributed to data curation and methodology. Shixian Zhou contributed to resources and software and validation. Zongyue Zeng contributed to conceptualization,funding acquisition, project administration, supervision and writing – review & editing.

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Figures

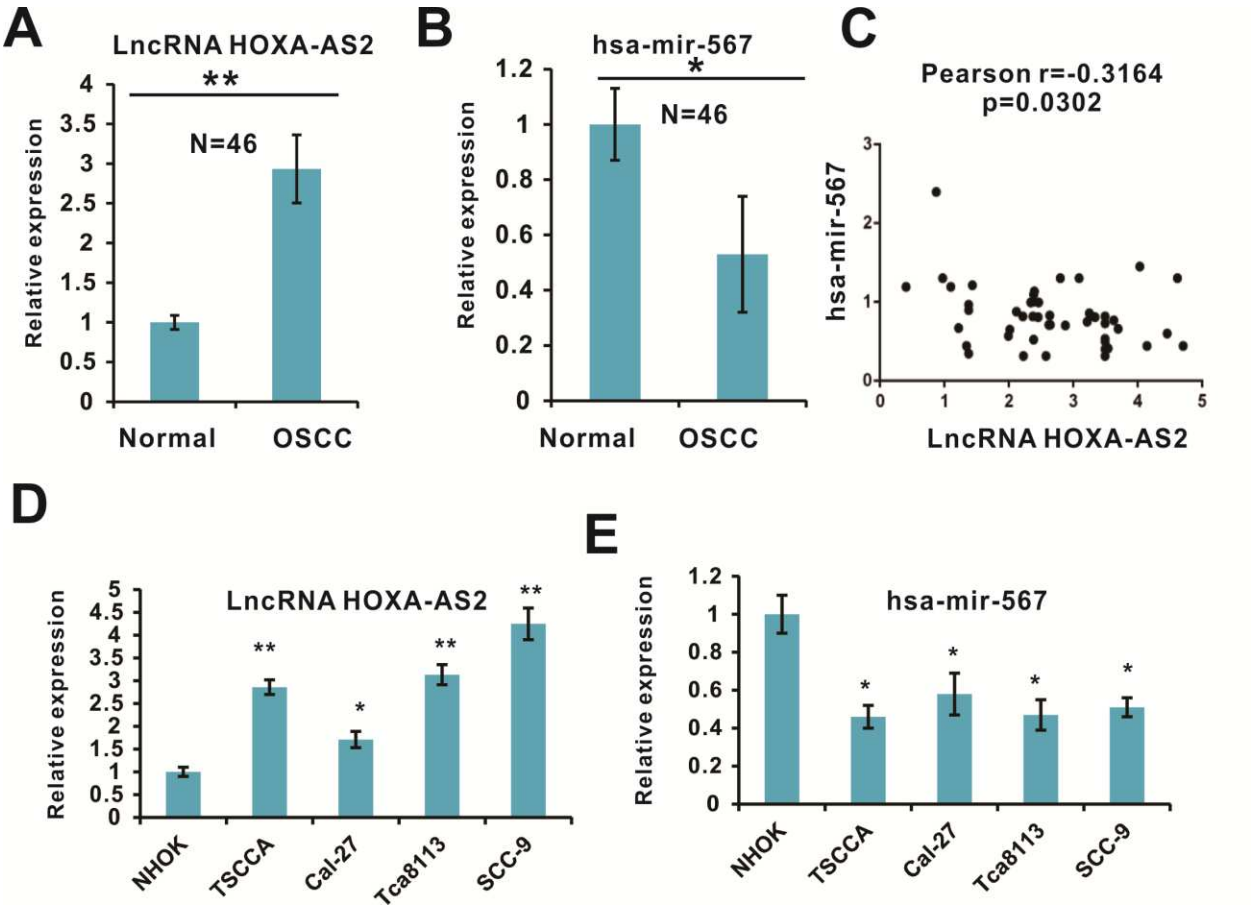


Figure.1 The expression of LncRNA HOXA-AS2 and miR-567 *in OSCC Clinical Tissue Samples and Cell Lines.* (**A**) The expressions of LncRNA HOXA-AS2 in OSCC clinical tissue samples and adjacent normal tissue samples were detected by qRT-PCR. (**B**) The expression of miR-567 in OSCC clinical tissue samples and adjacent normal tissue samples were detected by qRT-PCR. (**C**) A linear relationship between LncRNA HOXA-AS2 and miR-567 expression in OSCC samples were analyzed by GraphPad Prism. (**D**) The expressions of LncRNA HOXA-AS2 in four OSCC cell lines and NHOK normal oral cell line were detected by qRT-PCR. (**E**) The expressions of miR-567 in four OSCC cell lines and NHOK normal oral cell line were detected by qRT-PCR. * P <0.05, ** P<0.01.

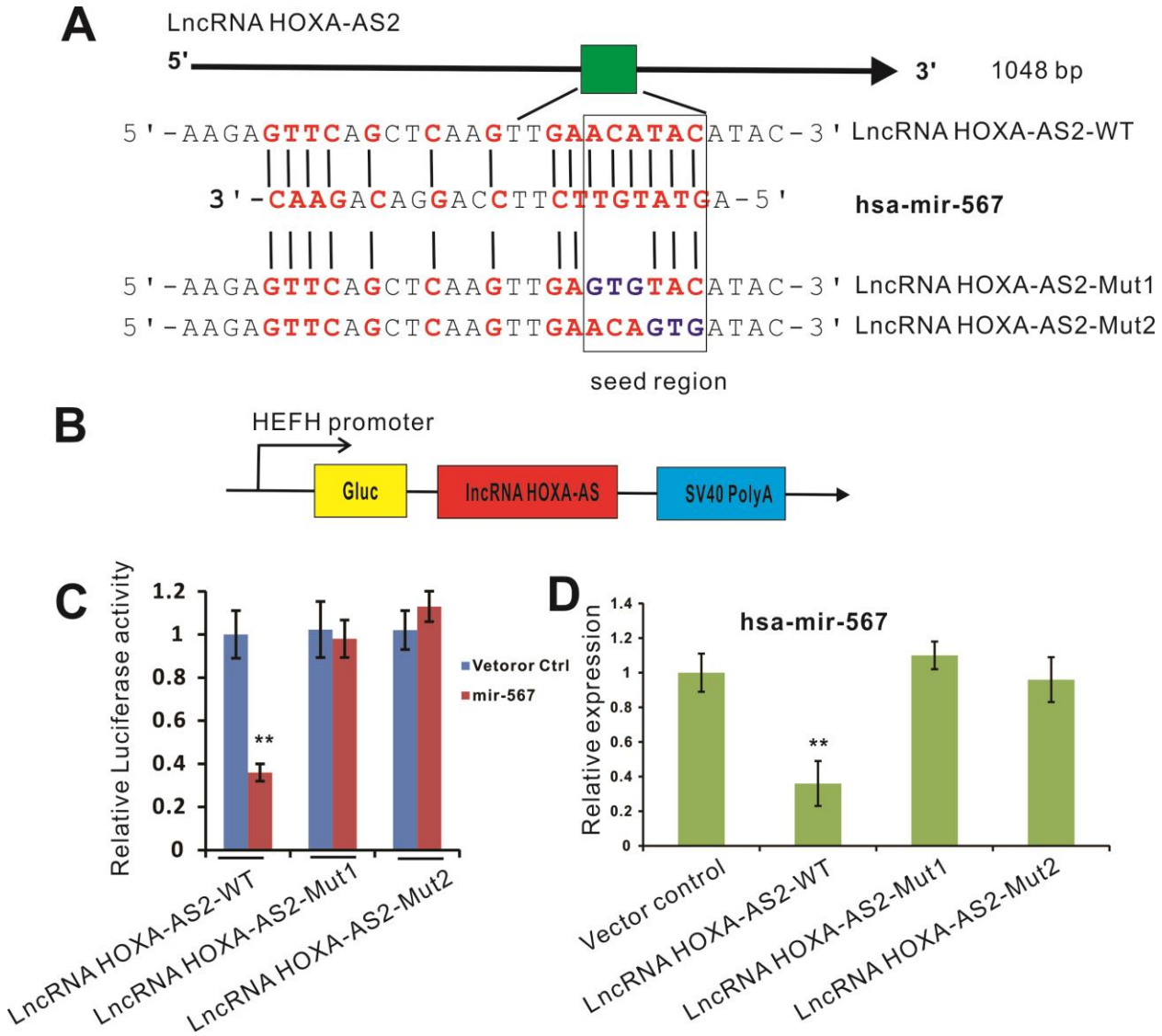


Figure.2 LncRNA HOXA-AS2 negatively regulated miR-567 expression by direct targeting. (**A**) The putative binding sites between LncRNA HOXA-AS2 and miR-567. (**B**) The construction of luciferase reporter expressing LncRNA HOXA-AS2. (**C**) The luciferase activities of LncRNA HOXA-AS2-WT、LncRNA HOXA-AS2-Mut1 or LncRNA HOXA-AS2-Mut2 with or without miR-567 co-transfected in TSCCA OSCC cells were evaluated by luciferase reporter assay. (**D**) The relative expression of miR-567 in LncRNA HOXA-AS2-WT, LncRNA HOXA-AS2-Mut1, LncRNA HOXA-AS2-Mut2 and their corresponding vector controls in TSCCA OSCC cells.

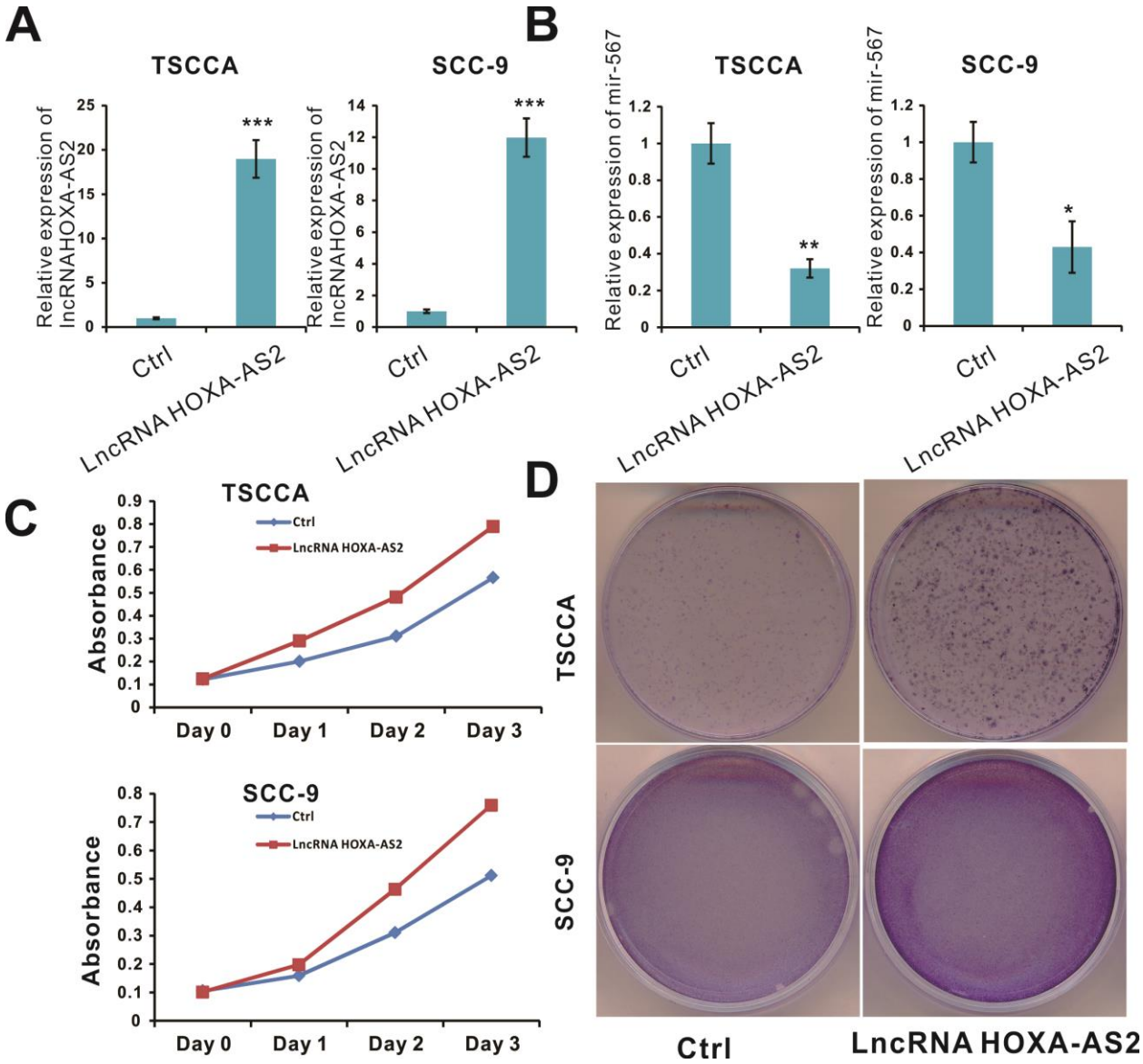


Figure.3 Cell proliferation was promoted in LncRNA HOXA-AS2 overexpressed OSCC cell lines. (**A**) The relative expression of LncRNA HOXA-AS2 in TSCCA and SCC-9 cells with LncRNA HOXA-AS2 transfected was examined by qRT-PCR. (**B**)The relative expression of miR-567 in TSCCA and SCC-9 cells with LncRNA HOXA-AS2 overexpressed. (**C**)& (**D**) Cell proliferation in TSCCA and SCC-9 cells with LncRNA HOXA-AS2 overexpressed was evaluated by WST-1 assay and crystal violet staining.

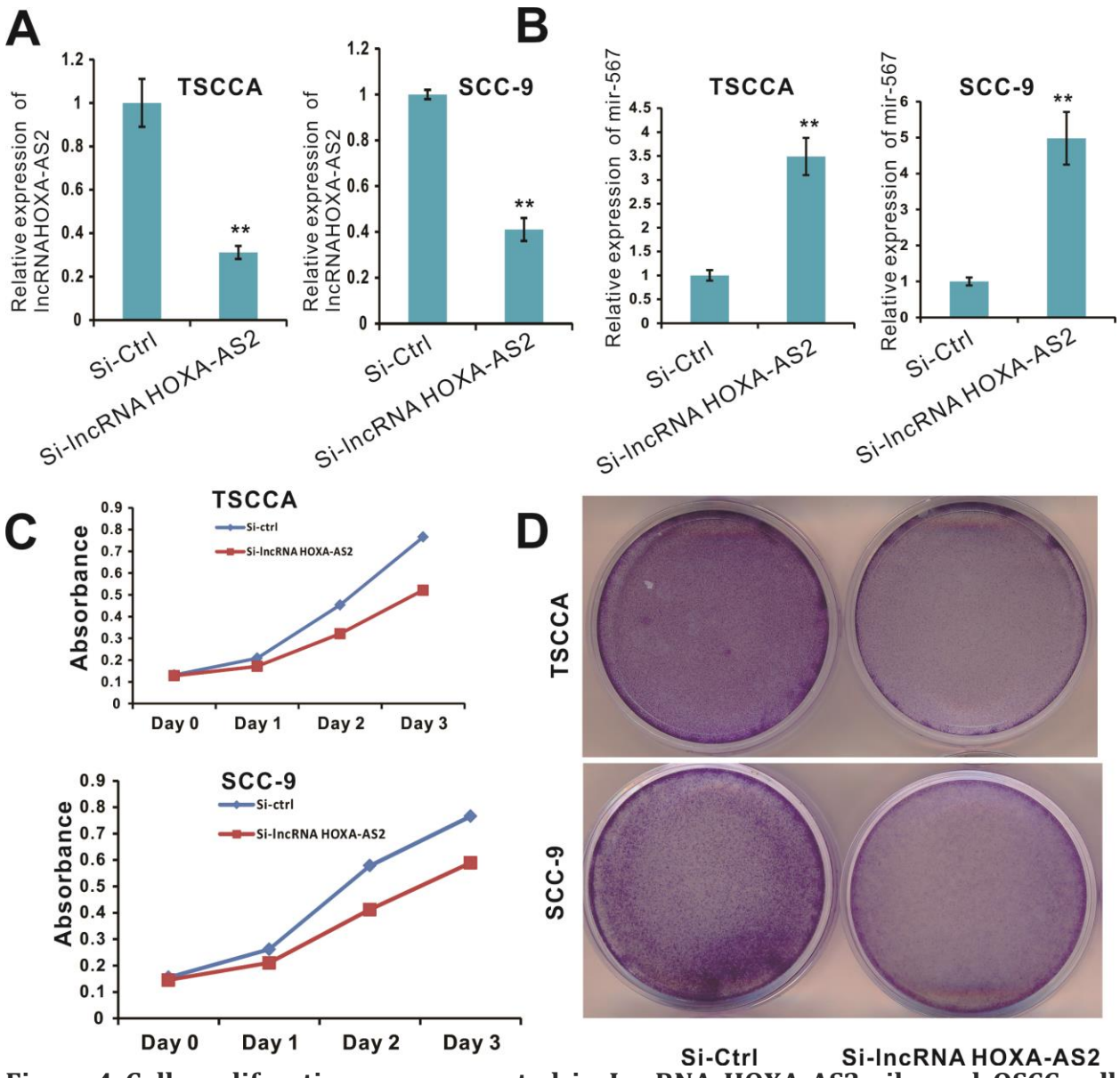


Figure.4 Cell proliferation was promoted in LncRNA HOXA-AS2 silenced OSCC cell lines. (**A**) The relative expression of LncRNA HOXA-AS2 in TSCCA and SCC-9 cells with LncRNA HOXA-AS2 silenced was examined by qRT-PCR. (**B**)The relative expression of miR-567 in TSCCA and SCC-9 cells with LncRNA HOXA-AS2 silenced. (**C**)& (**D**) Cell proliferation in TSCCA and SCC-9 cells with LncRNA HOXA-AS2 silenced was evaluated by WST-1 assay and crystal violet staining.

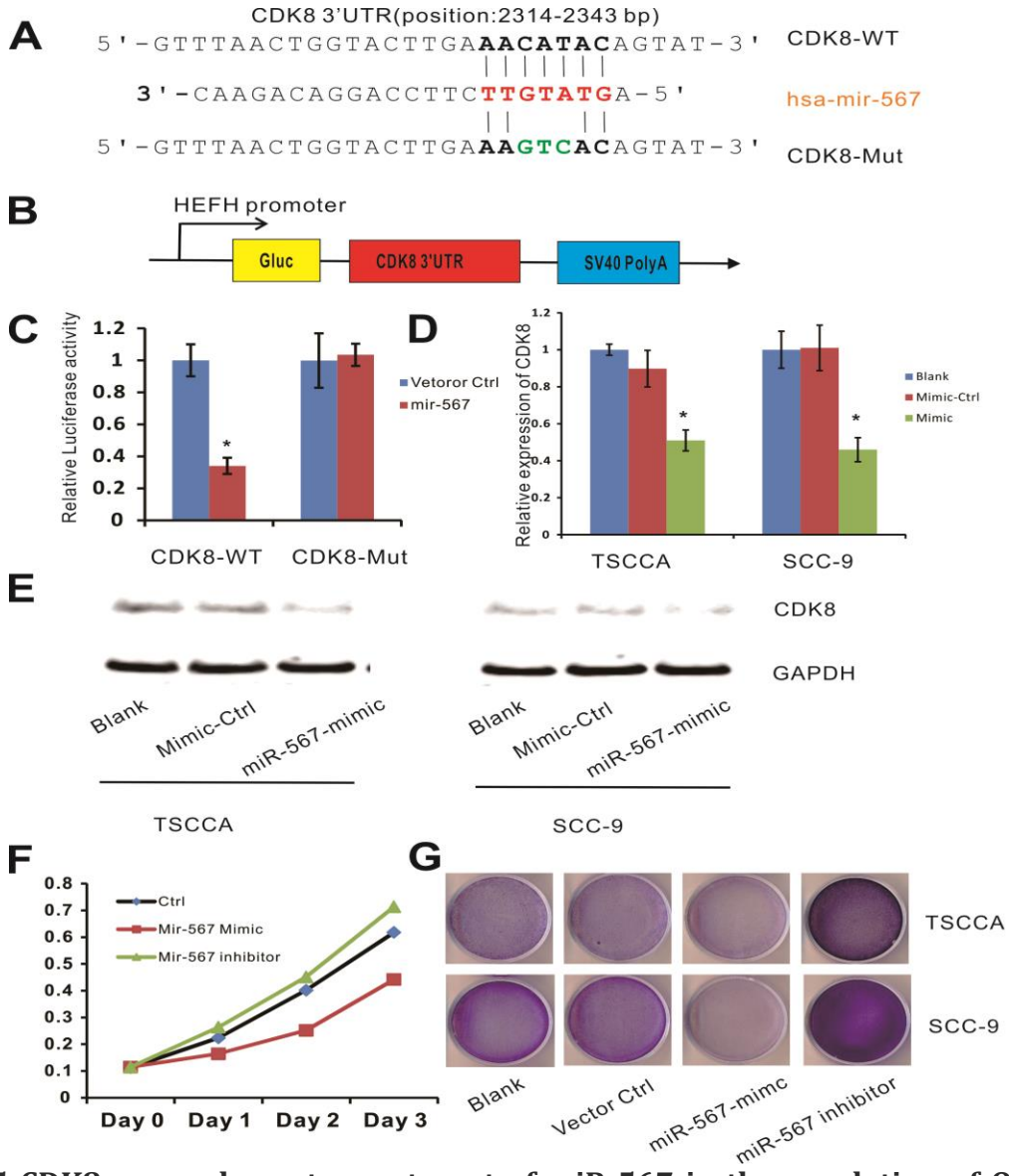


Figure.5 CDK8 was a downstream target of miR-567 in the regulation of OSCC Cell proliferation. (**A**) Bioinformatics analysis indicated the putative binding sites and corresponding mutant region for CDK8 within miR-567. (**B**) The construction of luciferase reporter that expresses CDK8. (**C**) The luciferase activities in CDK8-WT or CDK8-Mut and miR-567 or miR-Ctrl co-transfected OSCC cells were evaluated by luciferase reporter assay. (**D**) The relative expression of CDK8 in miR-567-mimic, Mimic-Ctrl and Blank treated OSCC cells. (**E**) The protein level of CDK8 in miR-567-mimic, Mimic-Ctrl and Blank treated OSCC cells.(GAPDH as control) (**F**)& (**G**) Cell proliferation in miR-567-mimic, miR-567-inhibitor and their corresponding controls treated OSCC cells were evaluated by WST-1 assay and crystal violet staining.

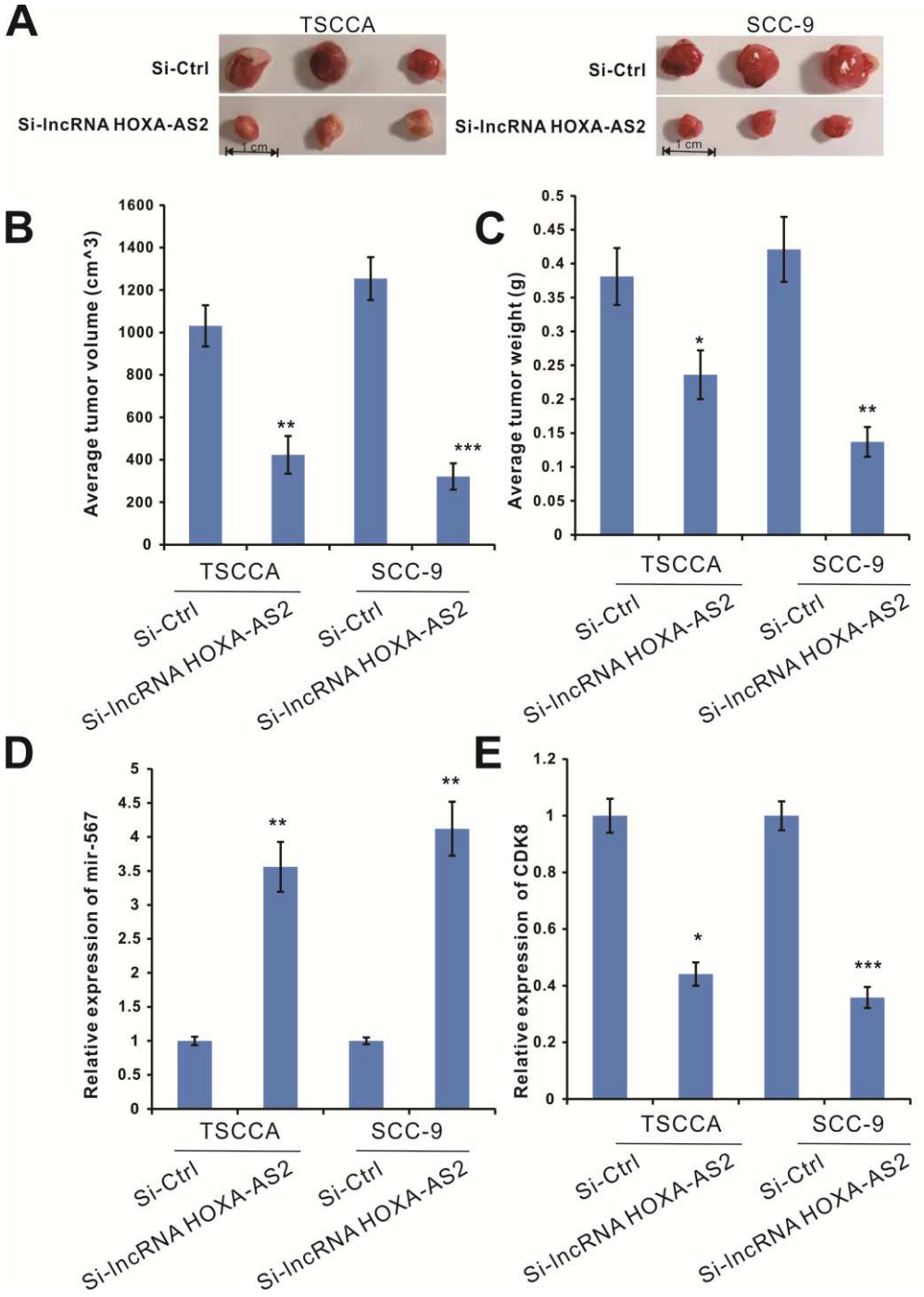


Figure.6 Silencing of LncRNA HOXA-AS2 inhibited OSCC tumor growth by releasing miR-567 to inhibit the expression of CDK8. TSCCA and SCC-9 cells with si-LncRNA HOXA-AS2 or its negative control were injected into nude mice to establish xenograft mice model, respectively. (**A**) Images of tumor nodes. (**B**)& (**C**) Tumor volume and tumor weight were analyzed. (**D**) miR-567 expression in tumor tissues. (**E**) CDK8 mRNA expression in tumor tissues.

Table.1 Correlation between LncRNA HOXA-AS2 expression and clinical characteristics of OSCC.

Characteristics	Number	LncRNA HOXA-AS2 expression		Chi-square valve	P-valve
		Low	High		
Age(years)					
<=55	20	12	8	0.456	0.5
>50	26	13	13		
Gender					
Male	28	15	13	0.365	0.546
Female	18	8	10		
Smoking status					
No	27	13	14	0.09	0.765
Yes	19	10	9		
Location					
Tongue	32	15	17	0.35	0.84
Cheek	9	4	5		
Gingiva	5	3	2		
TNM stage					
I+II	21	14	7	8.455	**0.004**
III+IV	25	6	19		
Differentiation					
low	22	8	14	3.577	0.059
Moderate and high	24	16	9		
Lymph node metastasis					
Yes	17	5	12	4.572	**0.032**
No	29	18	11		

Figures

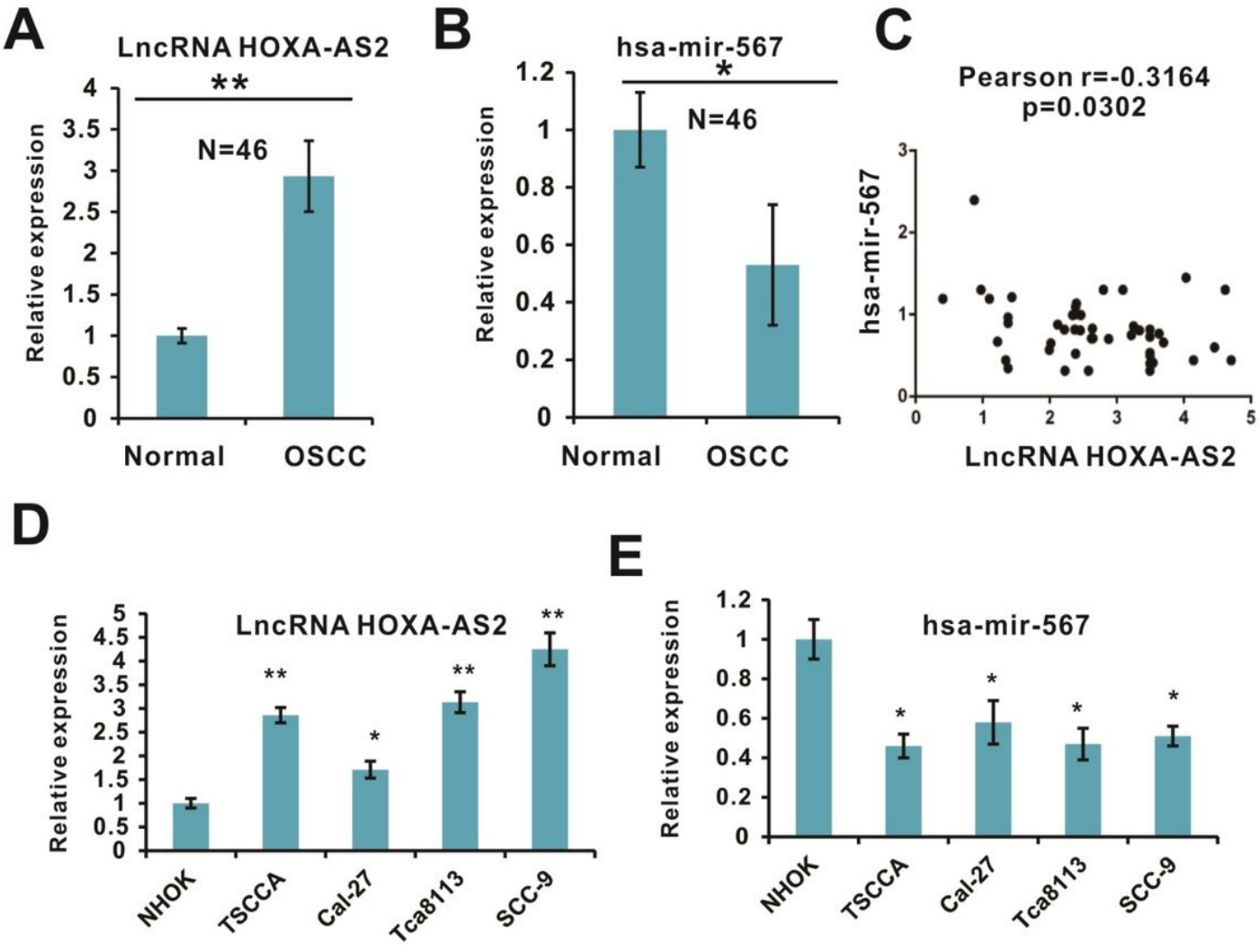


Figure 1

The expression of LncRNA HOXA-AS2 and miR-567 in OSCC Clinical Tissue Samples and Cell Lines. (A) The expressions of LncRNA HOXA-AS2 in OSCC clinical tissue samples and adjacent normal tissue samples were detected by qRT-PCR. (B) The expression of miR-567 in OSCC clinical tissue samples and adjacent normal tissue samples were detected by qRT-PCR. (C) A linear relationship between LncRNA HOXA-AS2 and miR-567 expression in OSCC samples were analyzed by GraphPad Prism. (D) The expressions of LncRNA HOXA-AS2 in four OSCC cell lines and NHOK normal oral cell line were detected by qRT-PCR. (E) The expressions of miR-567 in four OSCC cell lines and NHOK normal oral cell line were detected by qRT-PCR. * P <0.05, **P<0.01.

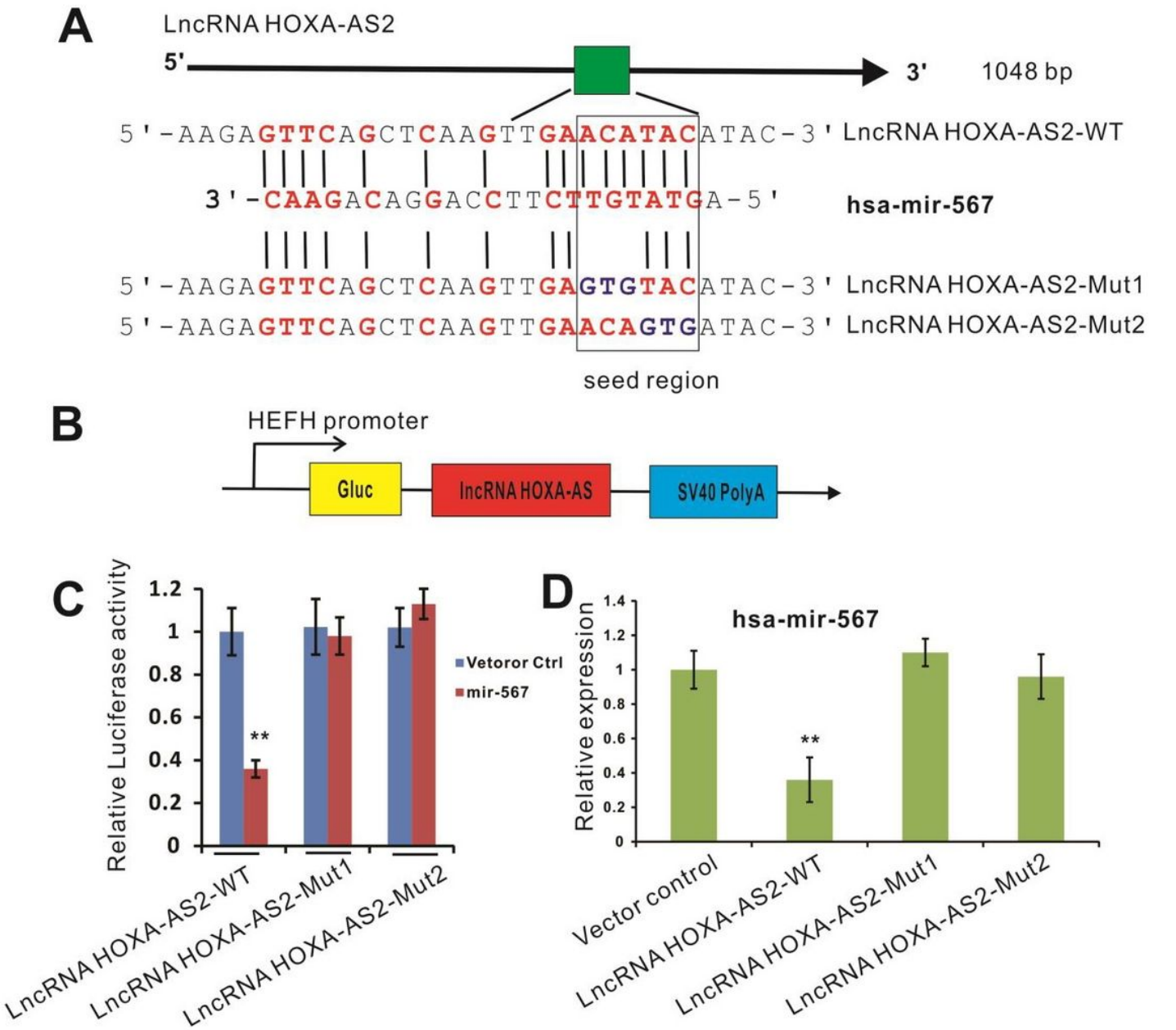
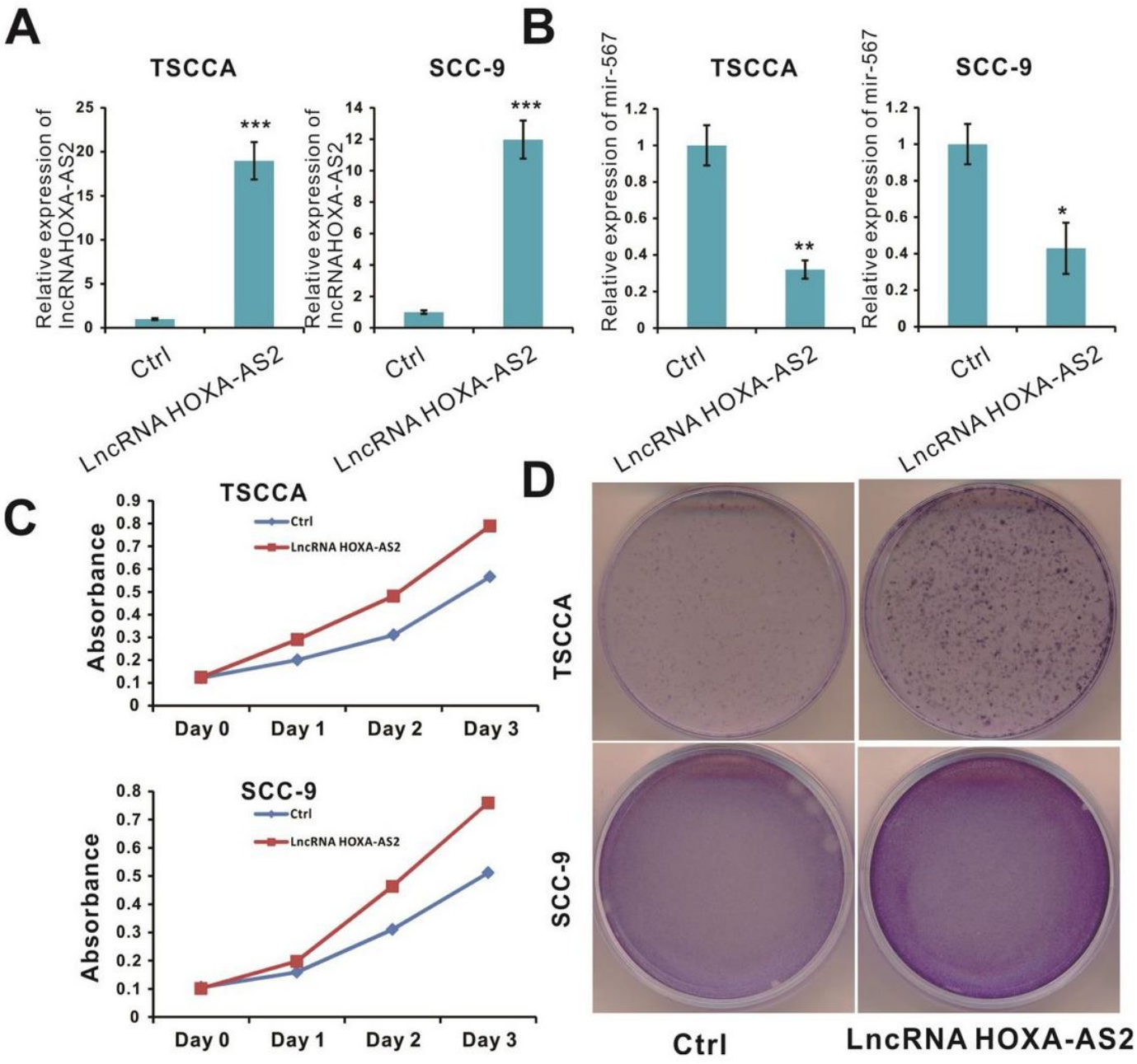


Figure 2

LncRNA HOXA-AS2 negatively regulated miR-567 expression by direct targeting. (A) The putative binding sites between LncRNA HOXA-AS2 and miR-567. (B) The construction of luciferase reporter expressing LncRNA HOXA-AS2. (C) The luciferase activities of LncRNA HOXA-AS2-WT□LncRNA HOXA-AS2-Mut1 or LncRNA HOXA-AS2-Mut2 with or without miR-567 co-transfected in TSCCA OSCC cells were evaluated by luciferase reporter assay. (D) The relative expression of miR-567 in LncRNA HOXA-AS2-WT, LncRNA HOXA-AS2-Mut1, LncRNA HOXA-AS2-Mut2 and their corresponding vector controls in TSCCA OSCC cells.



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Cell proliferation was promoted in LncRNA HOXA-AS2 overexpressed OSCC cell lines. (A) The relative expression of LncRNA HOXA-AS2 in TSCCA and SCC-9 cells with LncRNA HOXA-AS2 transfected was examined by qRT-PCR. (B)The relative expression of miR-567 in TSCCA and SCC-9 cells with LncRNA HOXA-AS2 overexpressed. (C)& (D) Cell proliferation in TSCCA and SCC-9 cells with LncRNA HOXA-AS2 overexpressed was evaluated by WST-1 assay and crystal violet staining.

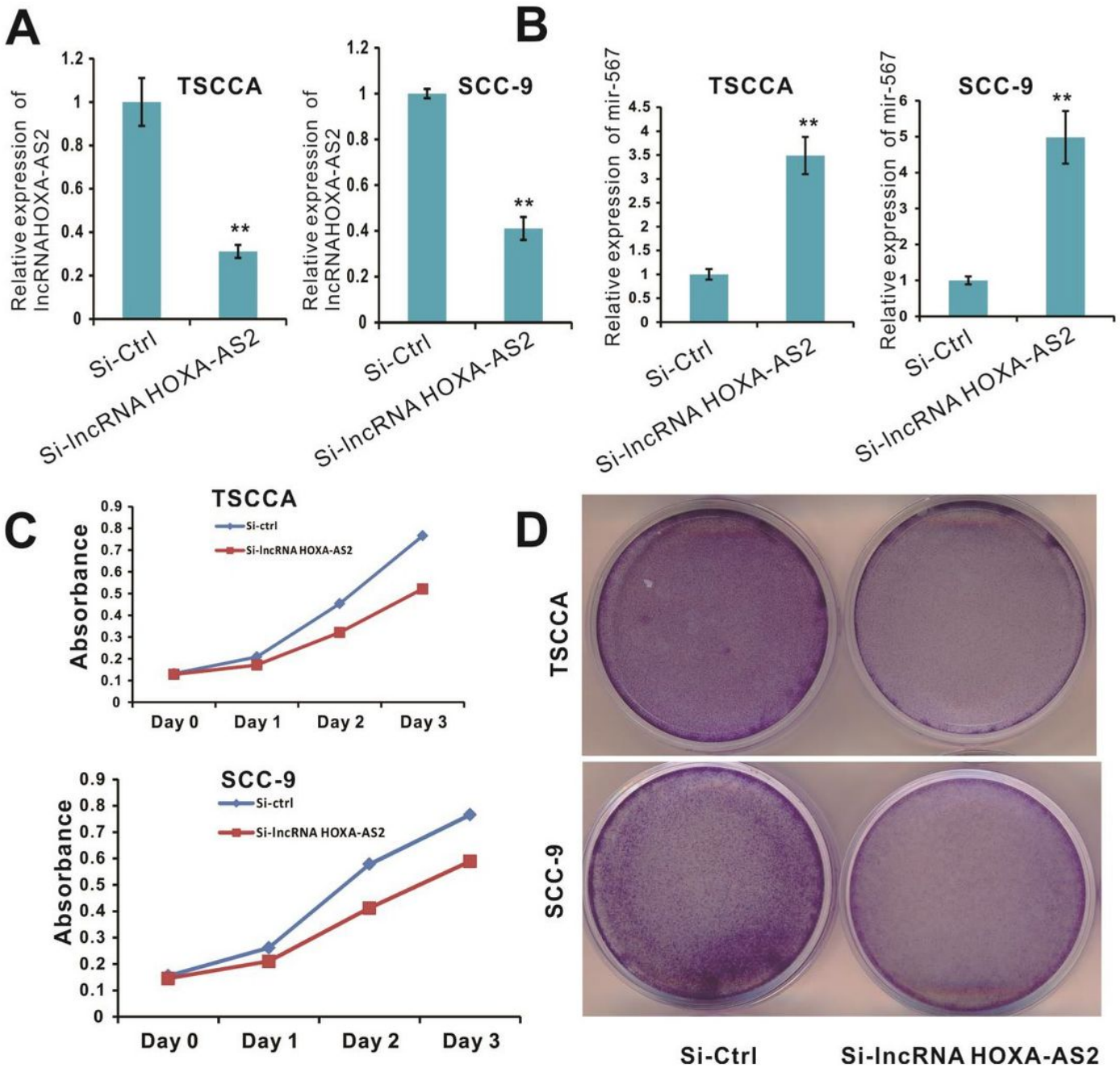

Figure 4

Cell proliferation was promoted in LncRNA HOXA-AS2 silenced OSCC cell lines. (A) The relative expression of LncRNA HOXA-AS2 in TSCCA and SCC-9 cells with LncRNA HOXA-AS2 silenced was examined by qRT-PCR. (B)The relative expression of miR-567 in TSCCA and SCC-9 cells with LncRNA HOXA-AS2 silenced. (C)& (D) Cell proliferation in TSCCA and SCC-9 cells with LncRNA HOXA-AS2 silenced was evaluated by WST-1 assay and crystal violet staining.

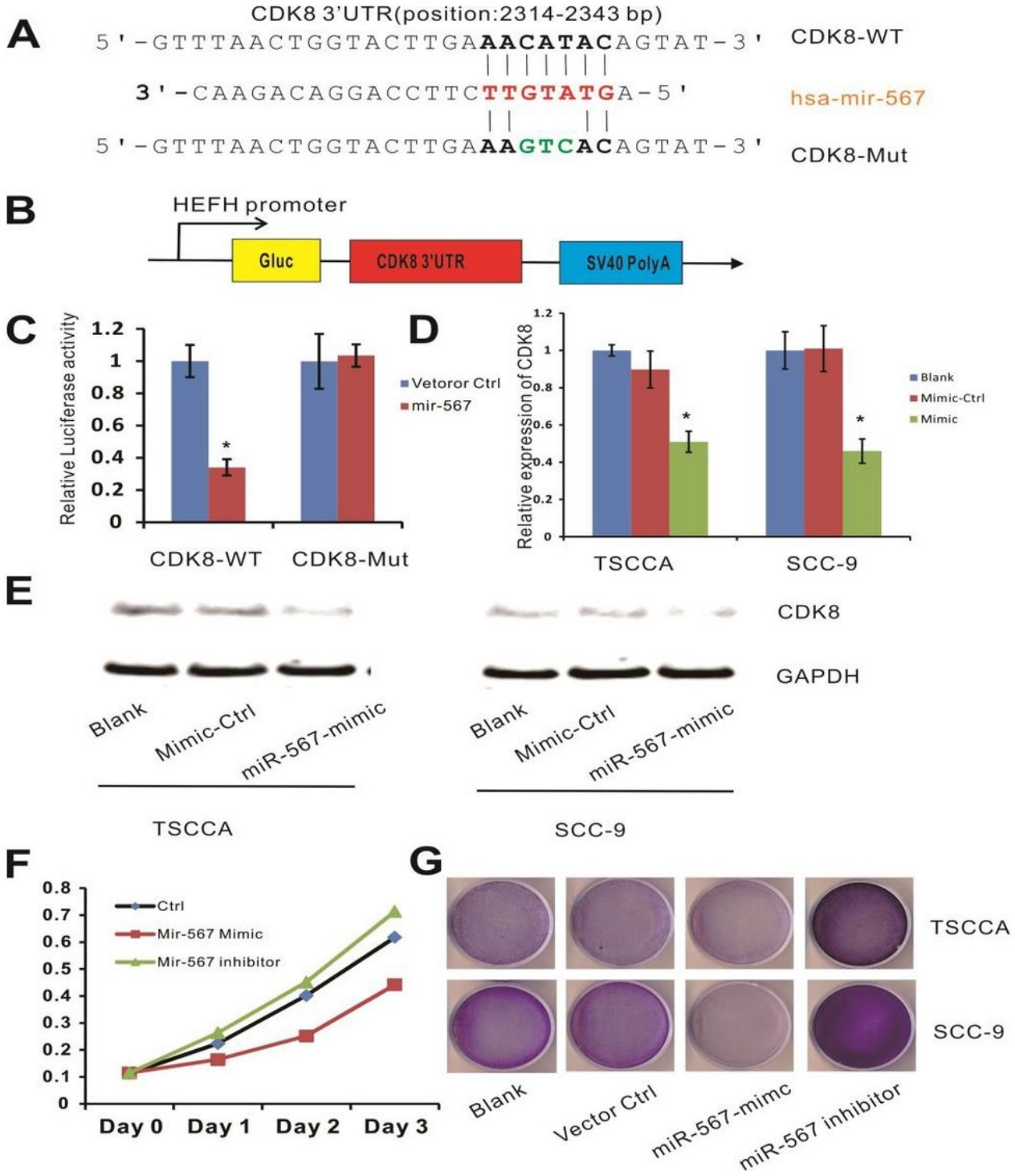

Figure 5

CDK8 was a downstream target of miR-567 in the regulation of OSCC Cell proliferation. (A) Bioinformatics analysis indicated the putative binding sites and corresponding mutant region for CDK8 within miR-567. (B) The construction of luciferase reporter that expresses CDK8. (C) The luciferase activities in CDK8-WT or CDK8-Mut and miR-567 or miR-Ctrl co-transfected OSCC cells were evaluated by luciferase reporter assay. (D) The relative expression of CDK8 in miR-567-mimic, Mimic-Ctrl and Blank

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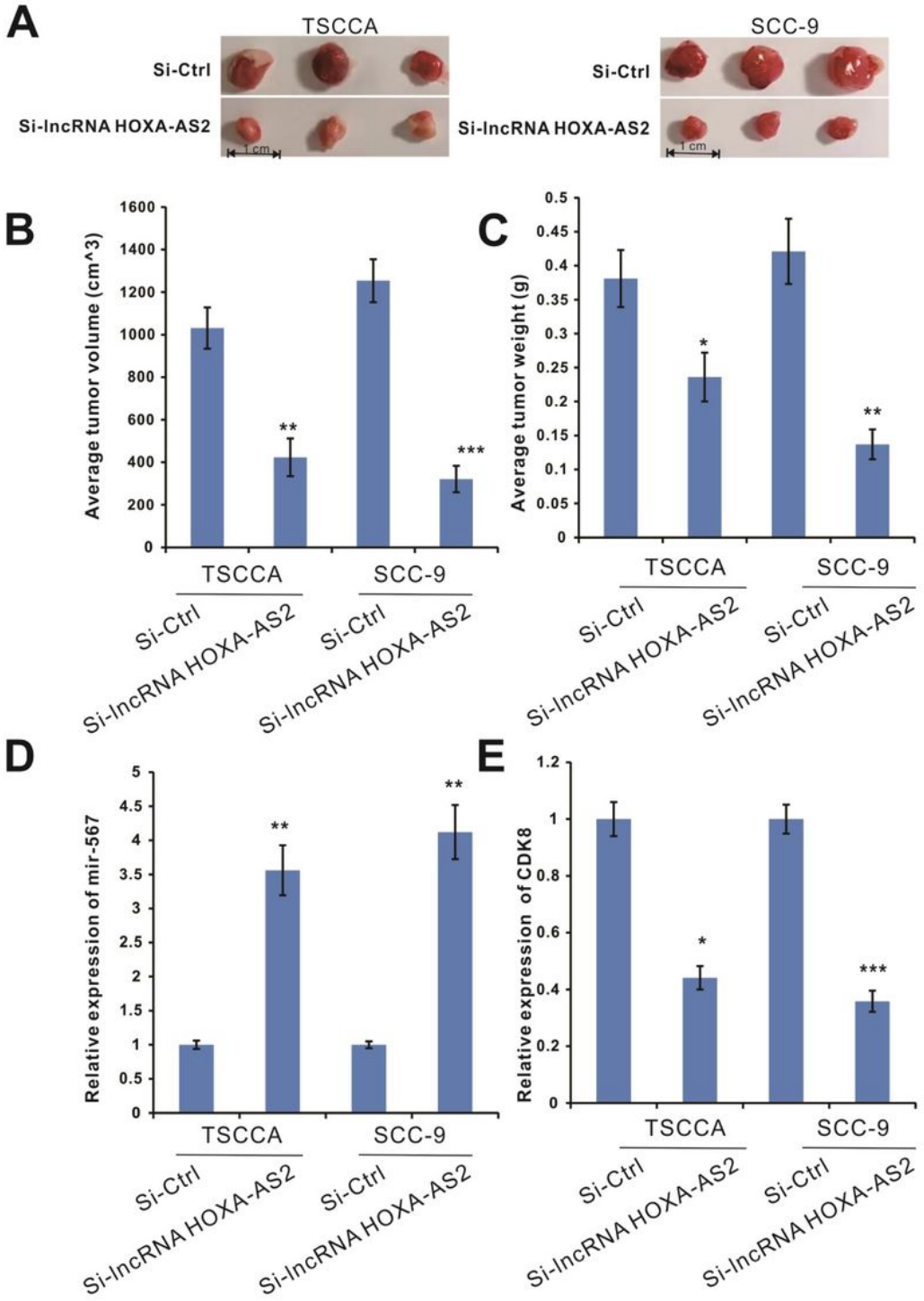

Figure 6

Silencing of LncRNA HOXA-AS2 inhibited OSCC tumor growth by releasing miR-567 to inhibit the expression of CDK8. TSCCA and SCC-9 cells with si-LncRNA HOXA-AS2 or its negative control were

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Supplementary Files

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