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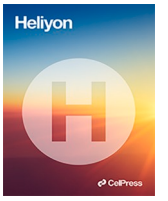

Research article

# Hedgehog/Gli2 signaling triggers cell proliferation and metastasis via EMT and wnt/β-catenin pathways in oral squamous cell carcinoma

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ABSTRACT

*Background:* Oral squamous cell carcinoma (OSCC) is the most lethal oral malignant tumor, however, clinical outcomes remain unsatisfactory. The Hedgehog/Gli2 pathway plays a pivotal role in tumor progression, yet the regulatory mechanism governing its involvement in the malignant evolution process of OSCC remains elusive.

*Methods:* OSCC animal tissue samples were used to detect the activation of the Hedgehog/Gli2 pathway in OSCC. Based on the clinical information of oral cancer patients in TCGA database, the role of this pathway in patients was analyzed, and the activation status of this pathway was verified in human OSCC cells. After activating or inhibiting the Hedgehog pathway, the effects of this pathway on the biological function of OSCC cells and its regulatory mechanism were examined. Interfering the expression of Gli2, a key transcription factor in this pathway, revealed the role of Hedgehog/Gli2 pathway in the malignant evolution of OSCC cells.

*Results:* The Hedgehog pathway exhibits abnormal activation in animal models of OSCC. Clinical data from TCGA demonstrate a significant enrichment of the Hedgehog pathway in patients with OSCC, and Gli2, a key downstream factor of this pathway, is closely associated with the occurrence and progression of OSCC. Cellular studies have revealed aberrant activation of this pathway in human OSCC cells, which exerts its function by modulating the activation of epithelial-mesenchymal transition (EMT) and Wnt/β-catenin pathways. Subsequent investigations further confirm the pivotal involvement of Gli2 in the Hedgehog pathway activation, underscoring its potential as a therapeutic target for inhibiting malignant proliferation and metastasis of OSCC cells through modulation of EMT and Wnt/β-catenin pathways.

*Conclusion:* The Hedgehog/Gli2 pathway induces EMT and activates Wnt/β-catenin pathway to trigger the malignant proliferation and metastasis of OSCC cells, and Gli2 plays a key role in this process, which suggests that targeting Gli2 may be a promising therapeutic strategy for inhibiting the proliferation and metastasis of OSCC.

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## 1. Introduction

The effective control of the increasing morbidity and mortality rates of cancer remains a significant global challenge in the 21st century. Oral cancer ranks as the sixth most prevalent form of cancer worldwide [1,2]. As an underestimated public health concern, oral cancer poses a serious threat to people's lives and well-being globally. Existing studies indicate that there will be an estimated 31,733 new cases and 15,745 deaths from oral cancer in China in 2022 [3]. Globally, over 350,000 new cases of oral cancer are diagnosed annually, resulting in approximately 145,000 deaths [4]. Among various types of oral cancer, oral squamous cell carcinoma (OSCC) is particularly detrimental and accounts for more than 90 % of all cases [5]. OSCC exhibits high malignancy potential along with susceptibility to metastasis and recurrence, leading to poor prognosis and adverse effects on patients' daily interpersonal communication [6]. Currently available clinical treatment methods for OSCC include surgery, chemotherapy, radiotherapy, and immunotherapy. These approaches have significantly advanced our understanding of OSCC pathogenesis and treatment options [7]. However their efficacy remains limited for patients with OSCC accompanied by lymph node metastasis. Therefore it is crucial to explore novel therapeutic strategies. Targeted therapy has demonstrated positive therapeutic outcomes in clinical tumor treatments; however effective targeted therapies specifically designed for OSCC are currently scarce. Further investigation into targeted therapies for OSCC is warranted.

Accumulating evidence has demonstrated the increasing significance of the Hedgehog signaling pathway in tumorigenesis. The aberrant activation of the Hedgehog pathway is closely associated with tumor proliferation, differentiation, apoptosis, angiogenesis, invasion, metastasis, and other cellular functions [8,9]. Guo et al. confirmed that Smad4 regulates TGF-Beta1-mediated Hedgehog activation by inhibiting Gli1 activity, thereby promoting epithelial-mesenchymal transition (EMT) of pancreatic cancer cells [10]. Yang et al. verified that oncogene MYBL2 promotes a malignant phenotype and suppresses apoptosis through the Hedgehog signaling pathway in clear cell renal cell carcinoma [11]. Wu et al. demonstrated that PARD3 drives tumorigenesis by activating the Hedgehog signaling pathway in liver cancer initiating cells [12]. Zhu et al. established that HERC4 regulates ovarian cancer cell proliferation by modulating Smo-triggered Hedgehog signaling [13]. Wu et al. validated USP5 as a promoter of tumorigenesis through activation of the Hedgehog signaling pathway in osteosarcoma [14]. Importantly, Gong et al. substantiated allotrypine's effect on OSCC proliferation and EMT via M6A-mediated modulation of the Hedgehog pathway [15]. Niu et al. confirmed EHMT2's promotion of OSCC's malignant phenotype and stemness properties by suppressing ARRB1 transcription and activating the Hedgehog signaling [16]. In summary, while it is evident that the Hedgehog pathway plays a crucial role in various cancers including OSCC; its regulatory mechanisms within OSCC lesions remain elusive and warrant further investigation.

It is important to emphasize that the Hedgehog pathway activation can be categorized into two distinct pathways: canonical and non-canonical Hedgehog pathway regulatory networks, depending on the activation of the Hedgehog pathway and its dependence on Gli protein for biological effects [17]. The canonical activation pathway refers to the signal transduction pathway mediated by Hedgehog's regulation of Gli family transcription factors. In the absence of Shh ligand protein, Ptch releases a protein that inhibits Smo activity, thereby blocking Smo activation and suppressing the Hedgehog pathway. Upon binding of Hedgehog ligand protein to Ptch, Ptch ceases secretion, leading to uninhibited Smo activity and subsequent activation of the Hedgehog signaling system. This ultimately results in the activation of Gli transcription factor members and their translocation into the nucleus, influencing downstream target gene expression within the Hedgehog pathway and promoting tumor proliferation, invasion, metastasis, and angiogenesis [18–20]. The non-canonical activation pathway refers to signal responses involving one or more components of the Hedgehog signaling cascade. It can be primarily classified into three categories based on its regulatory mechanism: Ptch-mediated signal transduction, Smo-mediated signal transduction, and Gli-mediated Hh signaling [21]. Ptch-mediated signal transduction encompasses Ptch's role in inducing apoptosis by recruiting pro-apoptotic factors, as well as its involvement in cell cycle regulation through interaction with CyclinB1 [22]. Smo-mediated signal transduction refers to the Smo-dependent pathway that operates independently of Gli, wherein it binds to heterotrimeric G proteins of the Gi family and activates several crucial protein kinases, second messengers, and $Ca2^{+}$ [21,22]. Gli-mediated signal transduction is not reliant on either Smo or upstream signals of Gli; instead, it engages multiple tumor-related signaling pathways such as TGF-β, MAPK, PI3K-AKT, and TNF-α to activate Gli activity [23]. Thus, the Hedgehog pathway represents a complex regulatory network rather than a linear cascade that synergistically interacts with various signaling pathways to regulate tumor progression.

However, in the two activation pathways of Hedgehog pathway, Gli family transcription factors play a key role as the convergence point of activation at all levels of Hedgehog pathway, which can transmit extracellular signals into the cell, regulate the expression of related genes at the transcriptional level by directly acting on downstream targets, and thus affect cell biological functions [24,25]. Gli2 plays a crucial role in embryonic cell differentiation, tissue development, organ formation and disease regulation (including tumorigenesis) [26]. Gli2 functions as a highly active transcription factor within the Hedgehog pathway [27]. It possesses five conserved zinc finger DNA binding domains in tandem repeats, predominantly localizes to the nucleus, and exhibits high affinity for its DNA binding sites [28]. Acting as a downstream transcription factor of this pathway, Gli2 serves as a convergence point for activation at all levels of the Hedgehog signaling cascade. Following activation, Gli2 undergoes post-translational modifications (such as phosphorylation and ubiquitination) to stabilize its nuclear presence [29,30]. This enables it to transmit extracellular signals to the nucleus and directly modulate downstream targets by regulating target gene expression at the transcriptional level, thereby affecting various biological processes including cell proliferation, differentiation and survival [31,32]. It is crucial to emphasize that Alexaki et al. have confirmed the direct involvement of Gli2 in melanoma invasion and metastasis [33]. Cannonier et al. have demonstrated a correlation between Gli2 levels in clinical OSCC samples and bone infiltration [34]. Yan et al. have revealed a close association between Gli2 expression and poor prognosis in patients with oral cancer [35]. These findings suggest that Gli2, as a pivotal downstream factor of the Hedgehog pathway, plays a fundamental role in the pathogenesis of this disease. However, the potential therapeutic

targeting of Gli2 for OSCC treatment remains uncertain, necessitating further investigation into its precise role in OSCC development.

To elucidate the underlying mechanism of the Hedgehog/Gli2 pathway regulation in OSCC development, we initially employed bioinformatics methods to predict Hedgehog/Gli2 pathway potential regulatory role in OSCC patients. Subsequently, we investigated the activation status of the Hedgehog pathway at both cellular and animal levels. Furthermore, we examined the impact of modulating Hedgehog pathway activation on the biological functions of OSCC cells. To ascertain whether Gli2, a key downstream transcription factor of this pathway, drives its activation in OSCC cells, we interfered with Gli2 expression and evaluated its influence on co-functional aspects of OSCC cells. Our findings confirm that aberrant Hedgehog pathway activation promotes malignant proliferation and metastasis in OSCC cells by inducing EMT and Wnt/β-catenin pathways. Importantly, Gli2 emerges as a pivotal driver mediating Hedgehog's regulatory role in tumorigenesis. These results suggest that targeting Gli2 could be a promising therapeutic strategy for inhibiting proliferation and metastasis in OSCC.

## 2. Materials and methods

### 2.1. Animal tissue samples

Female Chinese hamsters (8 weeks old, weighing 20–25g, n = 48) were procured and housed in a controlled environment at the Laboratory Animal Center of Taiyuan Medical University, Shanxi Province. The animals were randomly divided into two groups: control group (n = 24) and experimental group (n = 24). The control group received regular feeding, while the experimental group was subjected to thrice-weekly application of DMBA with a concentration of 0.005 g/L on the bilateral buccal pouch mucosa. To investigate OSCC pathogenesis comprehensively, eight Chinese hamsters from each group were randomly selected at the 9th, 15th, and 21st week of continuous DMBA application for collection of cheek pouch tissues and cancerous tissues. Some tissue samples underwent pathological examination while the remaining samples were stored at −80 °C. All animal procedures adhered to ethical guidelines approved by the Institutional Animal Care and Use Committee of Shanxi Medical University (IACUC 2021012), ensuring compliance with animal welfare principles based on the 3R principles without compromising experimental requirements or outcomes.

### 2.2. Data Source

Clinical data of patients with OSCC were downloaded from the Cancer Genome Atlas (TCGA) database (https://www.cancer.gov/ccg/research/genome-sequencing/tcga), excluding those without clinical information. Among the clinical data, samples from various oral cancer sites including alveolar ridge, tongue base, buccal mucosa, floor of mouth, hard palate, mouth, and tongue were retained while non-oral cancer site samples such as hypopharynx, larynx lip oropharynx tonsil were excluded. Pathway enrichment analysis for differential genes in patients with oral cancer was conducted using clusterProfiler package. Paired sample *t*-test was employed to analyze the expression level of functional genes in clinical samples.

### 2.3. Cell lines

The human oral cancer cell lines CAL27 and SCC9 were obtained from the American Type Culture Collection (Manassa, VA, USA). Both CAL27 and SCC9 cells were cultured using DMEM (Boster, China) supplemented with 10 % FBS (Gibco, USA), along with 1 % penicillin/streptomycin (Boster, China). Human normal oral epithelial cells (HOK) were generously provided by the Key Laboratory of the School of Stomatology at Shanxi Medical University. HOK cells were cultured using RPMI1640 (Boster, China) supplemented with 10 % FBS and 1 % penicillin/streptomycin. All cell lines were incubated at 37 °C under a $CO_2$ concentration of 5 %.

### 2.4. Cell treatment

Cells in the exponential growth phase were inoculated into a 6-well plate (50,000 cells/well) and cultured at 37 °C with 5 % $CO_2$. The Hedgehog pathway inhibitor, GANT61 (MCE, USA), and agonist, SAG (Beyotime, China), were employed to modulate the activity of this signaling cascade. Furthermore, we conducted a drug concentration gradient experiment to screen the optimal treatment concentration. The cells were treated with either GANT61 (20 μM) or SAG (5 μM). After 48h of drug treatment, other assays were performed to assess the impact of the Hedgehog pathway on various biological functions of oral cancer cells. Detailed information regarding these small-molecule drugs can be found in Supplementary Table 1.

### 2.5. Cell transfection

Cells in the exponential growth phase were inoculated into a 6-well plate (50,000 cells/well) and cultured at 37 °C with 5 % $CO_2$. Specific siRNAs targeting Gli2 and a negative control (si-NC) were designed and synthesized by GenePharma. The optimal concentration for siRNA transfection was determined through a drug concentration gradient experiment. Transfection of siRNA (100 nM) and si-NC (100 nM) was performed the following day using Lipofectamine™ 3000 (Invitrogen, USA). The medium was replaced with fresh medium after 6h of transfection. At 48h post-siRNA transfection, the efficiency of cellular transfection was assessed using quantitative reverse transcription polymerase chain reaction (qRT-PCR). Supplementary Table 2 provides the sequences of the siRNAs used to knockdown Gli2.

### 2.6. qRT-PCR

Total RNA was extracted by using a TRIzol kit (Takara, Japan), and cDNA was synthesized using a reverse transcription kit (Takara, Japan). qRT-PCR was performed on an Applied Biosystems 7500 (ABI 7500) system (Thermofisher, USA) using a SYBR Green PCR Kit (Takara, Japan). Using GAPDH as internal reference, the gene expression was normalized. The $2^{-\Delta\Delta CT}$ method was used to quantify gene expression. The primer sequences were obtained from Shanghai GenePharma Co., Ltd., China, and listed in Supplementary Table 3.

### 2.7. Western blot analysis

Protein collection were performed using RIPA Lysis Buffer (Boster, China). Total protein content was detected using a BCA Protein Quantification Kit (Boster, China). Total protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose filter membrane (Boster, China). After the nitrocellulose filter membranes were blocked using 5 % skim milk at room temperature for 60min, the primary antibody was added and incubated overnight in a shaker at 4 °C. The next day, the nitrocellulose filter membranes were washed with washing buffer (TBST) (Boster, China) and incubated with the secondary antibodies at 37 °C for 60min. An Enhanced Chemiluminescent Substrate Kit (Boster, China) and an automatic gel imaging system were used to detect the luminescent signals of the protein bands. β-actin was used as a standardization control. Details on the antibody is provided in Supplementary Table 4.

### 2.8. Cell counting Kit-8 (CCK-8) assay

Cell toxicity was detected using the CCK-8 assay: the cell suspension (5000 cells/well) was seeded into 96-well plates, and incubated at 37 °C and 5 % $CO_2$ for 24h. Each group had at least 3 replicate wells, and five concentration gradients were set for each siRNA and pathway agonist and inhibitor. In addition, CCK-8 assay was used to detect cell proliferation. Cell suspensions (5000 cells/well) were seeded onto 96-well plates and incubated at 37 °C with 5 % $CO_2$, with a minimum of three replicate wells per group. At predetermined time points (0h, 24h, 48h, and 72h), each well containing 90 μl of culture medium was supplemented with 10 μl CCK-8 solution (MCE, USA) and further incubated at 37 °C with 5 % $CO_2$ for a duration of 2 h. The absorbance at the wavelength of 450 nm was measured using a microplate reader (BioTek, USA).

### 2.9. Cell migration assay

Cell suspensions (20,000 cells/pores) were added to the upper chamber of the transwell chamber and cultured with 1 % FBS. Then, 600 μl medium containing 10 % FBS was added to the lower cavity. The cells were cultured at 37 °C and 5 % $CO_2$ with at least 3 repeat wells per group. After incubation for 24h, the cell medium and non-migrated cells in the upper chamber were removed and fixed with 4 % paraformaldehyde (Boster, China) for 30min, and 0.1 % crystal purple solution (Beyotime, China) was added to the migrated cells for 15min for staining. Images of migrating cells were captured under a microscope (Olympus, Japan) and counted using the ImageJ software.

### 2.10. Cell invasion assay

A pre-cooled 100 μL solution of matrigel (Corning, USA) was added to the upper chamber of a transwell system and incubated at 37 °C for 30min. Subsequently, a cell suspension containing 20,000 cells/well was introduced into the upper chamber and cultured with 1 % FBS. Additionally, 600 μl medium supplemented with 10 % FBS was added to the lower compartment. The cells were then incubated in a controlled environment at 37 °C with 5 % $CO_2$ concentration following identical steps as those employed for cell migration assays.

### 2.11. Colony formation assay

Cell suspensions (1000 cells/well) were added to 6-well plates and cultured at 37 °C and 5 % $CO_2$. Cells were grown in a medium containing 20 % FBS, and the medium was changed every five days. Two weeks later, cells were fixed with 4 % paraformaldehyde for 30min. The colonies were then stained with a 0.1 % crystal violet aqueous solution at room temperature for 15min. Images were acquired under a microscope, and the number of colonies (clusters of >50 cells) was counted.

### 2.12. Statistical analysis

The statistical analyses were conducted using SPSS Statistics 20.0, and the data were presented as mean ± deviation. The *t*-test was employed to assess differences between two groups, while a one-way analysis of variance followed by Tukey's post hoc test was used to determine differences among multiple groups. Statistical significance was defined as $^{*}P < 0.05$, $^{**}P < 0.01$. All experiments were performed at least three times.

## 3. Results

### 3.1. Hedgehog is abnormally activated in OSCC

Our preliminary histopathological analysis showed that after 9 weeks of continuous DMBA induction, the Chinese hamster OSCC model group exhibited simple epithelial hyperplasia. After 15 weeks of continuous DMEM induction, the Chinese hamster OSCC model group entered the abnormal proliferation stage. After 21 weeks of continuous DMBA induction, the Chinese hamster OSCC model group displayed the characteristics of squamous cell carcinoma and invasive carcinoma, and the OSCC animal model was successfully established. To further validate the involvement of the Hedgehog pathway in OSCC development, we built on our previous research and used qRT-PCR and western blotting to detect the mRNA and protein expression of key Hedgehog pathway factors in the simple hyperplasia, abnormal proliferation, and OSCC groups [36]. qRT-PCR results demonstrated increased expression of Shh, Ptch1, Gli1, and Gli2 in dysplasia and OSCC groups (Fig. 1A). Additionally, western blotting analysis indicated elevated protein expression of Gli2

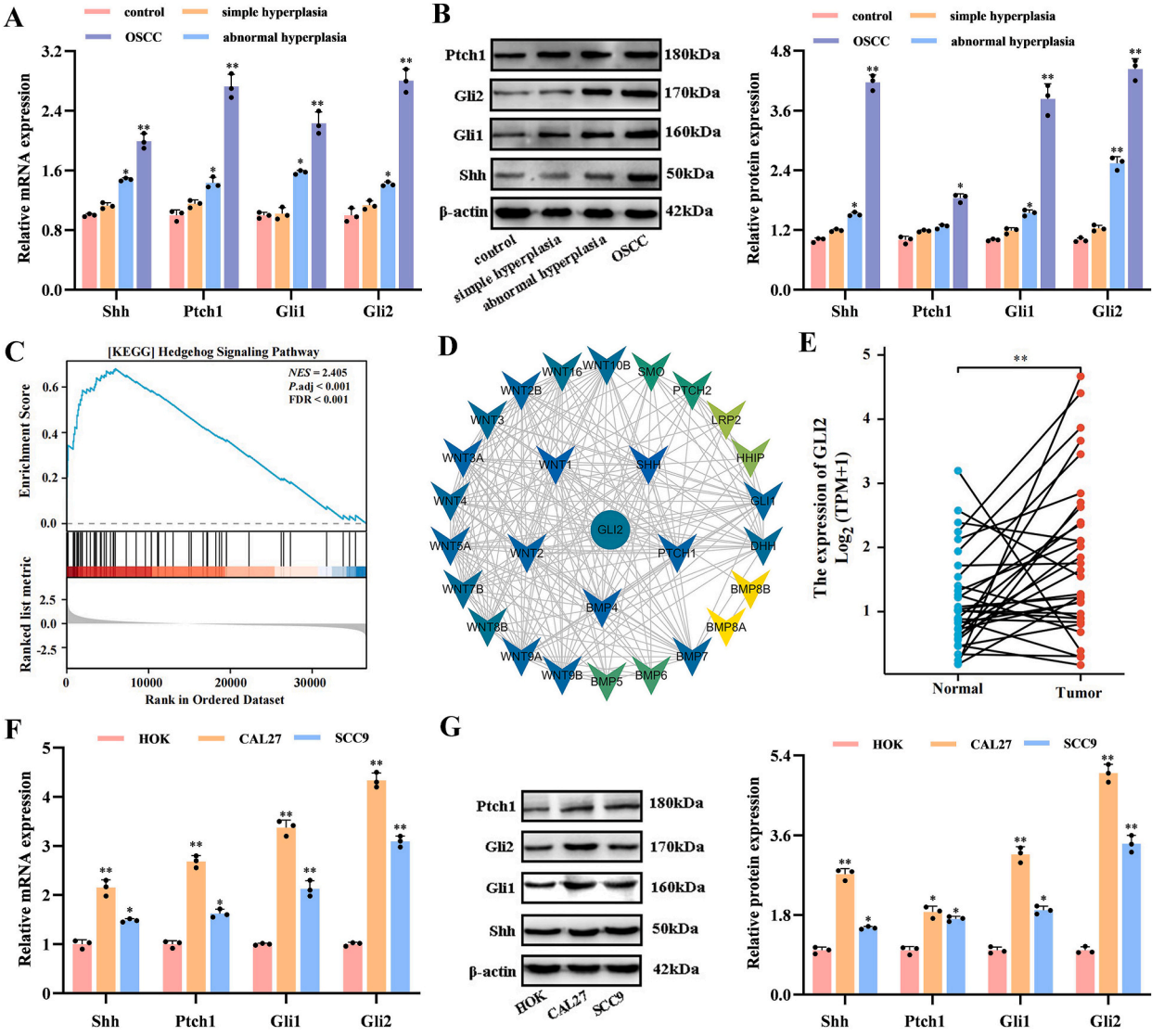


**Fig. 1.** Hedgehog/Gli2 is abnormally activated in OSCC (A) mRNA expression of key components of the Hedgehog pathway in OSCC animal samples. (B) Protein expression of key components of the Hedgehog pathway in OSCC animal samples (western blotting is related to Fig. S2). (C) GSEA analysis of oral cancer samples. (D) Key gene networks enriched by GSEA (the higher the degree, the bluer the color, and the lower the degree, the yellower the color). (E) Gli2 expression in oral cancer samples from TCGA database (the lines represent pairs of samples). (F) mRNA expression of key components of the Hedgehog pathway in OSCC cells. (G) Protein expression level of key components of the Hedgehog pathway in OSCC cell lines (western blotting is related to Fig. S3). (Data are represented as mean ± standard deviation, n = 3; *$P < 0.05$, **$P < 0.01$, compared with the control group). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

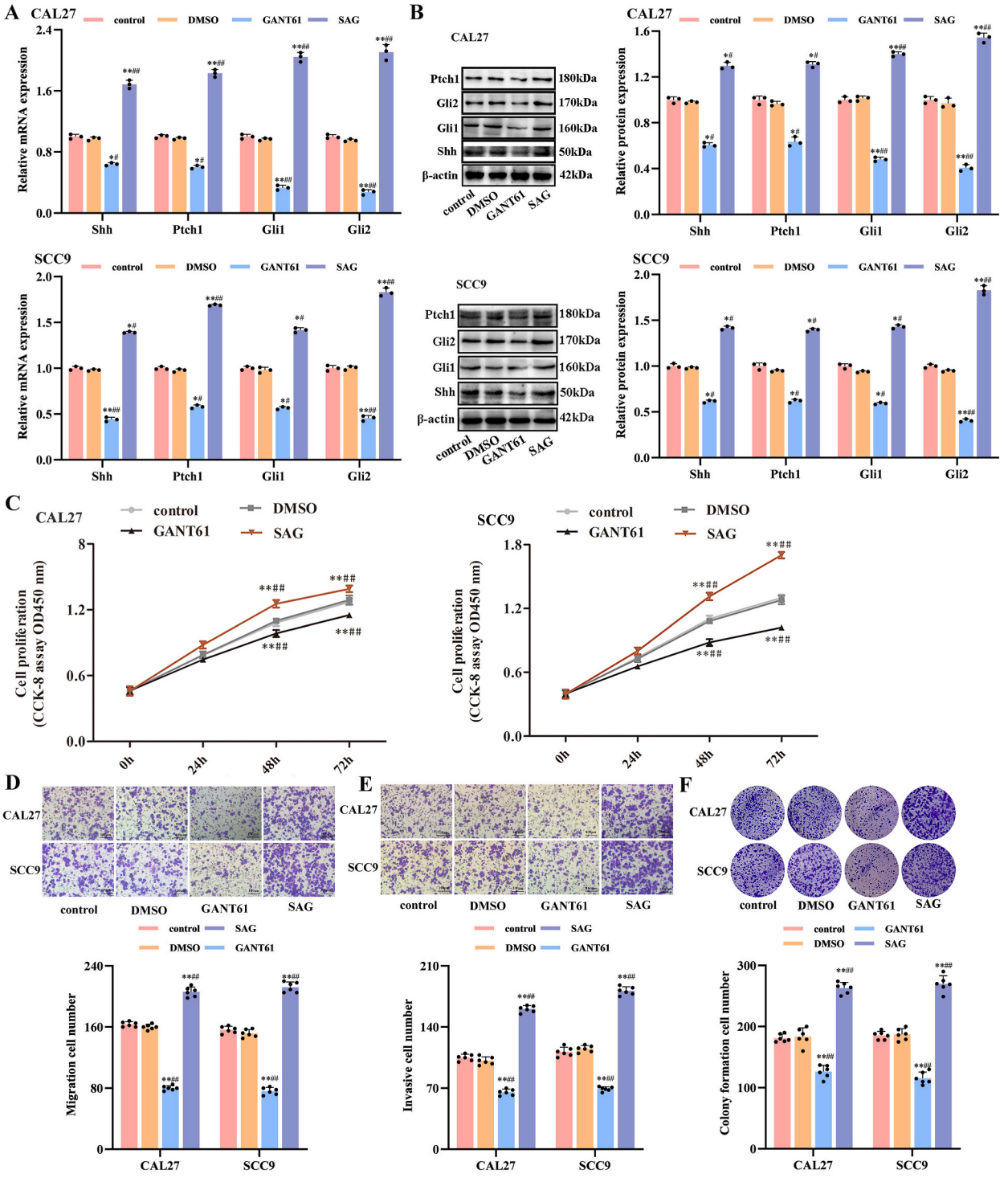


**Fig. 2.** Inhibiting the activation of Hedgehog pathway can prevent the malignant proliferation and metastasis of OSCC cells (A) mRNA expression of key components of the Hedgehog pathway in OSCC cells after SAG and GANT61 treatment (n = 3). (B) Protein expression of key components of the Hedgehog pathway in OSCC cells after SAG and GANT61 treatment (n = 3) (western blotting is related to Fig. S4). (C) CCK-8 assay was used to evaluate cell proliferation after SAG and GANT61 treatment (n = 3). (D) Representative images of cell migration after SAG and GANT61 treatment (the bar graphs display the percentage of cell migration in different groups, n = 6). (E) Representative images of cell invasion after SAG and GANT61 treatment (the bar graphs display the percentage of cell invasion in different groups, n = 6). (F) Representative image of cell colony formation after SAG and GANT61 treatment (the bar graphs represents the number of colonies formed by different groups, n = 6). (Data represented as mean ± standard deviation; $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with the DMSO group).

in simple hyperplasia group; increased protein expression of Shh, Gli1, and Gli2 in dysplasia group; as well as enhanced protein expression of Shh, Ptch1, Gli1, and Gli2 in OSCC group (Fig. 1B).

To investigate the potential role of the Hedgehog/Gli2 pathway in OSCC development, we retrieved clinical data of OSCC patients from TCGA database and performed gene-set enrichment analysis to identify genes significantly associated with the Hedgehog signaling pathway. Our results demonstrated a significant enrichment of core genes within the screened gene set (Fig. 1C). Furthermore, utilizing Cytoscape software, we further enriched this gene set and identified Gli2 as a key downstream transcription factor crucial for OSCC pathogenesis (Fig. 1D). Given its pivotal function, we conducted paired sample t-tests to analyze Gli2 expression in these clinical samples mentioned above, revealing a significant over-expression of Gli2, an important activating transcription factor downstream of the Hedgehog pathway, in oral cancer samples (Fig. 1E). To further elucidate the activation status of the Hedgehog/Gli2 pathway in OSCC, we investigated the gene and protein expression of key factors involved in the Hedgehog signaling pathway in OSCC cells. CAL27 and SCC9 cell lines, derived from highly invasive and metastatic OSCC, were used for this study, while HOK cells served as controls. CAL27 is an established OSCC cells that closely resembles human oral cancer characteristics both in vivo and in vitro [37]. SCC9 is a commonly used experimental model to investigate molecular mechanisms underlying oral cancer, such as cellular signaling, gene expression, and apoptosis due to its rapid growth rate in vitro [38]. HOK cells are immortalized cuticular epithelial cells isolated from normal tissue [39]. Our findings demonstrate a significant increase in mRNA levels of Shh, Ptch1, Gli1, and Gli2 in both CAL27 and SCC9 cell lines (Fig. 1H). Consistent trends were observed at the protein level using western blotting analysis (Fig. 1G). In conclusion, our results highlight the crucial role of the Hedgehog/Gli2 signaling pathway in oral cancer development and emphasize the importance of further investigating its underlying mechanism.

### *3.2. Inhibiting the activation of the hedgehog pathway can prevent the malignant proliferation and metastasis of OSCC cells*

GANT61, a selective inhibitor of Gli, effectively suppresses the Hedgehog signaling and exhibits potential anticancer effects. It primarily attenuates the expression of downstream targets in the Hedgehog pathway, leading to reduced Gli DNA interaction, transcription activity, and nuclear translocation in tumor cells. Consequently, it inhibits Gli-induced transcription and downregulates Gli expression in tumor cells [40,41]. SAG acts as an agonist for the Smo receptor by activating Smo and subsequently stimulating the Hedgehog pathway, resulting in increased Gli expression [42,43]. To investigate the regulatory role of the Hedgehog/Gli2 signaling pathway in oral cancer development and progression comprehensively, we initially examined how modulation of this pathway affects OSCC cell behavior under activated or inhibited conditions. We determined optimal drug treatment concentrations for SAG (5 μM) and GANT61 (20 μM) using CCK-8 assay on OSCC cells before proceeding with subsequent experiments at these concentrations (Supplementary Fig. 1A). Furthermore, our findings revealed that SAG significantly upregulated mRNA expression levels of Shh, Ptch1, Gli1,andGli2in OSCC cell lines; conversely, GANT61 notably downregulated their mRNA expression levels (Fig. 2A). Similar trends were observed at the protein level through western blotting analysis (Fig. 2B).

Consequently, we further treated OSCC cells with GANT61 and SAG to evaluate its effects on their biological behavior. CCK-8 assay revealed that SAG significantly enhanced CAL27 and SCC9 cell proliferation in vitro; however, treatment with GANT61 exerted an opposite effect (Fig. 2C). Moreover, our cell migration assay demonstrated that SAG markedly increased migratory ability while GANT61 noticeably suppressed migration of both CAL27 and SCC9 cells (Fig. 2D). Cell invasion experiments involve the use of coated membranes, onto which a layer of matrix glue (Matrigel) is applied to mimic the extracellular matrix and basement membrane. This allows for the assessment of cell invasion ability by evaluating their capacity to penetrate through the basement membrane, reflecting both migratory and invasive properties [44,45]. The cell invasion assay indicated that SAG prominently promoted invasive ability whereas treatment with GANT61 substantially inhibited invasion of both CAL27 and SCC9 cell lines (Fig. 2E). Furthermore, the colony formation assay validated the pro-proliferative effect of SAG while demonstrating the inhibitory effect of GANT61 on cell growth (Fig. 2F). Interestingly, our study revealed distinct expression patterns of key Hedgehog pathway factors in SCC9 and CAL27 cell lines following treatment with Hedgehog agonist and inhibitor, suggesting potential variations in drug sensitivity and tolerance between these two cell lines due to their different origins. In short, aberrant activation of the Hedgehog pathway plays a crucial role in regulating malignant proliferation and metastasis in OSCC cells. Suppression of this pathway activation effectively inhibits proliferation, growth, migration, and invasion of OSCC cells; conversely, its activation promotes these processes.

### *3.3. The hedgehog pathway influences the malignant proliferation and metastasis of OSCC cells by regulating the activation of Wnt/beta-catenin and EMT pathways*

According to the existing literature, the Hedgehog pathway is closely associated with EMT and Wnt/β-catenin pathways, which in turn are closely linked to the occurrence and progression of malignant tumors [46,47]. Therefore, we propose that modulation of the Hedgehog pathway influences OSCC cell proliferation and metastasis by affecting the activation of EMT and Wnt/β-catenin pathways. During EMT process, epithelial cells undergo a phenotypic transition from epithelial to mesenchymal state. The hallmark of this transition is the downregulation of E-cadherin and the upregulation of vimentin [48,49]. Moreover, MMP2 and MMP9 are often used as markers of tumor metastasis. During the development of OSCC, the changes in these biomarkers lead to the reorganization of the cytoskeleton, changes in cell polarity and adhesion properties, and enhanced motility; thus promoting the malignant metastasis of OSCC [50,51]. In addition, studies have pointed out after Wnt3a activates the classical Wnt/β-catenin pathway, it promotes the translocation of β-catenin from the cytoplasm to the nucleus, thereby initiating downstream gene transcription and promoting the expression of downstream target proteins C-myc and CyclinD1, thereby accelerating the proliferation and metastasis of tumor cells [52–54]. To investigate this possibility, we assessed the mRNA and protein expression levels of MMP2, MMP9, E-cadherin, and

vimentin after treatment with GANT61 and SAG as these molecules play crucial roles in EMT. qRT-PCR and western blotting results demonstrated that SAG upregulated mRNA and protein expression of MMP2, MMP9, and vimentin while downregulating mRNA and protein expression of E-cadherin. Conversely, GANT61 downregulated mRNA and protein expression of MMP2, MMP9, and vimentin while upregulating mRNA and protein expression of E-cadherin (Fig. 3A and B). Additionally, we determined mRNA and protein expression levels of Wnt3a, β-catenin, C-myc, and CyclinD1 which are important for the occurrence and metastasis of tumors. We found that SAG promoted mRNA and protein expression of Wnt3a, β-catenin, C-myc, and CyclinD1 while these were decreased after treatment with GANT61 (Fig. 3C and D). Taken together, the sefindings confirm that the Hedgehog pathway plays a key role in them alignant proliferation and metastasis of OSCC cells by mediating the activation of EMT and Wnt pathways in OSCC cells.

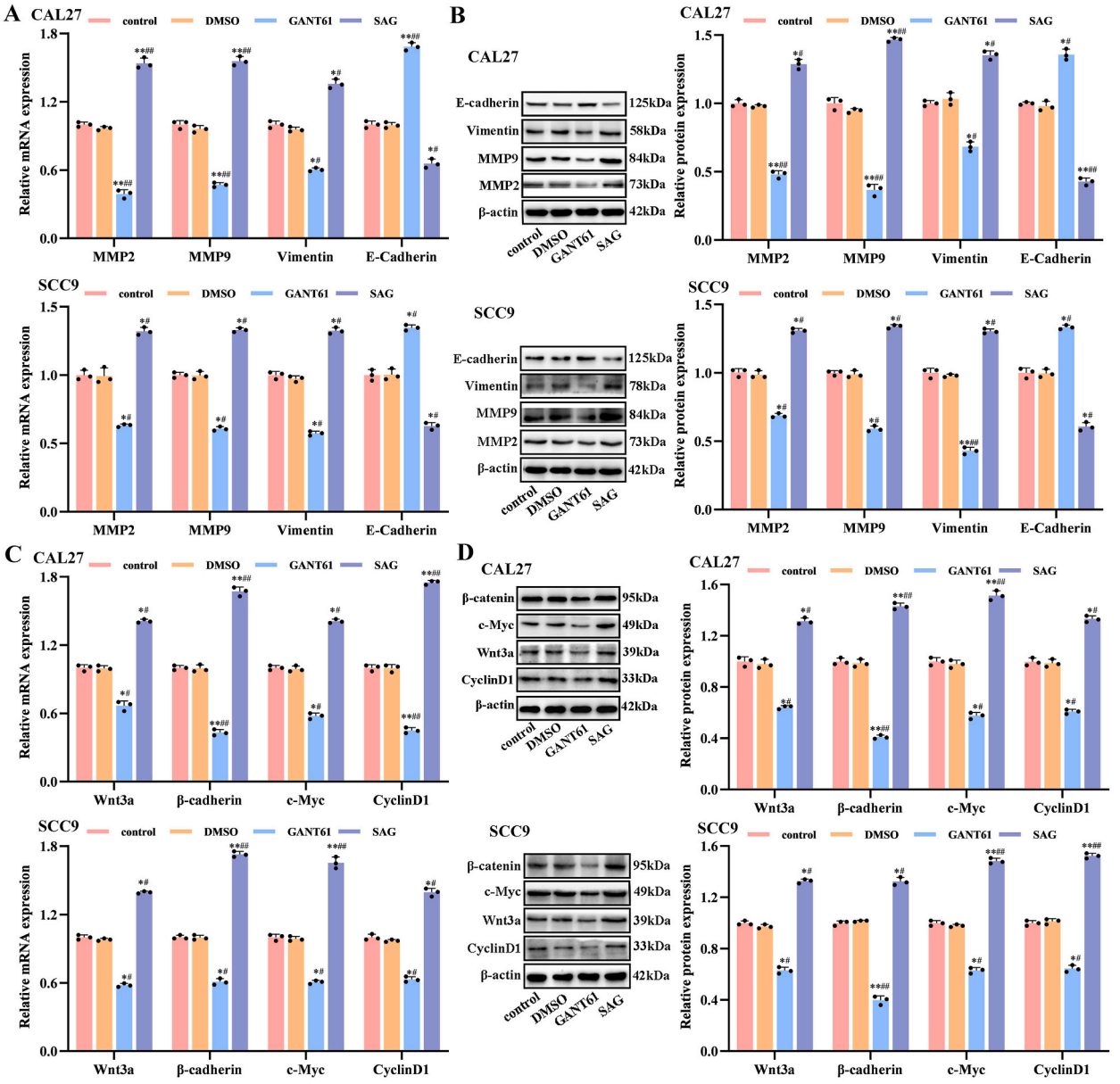


**Fig. 3.** Hedgehog pathway affects the malignant proliferation and metastasis of OSCC cells by regulating the activation of EMT and Wnt/β -catenin pathways (A) mRNA expression of key components of EMT pathway in OSCC cells after SAG and GANT61 treatment. (B) Protein expression of key components of EMT pathway in OSCC cells after SAG and GANT61 treatment (western blotting is related to Fig. S5). (C) mRNA expression of key components of Wnt/β-catenin pathway in OSCC cells after SAG and GANT61 treatment. (D) Protein expression of key components of Wnt/β-catenin pathway in OSCC cells after SAG and GANT61 treatment (western blotting is related to Fig. S6). (Data represented as mean ± standard deviation, n = 3; $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with the DMSO group).

### 3.4. Gli2 is a key factor that drives hedgehog pathway to regulate malignant proliferation and metastasis of OSCC cells

The aforementioned studies have provided preliminary evidence confirming the promotion of malignant proliferation and metastasis of OSCC cells through abnormal activation of the Hedgehog pathway. However, the specific role of Gli2 in this process remains unclear. To investigate whether Gli2 influences malignant proliferation and metastasis of OSCC cells via the Hedgehog pathway, we employed Gli2-targeting siRNA to interfere with its expression, aiming to elucidate the underlying mechanism behind Gli2's involvement in OSCC development. Initially, we determined the optimal interference concentration for siRNA (100 nM) using CCK-8 cytotoxicity assay and qRT-PCR analysis. Our results demonstrated that si-Gli2-1 and si-Gli2-2 exhibited superior interference efficiency at an siRNA concentration of 100 nM (Fig. 4A and Supplementary Fig. 1B). Subsequently, we detected the expression of key factors of the Hedgehog pathway in OSCC cells after interfering with Gli2 expression. The results from qRT-PCR and western blotting demonstrated that knockdown of Gli2 significantly attenuated the gene and protein expression of Shh, Ptch1, Gli1, and Gli2 in CAL27 and SCC9 cell lines (Fig. 4B and C). Our findings indicate that Gli2 plays a crucial role in regulating Hedgehog pathway activation in OSCC cell lines. Subsequently, we further investigated the impact of Gli2 on the biological behavior of OSCC cells. The CCK-8 assay revealed that knockdown of Gli2 markedly suppressed the proliferation of CAL27 and SCC9 cells in vitro (Fig. 4D). Cell migration assays indicated a significant inhibition in cell migration upon knockdown of Gli2 (Fig. 4E). Moreover, cell invasion assays demonstrated a suppressive effect on cell invasion following knockdown of Gli2 (Fig. 4F). Finally, the colony formation assay confirmed that down-regulation of Gli2 inhibited cell growth (Fig. 4G). These results suggest that down-regulation of Gli2 effectively inhibits the Hedgehog pathway activation by acting as a key transcription factor downstream this pathway in OSCC cells; consequently suppressing proliferation, migration, invasion and growth potentiality within CAL27 and SCC9 cell lines.

### 3.5. Gli2 is a key factor that drives the hedgehog pathway to regulate EMT and Wnt/β-catenin pathway activation in OSCC cells

Based on previous results, Gli2 has been identified as a crucial factor driving the aberrant activation of the Hedgehog pathway in OSCC cells and playing a pivotal role in their malignant proliferation and metastasis. Therefore, it is hypothesized that Gli2 may modulate the expression of activators involved in EMT and Wnt/β-catenin pathways by regulating Hedgehog pathway activity, thereby promoting OSCC cell proliferation and metastasis. To investigate this possibility, we silenced Gli2 expression to assess the mRNA and protein levels of key factors within EMT and Wnt/β-catenin pathways in CAL27 and SCC9 cell lines. Our findings demonstrate that knockdown of Gli2 significantly downregulates MMP2, MMP9, and vimentin at both mRNA and protein levels while upregulating E-cadherin expression in CAL27 and SCC9 cells (Fig. 5A and B). Furthermore, we observed reduced mRNA and protein expression of Wnt3a, β-catenin, C-myc, and CyclinD1 upon Gli2 knockdown (Fig. 5C and D). Our results indicate that Gli2 depletion enhances the activation of EMT and Wnt/β-catenin pathways leading to increased proliferation and migration capabilities of OSCC cells. These findings further validate the critical role played by Gli2 as a driver for the Hedgehog pathway activation in OSCC cells during tumor progression.

## 4. Discussion

Abnormal activation of the Hedgehog pathway is associated with tumor formation, metastasis, recurrence, and drug resistance in various cancers. Inhibition of this signaling pathway has a significant impact on certain cancers and its involvement in oral cancer development has been established [55]. However, the precise mechanism by which the Hedgehog pathway contributes to oral cancer remains unclear. Our study identified the Hedgehog pathway as one of the key pathways involved in differential gene enrichment. Furthermore, we observed increased expression of the Hedgehog pathway components in OSCC animal samples and cell lines, indicating abnormal activation of this pathway in OSCC. Therefore, elucidating the specific mechanism by which the Hedgehog pathway operates in OSCC is crucial for advancing our understanding of this disease. To investigate how the Hedgehog pathway regulates OSCC development, we modulated its activation in OSCC cells and assessed its impact on their biological functions. We found that inhibiting the Hedgehog pathway significantly suppressed malignant proliferation and metastasis of OSCC cells, while activating this pathway promoted these processes. These findings further support a pivotal regulatory role for the Hedgehog pathway in driving malignant proliferation and evolution of OSCC; however, additional studies are required to confirm its specific regulatory mechanisms.

OSCC malignant metastasis is one of the important reasons for its poor prognosis. EMT is a key event in tumor metastasis and plays an indispensable role in tumor invasion. During the EMT process, epithelial cells undergo a phenotypic change from epithelial-like state to mesenchymal state, and by regulating the expression of ECM proteins, they destroy the histological barrier, ultimately affecting the invasion and metastasis of tumor cells [56]. In addition, the aberrant activation of the Wnt/β-catenin pathway is closely related to the tumorigenesis and progression of various cancers, including OSCC. The abnormal activation of this pathway can affect the expression of downstream target proteins and accelerate the proliferation and metastasis of tumor cells [57,58]. Therefore, we hypothesize that EMT and the Wnt/β-catenin pathway are abnormally activated during the malignant proliferation and metastasis of OSCC. To elucidate the potential mechanism underlying Hedgehog-mediated regulation of malignant proliferation and metastasis in OSCC, we assessed the expression of key factors involved in the EMT pathway following abnormal activation/inhibition of the Hedgehog pathway. This investigation aimed to clarify how the Hedgehog pathway regulates OSCC cell migration and invasion. Additionally, we examined the expression of key factors in the Wnt/β-catenin pathway after abnormal activation/inhibition of the Hedgehog pathway to identify its potential role in regulating malignant proliferation of OSCC cells. Our findings demonstrate that inhibiting Hedgehog signaling suppresses malignant proliferation and growth by modulating Wnt/β-catenin signaling, while also inhibiting migration and invasion through EMT pathway regulation. Conversely, activating the Hedgehog pathway exerts opposite

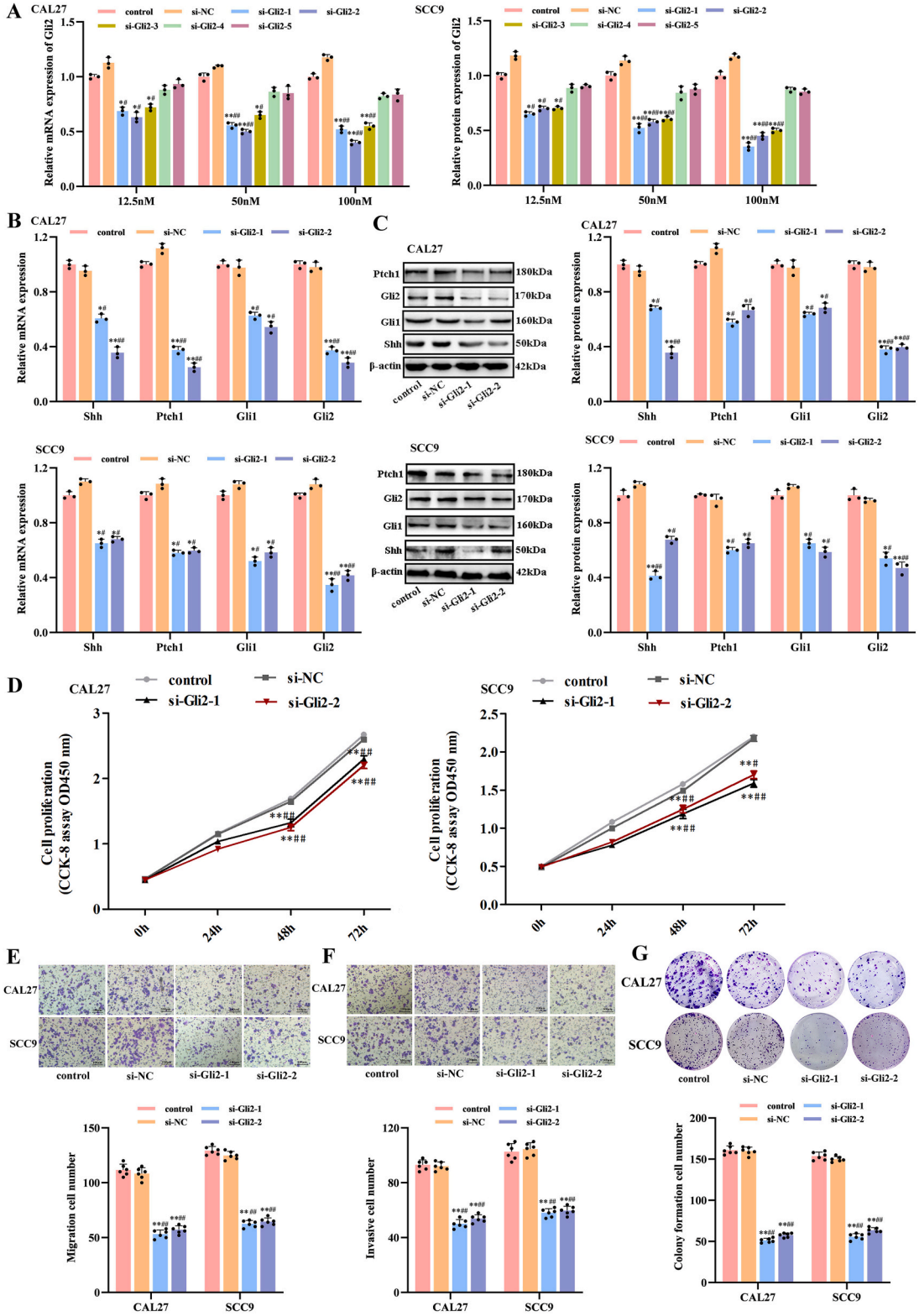


**Fig. 4.** Gli2 is a key factor that drives Hedgehog pathway to regulate malignant proliferation and metastasis of OSCC cells (A) Gli2 expression level after siRNA interference (n = 3). (B) mRNA expression of key components of the Hedgehog pathway in OSCC cells after Gli2 knockdown (n = 3). (C) Protein expressions of key components of the Hedgehog pathway in OSCC cells after Gli2 knockdown (n = 3) (western blotting is related to Fig. S7). (D) CCK-8 assay was used to evaluate cell proliferation after Gli2 knockdown (n = 3). (E) Representative image of cell migration after Gli2

knockdown (the bar graphs display the percentage of cell migration in different groups, n = 6). (F) Representative image of cell invasion after Gli2 knockdown (the bar graphs display the percentage of cell invasion in different groups, n = 6). (G) Representative image of cell colony formation after Gli2 knockdown (the bar graph represents the number of colonies formed in different groups, n = 6). (Data represented as mean ± standard deviation; $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with control group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with si-NC group).

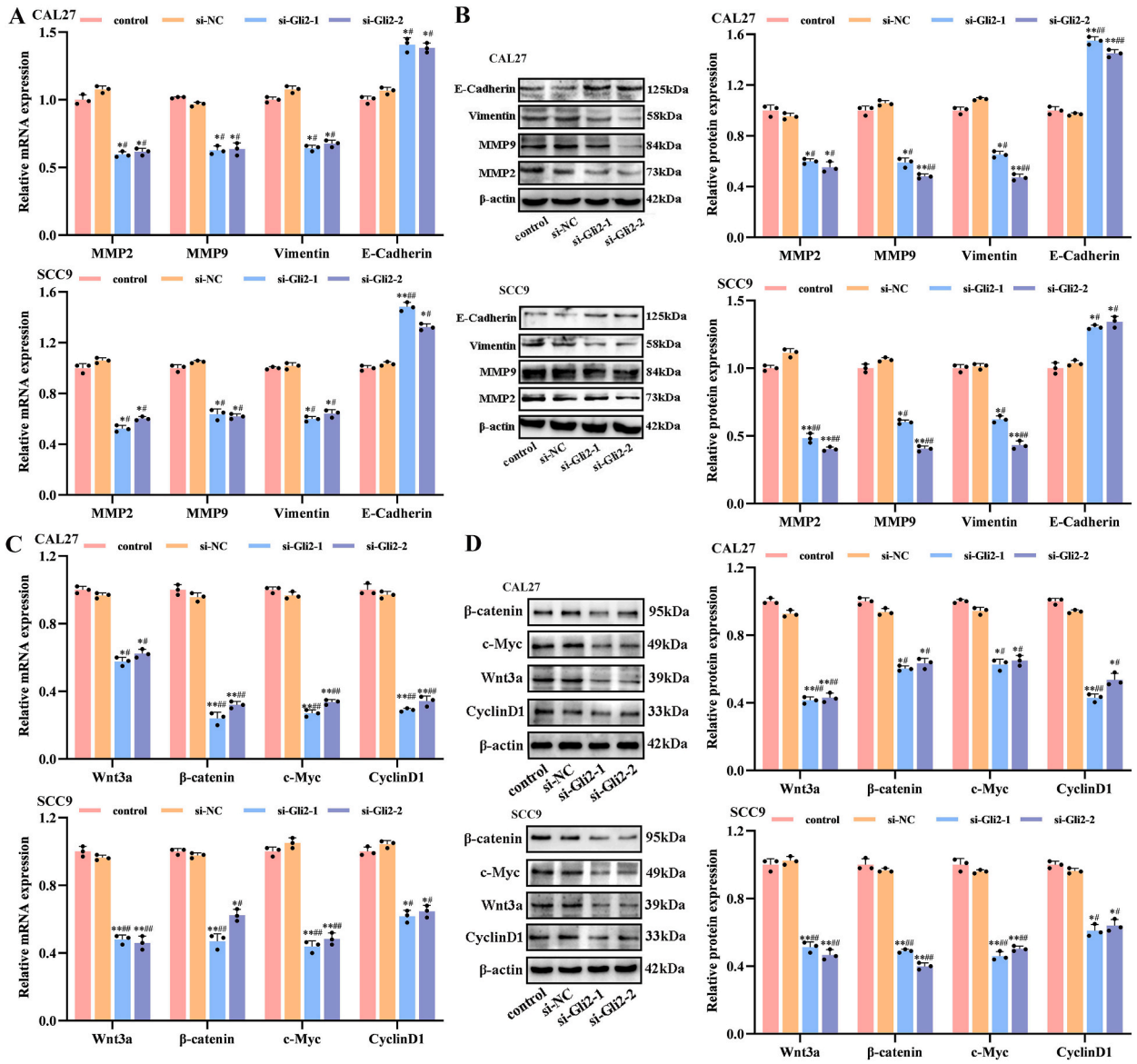


**Fig. 5.** Gli2 is a key factor driving Hedgehog pathway regulation of EMT and Wnt/β -catenin pathway activation in OSCC cells (A) mRNA expression of key components of EMT pathway in oral cancer cells following Gli2 knockdown. (B) Protein expression of key components of EMT pathway in OSCC cells following Gli2 knockdown (western blotting is related to Fig. S8). (C) mRNA expression of key components of Wnt/β-catenin pathway in OSCC cells following Gli2 knockdown. (D) Protein expression of key components of Wnt/β-catenin pathway in OSCC cells following Gli2 knockdown (western blotting is related to Fig. S9). (Data represented as mean ± standard deviation, n = 3; $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with the si-NC group).

effects. These results suggest that during OSCC progression, the Hedgehog signaling influences malignant proliferation and metastasis by regulating both EMT activation and Wnt/β-catenin pathways.

It is worth emphasizing that Gli2 has been previously documented and is essential for the initial Hedgehog signaling and ectopic activation of the Hedgehog pathway [59]. In this study, we observed a significantly higher mRNA and protein expression of Gli2 in OSCC cell lines and oral cancer animal models. Furthermore, analysis of clinical data from oral cancer patients using TCGA database

revealed a significant increase in Gli2 expression in OSCC patients. Based on these findings, we propose that Gli2 serves as a pivotal factor driving the aberrant activation of the Hedgehog pathway in OSCC cells. Subsequent interference with Gli2 expression resulted in a substantial decrease in Hedgehog pathway activity within OSCC cells, leading to inhibited proliferation, migration, invasion, and growth. Moreover, previous studies have shown that Gli2 is involved in regulating the activation of EMT and Wnt/β-catenin pathways. Zhang et al. found that FGF19/Gli2 signaling axis induces EMT to promote metastasis in lung squamous cell carcinoma [60]. Pantazi et al. proposed that Gli2 acts as a regulator of β-catenin which is associated with E-cadherin deletion and enhanced invasiveness [61]. In this study, we also found that interference with Gli2 expression affected the activation of EMT and Wnt/β-catenin pathways in OSCC cells. These observations collectively suggest that Gli2 plays a crucial role in promoting malignant proliferation and metastasis of OSCC through modulation of the Hedgehog pathway.

In conclusion, our study demonstrates that Gli2 facilitates the activation of EMT and Wnt/β-catenin signaling pathways by mediating the Hedgehog pathway activation, thereby enhancing proliferation and migration of oral cancer cells (Fig. 6A and B). Our findings not only elucidate the molecular mechanisms underlying Hedgehog pathway involvement in malignant proliferation and metastasis of oral cancer cells but also provide a theoretical foundation for personalized diagnosis and treatment strategies targeting Gli2 in oral cancer patients.

## 5. Limitations

Importantly, this study exclusively investigated the pivotal role of the Hedgehog/Gli2 pathway in malignant proliferation and metastasis of OSCC by regulating activation of EMT and Wnt pathways at the cellular level. However, the precise regulatory mechanism underlying Hedgehog/Gli2 interaction with EMT and Wnt signaling pathways in OSCC remains elusive within the scope of this study. Moreover, no elucidation was provided regarding the involvement of the Hedgehog/Gli2 pathway in OSCC progression in vivo. In our forthcoming research, we aim to comprehensively elucidate the regulatory mechanism through which the Hedgehog/Gli2 pathway influences EMT and Wnt pathways during OSCC development using both in vitro and in vivo experiments. Furthermore, considering its critical implication across various tumors, continuous exploration into this pathway is warranted alongside investigating potential targeted therapeutics tailored to individualized antitumor therapy requirements. This approach holds great promise for significantly enhancing cancer patients' prognosis. A comprehensive exploration of these issues will enhance our understanding of oral cancer's biological characteristics and pathogenesis while addressing more clinical challenges, ultimately facilitating the translation from basic research to clinical treatment.

## Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials.

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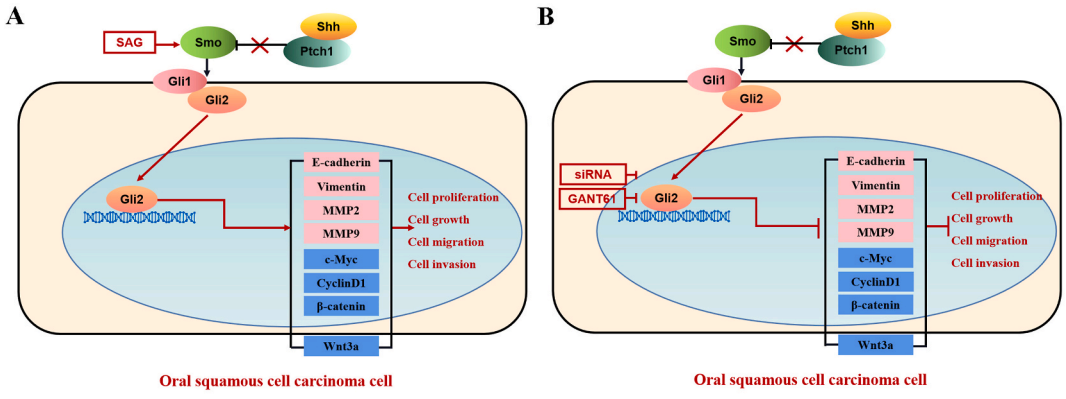


**Fig. 6.** The mechanism of the Hedgehog/Gli2 pathway in the malignant evolution of OSCC (A) Mechanism of action of the Hedgehog/Gli2 pathway in OSCC after activation. (B) Mechanism of action of the Hedgehog/Gli2 pathway in OSCC after inhibition.

## Ethical approval

All animal procedures were approved by the Institutional Animal Care and Use Committee of Shanxi Medical University (IACUC 2021012). All applicable institutional guidelines for the care and use of animals were followed. The study was performed in accordance with the Guidelines laid down by the National Institute of Health (NIH) regarding the care and use of animals for experimental procedures or with the European Communities Council Directive of November 24, 1986 (86/609/EEC) and in accordance with local laws and regulations.

## CRediT authorship contribution statement

**Xiaotang Wang:** Writing – original draft, Validation, Methodology, Investigation, Data curation. **Xiaona Song:** Validation, Methodology, Investigation. **Jiping Gao:** Methodology, Investigation, Formal analysis, Data curation. **Guoqiang Xu:** Validation, Resources, Methodology. **Xiaoru Yan:** Writing – review & editing, Formal analysis. **Junting Yang:** Writing – review & editing, Formal analysis. **Yiyan Yang:** Formal analysis, Data curation. **Guohua Song:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e36516.

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