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DZIP1 Promotes Proliferation, Migration, and Invasion of Oral Squamous Carcinoma Through the GLI1/3 Pathway

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Abstract

BACKGROUND: DZIP1 is an oncogene involved in the progression and stemness of carcinoma through the Wnt/β-catenin pathway, and the potential mechanism of DZIP1 in oral squamous cancer remains unknown. The aim of this study was to uncover the effect and mechanism of DZIP1 in the progression of oral squamous carcinoma. *METHODS:* TCGA database scanning was applied to verify dysregulated genes in oral squamous carcinoma. quantitative real-time polymerase chain reaction, immunohistochemistry, and Western blotting assays were used to detect the expression of DZIP1 in tissues and cell lines. We established stable DZIP1-overexpressing and DZIP1 knockdown cell lines. We investigated the biological function and the underlying mechanism of DZIP1 through a series of experiments. *RESULTS:* DZIP1 was one of the genes discovered by the scanning strategy to be upregulated in cancer tissue and negatively correlated with the overall survival (OS) of patients. DZIP1 promotes proliferation, migration, and invasion in an oral squamous carcinoma cell line through EMT in a GLI1/3-dependent manner. *CONCLUSIONS:* DZIP1 promotes the proliferation, migration, and invasion of oral squamous carcinoma through the GLI1/3 pathway.

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Background

Oral squamous cell carcinoma (OSCC) is the most common type of cancer in the head and neck area. It is one of the 10 most common cancers, and approximately 300,000 new cases are diagnosed annually worldwide [1]. OSCC is associated with poor prognosis and high morbidity when diagnosed at advanced stages, and it is considered to be a very immunosuppressive cancer. Despite advances in radiotherapy and surgical therapy, patients with late-stage OSCC still suffer from metastasis and recurrence [2]. Therefore, efforts are still needed to develop effective targeted therapies for OSCC.

Hedgehog (Hh) signaling is an important pathway engaged into the stemness and differentiation of stem cell and is critical for the physiology progression [3]. In general, when Hh is activated, Hh ligands bind to Patched (PTCH1), causing the level of Smoothened (SMO) to be increased, which dissociated the interaction of Suppressor of fused (SUFU) and glioma-associated oncogene homologue (GLI) complex [4]. The Gli was cleaved into active form and enters the nuclear and serves as transcription factors at the downstream end of Hh pathway. Thus, the level of GLI was one of the important markers measuring the level of Hh pathway activation.

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Gli can be activated by other factors which contribute to the output of Hh signaling [5]. Daz interacting protein 1 (DZIP1) has bipartite positive and negative functions in the Hh pathway [6–9]. However, its biological function and underlying mechanism in OSCC remain unclear. In this study, we detected the expression levels of DZIP1 in tissue and cell lines by quantitative real-time polymerase chain reaction (qRT-PCR), immunohistochemistry, and Western blotting and uncovered its biological function through a series of experiments.

Materials and Methods

Tissue Samples

All human tissues were obtained from the surgical suite in the Department of Stomatology the First Affiliated Hospital of Sun Yat-sen University after confirmation by a pathologist. Tissues were obtained with the patients' written consent under a protocol approved by the institution's Institutional Review Board.

Cell Culture

The normal epithelial Hatcat cell line and the human OSCC cell lines CAL27, SCC-9, and SCC-25 were obtained from American Type Culture Collection (Manassas, VA). Other cell lines were gifts from Professor Anxun Wang. Hatcat and CAL27 cells were cultured in DMEM (Sigma-Aldrich, St. Louis, MO) with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) and penicillin–streptomycin, while other cell lines were cultured in DMEM F-12 medium containing 10% FBS at 37°C in a humidified tissue culture incubator under 5% CO_2.

qRT-PCR

Total RNA from cells or tissues was extracted using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA) according to the protocol, and 2 μg of RNA was used to obtain cDNA through reverse transcription by using PrimeScript RT Master Mix (Takara Bio, Kusatsu, Japan). qRT-PCR was conducted using SYBR Green PCR Master Mix (Takara Bio) on a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Relative expression was determined by normalizing to the expression of β-actin.

Western Blot Assay

Equal protein were separated on 12% SDS-PAGE, after transmembraned and blocked, primary antibody was added overnight at 4°C. After incubation with its respective horseradish peroxidase–conjugated secondary antibodies for 1 hour, the protein bands of interest were visualized using enhanced chemiluminescence reagents (Millipore, Burlington, MA).

Colony Formation Assay

To measure the proliferation of cells, we applied colony formation; 200 cells/well were seeded and incubated for 14 days to form colonies. The colonies were fixed using methanol and stained with 0.1% crystal violet for 10 minutes.

Transwell Assay

Migration and invasion were assessed using Transwell plates. For the invasion assay, 5×10^4 cells were resuspended in 250 μl of plain medium in the upper chamber (8-μm pore size, Costar, Corning, NY), while the lower chamber was filled with 0.75 ml of complete medium. After incubation for 24 hours at 37°C, the upper chamber was fixed with 100% methanol and stained with 0.1% crystal violet. For the migration assay, 5×10^4 cells were plated on chambers. The transmembrane cells were estimated under a microscope (Nikon, Tokyo, Japan) at 200× magnification.

Cell Counting Kit-8 (CCK-8) assay

Five hundred cells were seeded into 96-well plates per well. Cell viability was determined using a CCK-8 assay (Dojindo, Kumamoto, Japan) every 24 hours and by measuring absorbance at 450 nm using a plate reader (BioTek, Winooski, VT) following the manufacturer's instructions.

Lentiviral Production and Stable Cell Line Construction

Lentiviral vectors expressing shRNA and DZIP1 were co-transfected with packaging vectors psPAX2 and pMD2G (Addgene) into HEK293FT cells for lentivirus production using Lipofectamine 3000 in accordance with the manufacturer's instructions. To establish stable cell lines, cells were transduced by using the

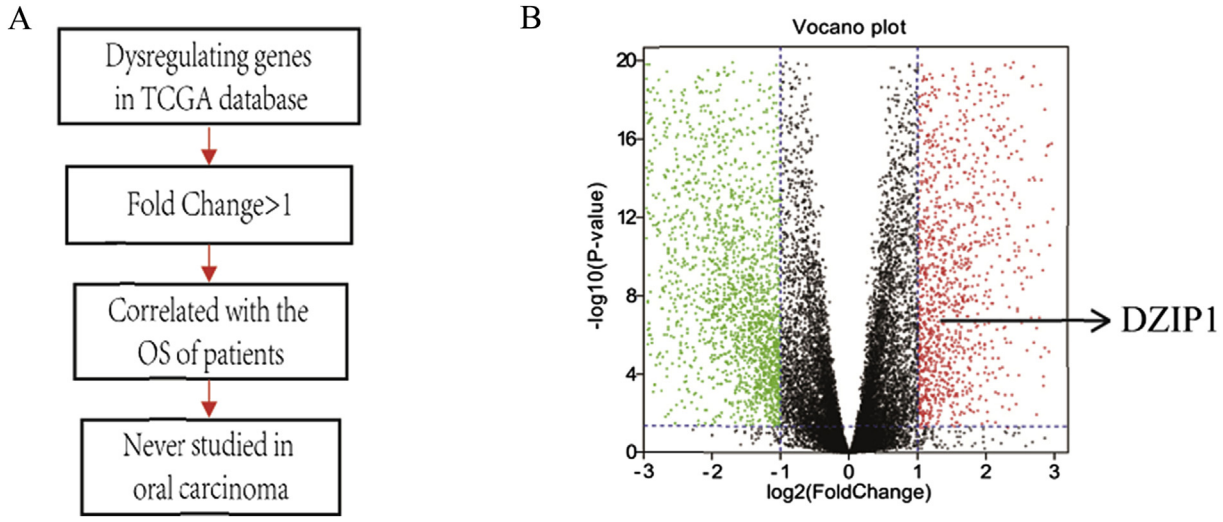

Figure 1. The scanning of dysregulated genes in OSCCA: (A) TCGA scanning strategy. (B) The volcano plot of dysregulated genes in OSCC.

above lentiviruses with polybrene (8 mg/ml, Sigma). After incubating for 72 hours, cells were selected with 2 mg/ml puromycin for 3 days.

Statistical Analysis

Statistical analyses were performed using SPSS version 18.0 (SPSS Inc., Chicago, IL). Data are presented as the mean ± SD. The χ^2 test

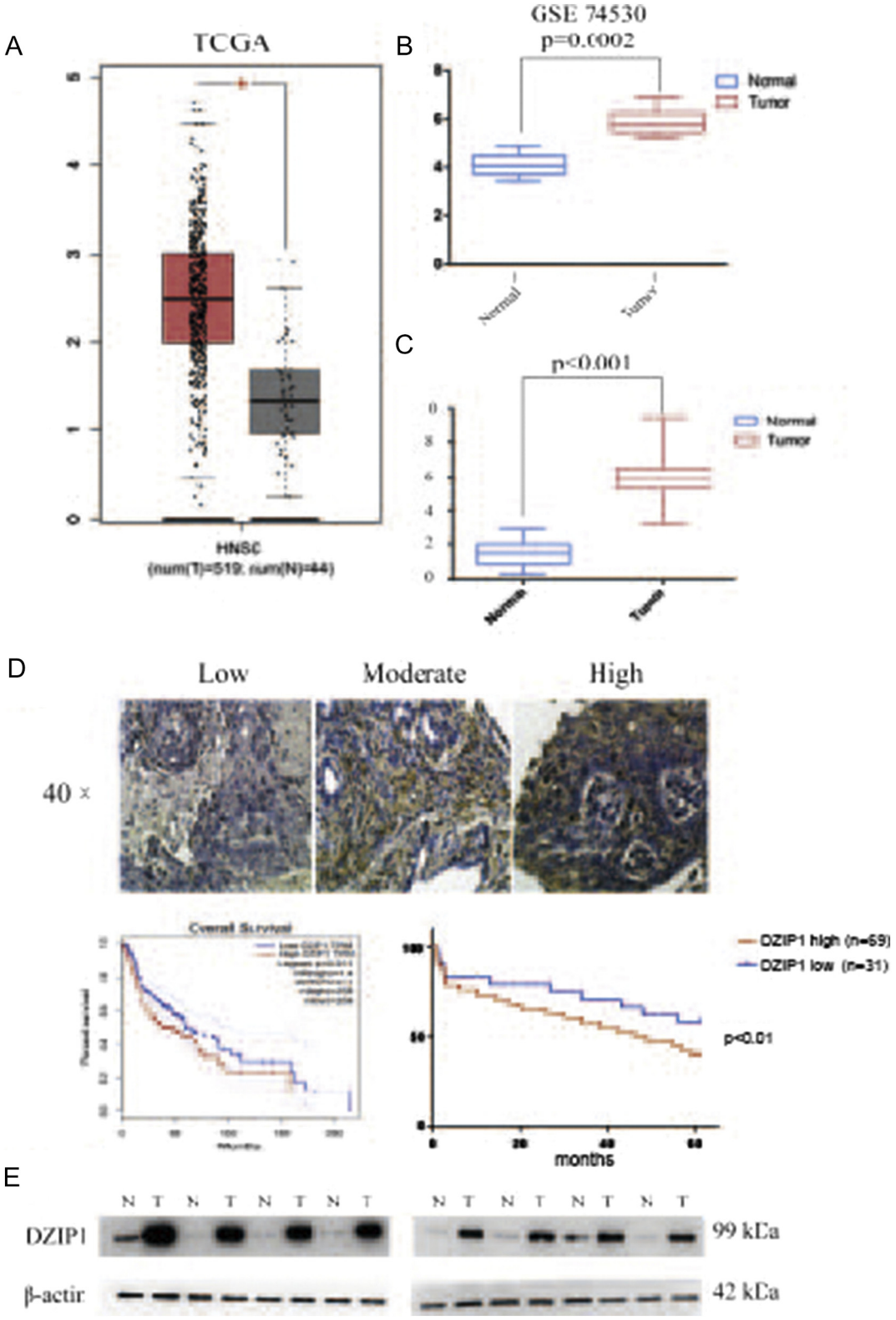

Figure 2. DZIP1 was upregulated in OSCC and negatively correlated with prognosis. The expression of DZIP1 in TCGA database (A), GSE74530 (B) and tumors of patients in the First Hospital Affiliated Hospital Of Sun Yat-sen University (C).(D) Upper: the IHC staining of DZIP1 in OSCC tissue.Lower: the survival analysis in TCGA database (left) and patients in the First Affiliated Hospital of Sun Yat-sen University (right)(E) Western blot of DZIP1 in eight patients.

Table 1. The Clinical Characteristic of Patients

Characteristics	Patients ($n = 100$)	Dzip1 lower($n = 31$)	Dzip1 higher($n = 69$)	*P* Value
Age (years)				
<60	45	12	33	>.05
≥60	55	19	36	
Gender				
Male	69	27	42	
Female	31	4	27	
Stage				
I + II	35	10	25	<.01
III + IV	65	21	44	
Lymph node				
Negative	61	18	43	<.01
Positive	39	13	26	

was used to analyze clinicopathological characteristics. Student's *t* test was applied for the difference of data. All experiments were repeated at least three times. A $P < .05$ was considered statistically significant.

Results

DZIP1: one of the most dysregulated genes in OSCC

We set a series of strategies to search for dysregulated genes in OSCC as described in Figure 1*A*. The volcano plot showing the overall genes identified from the TCGA database is shown in Figure 1*B*. DZIP1 was the best matched gene according to the scanning strategy.

DZIP1 Is Upregulated in OSCC and Negatively Correlated with Prognosis

We picked DZIP1 out as a target gene as previously described. We analyzed the expression of DZIP1 in 519 OSCC tumors and 44 normal tissues from the TCGA database and 100 OSCC tumors and paired normal tissues from the Department of Stomatology the First Affiliated Hospital of Sun Yat-sen University through qRT-PCR. DZIP1 was upregulated in OSCC tumors from both the TCGA database and our own database ($P < .001$), as shown in Figure 2*A*. We further analyzed the relationship between DZIP1 and prognosis. We applied an IHC assay to detect the protein level of DZIP1 and divided it into two groups. Tissues from both the TCGA database and our own database showed that patients with higher levels of DZIP1 had shorter overall survival times (Table 1). To further verify the expression difference in OSCC tumors and normal tissues, as shown in Figure 2*B*, we collected eight OSCC tumors and paired tissue samples and detected the protein level of DZIP1 through Western blot. The level of DZIP1 differs between patients in both tumors and normal tissues, and the level of DZIP1 was higher in tumors than in normal tissues (Figure 2*C*). Taken together, DZIP1 levels were higher in OSCC tumors than in normal tissues and negatively correlated with prognosis. (See Table 2.)

Table 2. Univariate and Multivariate Cox Regression Analysis of Risk Factors Associated with Overall Survival

Variables	Univariate Analysis			Multivariate analysis		
	HR	95% CI	*P* Value	HR	95% CI	*P* Value
DZIP1 expression (high vs. low)	3.18	1.42-7.10	**<.01**	3.383	1.51-7.58	**<.01**
Sex (male vs. female)	1.07	0.57-2.01	.84			
Age (≥60 vs. <60)	1.07	0.60-1.91	.82			
Stage (late vs. early)	1.72	0.91-3.26	.10			
Lymph node (positive vs. negative)	2.40	1.35-4.28	**<.01**	2.56	1.44-4.55	**<.01**

Stable Cell Lines Were Successfully Constructed

To explore the biological function of DZIP1, we detected its mRNA and protein levels in OSCC cell lines and found that OSCC cell lines harbor higher levels of DZIP1 than normal epithelium cells. Among the OSCC cell lines, UM1 had the highest level of DZIP1, while UM2 had the lowest level of DZIP1 (Figure 3, *A* and *B*). We established a stable knockdown cell line of UM1 cells and DZIP1-overexpressing UM2 cells. The mRNA protein levels of DZIP1 in different cell lines were tested through qRT-PCR and Western blot (Figures 3*C*).

DZIP1 Promotes the Proliferation of OSCC

To uncover the biological function of DZIP1 in OSCC, we applied the CCK-8 assay, the colony formation assay, and the EdU assay. Cells with higher levels of DZIP1 showed higher proliferation rates in both the UM1 and UM2 cell lines in the CCK-8 assay and the colony formation assay. DZIP1-overexpressing cells harbor a high percentage of EdU-positive cells, while DZIP1 knockdown cells harbor a low percentage of Edu-positive cells (Figure 4). Taken together, DZIP1 promotes the proliferation of OSCC in both short and long time periods.

DZIP1 Promotes the Migration and Invasion of OSCC

DZIP1 was shown to be negatively correlated with prognosis as previously described. Metastasis and recurrence are the most important reasons responsible for the deaths of patients. We next examined the function of DZIP1 in migration and invasion. We applied the wound healing assay, the Transwell assay, and the invasion chamber assay as described above. The wound healed more rapidly in the DZIP1-overexpressing cell line than in the DZIP1 knockdown cell line, and the DZIP1-overexpressing cells moved more easily through the well than the DZIP1 knockdown cells in both the Transwell assay and the invasion chamber assay (Figure 5). As shown by the above data, DZIP1 promotes the migration and invasion of OSCC.

DZIP1 Promotes the Expression of Mesenchymal Markers and Proliferation Markers in OSCC

DZIP1 promotes proliferation, migration, and invasion in OSCC. EMT markers are the most common markers reflecting cell mesenchymal or epithelial status. PCNA is a basic marker reflecting proliferation. We detected the expression of EMT markers and PCNA in the DZIP1 knockdown UM1 cell line and the DZIP1-overexpressing UM2 cell line. Mesenchymal markers such as N-cadherin, Snail, and Vimentin decreased, but epithelial markers such as E-cadherin increased, in the shDZIP1 cell line; however, mesenchymal markers increased but epithelial markers decreased at both the RNA and protein level in the DZIP1-overexpressing cell line (Figures 6, *A* and *B*). Studies have shown that DZIP1 is correlated with GLI1/3 and stemness [10]. However, the relationship differs depending on cell type. The correlation of DZIP1 with GLI1/3 is still unknown in OSCC [6,8]. We next detected the level of GLI1/3 in the cell lines described above. GLI1/3 was positively correlated with DZIP1 expression. GLI1/3 decreased with DZIP1 knockdown but increased with DZIP1 overexpression. Taken together, DZIP1

promotes the expression of mesenchymal markers and proliferation markers in OSCC.

DZIP1 Promotes the Proliferation of OSCC in a GLI1/3-Dependent Manner

We previously proved that DZIP1 promotes the proliferation and expression of PCNA and positively correlates with GLI1/3. To further uncover the molecular mechanism of DZIP1 in OSCC, we established two rescue cell lines: dual expressing GLI1/3 in the DZIP1 knockdown cell line and dual knockdown of GLI1/3 in the DZIP1-overexpressing cell line. The rescue cell lines are named UM1-Rescue and UM2-Rescue. We next applied the CCK-8 assay, the colony formation assay, and the Edu assay to the rescue cell lines. DZIP1 promotes the proliferation of OSCC as described above. The function of DZIP1 in the proliferation of OSCC was completely restored and abolished in DZIP1 knockdown and DZIP1-overexpressing cells, respectively (Figure 7).

DZIP1 Promotes Migration and Invasion of OSCC in a GLI1/3-Dependent Manner

We previously proved that DZIP1 promotes migration, invasion, and the expression of mesenchymal markers. We previously proved that DZIP1 promotes the proliferation of OSCC in a GLI1/3-dependent manner. We next tested migration and invasion in the rescue cell lines. The function of DZIP1 in promoting migration and invasion in OSCC was completely restored and abolished in DZIP1 knockdown and DZIP1-overexpressing cells, respectively (Figure 8).

PCNA and EMT Markers Are Restored with the Rescue of GLI1/3

We have previously shown that DZIP1 exerts its function in a GLI1/3-dependent manner. We next detected changes in EMT markers and PCNA in the rescue cell lines. The levels of EMT markers and PCNA in the rescue cell lines returned to the levels observed in control cells at both the RNA and protein levels (Figure 9).

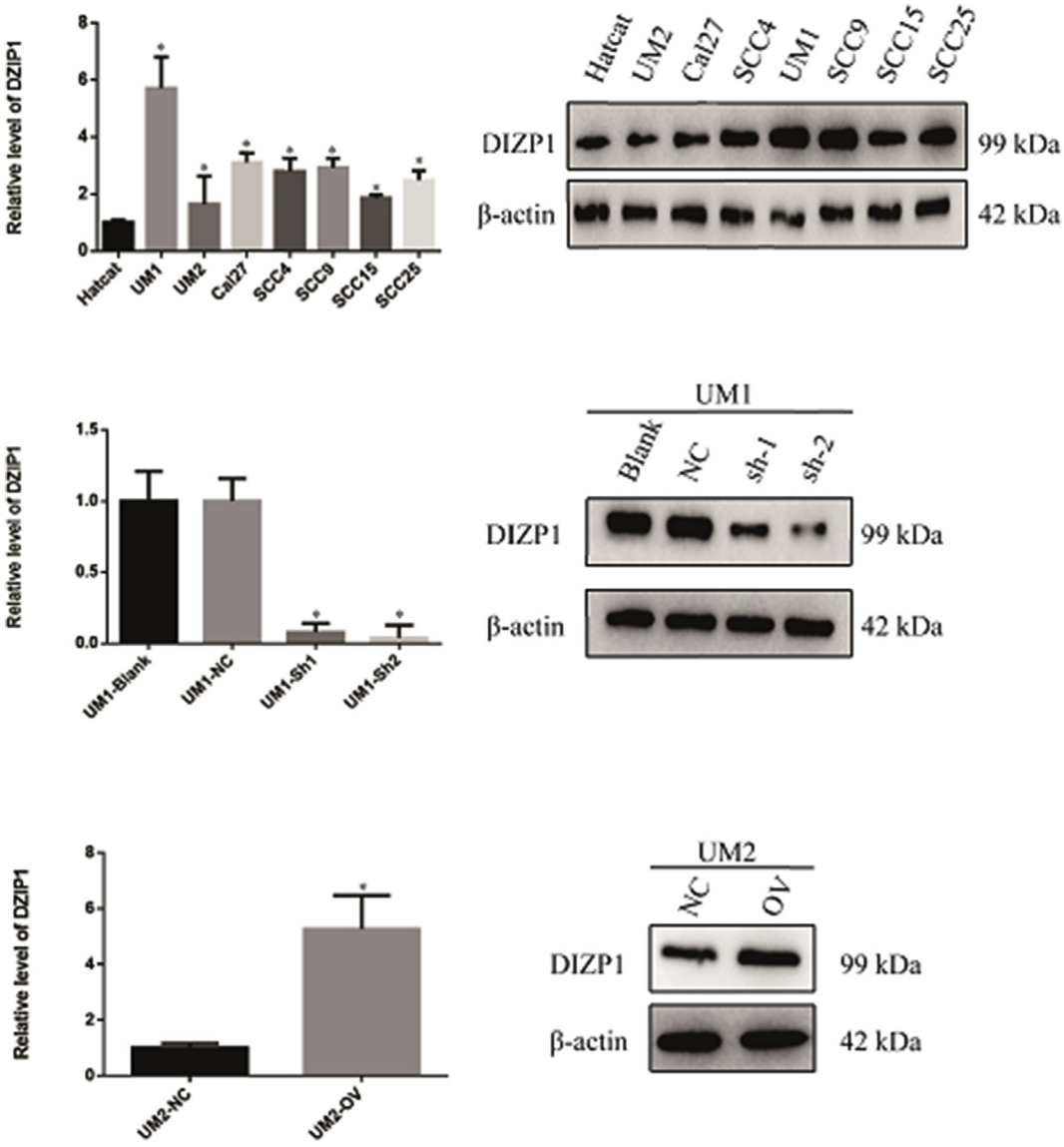

Figure 3. Stable cell line establishment.(A) The relative mRNA level of DZIP1 by qRT-PCR (left) and protein level by Western blot in OSCC cell line.(B) The relative mRNA level of DZIP1 by qRT-PCR (left) and protein level by Western blot in the stable cell line.

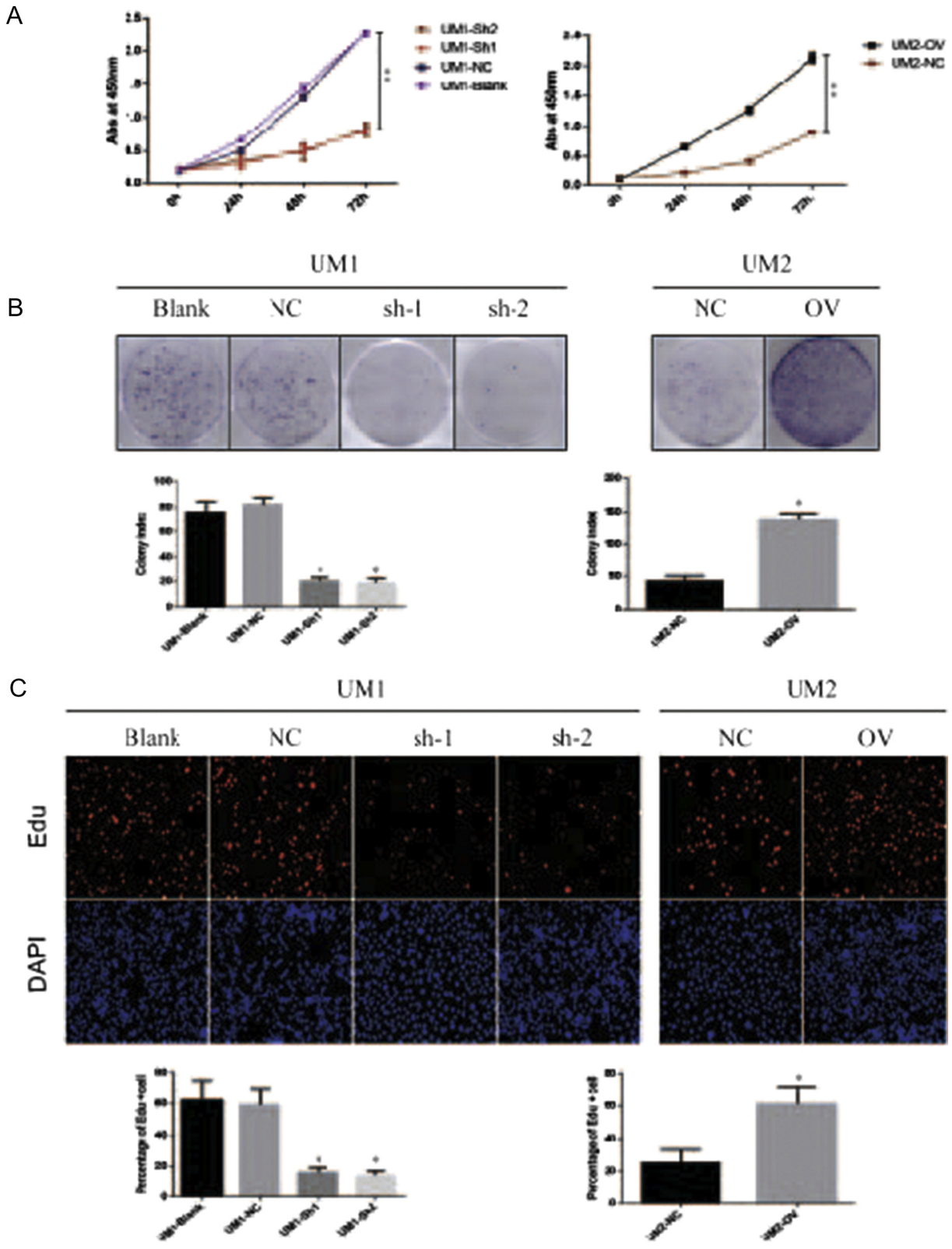

Figure 4. DZIP1 promotes the proliferation of OSCC.(A) CCK-8 analysis of cell lines.(B) Upper: the CFA of stable cell line.Lower: the colony index of stable cell line.(C) Upper: the Edu assay of stable cell line.Lower: the percentage of Edu-positive cells of stable cell line.

Discussion

OSCC is one of the 10 most common cancers worldwide, and current therapy is surgical operation with adjuvant chemotherapy and radiotherapy [11]. Some patients still suffer from metastasis and recurrence. OSCC is known to be an immunosuppressive cancer, and immunotherapy has been reported to no longer be effective [12,13]. Therefore, the identification of more biomarkers and therapeutic targets is needed.

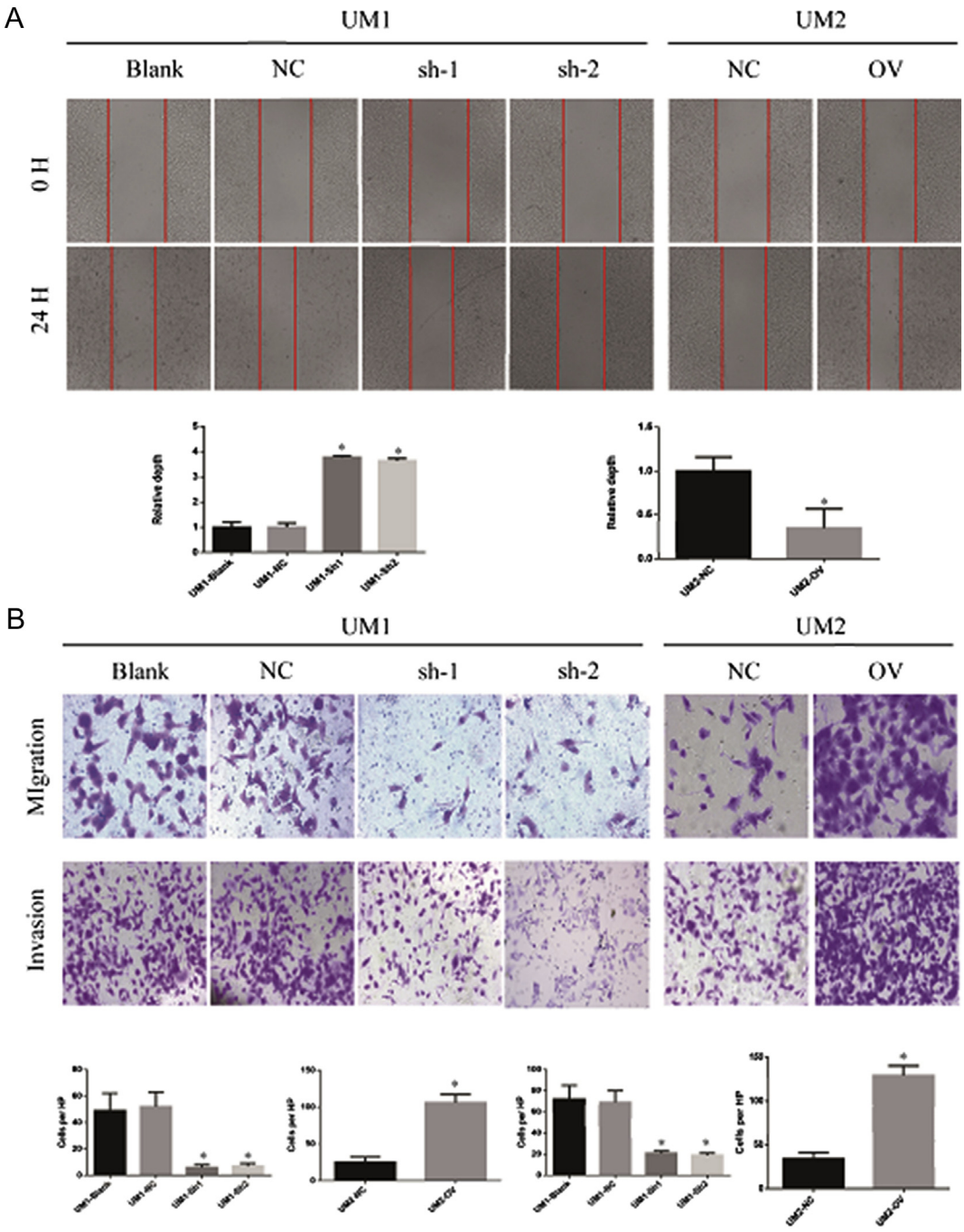

Figure 5. DZIP1 promotes the migration and invasion of OSCC.(A) Upper: wound healing assay of cell lines.Lower: the relative width of cell lines.(B) Upper: the migration and invasion of stable cell line.Lower: the statistics of stable cell line.

The TCGA database is one of the most authoritative databases describing the prognosis of different cancer patients. We scanned the database following the protocol described above to determine the unidentified genes contributing to prognosis. DZIP1 was one of the best matched genes. We next examined the level of DZIP1 in 100 OSCC and paired normal tissues and its correlation with prognosis in patients diagnosed with OSCC in the Department of Stomatology at the First affiliated hospital of Sun Yat-sen University. The results showed that DZIP1 was upregulated in cancer tissues and negatively correlated with prognosis. We next examined the level of DZIP1 in cell lines at both the RNA and protein levels. We established stable DZIP1 knockdown and overexpression cell lines. To uncover the biological function of DZIP1 in OSCC, we applied a series of experiments measuring proliferation, migration, and invasion in the cell lines. We found that DZIP1 promotes the proliferation, migration, and invasion of OSCC in both the UM1 and UM2 cell lines. PCNA and EMT markers are the most common markers reflecting proliferation, migration, and invasion abilities. We next detected the levels of PCNA and EMT markers. PCNA and mesenchymal markers such as N-cadherin, Snail, and vimentin increased with the overexpression of DZIP1 and decreased with DZIP1 knockdown. We have proven that DZIP1 promotes

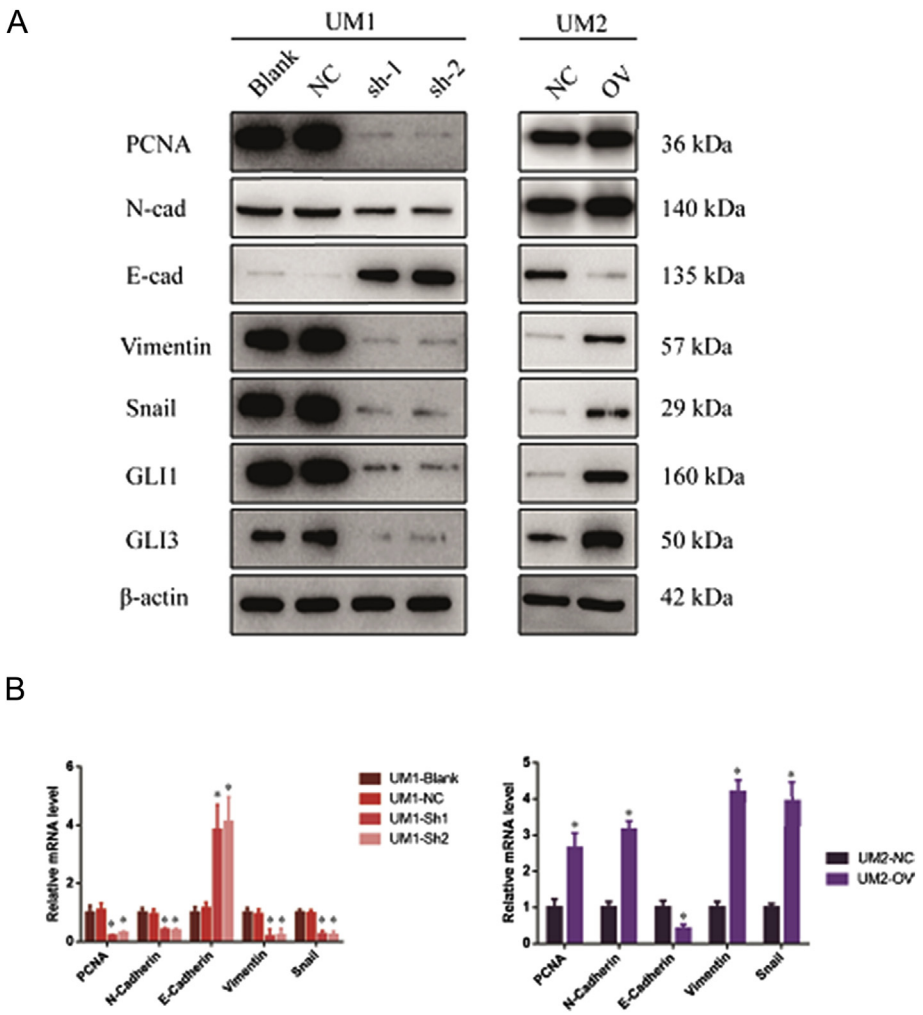

Figure 6. DZIP1 promotes GLI1/3, the EMT, and proliferation marker of OSCC.(A) The western blot of PCNA and EMT markers of cell line.(B) The relative mRNA level of PCNA and EMT markers of cell line.

proliferation, invasion, and migration through the EMT pathway. To further uncover the biological mechanism of DZIP1, we found that DZIP1 promotes stemness in multiple cancers through the GLI1/3 pathway, but the effect of DZIP1 on GLI was not clear. DZIP1 binds to GLI directly and catalyzes to activation form or inhibition form on cell type. The biological function of DZIP1 and its relationship with GLI in OSCC cancer are still unclear. We next established rescue cell lines by the overexpression of GLI1/3 in the DZIP1 knockdown cells and the knockdown of GLI1/3 in the DZIP1-overexpressing cells. DZIP1 levels in the rescue cell lines were unchanged, indicating that GLI1/3 is downstream of DZIP1. We next examined the proliferation, migration, and invasion ability of the rescue cell lines. Proliferation, migration, and invasion were completely restored with the rescue of GLI1/3, indicating that DZIP1 exerts its function in a GLI1/3-dependent manner. DZIP1 may directly bind to GLI1/3 and promote its activation in OSCC cells. Active GLI1/3 was transported into the nucleus and promoted the transcription of stemness, thereby promoting proliferation, migration, and invasion in the OSCC cell line through the Hh pathway.

Overactivation of the Hh pathway plays an initial role in the initiation and progression of multiple cancers such as breast cancer and hepatic cancer [14,15]. Hh promotes the progression of cancer by promoting the stemness of cancer stem cell and the proliferation of differential cancer cell [16]. The effect of Hh pathway on the self-renewal of stem cell may explain why Hh was overactivated in multiple cancers such as skin cancer basal cell carcinoma and cerebellum cancer medulloblastoma [17]. In view of the importance of Hh pathways, research aimed to treat the tumor through blocking the activation of Hh pathway [18,19]. Interestingly, the expression of ligands in many tumors was equal and does not contain any mutations in pathway components. We hypothesized that Hh depends on the ligand production which can be produced by both the tumor cells themselves and the surrounding stroma [20] [21]. Disappointingly, however, recent clinical trials for drugs that target the Hh pathway in patients with tumors have been discouraging, suggesting that more research should be done in the pathway and the cancer.

However, our research showed that DZIP1 promotes the proliferation, migration, and invasion and progression of OSCC through EMT in a GLI1/3-dependent manner. It may be a potential therapeutic target in the future.

Conclusion

In this study, we proved that DZIP1 promotes the proliferation, migration, and invasion of oral squamous carcinoma through the

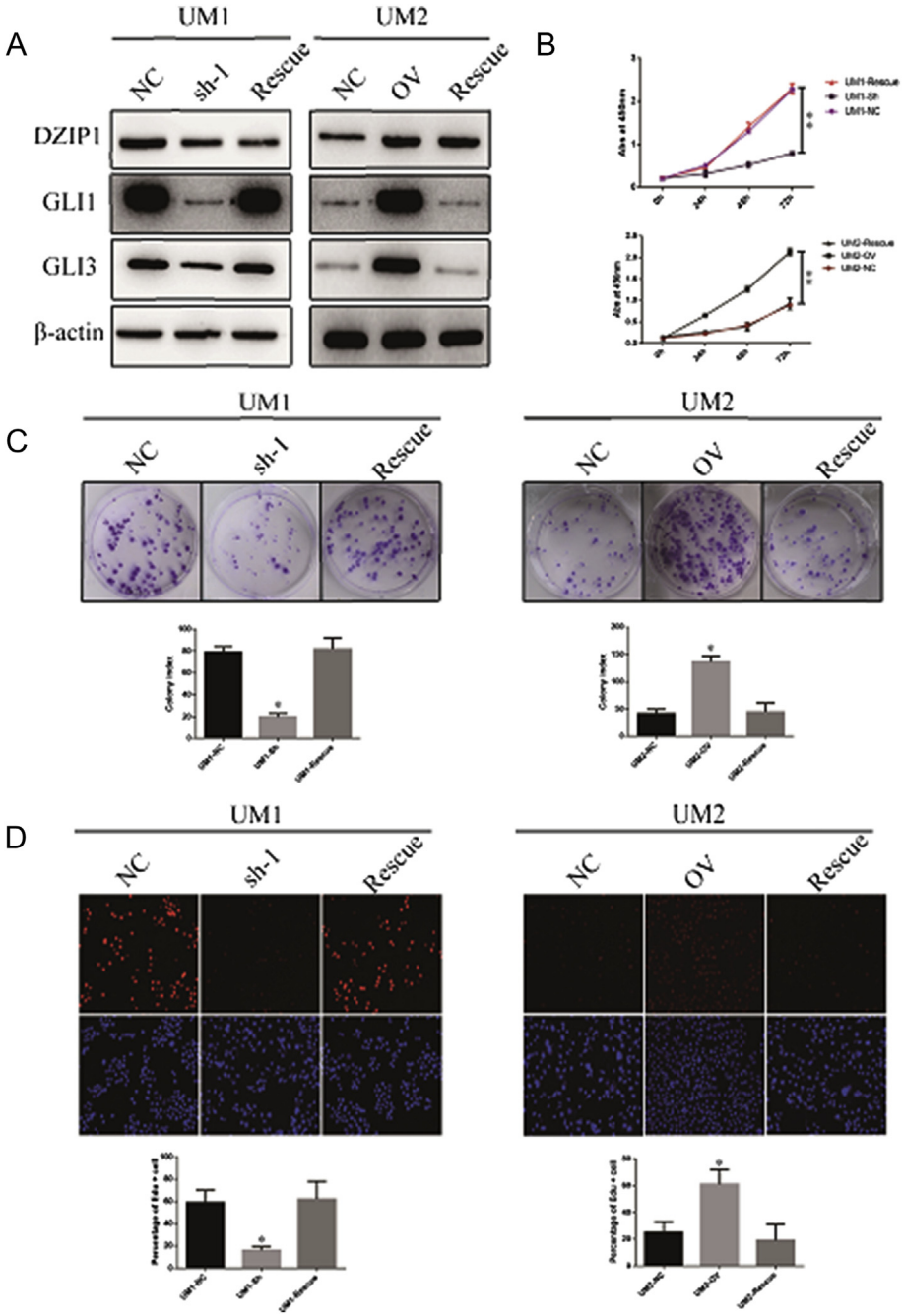

Figure 7. DZIP1 promotes the proliferation of OSCC in GLI1/3-dependent manner.(A) Western blot of DZIP1 and GLI1/3 of cell lines.(B) CCK-8 analysis of cell lines.(C) Upper: the CFA of stable cell line.Lower: the colony index of stable cell line.(D) Upper: the Edu assay of stable cell line.Lower: the percentage of Edu-positive cells of stable cell line.

GLI1/3 pathway and may be a potential therapeutic target in the future.

Declarations

Ethics Approval

This study was approved by the Medical Ethics and Human Clinical Trial Committee, and Animal Use and Management Committee at First Affiliated Hospital of Sun Yat-Sen University.

Research Involving Animals

No animal research was done.

Research Involving Plants

No plant research was done.

Consent for Publication

All authors agreed to publish the article in the journal.

Trial Registration

No trial registration was done.

Availability of Data and Materials

All the datasets supporting the conclusions of this article are included within the article.

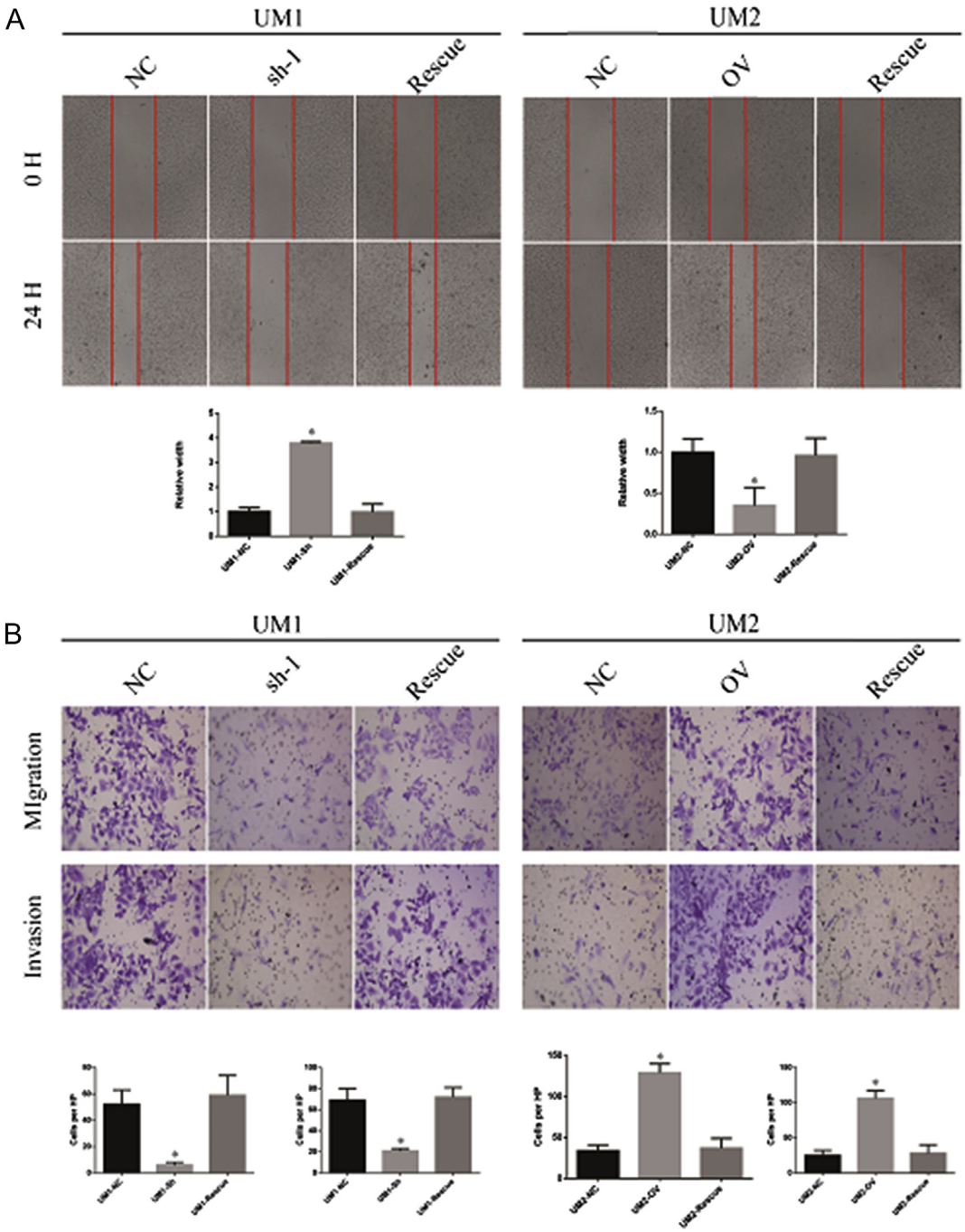

Figure 8. DZIP1 promotes the migration and invasion of OSCC in GLI1/3-dependent manner.(A) Upper: wound healing assay of cell lines.Lower: the relative width of cell lines.(B) Upper: the migration and invasion of stable cell line.Lower: the statistics of stable cell line.

Standards of Reporting

No reports were needed.

Competing Interests

The authors declare that they have no competing interests.

Authorship

Y. C. and L. J. M.: experimental design and manuscript draft; Y. C. was the corresponding author, while L. J. M. was the co-corresponding author. W. X. Y. and Y. D.: experimental application and result analysis. Y. H. Z., J. L., D. L. L., and Q. W.: clinical sample collection, interpretation, and analysis.

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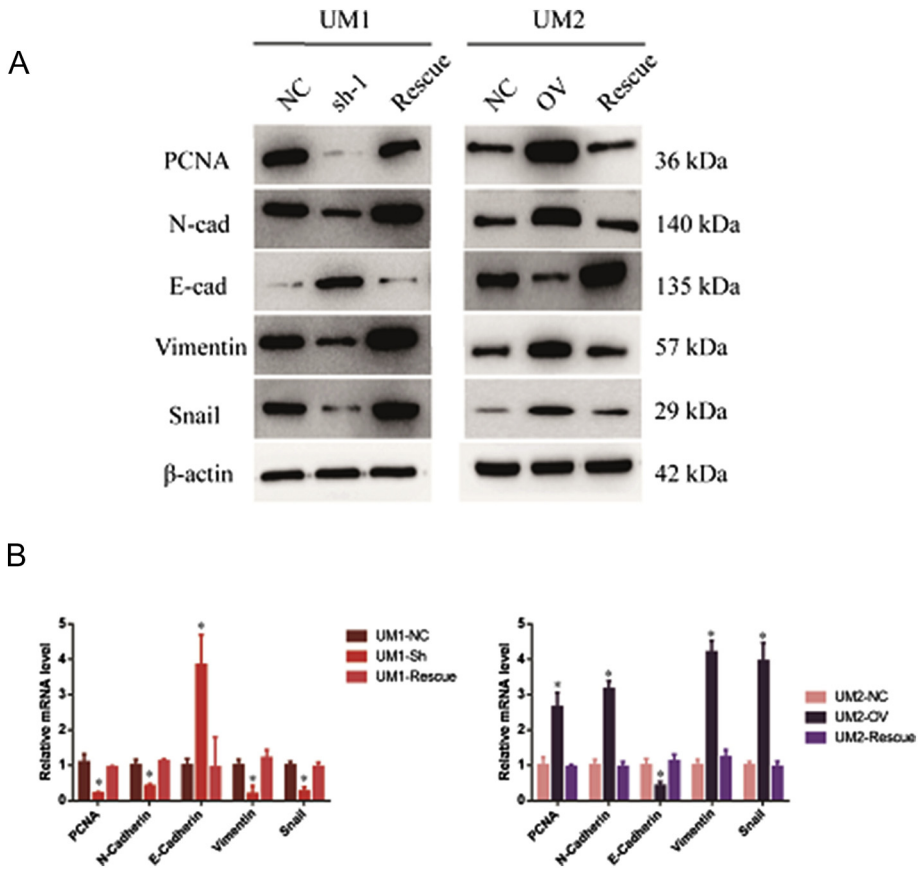

Figure 9. DZIP1 promotes the EMT and proliferation marker of OSCC in GLI1/3-dependent manner.(A) The western blot of PCNA and EMT markers of cell line.(B) The relative mRNA level of PCNA and EMT markers of cell line.

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Duplicate Publication.

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