

Galectin-7 promotes the invasiveness of human oral squamous cell carcinoma cells via activation of ERK and JNK signaling

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Received January 27, 2016; Accepted October 28, 2016

DOI: 10.3892/ol.2017.5649

Abstract. Galectin-7 is a member of the β -galactoside-binding protein family, and is highly expressed in oral squamous cell carcinoma (OSCC). The aim of the present study was to investigate the effects of manipulating galectin-7 expression on the biological phenotype of human OSCC cells and the associated molecular mechanisms. Knockdown of endogenous galectin-7 via small interfering RNA (siRNA) was performed and cell proliferation, apoptosis, migration, and invasion were subsequently assessed. The data indicated that galectin-7 silencing had no impact on the proliferation or apoptosis of OSCC cells. However, compared with non-transfected cells, percentage wound closure was significantly lower in galectin-7-silenced cells following 24 h incubation, indicating decreased cell migration. Furthermore, Matrigel invasion assays demonstrated that galectin-7 knockdown significantly reduced the number of invaded cells, compared with the number in non-transfected cells. Western blot analysis indicated that galectin-7 overexpression resulted in a significant increase in the expression of the proteins matrix metalloproteinase (MMP)-2 and MMP-9. The invasive abilities of cells overexpressing galectin-7 significantly decreased following co-transfection with MMP-2- or MMP-9-specific siRNA. Increasing galectin-7 expression significantly enhanced the phosphorylation of extracellular signal-related kinase (ERK) 1/2 and c-Jun N-terminal kinase (JNK) 1/2. Pharmacological inhibition of ERK or JNK activity significantly suppressed the invasiveness of galectin-7-overexpressing cells and abrogated the upregulation of MMP-2 and MMP-9. Taken together, the results of the current study provide novel evidence for the pro-invasive activity of galectin-7 in OSCC cells, which is

associated with activation of ERK and JNK signaling and the induction of MMP-2 and MMP-9.

Introduction

Oral squamous cell carcinoma (OSCC) is one of the most common malignancies in the world, and has a high mortality rate (1). Invasive growth via the lymphatic route is a typical feature of OSCC (2) and lymph node involvement status has been identified as a reliable prognostic indicator in OSCC patients (3,4). Therefore, a number of studies are underway in order to investigate the molecular mechanisms involved in regulating OSCC invasiveness (5).

Galectin-7 is a member of the β -galactoside-binding protein family. It is predominantly expressed in epithelial cells within healthy tissue and plays an important role in epithelial development and homeostasis (6,7). Galectin-7 expression may be altered in epithelial cancer, therefore it may serve an important role in cancer progression (8). The exact role of galectin-7 may vary in different types of cancers; it may play distinct and even opposing roles in tumor development. For example, in human gastric cancer specimens, galectin-7 is underexpressed due to epigenetic modifications and this suppresses the proliferation and invasion of gastric cancer cells (9). By contrast, in high-grade breast cancer galectin-7 is overexpressed, facilitating the spontaneous metastasis of breast cancer cells in preclinical mouse models (10). The tumor-promoting role of galectin-7 has also been noted in ovarian cancer cells (11) and cervical cancer cells (12).

Previous studies have demonstrated that galectin-7 increases the expression of matrix metalloproteinases (MMPs), especially MMP-9, thus modulating the invasiveness of cancer cells (11,12). Additionally, OSCC tissues exhibit increased MMP-2 and MMP-9 activity compared with adjacent healthy tissues (13). It has previously been demonstrated that MMPs serve a critical role in the invasion and metastasis of oral cancer (14). Alves *et al* (15) reported that galectin-7 is highly expressed in OSCC and its expression is significantly correlated with the histological grade of disease. These findings suggest that galectin-7 may contribute to OSCC invasiveness by modulating the expression of MMP-2 and MMP-9. The present study investigated the effects of manipulating galectin-7 expression on the biological phenotypes of human

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Key words: cell proliferation, tumor invasion, oral squamous cell carcinoma, signaling pathways

OSCC cells and evaluated the involvement of MMP-2 and MMP-9 on the action of galectin-7.

Materials and methods

Cell culture and treatment. The human OSCC cell lines SCC-4 and SCC-9 were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were maintained at 37°C in 5% CO₂ in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (FBS), 1 mmol/l L-glutamine, and 100 U/ml penicillin, 100 µg/ml streptomycin (all from Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). For inhibitor experiments, cells were pretreated with the c-Jun N-terminal kinase (JNK) inhibitor SP600125 (10 µM; Calbiochem; EMD Millipore, Billerica, MA, USA), extracellular signal-related kinase (ERK) inhibitor PD98059 (10 µM; Calbiochem; EMD Millipore), or 0.1% dimethyl sulfoxide (DMSO) used as vehicle control 1 h before transfection of galectin-7-expressing plasmid.

Plasmids, small interfering RNA (siRNA), and transfection. A galectin-7-expressing plasmid (pCEP4-GAL7) was purchased from Addgene (Cambridge, MA, USA) and an empty vector (pCEP4) was also purchased (Invitrogen; Thermo Fisher Scientific, Inc.). Galectin-7 siRNA, MMP-2 siRNA, MMP-9 siRNA, and negative control siRNA were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). For overexpression or knockdown of galectin-7, cells were seeded onto 6-well plates (4x10⁵ cells/well) and transfected with 1 µg pCEP4-GAL7, 1 µg pCEP4 and 50 nM galectin-7 siRNA, or 50 nM control siRNA using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Cells were incubated for 24 h, and subsequently collected for further experiments. To validate the involvement of MMP-2 and MMP-9, cells were co-transfected with 1 µg pCEP4-GAL7 and 50 nM MMP-2 siRNA, MMP-9 siRNA, or control siRNA, and tested for invasive ability following incubation for 24 h.

Cell proliferation assay. Cell proliferation was measured using the MTT assay. Transfected cells were detached and re-seeded onto 96-well plates (2x10³/well). Following incubation for 1, 3, and 5 days, 0.5 mg/ml MTT (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) was added to the culture and incubated for additional 4 h at 37°C. Formazan crystals were dissolved in DMSO. Absorbance was measured at 570 nm using a multi-plate reader.

Apoptosis detection assay. Apoptosis analysis was performed using the Annexin V-FITC Apoptosis Detection kit (Nanjing KeyGen Biotech Co., Nanjing, China), according to the manufacturer's instructions. In brief, cells were incubated with a staining solution containing fluorescein isothiocyanate (FITC)-conjugated annexin-V and propidium iodide (PI) for 10 min at 4°C in the dark. The percentage of apoptotic cells was determined using a FACScan flow cytometer with the CellQuest software (BD Biosciences, San Jose, CA, USA).

Wound-healing assay. Cells were seeded onto 6-well plates and allowed to grow to ~95% confluence. A wound was made in the monolayer using a 100-µl pipette tip. The culture was

washed to remove cellular debris and incubated for 24 h at 37°C. Cells were imaged using a phase contrast microscope at different time points. The extent of wound closure was quantified by measuring its area before migration, and 24 h after migration. Results were expressed as percentage of wound closure.

Transwell invasion assay. Invasion assays were performed using Transwell chambers, which were coated with Matrigel (BD Biosciences) 24 h prior to use. The cells were subsequently harvested and resuspended in serum-free medium containing 1% bovine serum albumin (Sigma-Aldrich; Merck Millipore). The cell suspension was added to the upper chamber and the lower chamber was filled with culture medium containing 10% FBS. After incubation for 24 h at 37°C, cells on the upper surface of the chamber were removed using a cotton swab. Invaded cells on the lower surface were fixed in 4% formaldehyde, stained with 0.5% crystal violet, and counted under a microscope.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was extracted from cells using the TRIzol reagent following the manufacturer's instructions (Invitrogen; Thermo Fisher Scientific, Inc.). Reverse transcription was performed using the PrimeScript First Strand cDNA Synthesis kit (Takara Biotechnology Co., Dalian, China). RT-qPCR was performed on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) with SYBR-Green detection mix (Takara Biotechnology Co.). The following primers were used in the current study: Galectin-7 forward 5'-TTGCTCCTTGCTGTTGAAGACCAC-3', and reverse 5'-AGGTTCCATGTAAACCTGCTGTGC-3' (16); glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward, 5'-TGACTTCAACAGCGACACCCA-3'; and reverse, 5'-CACCTGTGTGCTGTAGCCAAA-3'. PCR conditions were as follows: 95°C for 5 min, followed by 40 cycles of 95°C for 10 sec, 64°C for 30 sec, and 72°C for 30 sec. The relative galectin-7 mRNA level was calculated using the 2^{-ΔΔC_q} method (17) following normalization against the level of GAPDH.

Western blot analysis. Cells were lysed in radioimmunoprecipitation assay buffer (phosphate buffer solution, 1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate) containing a protease inhibitor cocktail (Cell Signaling Technology, Inc., Danvers, MA, USA) on ice for 30 min. After centrifugation at 15,000 x g for 20 min, the supernatant was collected and protein concentrations were measured using a protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Membranes were probed with the following antibodies at 1:300 dilution: Rabbit anti-galectin-7 monoclonal antibody (cat. no. ab108623), rabbit anti-MMP-2 polyclonal antibody (cat. no. ab97779), mouse anti-MMP-9 monoclonal antibody (cat. no. ab119906), rabbit anti-GAPDH monoclonal antibody (cat. no. ab181602; all from Abcam, Cambridge, MA, USA), rabbit anti-phospho-ERK1/2 polyclonal antibody (cat. no. 9101), rabbit anti-ERK1/2 polyclonal antibody (cat. no. 9102), rabbit anti-phospho-JNK monoclonal

antibody (cat. no. 4668), rabbit anti-JNK polyclonal antibody (cat. no. 9252), rabbit anti-phospho-p38 monoclonal antibody (cat. no. 4511) and rabbit anti-p38 monoclonal antibody (cat. no. 8690; all from Cell Signaling Technology, Inc.). Horseradish peroxidase-conjugated secondary antibodies (cat. nos. sc-2004 and sc-2005; Santa Cruz Biotechnology, Inc.) were diluted at 1:2,000 prior to use. Proteins were visualized using an enhanced chemiluminescence kit (Pierce Biotechnology, Inc., Rockford, IL, USA). The blots were quantified by densitometry with the Quantity One software (Bio-Rad Laboratories).

Statistical analysis. Data are expressed as mean \pm standard deviation. Statistical differences were examined using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Galectin-7 silencing has no impact on cell proliferation or apoptosis in OSCC cells. To analyze the role of galectin-7 in the proliferation of OSCC cells, galectin-7-specific siRNA was transiently transfected into SCC-4 and SCC-9 cell lines. The delivery of galectin-7 siRNA significantly decreased mRNA and protein levels of endogenous galectin-7 in both SCC-4 and SCC-9 cells (Fig. 1A and B; $P < 0.05$). The results of the MTT assay demonstrated that this downregulation of galectin-7 did not significantly affect SCC-4 and SCC-9 cell proliferation compared with non-transfected cells over a 5-day period (Fig. 1C). Annexin-V/PI staining analysis identified comparable percentages of apoptotic cells in non-transfected and galectin-7 siRNA-transfected cells (Fig. 1D).

Galectin-7 knockdown attenuates the migration and invasion of OSCC cells. The effect of galectin-7 downregulation on the invasive properties of OSCC cells was then analyzed. Galectin-7 silencing caused a significant decline in cell motility during *in vitro* wound-healing assays. Compared to non-transfected SCC-4 cells, the percentage wound closure was significantly lower in galectin-7-silenced SCC-4 cells 24 h following incubation ($18.5 \pm 3.2\%$ vs. $54.4 \pm 6.4\%$, $P < 0.05$; Fig. 2A). Similarly, galectin-7 siRNA transfection resulted in a significant reduction in the motility of SCC-9 cells ($P < 0.05$). Matrigel invasion assays demonstrated that galectin-7 knockdown significantly reduced the numbers of invaded cells by $>60\%$, compared with non-transfected cells ($P < 0.05$; Fig. 2B).

Overexpression of galectin-7 accelerates the migration and invasion of OSCC cells. Further tests confirmed the effect of increased galectin-7 on the migration and invasion of OSCC cells. Transfection of the plasmid pCEP4-GAL7 into SCC-4 and SCC-9 cells led to a significant increase in galectin-7 expression compared with non-transfected cells (Fig. 3A). This increase in galectin-7 expression in turn significantly increased OSCC cell migration and invasion ($P < 0.05$; Figs. 3B and C).

Upregulation of MMP-2 and MMP-9 mediates the pro-invasive activity of galectin-7. A possible association between galectin-7-mediated invasiveness and MMP-2 and MMP-9

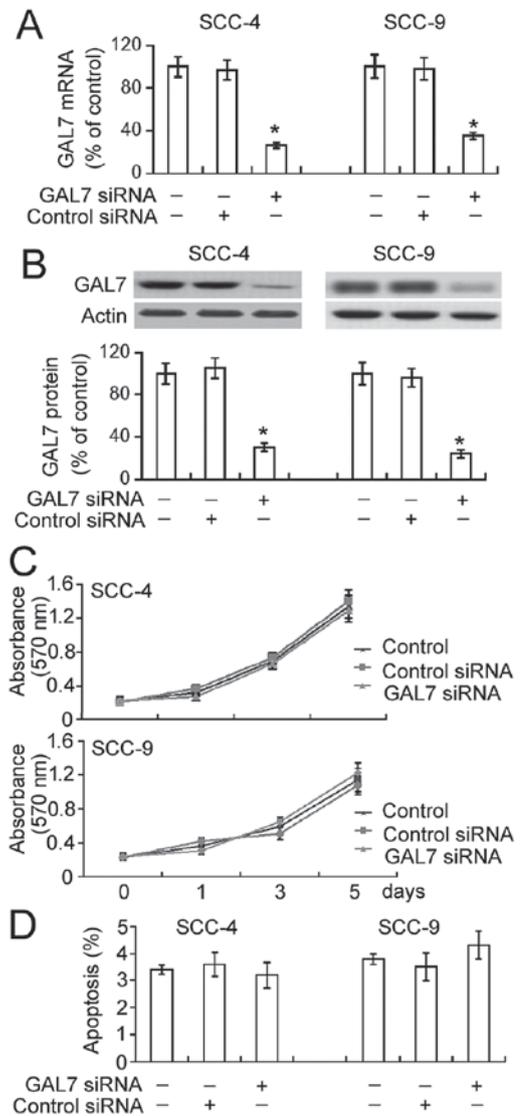


Figure 1. GAL7 silencing does not affect the proliferation and apoptosis of OSCC cells. (A) RT-qPCR and (B) western blot analysis of galectin-7 mRNA and protein levels, respectively, in SCC-4 and SCC-9 cells transfected with control or galectin-7 siRNA. Bar graphs represent mean \pm SD of three independent experiments. * $P < 0.05$ vs. non-transfected cells. (C) The viability and (D) apoptosis of SCC-4 and SCC-9 cells transfected with control or galectin-7 siRNA were determined by MTT assay and annexin-V/PI staining analysis, respectively. GAL7, galectin-7; OSCC, oral squamous cell carcinoma; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; PI, propidium iodide; siRNA, small interfering RNA.

upregulation was investigated. Western blot analysis demonstrated that galectin-7 overexpression resulted in a 3-5-fold increase in MMP-2 protein and 2-3-fold increase in MMP-9 protein expression in both SCC-4 and SCC-9 cells (Fig. 4A). Transwell invasion assay demonstrated that the invasiveness of SCC-4 and SCC-9 cells overexpressing galectin-7 was significantly decreased by co-transfection with MMP-2 or MMP-9-specific siRNA ($P < 0.05$; Fig. 4B).

Galectin-7 promotes OSCC cell invasion via activation of ERK and JNK signaling. Finally, the signaling pathways involved in the action of galectin-7 were investigated. As shown in Fig. 5A, increasing galectin-7 expression markedly enhanced the phosphorylation of ERK1/2 and JNK1/2

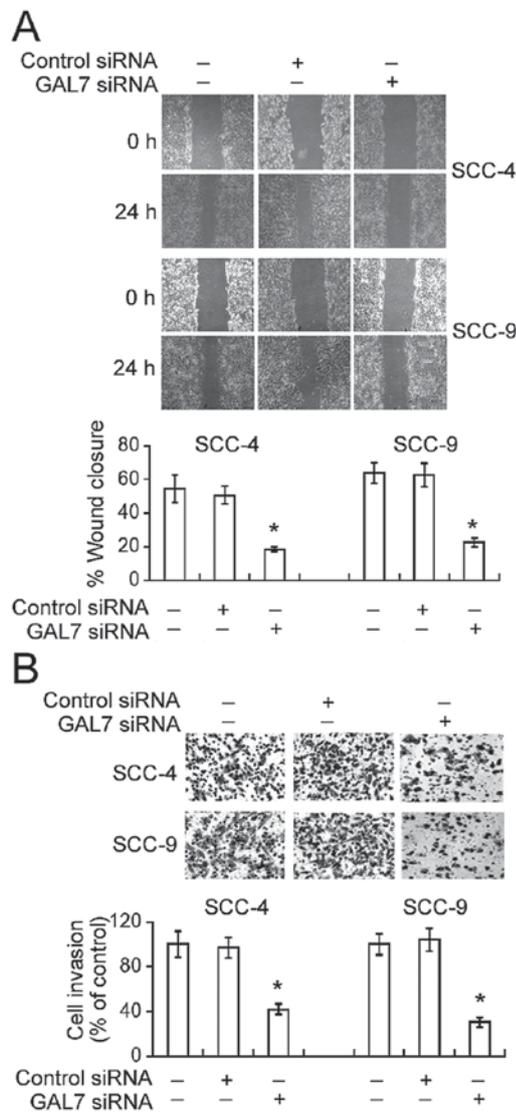


Figure 2. GAL7 knockdown attenuates OSCC cell migration and invasion. (A) Cells transfected with control or GAL7 siRNA were subjected to a wound-healing assay. Top panels: Representative images of cells were taken before and 24 h after scratching. Bottom panels: Bar graphs show the mean percentage of three independent experiments. (B) Transwell invasion assay. Cells transfected with control or GAL7 siRNA were tested for invasiveness in Matrigel-coated transwells. Top panels: Representative images of invasion assay are shown. Bottom panels: Bar graphs represent quantitative data from three independent experiments. *P<0.05 vs. non-transfected cells. GAL7, galectin-7; OSCC, oral squamous cell carcinoma; siRNA, small interfering RNA.

in SCC-4 and SCC-9 cells, without altering total levels of ERK1/2 and JNK1/2. No change in p38 phosphorylation levels was detected. Notably, the pharmacological inhibition of ERK or JNK activity significantly suppressed the invasiveness of galectin-7-overexpressing SCC-4 and SCC-9 cells (P<0.05; Fig. 5B) and abrogated the upregulation of MMP-2 and MMP-9 (P<0.05; Fig. 5C).

Discussion

Matsukawa *et al* (18) reported previously that adenoviral delivery of the galectin-7 gene may induce modest apoptosis and reduce the viability of human OSCC HSC3 cells.

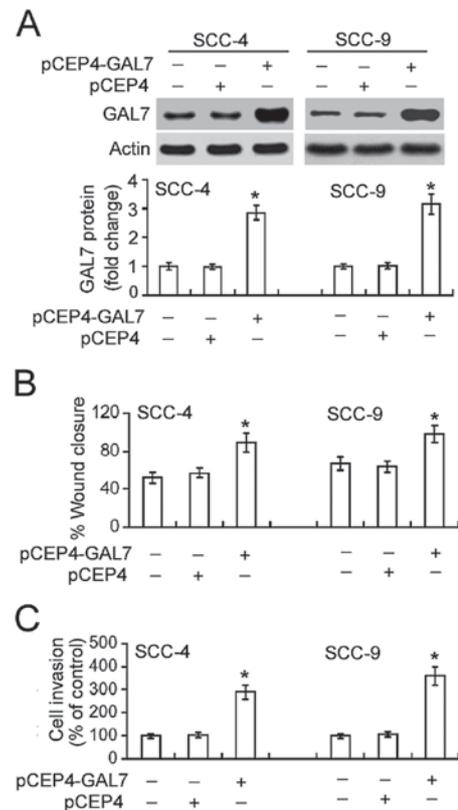


Figure 3. Overexpressing GAL7 accelerates the migration and invasion of OSCC cells. (A) Western blot analysis of galectin-7 in OSCC cells transfected with vector (pCEP4) or GAL7-expressing plasmid (pCEP4-GAL7). Bar graphs represent quantitative data from three independent experiments. (B) The migration and (C) invasion capacities of vector-or GAL7-expressing cells were determined by wound-healing assay and Transwell invasion assay, respectively. Bar graphs represent quantitative data from three independent experiments. *P<0.05 vs. non-transfected cells. GAL7-galectin-7; OSCC, oral squamous cell carcinoma.

However, knockdown of galectin-7 using antisense galectin-7 oligonucleotides demonstrated no significant effects on cell viability. In the current study, the biological roles of galectin-7 in two other OSCC cell lines were explored, and targeted reduction of galectin-7 via siRNA technology did not alter viability and spontaneous apoptosis in SCC-4 and SCC-9 cells. These results suggest that galectin-7 is not required for the maintenance of OSCC cell viability. The anti-viability effect elicited by overexpression of galectin-7 may only reflect a non-specific cytotoxicity, as the potential cytotoxic activity of galectin-7 overexpression on healthy human cells was not tested in the current study.

The ability of galectin-7 to modulate cell behavior seems to be cell-dependent. Previous studies have demonstrated that overexpressing galectin-7 inhibits the proliferation of several specific cancer cells such as gastric cancer cells (9) and colon carcinoma cells (19). However, in other cancer cells including epithelial ovarian cancer (20), galectin-7 was involved in cell proliferation, as its downregulation inhibited the proliferation of A2780-PAR ovarian cancer cell.

Metastasis is the main cause of cancer-associated mortality. Galectin-7 exhibits the ability to modulate the metastatic phenotype of several types of cancer cells (10,12,21). Demers *et al* (21) demonstrated that ectopic expression of

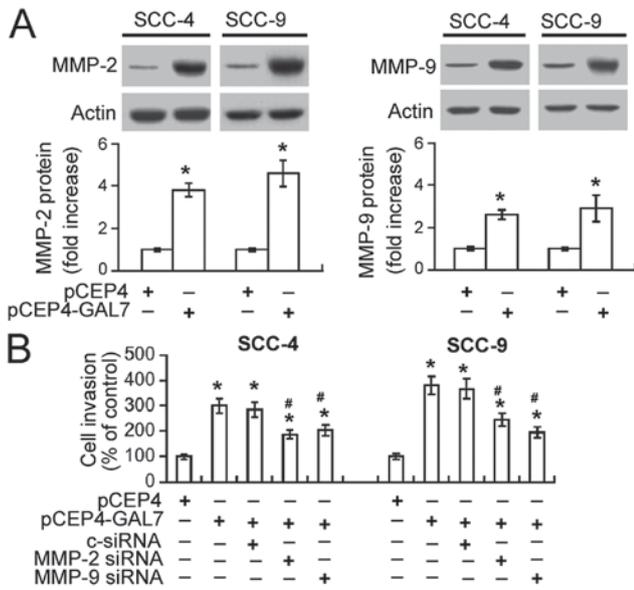


Figure 4. Upregulation of MMP-2 and MMP-9 mediates the pro-invasive activity of GAL7. (A) Western blot analysis of MMP-2 and MMP-9 protein expression in cells transfected with vector or GAL7-expressing plasmid. Bar graphs represent quantitative data from three independent experiments. *P<0.05 vs. vector-transfected cells. (B) The invasiveness of cells transfected with indicated constructs was determined by Transwell invasion assay. *P<0.05 vs. vector-transfected cells; #P<0.05 vs. cells transfected with GAL7-expressing plasmid alone. GAL7, galectin-7; MMP, matrix metalloproteinase.

galectin-7 increases the invasiveness of lymphoma, accelerates the development of thymic lymphoma, and previously identified that overexpressing galectin-7 enhances the metastatic growth of breast cancer cells in the lungs and bones in two different mouse models (10). Enforced expression of galectin-7 also promotes the invasiveness of human HeLa cervical epithelial adenocarcinoma cells (12). The results of the present study are consistent with results from previous studies, as they demonstrated that galectin-7 has the ability to modulate the invasive properties of OSCC cells. Knockdown of galectin-7 suppressed the migration and invasion of SCC-4 and SCC-9 cells, whereas overexpressing galectin-7 increased them. Taken together, these findings indicate that galectin-7 is a potential target for the treatment of tumor dissemination in OSCC.

Compelling evidence suggests that the induction of MMPs plays a pivotal role in the OSCC invasiveness. For instance, Bedal *et al* (22) have previously reported that collagen XVI facilitates the invasion of OSCC cells by inducing MMP-9 expression. It has previously been suggested that the downregulation of MMP-2 and MMP-9 may account for the decreased invasiveness of OSCC cells due to the knockdown of BubR1, a critical component of spindle assembly checkpoint (23). Inhibiting MMP-2 and MMP-9 expression has also been demonstrated to mediate the anti-invasive effects of curcumin (a natural polyphenolic compound) in OSCC cells (24). In line with its pro-invasive activity, galectin-7 expression increases the expression of MMP-9 in several cancer cells (12,16,21). The current study investigated the effects of MMP-2 and MMP-9 on galectin-7 action in OSCC cells. Galectin-7 overexpression resulted in the significant upregulation of MMP-2 and MMP-9.

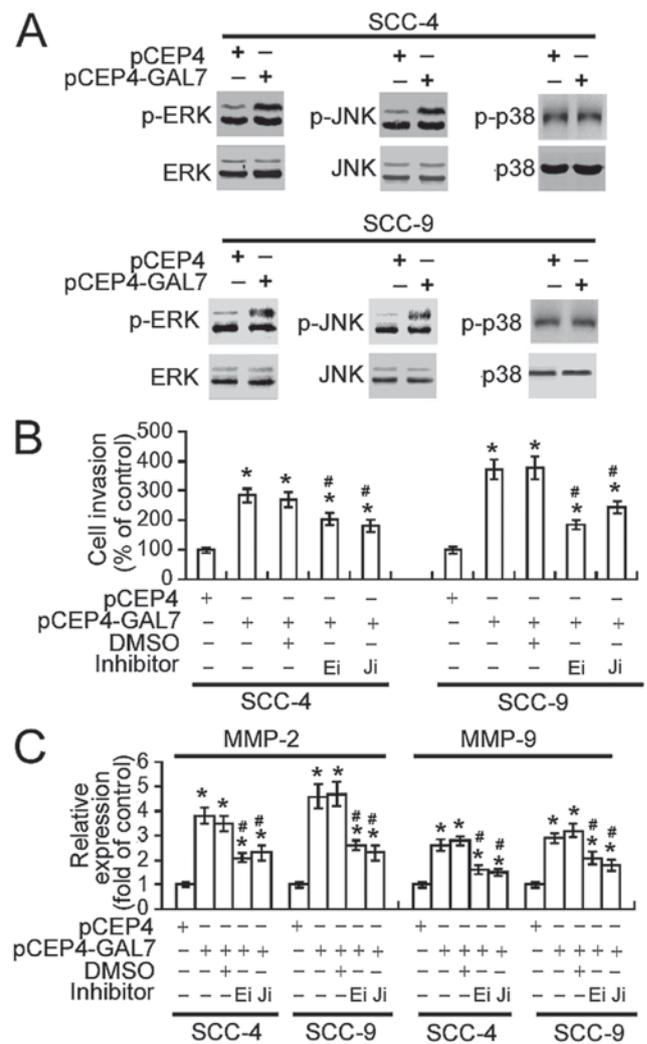


Figure 5. GAL7 promotes OSCC cell invasion via activation of ERK and JNK signaling. (A) Western blot analysis of signaling proteins in cells transfected with vector or GAL7-expressing plasmid. Representative blots from three independent experiments are shown. (B and C) Cells were transfected with vector or GAL7-expressing plasmid with or without prior treatment with an ERK inhibitor (Ei) or JNK inhibitor (Ji). (B) Cell invasiveness and (C) MMP-2 and MMP-9 expression were determined by Transwell invasion assay and Western blot analysis, respectively. Bar graphs represent quantitative data from three independent experiments. *P<0.05 vs. vector-transfected cells; #P<0.05 vs. cells transfected with GAL7-expressing plasmid alone. GAL7, galectin 7; OSCC, oral squamous cell carcinoma; MMP, matrix metalloproteinase; ERK, extracellular signal-related kinase; JNK, c-jun N-terminal kinase.

Most importantly, silencing MMP-2 or MMP-9 significantly impaired the invasiveness of OSCC cells that overexpressed galectin-7. Thus MMP-2 and MMP-9 may be required for the galectin-7-mediated invasiveness of OSCC cells.

To gain a better insight into the function of galectin-7 in OSCC cell invasiveness, the signaling pathways involved were analyzed. Since mitogen-activated protein kinase (MAPK) pathways are implicated in the invasion of oral cancer cells (25,26) and galectin-7 may activate p38 MAPK signaling in cervical cancer cells (12), the current study investigated the importance of MAPK signaling in mediating galectin-7 action. The results of the present study demonstrated that galectin-7 overexpression leads to the phosphorylation and activation of ERKs and JNKs, but not p38 MAPK, in SCC-4

and SCC-9 cells. Interestingly, the pharmacological inhibition of ERK or JNK activity significantly attenuated OSCC cell invasiveness induced by galectin-7 overexpression. Moreover, galectin-7-mediated upregulation of MMP-2 and MMP-9 was compromised by pretreatment with the ERK or JNK inhibitors. Taken together, these results suggest that galectin-7 promotes the invasiveness of OSCC cells largely by inducing the expression of MMP-2 and MMP-9 via activation of ERK and JNK signaling.

In conclusion, the current study provides novel evidence demonstrating the pro-invasive activity of galectin-7, which is associated with increased MMP-2 and MMP-9 expression, in OSCC cells. Further studies are required to investigate the utility of galectin-7 as a target for the treatment of metastatic OSCC.

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