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Original Article

Sox3 silencing inhibits metastasis and growth of esophageal squamous cell carcinoma cell via down-regulating GSK-3β

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Abstract: The early stages of localized esophageal squamous cell carcinoma (ESCC) can be effectively treated by surgical resection of the early esophageal cancer, but there is still no effective treatment once it has been metastasis to distant tissues. Although a number of both metastasis promoting and metastasis suppressor genes have been reported, the underlying molecular mechanisms responsible for ESCC metastatic cascade are still not fully understood. Therefore, defining the potential molecules that govern ESCC metastasis may aid the development of new therapeutic strategies for combating ESCC. In the present study, we found Sox3 is involved in the metastasis of ESCC cells and demonstrated that Sox3 (Sex determining region Y-box 3) disruption impaired ESCC cells migration and invasion. The requirement of Sox3 in the metastasis of ESCC cells was further confirmed by gene silencing in vitro. Moreover, down-regulation of Sox3 expression strikingly inhibited ESCC cellular growth both in vitro and vivo. Finally, we found that Sox3 promotes ESCC migration and invasion through the GSK-3β signaling pathway. To conclude, our findings suggest a novel mechanism underlying the metastasis of ESCC cells which might serve as a new intervention target for the treatment of ESCC.

Keywords: Esophageal squamous cell carcinoma, migration, invasion, Sox3, GSK-3β

Introduction

Esophageal squamous cell carcinoma (ESCC) is the predominant subtype of esophageal cancer in eastern Asia and southern Africa [1]. The incidence of ESCC is increasing recently, and the overall five-year survival rate of ESCC is only 20-30% [2]. ESCC patients often have significantly different outcomes due to early metastasis happening or not, although the patients are at the same pathological stage and receive the similar surgical therapy [3]. Currently, there is still no effective treatment for ESCC once it is progressed to metastatic phases [4]. Metastasis is the spread of tumor cells from a primary site to distant organs and is the most life-threatening factor of ESCC. Metastasis is a multistep progress that can be envisioned a succession of cell biological changes, including cancer cells separating from original tumor, local invasion through surrounding tissues, intravasation into and transferring through blood stream, arresting in the parenchyma of distant tissues, formation of small nodules (micro-metastasis), and finally, growth of micro-metastatic lesions into macroscopic tumors [5].

Although a variety of metastasis-promoting and metastasis suppressor genes have been recently identified to be related to tumor metastasis, the molecular mechanisms governing ESCC metastasis process are still not completely understood and the treatment efficiency of metastatic ESCC has not been significantly improved [6]. Hence, further understanding the underlying related mechanism is urgently required to find new potential targets for improving disease diagnosis, treatment and prevention. In the present study, in order to identify genes involved in the metastasis of ESCC, we transfected ECA109 esophageal squamous cell carcinoma cells with a specifically designed ret-

roviral vector pDisrup 8 [7] that can randomly disrupt genes in genome. The vector bearing ECA109 clones were selected by blasticidin and then screened by wound healing and migration assay to determine migration potential. Cell clones with increased or decreased migratory potential were subjected to 3'RACE to identify genes that were involved in the metastasis of ECA109 cells [8]. As a result, we found that Sox3 is involved in the cell migration of ECA109 cells.

In this study, we reported the identification of a novel role of Sox3 whose disruption impaired the metastasis of ESCC cell ECA109. The mRNA and protein expression of Sox3 is over-expression in a panel of ESCC cell lines compared to non-tumorigenic human bronchial epithelial cells. Moreover, the expression level of Sox3 genes in ESCC was also analyzed using public database Oncomine (https://www.oncomine.org/) [9]. Furthermore, by silencing Sox3, we further confirmed the role of Sox3 in the migration of ESCC cell in vitro. In addition, reduction of Sox3 expression in ECA109 cells significantly impaired their growth in vitro and vivo. Further investigation demonstrated that GSK-3β signaling pathway is involved in Sox3-promoted ESCC migration and growth. All results demonstrate that Sox3 may be a potential molecular target for novel therapeutic strategies for ESCC.

Materials and methods

Cell culture and reagents

Esophageal squamous cell carcinoma cells ECA109, SKGT-5, SKGT-4, TE-1, TE-3, TE-8 cells, and SV40-immortalized non-tumorigenic human bronchial epithelial cells BEAS-2B were purchased from Cell Resource Center, Shanghai Institutes for Biological Sciences. Cells were cultured in DMEM or 1640 with 10% FBS (Gibco, Invitrogen, USA), and maintained at 37°C in a humidified atmosphere containing 5% CO_2. Lipofectamine RNAi MAX reagent was purchased from Invitrogen (USA).

Plasmids and transfection

The pDisrup retroviral vector was constructed based upon MMLV retroviral vector pLNCX as backbone. The splicing donor and acceptor were designed according to human adenovirus type 2 major late mRNA intron sequence [8]. The short small interfering RNA (siRNA) was constructed with sequence specifically targeted to Sox3 gene: 5'-CAAGGAGTTAGTTAAATGC-3'. Hemagglutinin (HA)-tagged constitutively active (S9A) GSK3β (Plasmids 14754, Addgene, Cambridge, MA) was deposited by Dr. Jim Woodgett [10]. Transient transfection was performed using the Lipofectamine RNAi MAX reagent (Invitrogen) and following the manufacturer's instructions. For pDisrup clone selections, cells were selected with 25 μg/ml Blasticidin S. HCl (Invitrogen, USA).

One solution cell proliferation assay

The cell viability was determined by CellTiter 96® Aqueous One Solution cell proliferation assay (Promega, Madison, WI, USA). Briefly, ECA109 cells were seeded in 96-well cell culture plates and treated with indicated agents. After incubation for indicated time period, 20 μL of One Solution reagent were added to each well and incubation was continued for additional 4 h. The absorbance was measured at 490 nm using Synergy™ HT Multi-Mode Microplate Reader (Bio-Tek, Winooski, VT, USA). The effect of siRNA on cell viability was assessed as the percent of cell viability compared with vehicle-treated control cells, which were arbitrarily assigned 100% viability. The data were presented as mean ± SE. Differences in the results of different groups were evaluated using either two-tailed Student's t test or one-way ANOVA followed by post hoc Dunnett's test.

Lactate dehydrogenase (LDH) toxicity assay

The LDH released into cell cultures is an index of cytotoxicity and evaluation of the permeability of cell membrane. Cells were seeded in 96-well plate at a density of 1×10^5 cells per well. After incubation with vehicle (0.1% DMSO) or 1% Triton X-100 for 24 h, cell supernatants were collected and analyzed for LDH activity using LDH cyto-toxicity assay kit from Keygen biotech [11]. The absorbance of formed formazan was read at 490 nm on a microplate reader.

Oncomine analysis

The expression level of Sox3 genes in the melanoma was analyzed using Oncomine [9]. For this, we compared clinical specimens of cancer vs. normal patient from the Kimchi Esophagus database [12]. In order to reduce our false discovery rate, we selected $P < 0.01$ as a threshold. We analyzed the results for their *p*-values and fold change.

Wound healing assay

Briefly, ECA109 cells were seeded in 60 mm dishes and cultured at 37°C overnight to produce a confluent monolayer. After starvation in serum-free medium for 24 hours, a wound was created by scratching the monolayer with a 200 μl sterile pipette tip. The wounded monolayer was then washed three to remove cell debris and incubated with fresh medium. The area of cell-free scratch was photographed at 0 h and 24 h after scratching respectively. The wound healing effect was determined by measuring the percentage of the remaining cell-free area compared with the area of the initial wound [13]. The data were presented as mean ± SE. Differences in the results of different groups were evaluated using either two-tailed Student's t test or one-way ANOVA followed by post hoc Dunnett's test.

Migration assay

Migration of cells was determined by BD Transwell Migration Chamber (BD Biosciences, USA) assay in vitro according to the manufacturer's instructions. In brief, 1×10^5 cells with 500 μl in serum-free medium were added into the upper chamber and 750 μl of cells conditioned medium was added into the lower chamber. After incubation in humidified tissue culture incubator, 37°C, 5% CO_2 atmosphere for 24 h, the non-migration cells in the upper surface of the membrane were removed and the cells migrating to the lower surface of the membrane were stained with 0.5% crystal violet. Cell counting was then carried out by photographing the membrane through the microscope. Five random fields under microscope were taken and migration cells were quantified [14]. The data were presented as mean ± SE. Differences in the results of different groups were evaluated using either two-tailed Student's t test or one-way ANOVA followed by post hoc Dunnett's test.

Invasion assay

Assay was performed with Matrigel-coated chambers from a BioCoat Matrigel Invasion Chamber Kit (BD Biosciences). Cells with 500 μl in serum-free medium were added into the upper chamber and complete medium was added into the lower chamber. After incubation for 24 h, non-invasive cells in the upper surface of the membrane were removed and the cells invasion to the lower surface of the membrane was fixed. Cell counting was then carried out by photographing the membrane through the microscope [15] and five random fields were taken. The data were presented as mean ± SE. Differences in the results of different groups were evaluated using either two-tailed Student's t test or one-way ANOVA followed by post hoc Dunnett's test.

Western blot

After washing with PBS twice, cells were extracted with cold lysis buffer and centrifuged at 15,000 g for 15 min at 4°C. Protein concentration of the supernatants was determined with Bradford assay (Biorad, USA). 30 μg of samples was separated by electrophoresis on 10% SDS-PAGE and transferred to Polyvinylidene fluoride membrane (Millipore, USA). After blocking with 5% skimmed milk for 1.5 h, membranes were incubated with different specific primary antibodies in 5% bovine serum albumin (BSA). Anti-Sox3, FAK, phospho-FAK-^{Tyr397}, GSK-3β, phospho-GSK-3β^{Ser9}, Akt, phospho-Akt^{Ser473}, Src, and phospho-Src^{Tyr416} from Cell Signaling Technology. After washing with TBST for 30 min, the membranes were further incubated with corresponding HRP-conjugated secondary antibodies and developed with Pierce's West Pico chemiluminescence substrate (Millipore, USA). All results were obtained from 3 independent experiments.

Matrix metalloproteins (MMPs) activity assay

The activity of MMP-9 and MMP-2 were determined by QuickZyme MMPs activity assay (QucikZyme BioSciences) according to the manufacturer's instructions [16]. Briefly, after transfection, cells were washed with fresh medium and replaced with serum-free medium. After additional 24 h, the medium was collected and centrifuged at 10000 g for 10 min. Respective supernatant was added to the 96-well strip coated with MMP-9 antibody or MMP-2 antibody and incubated at 4°C overnight. After washing with wash buffer for 3 times, 50 μl assay buffer was added into the well, followed by adding 50 μl detection reagent. After incubation at 37°C for 1 h, OD405 was measured with Microplate Reader (Bio-Tek).

Real-time PCR

Total RNA was isolated using TRIzol according to the manufacturer's instructions (Invitrogen, USA) and the concentration of total RNA was

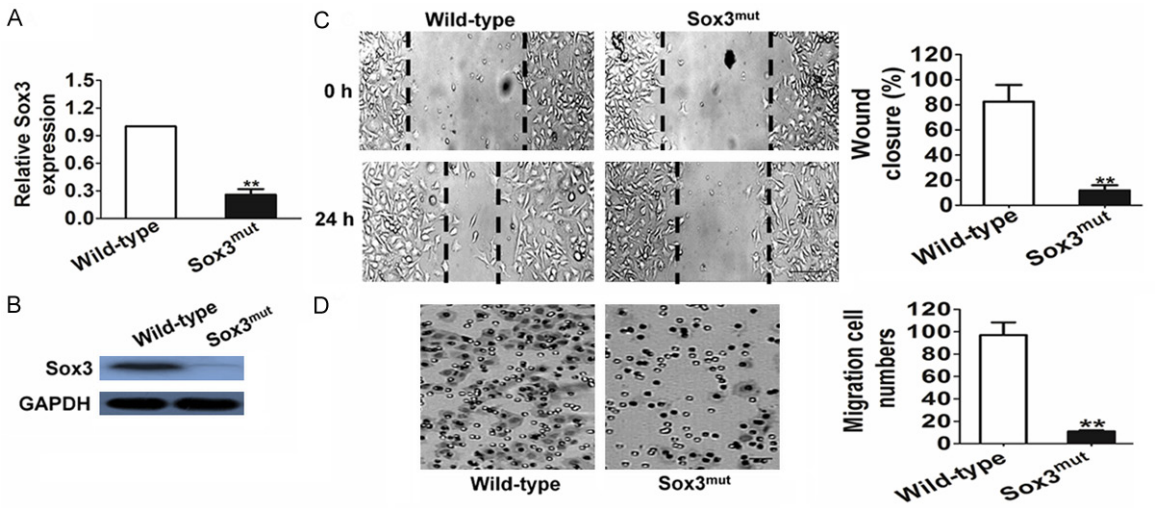

Figure 1. Identification of a novel role of Sox3 in the metastasis of esophageal squamous cell carcinoma. A and B. Sox3 expression in Sox3^{mut} cells was analyzed by real-time PCR and Western blotting. C. Wound healing of control and Sox3^{mut} cells was performed and representative pictures of the wound distance were taken at each time point as indicated. Scale bars: 50 μm. D. The cell motility was evaluated by Transwell migration assay. Representative pictures were taken after staining with crystal violet. Scale bars: 50 μm. Data were collected from three independent experiments and were average ± S.E. values. $^{**}P < 0.01$, compared to wild type cells.

detected by spectrophotometry at OD260. Reverse transcription (RT) was carried out using superscript III reverse transcriptase (Invitrogen, USA) as described in the manufacturer's manual. The real-time PCR was performed on ABI Prism 7500 Sequence detection system (Applied Biosystems, CA) with the KAPA SYBR® qPCR Kit (KAPA Biosystems, USA) according to the manufacturer's instructions. The primers used was as follow: Sox3 (Forward: 5'-GGTGCCAACGCTGTCAACAAC-3', Reverse: 5'-TCTCTCAGGTCTCCCAAACAAGC-3'), β-actin (Forward: 5'-GCTCTTTTCCAGCCTTCCTT-3', Reverse: 5'-TGATCCACATCTGCTGGAAG-3'). The target mRNA level of control cells normalized to the level of β-actin mRNA, was defined as 1. Results were obtained from three independent experiments. The data were presented as mean ± SE. Differences in the results of different groups were evaluated using either two-tailed Student's t test or one-way ANOVA followed by post hoc Dunnett's test.

Xenograft model

After Sox3 knockdown, 3 × 10^6 ECA109 cells were subcutaneously implanted into female, BALB/c-nu mice to build ESCC xenograft [17]. Mice at 6-8 weeks old (15-20 g) were purchased from Shanghai Slack laboratory animal co., LTD. Mice were maintained at dark/light cycles of 12 h duration with food and water available. Tumor volume and mice body weight were measured every 3 days. Tumor volume was calculated as $mm^3 = 0.5 \times length\ (mm) \times width\ (mm)^2$. Animal handling and experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Third Military Medical University. The data were presented as mean ± SE. Differences in the results of two groups were evaluated using either two-tailed Student's t test or one-way ANOVA followed by post hoc Dunnett's test.

Statistical analysis

The data were presented as mean ± SE. Differences in the results of two groups were evaluated using either two-tailed Student's t test or one-way ANOVA followed by post hoc Dunnett's test. The differences with $P < 0.05$ were considered statistically significant.

Results

Sox3 is a novel regulator of ESCC metastasis

The esophageal squamous cell carcinoma (ESCC) cell line ECA109 is widely used to investigate metastasis of ESCC for its high metastatic potential [18]. To identify the key gene involved in ESCC metastasis, we transfected ECA109 cells with pDisrup vector to randomly

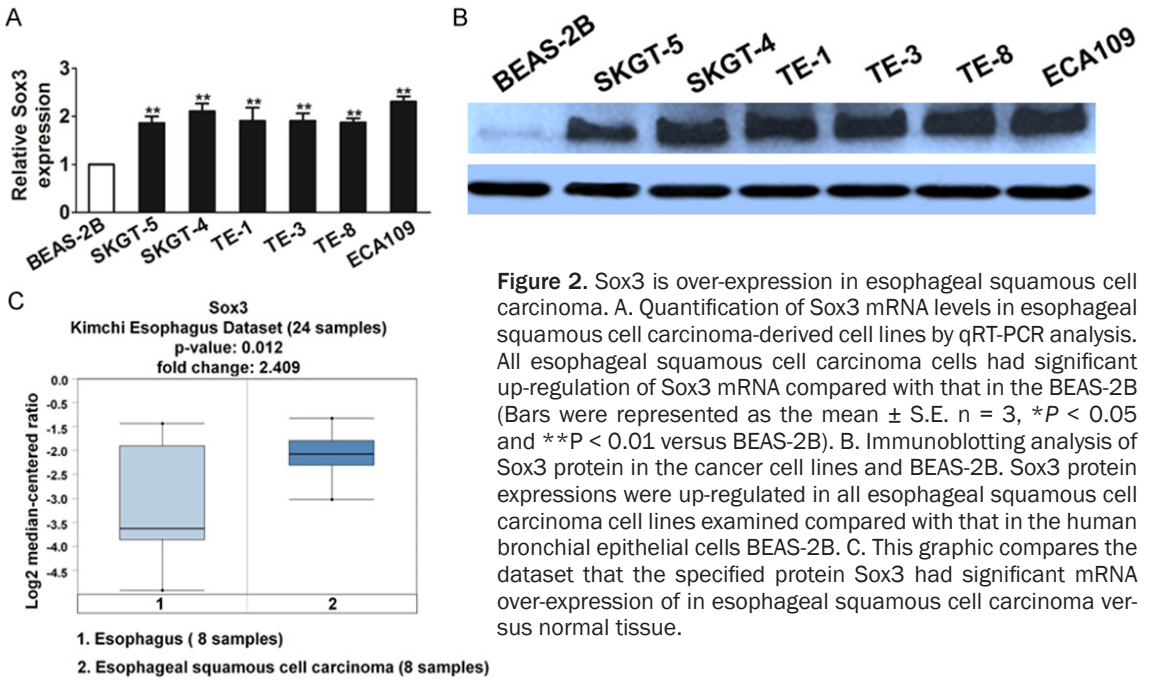

Figure 2. Sox3 is over-expression in esophageal squamous cell carcinoma. A. Quantification of Sox3 mRNA levels in esophageal squamous cell carcinoma-derived cell lines by qRT-PCR analysis. All esophageal squamous cell carcinoma cells had significant up-regulation of Sox3 mRNA compared with that in the BEAS-2B (Bars were represented as the mean ± S.E. n = 3, $*P < 0.05$ and $**P < 0.01$ versus BEAS-2B). B. Immunoblotting analysis of Sox3 protein in the cancer cell lines and BEAS-2B. Sox3 protein expressions were up-regulated in all esophageal squamous cell carcinoma cell lines examined compared with that in the human bronchial epithelial cells BEAS-2B. C. This graphic compares the dataset that the specified protein Sox3 had significant mRNA over-expression of in esophageal squamous cell carcinoma versus normal tissue.

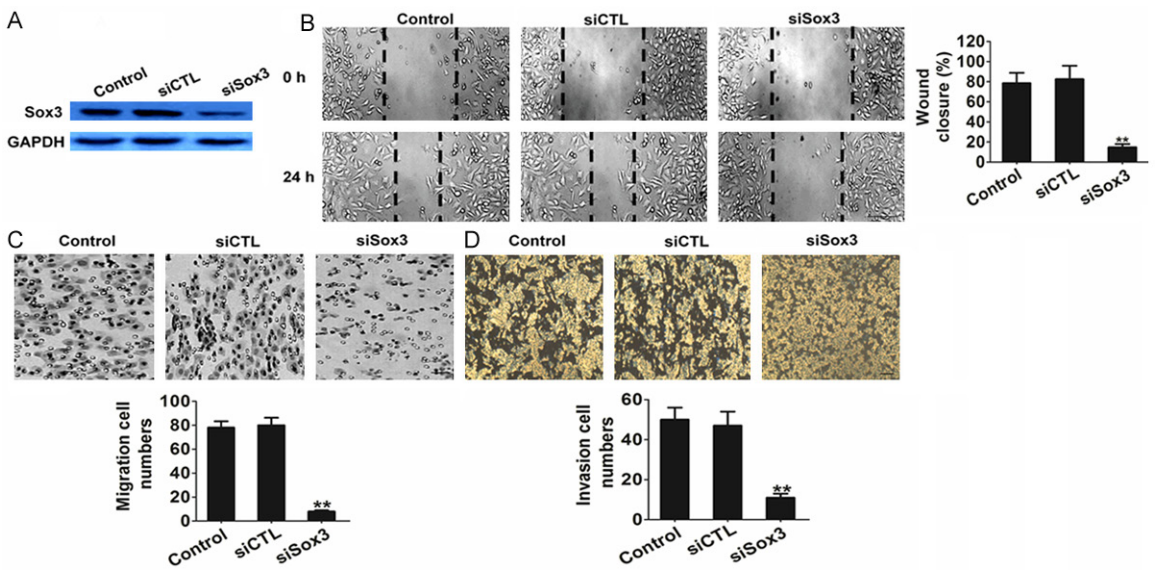

Figure 3. Sox3 silencing decreases the migration of esophageal squamous cell carcinoma cells. A. Western blot shows that the Sox3 was elevated in control cells and siCTL transfected cells than siSox3 transfected cells. GAPDH was used as a loading control. B. Both siSox3 and siCTL transfected cells were plated in 6 cm dishes. Wound healing assay was performed to determine the metastatic potential of cells and representative pictures of the wound distance were taken at 0 and 24 h post scratching as indicated. The percentage of wound closure was quantified. Scale bars: 50 μm. C. The cell motility was evaluated by transwell migration assays. Data were collected from three independent experiments and were average ± S.E. values. $**P < 0.01$, compared to control cells. Scale bars: 50 μm. D. The cell motility was evaluated by Transwell invasion assay and the number of invasion cells was quantified. Scale bars: 50 μm. Data were from three independent experiments and are average ± S.E. values. $*P < 0.01$, compared to control cells.

produce insertions into the genomic DNA, followed by selection with blasticidin to obtain mutated ECA109 cell clones. The mobility and migration ability of mutant ECA109 cell clones was then determined by wound healing and migration assay. Finally, cell clones with

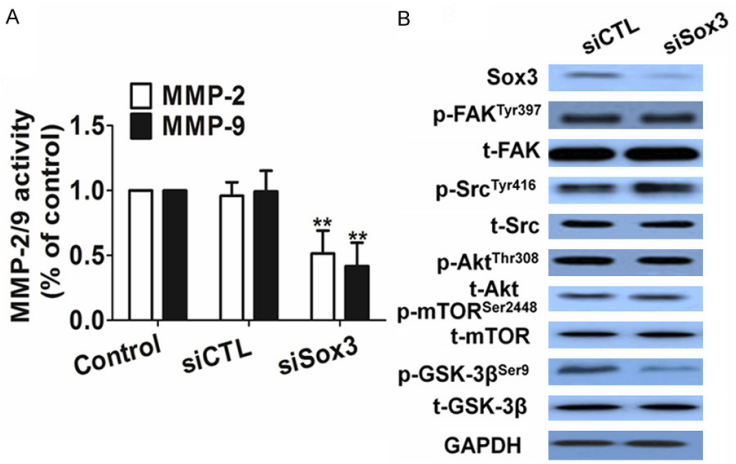

Figure 4. Sox3 regulates GSK3β pathway in esophageal squamous cell carcinoma cells. A. Quantification of MMP-2/9 activity in ECA109 cells transfected with siSox3. Bars were represented as the mean ± S.E. n = 3, $^{**}P < 0.01$ versus control cells. B. Western blot shown that the phosphorylation of GSK3β was inhibited in cells transfected with siSox3. Data were from three independent experiments.

increased or decreased migration potential were further analyzed by the RT-PCR and 3'RACE to identify the genes disrupted by pDisrup vector. With this strategy, several candidate genes were identified, including a gene named Sox3 and this candidate was designated as *Sox3*^{mut} which exhibited decreased migration potential. To verify whether the identified gene was indeed disrupted in ECA109 cells, real-time PCR and western blot assay were carried out to determine Sox3 gene expression in *Sox3*^{mut} cells. As shown in **Figure 1A** and **1B**, the expression of Sox3 was greatly reduced in *Sox3*^{mut} cell clone compared to wild-type cells. To confirm if loss function of Sox3 affected ECA109 migration, we performed wound healing and Transwell migration assay to evaluate the cell metastasis. As shown in **Figure 1C**, 24 h after scratching, the area of wound recovered by the migration of Sox3^{mut} cells was not significant. 24 h later, the wild type ECA109 cells had almost closed up the wound. Consistently, there were less Sox3^{mut} cells that migrated across the membrane of the Transwell chamber compared to the wide type ECA109 cells (**Figure 1D**). In summary, disruption of Sox3 led to reduced ECA109 cells mobility and significantly impaired the migration of ECA109 cells.

Sox3 is over-expression in ESCC

To investigate the mRNA expression of Sox3, we performed quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis in human bronchial epithelial cells BEAS-2B and a panel of ESCC cell lines. Sox3 mRNA was up-regulated in all ESCC cell lines compared with that in the BEAS-2B (**Figure 2A**). We also performed western blot analysis to investigate the Sox3 protein expression status in the ESCC cells and bronchial epithelial cells. A significant increase in Sox3 protein expression was seen in ESCC cell lines compared with BEAS-2B (**Figure 2B**). These analyses indicated that both transcription and translational products of Sox3 were highly expressed in ESCC cells. The expression level of Sox3 genes in ESCC was also analyzed using Oncomine (https://www.oncomine.org/). For this, we compared clinical specimens of cancer vs. normal patient from the Kimchi Esophagus database [12]. We analyzed the results for their *p*-values and fold change. Oncomine analysis of neoplastic vs. normal tissue showed that Sox3 was significantly over-expressed in ESCC (**Figure 2C**).

Silencing Sox3 inhibits ESCC metastasis

To ascertain Sox3 was indeed responsible for reduced migration in ESCC cells, we investigated whether reduced ECA109 cells migration could be reproduced by gene silencing with independent siRNA specific for Sox3. To perform this experiment, we silenced the expression of Sox3 with siRNA-incorporated plasmid. As shown in **Figure 3A**, the expression of Sox3 in ECA109 transfected with Sox3-specific siRNA (siSox3) was significantly decreased compared with the cells transfected with scrambled siRNA (siCTL) and control cells. Then, the transfected cells were subjected to wound healing and Transwell migration assay to evaluate their migratory potential. ECA109 cells transfected with siCTL were able to close a wound by 24 h. However, the wound inflicted on cells transfected with Sox3 siRNA had not yet closed up at 24 h (**Figure 3B**). Consistently, transwell migration assay results also showed that the number of siSox3 cells moved across the membrane was fewer than the siCTL cells (**Figure 3C**). Invasion is the hallmark of tumor metastasis. Using inva-

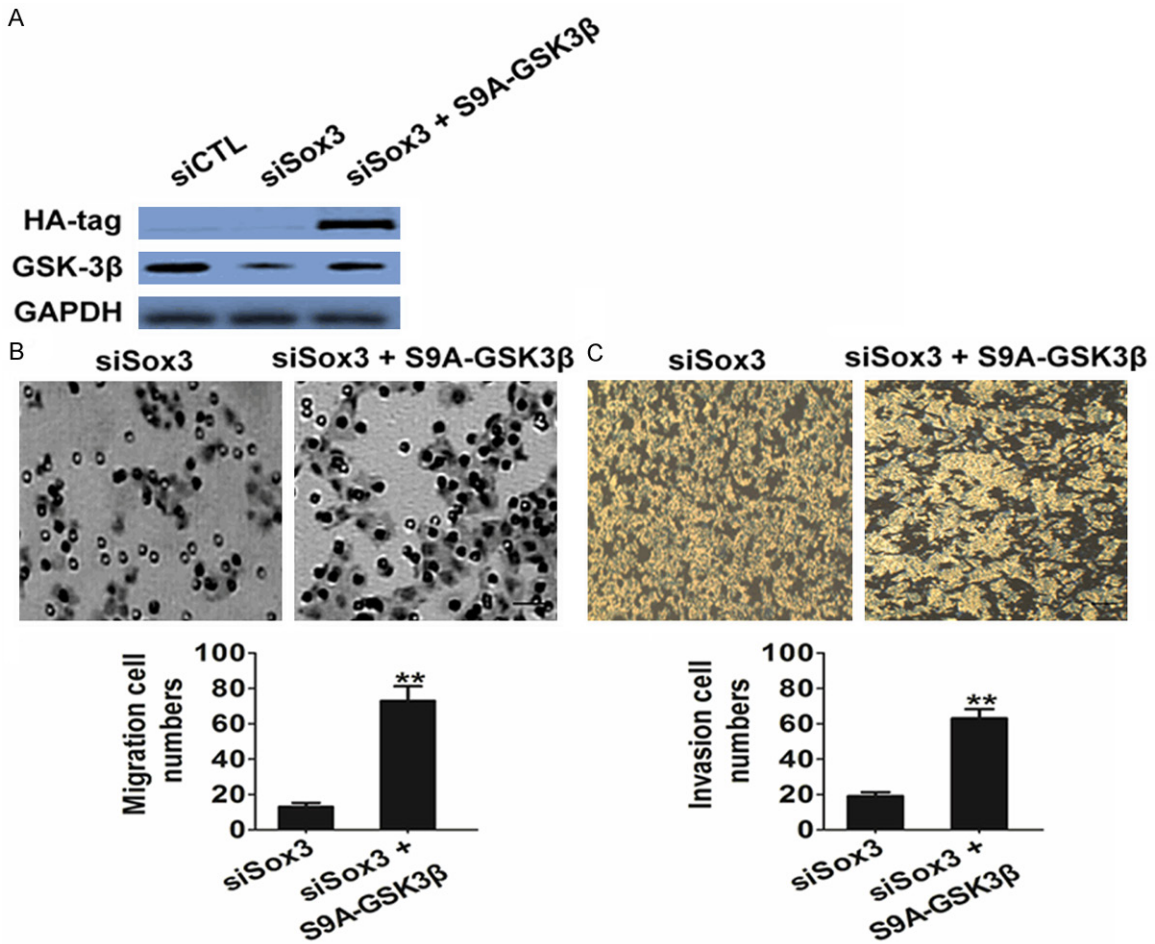

Figure 5. Sox3 facilities the migration of esophageal squamous cell carcinoma cells via GSK3β pathway. A. Expression of S9A-GSK3β was confirmed by western blot with antibody against HA-tag and GSK3β, and GAPDH was used as loading control. B. Transwell migration assay was performed to determine the motility of cells co-transfected with Sox3 silencing plasmid and GSK3β plasmid. Columns were data collected from three independent experiments. Representative pictures were taken after staining with crystal violet. Scale bar represents 50 μm. Data were from three independent experiments and are average ± S.E. values. $^{**}P < 0.01$, compared to siSox3 cells. C. Transwell invasion assay was performed to determine the motility of cells co-transfected with Sox3 silencing plasmid and GSK3β plasmid. Columns were data collected from three independent experiments. Representative pictures were taken after staining with crystal violet. Scale bar represents 50 μm. Data were from three independent experiments and were average ± S.E. values. $^{**}P < 0.01$, compared to siSox3 cells.

sion analysis, we investigated the effects of Sox3 siRNA on invasion in tumor cells. In accordance with migration result, we found that silencing of Sox3 in ECA109 resulted in significantly suppressed invasion (**Figure 3D**). Taken together, these results indicated that silencing of Sox3 could reproduce the effect of Sox3 disruption by pDisrup 8 plasmid and drastically reduced ESCC cells motility.

GSK3β mediates the role of Sox3 in ESCC cell metastasis

Matrix metalloproteinases (MMP), which are capable of degrading the various structural components of the ECM, play a critical role in tumor invasion and metastasis and the up-regulation of MMP has been considered as the markers for the metastasis of tumor cells [19]. We then tested the effect of Sox3 silencing on the activity of MMP-2/9. Consistent with cells assay result, the activity of MMP-2/9 was also markedly decreased with the down-expression of Sox3 (**Figure 4C**). Collectively, these results suggest that Sox3 promotes ESCC cells metastasis and MMPs activity. To determine the signaling pathways which are involved in Sox3-mediated ESCC cell migration, multiple potential signaling pathways related to migration and

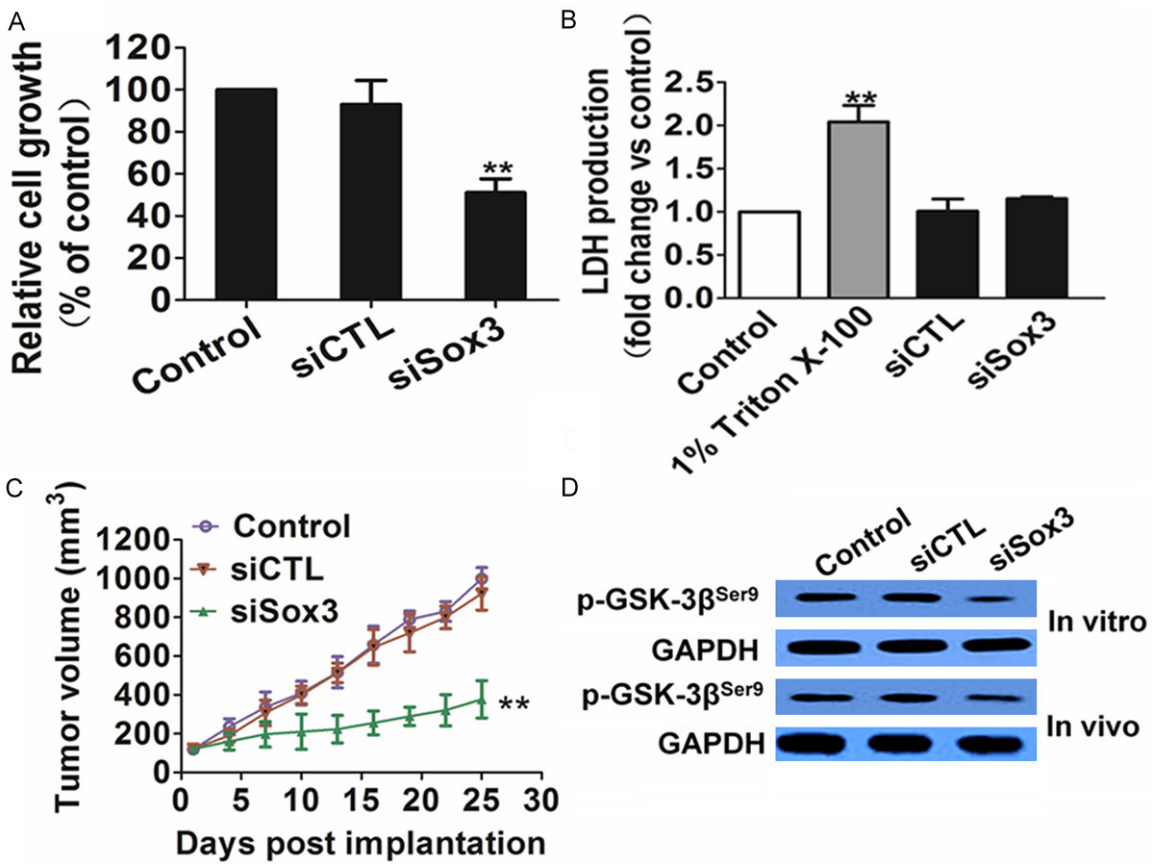

Figure 6. Sox3 siRNA inhibits esophageal squamous cell carcinoma growth in vitro and in vivo. A. One solution cell proliferation assay analyzed cell proliferation in ECA109-siSox3 and control cells. Data were from three independent experiments and were average ± S.E. values. $^{**}P < 0.01$, compared to control cells. B. Sox3 siRNA did not result in LDH release, indicating Sox3 siRNA brought little toxic effects on esophageal squamous cell carcinoma cells. Data were from three independent experiments and were average ± S.E. values. $^{**}P < 0.01$, compared to control cells. C. ECA109-siSox3 or control cells were subcutaneously implanted into mice to build esophageal squamous cell carcinoma xenograft. On day 25 post injection, tumors were removed and photographed. Sox3 siRNA resulted in significantly tumor growth inhibition versus control mice. Data were from three independent experiments and were average ± S.E. values. N = 6, $^{**}P < 0.01$, compared to mice injected with control cells. D. Western blot showed that the phosphorylation of GSK3β was inhibited in cells transfected with Sox3 siRNA in vitro. GSK3β expression in tumor sections from different mice group was detected by western blot with the anti-phospho-GSK3β^{Ser9} antibody.

invasion of cancer cells were screened. As shown in **Figure 4B**, only the basal level of GSK-3β activation was found to be significantly down-regulated in Sox3 siRNA cells. In contrast, no obvious difference could be observed for many other signaling pathways, such as Focal Adhesion Kinase (FAK), mTOR and Src. In combination, these results strongly suggest that Sox3 facilitated the activation of GSK3β signaling pathway in ESCC cells.

Confirming GSK3β is involved in Sox3 promoting ESCC cell metastasis

To confirm the role of GSK-3β in Sox3-mediated cell migration, constitutively active form of GSK3β (S9A-GSK3β) was introduced into Sox3-silenced cells. The expression of S9A-GSK3β was confirmed by western blot with anti-HA-tag and anti-GSK3β antibody (**Figure 5A**). The migration and invasion of cells was then examined by Transwell migration and invasion assay. As expected, active GSK3β largely restored the impaired metastasis in Sox3-silenced ECA109 cells (**Figure 5B** and **5C**). To conclude, these data indicated that GSK3β signaling was involved in Sox3 promoted metastasis of ESCC cells.

Sox3 knock-down inhibits growth of ESCC cells

As GSK3β activity regulates many processes including tumor growth and considered to be hallmarks of cancer, the GSK3β pathway has

become an important new therapeutic target. GSK3β activation promotes resistance to standard chemotherapy and radiation therapy, and inhibition of GSK3β signaling induces apoptosis and decreased growth of tumor cells dependent on elevated GSK3β signaling for survival and growth. We observed proliferation of the cell lines after transfected. In ECA109 cell line tested, Sox3 knockdown lead to a statistically significant decrease in growth compared to siCTL treated cells (**Figure 6A**). To validate whether the growth inhibition of Sox3 siRNA was due to its toxicity effects on melanoma cells, LDH cytotoxicity assay was carried out. As shown in **Figure 6B**, Triton X-100 significantly increased LDH release, and Sox3 knockdown as well as siCTL treated brought little toxic effects on ECA109 cells when compared to vehicle control. To elucidate its mechanism of action, we assessed the effect of Sox3 knockdown on GSK3β activity in vitro. ECA109 cells were transfected with siCTL or Sox3 siRNA and western blot analysis was performed. As shown in **Figure 6D**, Sox3 knockdown was associated with a decline in GSK3β activity.

To evaluate the effects of Sox3 knockdown on esophageal squamous cell carcinoma cells growth in vivo, we further constructed an experiment using ECA109 cells xenograft mouse model. It was found that Sox3 knockdown dramatically suppressed tumor volumes compared with the siCTL control group (**Figure 6C**). To further examine whether Sox3 knockdown suppress ECA109 cells growth in vivo via GSK3β, GSK3β activity in tumor tissues were assayed by western blot analysis with specific antibody against p-GSK-3β^{Ser9}. Mice implantation with Sox3 knockdown cells showed a significant reduction of -GSK-3β^{Ser9} in tumors (**Figure 6D**). All the results demonstrated that Sox3 knockdown inhibited growth of ESCC cells via suppressed GSK-3β signaling pathway.

Discussion

Esophageal squamous cell carcinoma (ESCC) remains one of the most aggressive carcinomas of the gastrointestinal tract with a poor prognosis and the highest risk of death [1]. Advances in surgical technique and perioperative management have improved survival to some extent. Metastasis is the principal negative prognostic factor in this disease. Therefore, there have been studies of the effects of various biological factors on the malignant potential of ESCC [20]. Thus, the identification of a marker that predicts the metastasis, and hence prognosis, is highly desirable. In the present study, we identified the role for Sox3 in the metastasis of esophageal squamous cell carcinoma cells. We found that Sox3 promotes the metastasis of ESCC cells in vitro and ESCC cells growth through the GSK3β signaling pathway. Our results may provide a new target for intervention in the ESCC treatment and may improve the future treatment of ESCC. In this work, we firstly reported a novel role for Sox3 in the metastasis of ESCC cells. We found that the metastasis of ECA109 ESCC cells was significantly inhibited, when the Sox3 gene was disrupted by insertional mutagenesis. Further investigation with gene silencing of Sox3 showed that the metastasis of ECA109 cells was significantly decreased as revealed by the wound healing assay and migration assay. All these data presented that Sox3 is involved in the metastasis of ESCC cells. In addition, we proofed Sox3 is up-expression in cancer using cell lines and cancer public database (Oncomine), which is consistent with previous results [21].

In humans, SOX3, an HMG box protein, is implicated in a syndrome of X-linked hypopituitarism and mental retardation. The Sox genes encode a family of high-mobility groups that are a family of transcriptional factors and have emerged as potent modulators involved in orchestrating embryonic development and cell fate, organogenesis, stem cells maintenance, and cancerogenesis in multiple processes. Improper regulation of Sox genes have been demonstrated to be associated with cancerous development of various types of cancer [22], such as gastric cancer, brain tumors, ovarian cancer, adenocarcinoma, hepatocarcinoma, breast cancer, melanomas, prostate cancer, colon carcinoma, and the lung cancer. The role of the Sox gene family in the cancerogenesis has been attributed to their properties involving in the regulation of cell differentiation, proliferation, and survival in multiple essential processes.

The underlying molecular mechanism for Sox3-regulated ESCC metastasis is identified to be related to GSK3β signaling pathway. Our results showed that Sox3 silencing leads to down-regulation of GSK3β phosphorylation in ECA109 cells. Furthermore, restored GSK3β activity by an active form GSK3β plasmid could rescue the

impaired invasion and migration of ECA109 cells induced by Sox3 silencing as revealed by the Transwell migration and invasion assay. As GSK3β activity regulates many processes considered to be hallmarks of cancer [23], we observed cell proliferation of the cell lines after transfected. Sox3 knockdown lead to a statistically significant decrease ESCC cells growth both in vitro and vivo via suppressed GSK3β signaling pathway. Due to the important roles of Sox3 in the metastasis and growth of ESCC, it may serve as an attractive target for molecular targeting cancer therapy.

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Disclosure of conflict of interest

None.

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