

TGF- β 1 induces N-cadherin expression by upregulating Sox9 expression and promoting its nuclear translocation in human oral squamous cell carcinoma cells

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Received November 27, 2019; Accepted January 29, 2020

DOI: 10.3892/ol.2020.11582

Abstract. Squamous cell carcinoma (SCC) is the most frequent cancer that develops in the oral cavity. Epithelial-mesenchymal transition (EMT) is known to play an important role in the process of metastasis of SCC cells. In our previous study, we demonstrated that TGF- β 1 induced EMT in the human oral SCC (hOSCC) cell line HSC-4. We also found that Slug plays an important role in suppressing E-cadherin expression and promotion of the migratory activity of HSC-4 cells. However, we also demonstrated that Slug does not participate in upregulation of N-cadherin expression, suggesting that EMT-related transcription factors other than Slug also play an important role in the process. In the present study, we aimed to elucidate how the transcription factor Sox9 affects the TGF- β 1-induced upregulation of N-cadherin expression in HSC-4 cells. We found that TGF- β 1 upregulated Sox9 expression in HSC-4 cells. In addition, Sox9 siRNA significantly abrogated the TGF- β 1-induced upregulation of N-cadherin expression and inhibited the TGF- β 1-promoted migratory activity in HSC-4 cells. We also demonstrated that TGF- β 1 upregulated the phosphorylation status of Sox9 and then promoted nuclear translocation of Sox9 from the cytoplasm, possibly resulting in an increase in N-cadherin expression. The cyclic AMP-dependent protein kinase A inhibitor H-89, which is known to suppress phosphorylation of Sox9, significantly

abrogated the TGF- β 1-induced upregulation of N-cadherin expression. These results suggested that TGF- β 1 induced N-cadherin expression by upregulating Sox9 expression and promoting its nuclear translocation, which results in EMT progression in hOSCC cells.

Introduction

Transforming growth factor- β (TGF- β) has been shown to possess a contradictory dual-faceted nature; it plays both as a tumor suppressor during the initial stages of tumorigenesis as well as an activator in tumor progression. In early-stage cancer cells, TGF- β inhibits cell proliferation, while promoting apoptosis; however, in the late stage of cancer, TGF- β induces invasion and metastasis of cancer through epithelial-mesenchymal transition (EMT), escape from immune system and facilitating angiogenesis (1). TGF- β binds to TGF- β receptor type I (T β R-I) and type II (T β R-II), which are transmembrane serine/threonine kinases. Smad2 and Smad3 when phosphorylated by TGF- β receptor, T β R-I/T β R-II hetero-tetramer, bind to Smad4 and translocate to the nucleus. The transcription of several target genes is regulated by the Smad2/3/4 complex in cooperation with other cofactors (2,3). Recent preclinical and clinical trials in tumor carcinogenesis have focused on testing inhibitors, such as small-molecule tyrosine kinase inhibitors, antibodies and antisense molecules, which block the TGF- β signaling pathway and TGF- β synthesis by small compounds, antibodies and antisense molecules (4).

Squamous cell carcinoma (SCC) is the most frequent cancer in the oral cavity (5). EMT is known to play an important role in cancer metastasis (6). In addition, bone morphogenetic protein (BMP), which belongs to TGF- β superfamily, seems to be involved in mesenchymal-epithelial transition (MET) after metastasis, but the mechanisms have not yet been clarified (7).

EMT induces the loss of characteristics of epithelia and the gain of characteristics of mesenchyme in differentiated epithelial cells, which leads to increased cell migration and invasion (8). EMT is not only an important process in development, adult tissue maintenance and reproduction (9,10), but also in cancer and desmoplasia in disease (11). In general, TGF- β is a crucial inducer of EMT (12,13). Cadherin switch (expression

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Abbreviations: BMP, bone morphogenetic protein; EMT, epithelial-mesenchymal transition; hOSCC, human oral squamous cell carcinoma; TGF- β , transforming growth factor- β ; RT-qPCR, reverse transcription-qPCR; T β R-I, TGF- β receptor type I; T β R-II, TGF- β receptor type II

Key words: EMT, Sox9, squamous cell carcinoma, TGF- β , N-cadherin

changes from E-cadherin to N-cadherin) is known to play an important role in the malignant transformation of cancer cells in the EMT process (14). The mechanism underlying regulation of the cadherin switch in human oral squamous cell carcinoma (hOSCC) cells remains to be elucidated, whereas previous studies have reported changes to the expression of various genes related to the cadherin switch in many kinds of SCC cells other than hOSCC cells (15,16).

In our previous study, we demonstrated that TGF- β 1 induced EMT in hOSCC cell line HSC-4. We also showed that the migratory activity of HSC-4 cells was promoted through TGF- β 1-induced integrin α 3 β 1/FAK activation (16). In addition, we found that the TGF- β 1-induced upregulation of Slug expression, which positively regulated the migratory activity of HSC-4 cells. TGF- β 1 also stimulates the invasion ability of HSC-4 cells through the Slug/Wnt-5b/MMP-10 signaling axis (17). These results suggested that Slug might be an important EMT-related transcription factor which promotes metastasis of hOSCC cells. However, we also demonstrated that Slug did not participate in the upregulation of N-cadherin expression (16), suggesting that EMT-related transcription factors other than Slug played an important role in the process.

Sox9, also known as sex-determining region Y (SRY) protein, is a transcription factor that regulates chondrocyte differentiation and cartilage formation (18). Sox9 positively regulates cell stemness (19), in conjunction with intracellular signaling pathways, such as Wnt signaling (20). Further, it promotes N-cadherin gene transcription in chondrocytic CFK2 cells (21). Sox9 also induces EMT, which in turn results in neural crest formation (22) and nephrolithiasis in primary renal tubular epithelial cells (23). In lung adenocarcinoma, Sox9 mediates Notch-1-induced mesenchymal phenotypes (24), and coexpression of Sox9 and collagen type X alpha 1 in presence of TGF- β 1 is associated with tumor progression in gastric cancer (25). In contrast, knockdown of Sox9 inhibits EMT in thyroid cancer cells (26). However, it is unclear whether Sox9 is involved in TGF- β 1-induced EMT and N-cadherin expression in hOSCC.

In this study, we aimed to identify whether the EMT-related transcription factor Sox9 upregulates N-cadherin expression in hOSCC cells. In addition, we also aimed to elucidate the TGF- β 1-induced signals that affect the function of Sox9 in HSC-4 cells at a molecular level.

Materials and methods

Materials. Cultured cell lines were obtained from the Japanese Collection of Research Bioresources Cell Bank. Recombinant human TGF- β 1 was purchased from PeproTech. Protease inhibitor cocktail, for use with mammalian cell and tissue extracts, and phosphatase inhibitor cocktails 1 and 2 were purchased from Sigma-Aldrich. The Protein kinase inhibitor (PKA), H-89 was obtained from Santa Cruz Biotechnology Inc. and okadaic acid (OA) was procured from Merck (Calbiochem, KGaA). All other purchased reagents were of analytical grade.

Cell culture. All cell lines were grown at 37°C and 5% CO₂. Human HSC-4 SCC cells (JCRB0624) were cultured in Eagle's minimum essential medium (MEM; Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Gibco BRL).

SAS cells (JCRB0260) were cultured in PRIM1640 medium (Gibco BRL) supplemented with 10% FBS. HO-1-N1 cells (JCRB0831) were cultured in Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 medium (1:1; Gibco BRL) with 10% FBS. The culture medium was removed and replaced with serum-free medium 24 h prior to the TGF- β 1-stimulated experiments. For time-course experiments, 2.0x10⁵ hOSCC cells were cultured in 500 μ l of medium without serum containing 10 ng/ml TGF- β 1, for 1 to 48 h in 12 or 24-well tissue culture plates.

Reverse transcription-quantitative PCR (RT-qPCR). For total RNA preparation, 2.0x10⁵ cells were cultured in 24-well tissue culture plates. Total RNA was isolated using the ISOGEN reagent (Nippon Gene), according to manufacturer's instructions. RNA was reverse transcribed into first-strand cDNA using a RT-PCR System kit (Takara Bio Inc.). qPCR was performed on a Thermal Cycler Dice Real Time System (Takara Bio) using SYBR Premix Ex Taq II (Takara Bio) with human gene-specific primers (Table I). Target gene expression was normalized to an internal β -actin reference and expressed in terms of fold-change relative to the control sample (27).

Suppression of gene expression by small interfering RNAs (siRNA). The sense sequences of human Slug siRNA (MISSION siRNA, Hs_SNAIS_9785, Sigma-Aldrich), and Sox9 siRNA (siRNA, Life Technologies) are 5'-GCAUUUGCAGACAGG UCAATT-3' and 5'-UGAAGAAGGAGAGCGAGGAGG ACAA-3', respectively. Logarithmically growing cells were seeded at a density of 1x10⁵ cells in 24-well tissue culture plates and transfected with 10 nM of a specific siRNA using Lipofectamine RNAiMAX (Life Technologies), according to manufacturer's instructions. Forty-eight hours after transfection, cells were stimulated using 10 ng/ml TGF- β 1 and then were used for RT-qPCR analysis to analyze vimentin gene expression or for wound healing assay, as described below. Stealth™ RNAi Negative Control High GC Duplex (Life Technologies), which does not possess significant homology to vertebrate gene sequences, was used as a negative control. Suppression of gene expression by siRNA was evaluated by RT-qPCR and western blot analyses were performed for targeted molecules.

Western blot analysis. For western blot experiments, 3.0x10⁶ cells were lysed in RIPA buffer (Sigma-Aldrich) containing a protease and phosphatase inhibitor cocktail (Sigma-Aldrich). The protein content of the samples was measured using BCA reagent (Thermo Fisher Scientific, Inc.). For the preparation of cell lysates to examine marker proteins, 1.0x10⁶ cells were cultured in a 6-well plate in serum-free MEM with or without 10 ng/ml TGF- β 1 for the indicated times. Cells were dissolved in SDS sample buffer containing a protease and phosphatase inhibitor cocktail (Sigma-Aldrich). Acrylamide gels of 12.5% (ATTO Co.) for SDS-PAGE were used for protein separation, and the proteins were subsequently transferred onto PVDF membranes (Merck). Membranes were probed with primary antibodies, including mouse anti-N-cadherin (1:250, H-2; Santa Cruz Biotechnology) and rabbit anti-Sox9 (1:1,000, AB5535; Chemicon International Inc.) antibodies, while a mouse anti- β -actin antibody (1:1,000,

Table I. Primer sequences for reverse transcription-quantitative PCR.

Target mRNA	Oligonucleotide sequence, 5'-3'	Predicted size, bp
E-cadherin	(F) TACTACTGCCAGGAGCCAGA (R) TGGCACCAGTGTCCGGATTA	103
Fibronectin	(F) AACTTCGAATTATGAGCAGGACCAG (R) GCCCTCAGAAGTGCAATCAGTGTA	151
Laminin α 3	(F) TCGGTCACACCAAAGCAGTCTC (R) TGTGTCCAGTTCAGGTGCAG	93
N-cadherin	(F) CGAATGGATGAAAGACCCATCC (R) GCCACTGCCTTCATAGTCAAACACT	171
Slug	(F) TGTTGCAGTGAGGGCAAGAA (R) GACCCTGGTTGCTTCAAGGA	158
Sox9	(F) GGAGATGAAATCTGTTCTGGGAATG (R) TTGAAGGTTAACTGCTGGTGTCTG	149
Thrombospondin-1	(F) GGAGACAAAGACTGGCTTCTGGAC (R) GGCCACTGCAGGTGATGAGTAA	66
β -actin	(F) GGAGATTACTGCCCTGGCTCCTA (R) GACTCATCGTACTCCTGCTTGCTG	89

F, forward; R, reverse.

clone C4; Santa Cruz) was used as a loading control in siRNA experiments. The blots were then incubated with alkaline phosphatase-conjugated secondary antibody, and subsequently, signals were detected using an alkaline phosphatase substrate kit (BCIP/NBT Substrate kit; Vector Laboratories Inc.).

Cell migration assay with a Boyden chamber. The Boyden chamber-based cell migration assays were performed as follows. First, the cells were transfected with Slug siRNA as described above. Then, they were treated with 10 ng/ml TGF- β 1 under serum-free conditions for 48 h. Subsequently, the cells were plated at a density of 1.0×10^5 cells in the upper chamber of a Boyden chamber apparatus in serum-free media and were allowed to migrate into a medium containing 10% FBS in the lower chamber for 24 h at 37°C. Following the 24 h incubation period, the filter was fixed in 4% paraformaldehyde and stained with DAPI for 10 min. The cells that migrated to the underside of the membrane were counted in nine random fields under a fluorescence microscope. Data are the average of triplicate experiments. The values indicate the mean number of migrating cells compared with control. The level of significance was determined using the Tukey's multiple comparison test.

Immunofluorescence analysis of cultured cells. Cells plated on 8-well chamber slides were incubated at 37°C for 24 h and then stimulated with 10 ng/ml TGF- β 1 for an additional time period of 48 h. Slides were fixed with 4% paraformaldehyde at room temperature for 15 min. Cells were then incubated with specific antibodies with 1:200 dilutions of mouse anti-Slug (A-7; Santa Cruz), rabbit anti-Sox9 (H-90; Santa Cruz) and rabbit anti-phospho-Sox9 (Ser181) (CSB-PA050120; Cusbio Technology) antibodies for 16 h at 4°C. After rinsing with phosphate-buffered saline (PBS), cells were incubated with secondary antibodies Alexa Fluor® 488 goat anti-mouse or

anti-rabbit antibodies (1:1,000; Life Technologies) for 1 h at room temperature and then stained with DAPI (1:500, Sigma-Aldrich) for 10 min. Slides were then washed and imaged using a fluorescence microscope (IX70; Olympus).

Statistical analysis. All experiments were performed at least in triplicate. Results are expressed as mean \pm standard deviation (SD). Differences between two groups (control and TGF- β 1-treated cells) for the time course of Sox9 expression, and the expressions of Slug, Sox9 and N-cadherin in hOSCC cells were analyzed using unpaired two-tailed Student's t-test. Differences among multiple samples for the siRNA- or inhibitor-treated experiments were compared using Tukey's multiple comparison test following ANOVA with IBM SPSS Statistics 24 software. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

EMT-inducible transcription factors other than Slug is involved in the TGF- β -induced EMT in hOSCC cells. We reported that Slug plays an important role in the TGF- β -induced downregulation of E-cadherin expression in HSC-4 cells (16,17). We also found that Slug increased the expression of the mesenchymal marker, vimentin, but not N-cadherin. We previously demonstrated that the same Slug siRNA significantly downregulated mRNA expression of Slug in HSC-4 cells (16). Therefore, we further examined whether Slug increased the expression of mesenchymal markers other than vimentin to verify the extent to which Slug affected the TGF- β -induced EMT in HSC-4 cells. RT-qPCR analysis revealed that the TGF- β -induced expression of mesenchymal markers fibronectin (Fig. 1A) and thrombospondin-1 (Fig. 1B) were significantly downregulated following the administration

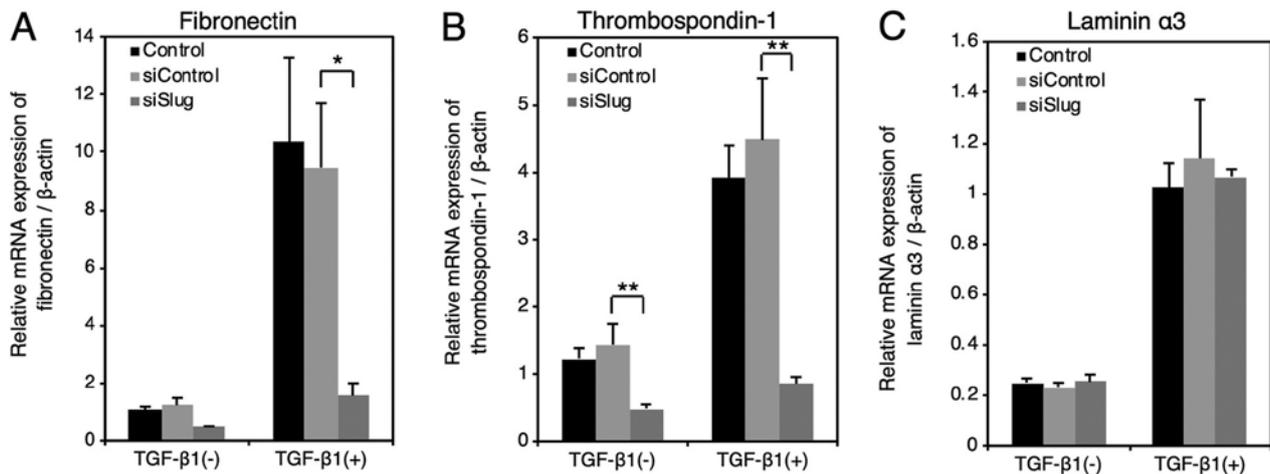


Figure 1. Effect of Slug siRNA on the expression of EMT-related mesenchymal genes in HSC-4 cells. HSC-4 cells were transfected with Slug siRNA (siSlug, dark gray bar) or negative control siRNA (siControl, light gray bar) or without siRNA (Control, black bar). mRNA expression levels of (A) fibronectin, (B) thrombospondin-1 and (C) Laminin α 3 in HSC-4 cells stimulated with or without 10 ng/ml TGF- β 1 for 24 h following siRNA treatment for 48 h were evaluated by reverse transcription-quantitative PCR. Values have been normalized to β -actin mRNA levels. Results were analyzed using Tukey's multiple comparison test. Data are presented as the mean \pm SD from quadruplicate experiments. * P <0.05, ** P <0.01. EMT, epithelial-mesenchymal transition; si, small interfering; TGF- β 1, transforming growth factor- β .

of Slug siRNA. The status of Laminin α 3 was not affected by Slug siRNA (Fig. 1C), suggesting that transcription factors other than Slug must also be involved in the TGF- β -induced EMT in HSC-4 cells.

TGF- β 1 upregulates expression of transcription factor Sox9. The mRNA expression of Sox9 was found to be significantly upregulated at 3-48 h after TGF- β 1 stimulation (10 ng/ml) in HSC-4 cells (Fig. 2). The expression of Sox9 mRNA continuously increased between 3 and 24 h following TGF- β 1 stimulation, peaking at 24 h and then decreased at 48 h after stimulation. We previously reported that TGF- β 1 (10 ng/ml) increased the mRNA expression of EMT-related transcription factor, Slug, at 1.5 h following stimulation, in a Smad signal transduction mechanism-dependent manner, in HSC-4 cells (17). These results indicate that Sox9 is not a direct target of Smad signaling.

TGF- β 1 upregulates expression of N-cadherin, and promotes the migration of HSC-4 cells. As shown in Fig. 3A, we confirmed that control siRNA did not affect Sox9 mRNA expression, and that Sox9 siRNA significantly suppressed Sox9 mRNA expression in HSC-4 cells. Notably, Sox9 siRNA significantly abrogated the TGF- β 1-induced upregulation of N-cadherin mRNA expression (Fig. 3B). In addition, we confirmed that Sox9 siRNA suppressed the TGF- β 1-induced upregulation of N-cadherin expression at protein level (Fig. 3C). TGF- β 1-induced cell migration was significantly and incompletely decreased by Sox9 siRNA in HSC-4 cells (Fig. 3D). These results suggest that N-cadherin promotes the migration activity of HSC-4 cells partially through Sox9-dependent pathway.

TGF- β 1 upregulates expression levels of Sox9 and/or N-cadherin in hOSCC cells other than HSC-4 cells. We previously reported that SAS and HO-1-N1 were TGF- β 1-responsive hOSCC cells: TGF- β 1 upregulated expression of fibronectin

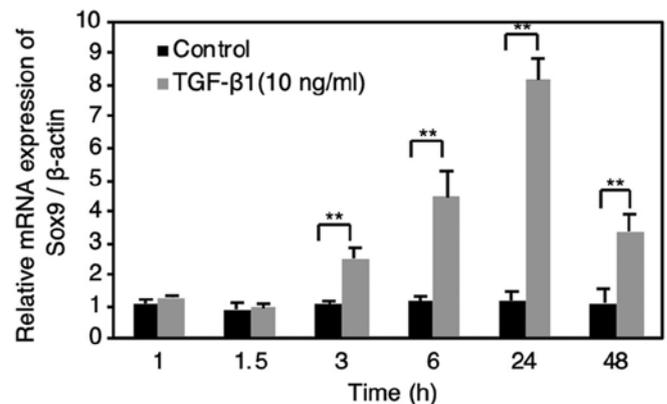


Figure 2. Time course of Sox9 expression after TGF- β 1 stimulation in HSC-4 cells. Sox9 gene expression was examined in cells treated with 10 ng/ml TGF- β 1 (dark gray bar) or control (black bar) for the indicated times up to 48 h with reverse transcription-quantitative PCR analysis. Differences between control and TGF- β 1-treated cells at each time points were analyzed using Student's t-test. Data are presented as the mean \pm SD from quadruplicate experiments. ** P <0.01. TGF- β 1, transforming growth factor- β .

and plasminogen activator inhibitor-1 (16). Here, we examined whether TGF- β 1 upregulated Slug, Sox9, and N-cadherin in SAS cells and HO-1-N1 cells as in HSC-4 cells. We found that TGF- β 1 (10 ng/ml) significantly upregulated the mRNA expressions of Slug, and N-cadherin at 24 h following stimulation with TGF- β 1 (Fig. 4A, B and C). In contrast, TGF- β 1 (10 ng/ml) significantly upregulated the mRNA expression of Sox9 in both HSC-4 cells and SAS cells, but not in HO-1-N1 cells (Fig. 4A, B and C), suggesting that the TGF- β 1-induced upregulation of N-cadherin expression is not always dependent on the upregulation of Sox9 expression in hOSCC cells.

TGF- β 1 promotes phosphorylation and nuclear translocation of Sox9 in HSC-4 cells. The transcription factor Sox9 is known to translocate from cytoplasm into the

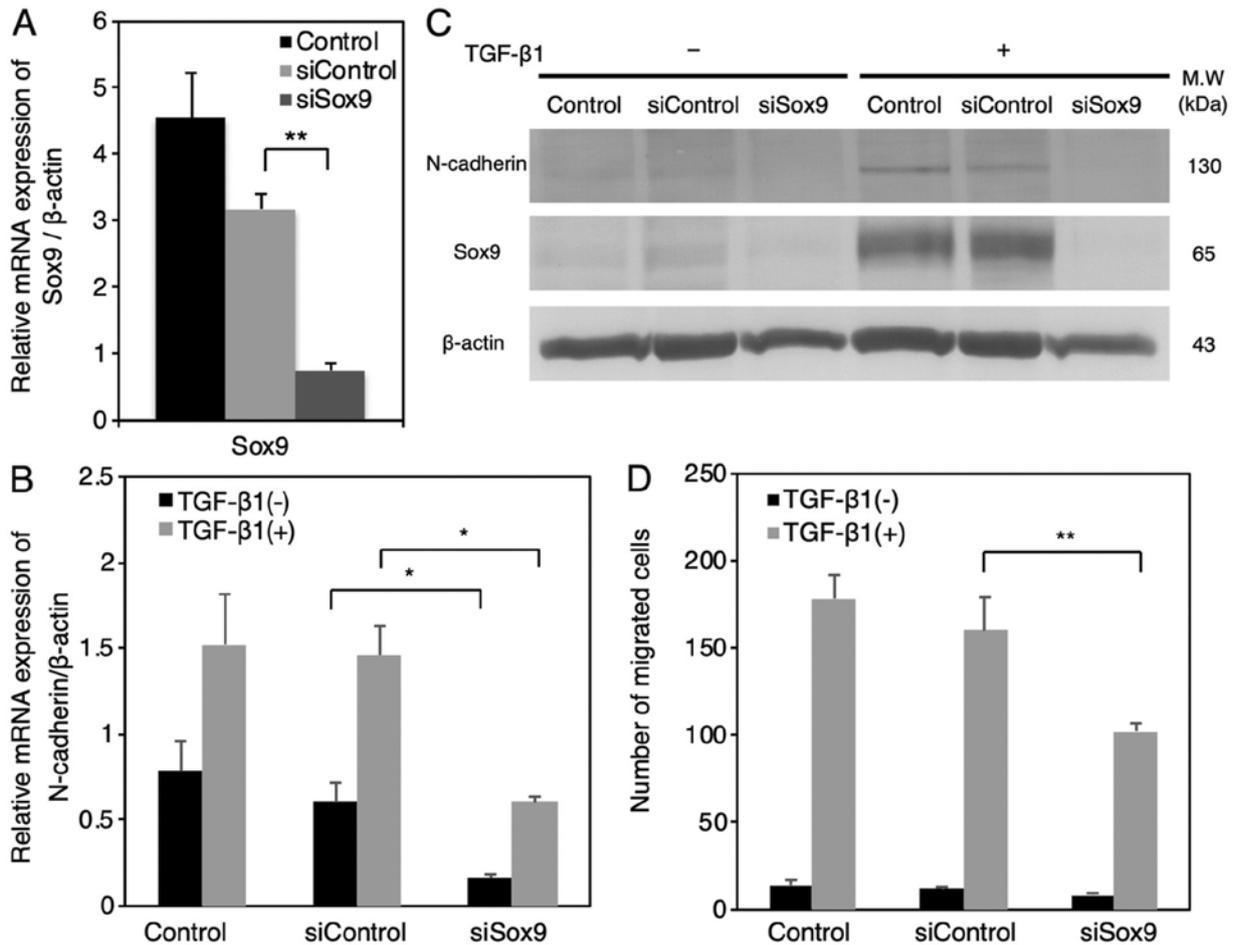


Figure 3. TGF- β 1 upregulates expression of N-cadherin, and promotes the migration of HSC-4 cells. HSC-4 cells were transfected with Sox9 siRNA (siSox9), control siRNA (siControl) or without siRNA (Control). (A) mRNA expression of Sox9 in HSC-4 cells stimulated with 10 ng/ml TGF- β 1 for 24 h in serum-free medium was determined by RT-qPCR. (B) Cells transfected with Sox9 siRNA or control siRNA were stimulated with (gray bar) or without (black bar) 10 ng/ml TGF- β 1 for 24 h in serum-free medium. Expression level of N-cadherin was analyzed by RT-qPCR. Values have been normalized to β -actin mRNA levels. Data are presented as the mean \pm SD from quadruplicate experiments for each time point. (C) Protein expression levels of N-cadherin or Sox9 in HSC-4 cells transfected with Sox9 siRNA or control siRNA were determined by western blotting with anti-N-cadherin or Sox9 antibodies, respectively. β -actin in cell lysate was used as a loading control. (D) Cell migration ability of HSC-4 cells transfected with Sox9 siRNA or control siRNA was evaluated in a Boyden chamber assay after stimulation with (gray bar) or without (black bar) 10 ng/ml TGF- β 1 under serum-free conditions for 48 h. After 24 h incubation in a Boyden chamber, the cells which moved from the upper surface of membrane to the lower surface of the membrane were stained with DAPI. Subsequently, the number of stained cells were counted. Results were analyzed using Tukey's multiple comparison test. Data are presented as the mean \pm SD of three wells for each time point. * P <0.05, ** P <0.01. TGF- β 1, transforming growth factor- β ; si, small interfering; RT-qPCR, reverse transcription-quantitative PCR.

nucleus in response to TGF- β 1 stimulation in interstitial cells (28). In addition, cyclic AMP-dependent protein kinase (PKA)-mediated phosphorylation of Sox9 plays an important role in its transcriptional activity (29). We confirmed that TGF- β 1 (10 ng/ml) induced nuclear translocation of Slug, as previously reported (30) (Fig. 5A). Similarly, we found that TGF- β 1 (10 ng/ml) promoted nuclear translocation of total Sox9 and Ser-181-phosphorylated, which is known to activate the transcriptional activity of Sox9-target genes in chondrocytes (31) (Fig. 5A and B). Furthermore, we found that TGF- β 1 stimulation resulted in an increase in total Sox9 and pSox9 levels (Fig. 5A and B). Interestingly, the pretreatment of HSC-4 cells with the PKA inhibitor, H-89 (15 μ M), before TGF- β 1 stimulation inhibited the TGF- β 1-induced phosphorylation and nuclear translocation of pSox9 (Fig. 5B). Further, we observed that the PKA inhibitor significantly abrogated the TGF- β 1 (10 ng/ml)-induced upregulation of N-cadherin mRNA expression (Fig. 5C). It was previously reported that

protein phosphatase A2 (PP2A) negatively regulates PKA activity (32,33). We confirmed that the PP2A inhibitor okadaic acid (50 nM) clearly and significantly enhanced TGF- β 1 (10 ng/ml)-induced upregulation of N-cadherin mRNA expression (Fig. 5D). These results suggest that the TGF- β 1 promoted phosphorylation and nuclear translocation of Sox9 occurs in a PKA-dependent manner, possibly resulting in the upregulation of N-cadherin expression in HSC-4 cells.

Discussion

We demonstrate that TGF- β 1 increased N-cadherin expression, and migratory activity in HSC-4 cells through upregulation of Sox9 expression, and promotion of Sox9 nuclear translocation. Interestingly, Zhang *et al* reported that TGF- β , secreted from tumor-associated macrophages, induces EMT in non-small lung cancer through activation of Sox9-mediated signals (34). In contrast, Wnt and/or Hippo pathways are known to play

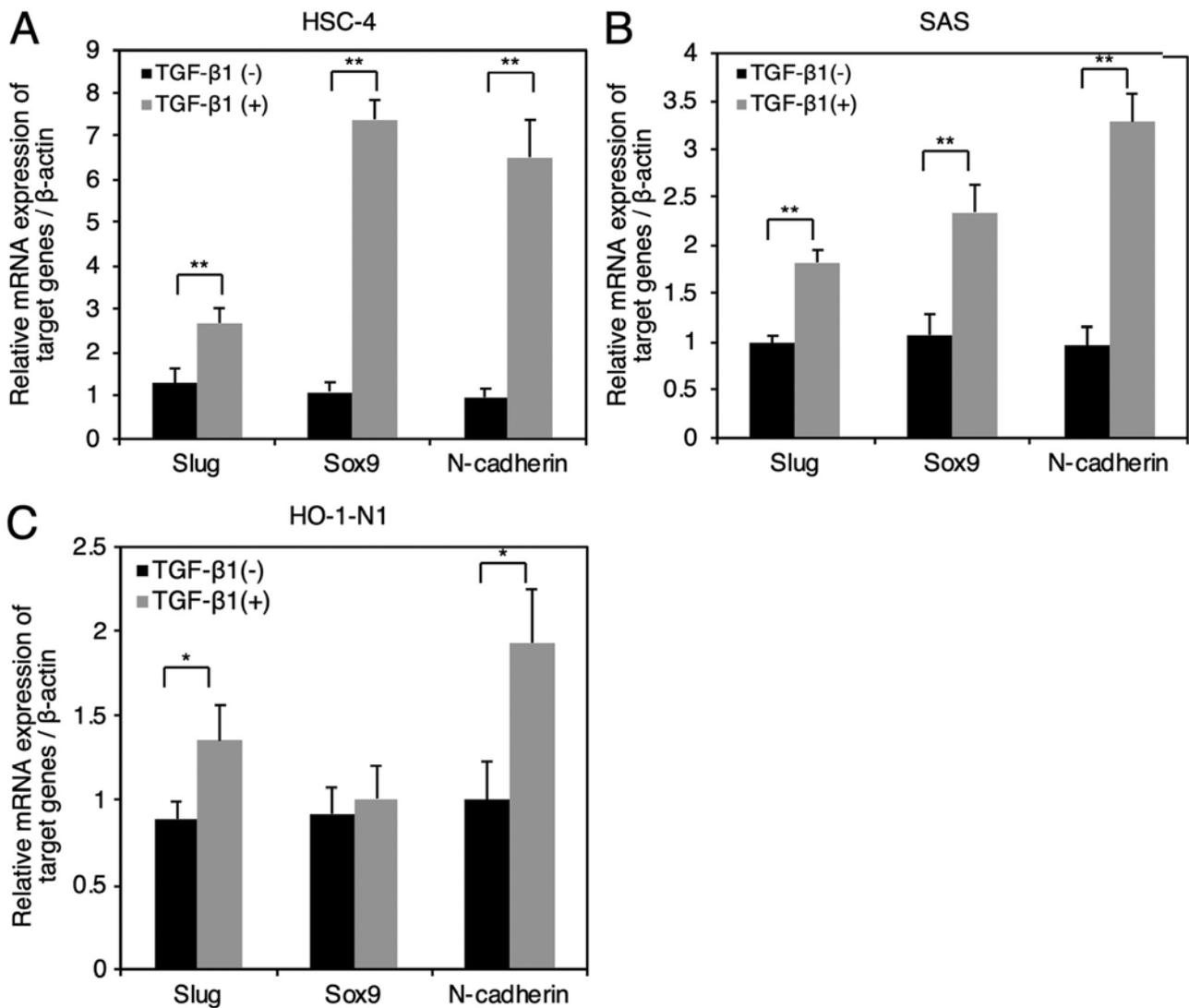


Figure 4. Effect of TGF- β 1 on the expression of Slug, Sox9 and N-cadherin in hOSCC cells. mRNA expression levels of the TGF- β 1 target genes Slug, Sox9, and N-cadherin were analyzed by reverse transcription-quantitative PCR analysis in (A) HSC-4, (B) SAS and (C) HO-1-N1 hOSCC cell lines following treatment with 10 ng/ml TGF- β 1 for 24 h (gray bar) compared with untreated control (black bar). Differences between control and TGF- β 1-treated cells were analyzed using Student's t-test. Data are presented as the mean \pm SD from quadruplicate experiments. * P <0.05, ** P <0.01. TGF- β 1, transforming growth factor- β ; hOSCC, human oral squamous cell carcinoma.

important roles in TGF- β 1-induced expression of Sox9 (20,35). In addition, Dyer *et al* reported that BMP-2-induced Smad1/5/8-mediated signal increased Sox9 protein levels in the atrioventricular cushions during EMT (36). However, we confirmed that BMP-2 (10 ng/ml) did not increase Sox9 mRNA levels in HSC-4 cells (data not shown).

We previously reported that Slug is an EMT-related transcription factor that upregulates expression of vimentin, Wnt-5B, and MMP-10 (16,17). Similarly, in this study, transfection of HSC-4 cells with Slug siRNA demonstrated that Slug promotes gene expressions of fibronectin and thrombospondin-1. Notably, the expression levels of thrombospondin-1 were found to be significantly downregulated by siSlug in the absence of TGF- β 1 stimulation. Collectively, these findings suggest two possibilities; that Slug mediated the fundamental machinery of transcription of fibronectin and thrombospondin-1 genes, or that HSC-4 cells autonomously secreted TGF- β 1.

On the contrary, we found that TGF- β 1-induced expression of mesenchymal marker, Laminin α 3, was not abrogated by Slug siRNA, indicating that Slug does not participate in the TGF- β 1-induced expression of Laminin α 3. However, RT-qPCR analysis revealed that the TGF- β 1-induced expression of Laminin α 3 was significantly downregulated by Sox9 siRNA (data not shown), suggesting that TGF- β 1-induced expression of Laminin α 3 was mediated by Sox9 and not by Slug. Interestingly, a cooperative interplay of Slug and Sox9 in EMT was observed in early neural crest development (22) and in mammary stem cells (19). Moreover, Slug and Sox9 were found to cooperatively and positively regulate the expressions of tenascin-C and periostin, which are tumor-initiating niche factors in breast cancer cells (37). Slug also regulates Sox9 stability in lung carcinoma cells (38). Whether the signal crosstalk between Slug- and Sox9-mediated signals played an important role in the TGF- β 1-induced EMT in hOSCC cells remains under investigation.

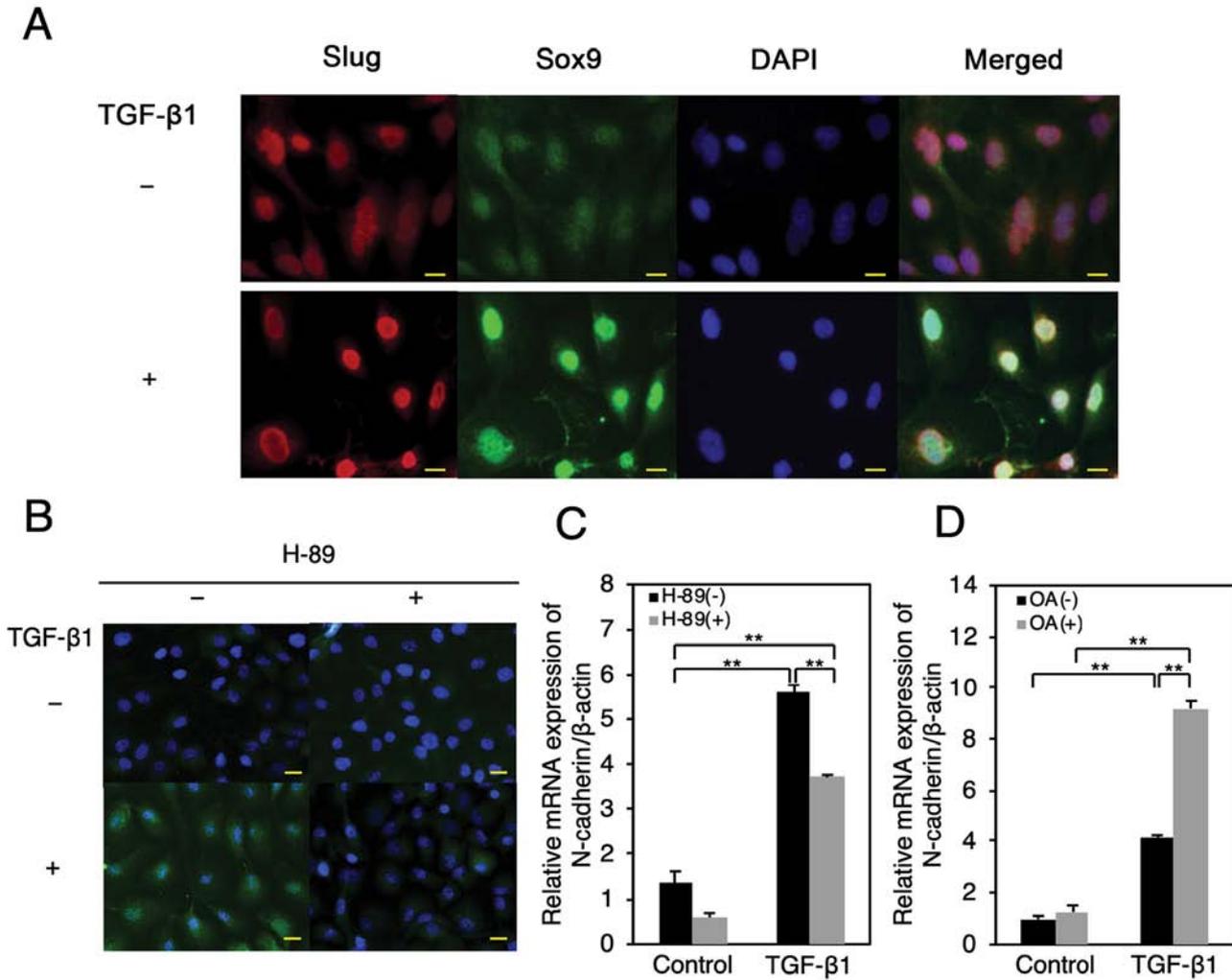


Figure 5. TGF-β1 promotes phosphorylation and nuclear translocation of Sox9 in HSC-4 cells. (A) HSC-4 cells were cultured with or without 10 ng/ml TGF-β1 under serum-free conditions for 24 h. (B) HSC-4 cells were stimulated with or without 10 ng/ml TGF-β1 for 48 h following treatment with or without 15 μM H-89 for 60 min. Cells were immunostained with specific antibodies and were then counterstained with DAPI to detect nuclei. Scale bar, 50 μm. HSC-4 were treated with the inhibitor of (C) 15 μM H-89 or (D) 50 nM OA, or DMSO as a vehicle for 60 min before TGF-β1 treatment. Cells were stimulated with 10 ng/ml TGF-β1 for 48 h (TGF-β1) or untreated control (Control). mRNA expression levels of N-cadherin treated with inhibitor (grey bars) or without inhibitor (black bar) were examined by reverse transcription-quantitative PCR. Values have been normalized to β-actin mRNA levels. Results were analyzed using Tukey's multiple comparison test. Data are presented as the mean ± SD from quadruplicate experiments. **P<0.01. OA, okadaic acid; TGF-β1, transforming growth factor-β1.

The phosphorylation sites of Sox9 have been reported as serine (S) residues 64 and 181 (29,31). Particularly, the phosphorylation of S181 played a crucial role in the nuclear translocation of Sox9 (31). We observed that Sox9 gets translocated into nuclei in response to TGF-β1-stimulation. In addition, we demonstrated that the nuclear-translocated Sox9 is phosphorylated at S181 by TGF-β1-stimulation. It was reported that Sox9 is phosphorylated by cyclic AMP-dependent protein kinase A (PKA), resulting in enhancement of transcriptional activity of Sox9 (29). This led us to examine whether PKA was involved in the TGF-β1-induced upregulation of N-cadherin expression. The results of our study showed that the PKA inhibitor, H-89, partially, but significantly suppressed the TGF-β1-induced upregulation of N-cadherin expression, suggesting that TGF-β1-induced upregulation of N-cadherin expression was only partly mediated by a PKA-dependent signal. In addition, these results further implicated that the TGF-β1-induced phosphorylation of Sox9 (S181) could be

possibly mediated by PKA. In contrast, it was demonstrated that TGF-β1-stimulated Smad3/4 directly activated PKA through an interaction between Smad4 and a regulatory subunit of PKA (39,40). In addition, Chowdhury *et al* also reported TGF-β activated PKA in colon cancer cells (33). Corroborating these findings, we previously showed that TGF-β1 induced activation of Smad2/3 in HSC-4 cells (16), suggesting the possible involvement of Smad2/3 in activation of PKA in TGF-β1-stimulated HSC-4 cells.

In summary, we have demonstrated that TGF-β1 induces N-cadherin expression through upregulated expression and promotion of nuclear translocation of Sox9, thus resulting in the progression of EMT in hOSCC cells.

Acknowledgements

The authors would like to thank Dr Takahiro Chiba (Division of Oral and Maxillofacial Surgery, Department of Reconstructive

Oral and Maxillofacial Surgery, Iwate Medical University School of Dentistry) for assistance with the cell cultures of hOSCC cell lines and RT-qPCR analysis.

Funding

The present study was supported in part by JSPS KAKENHI (grant nos. JP18K17237, JP16H05534 and JP17K11851) from The Ministry of Education, Culture, Sports, Science and Technology of Japan.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TH, DS and MK performed western blotting and reverse transcription-quantitative PCR analyses, fluorescence immunostaining and cell migration assays. TH, HY, AI and MK designed the present study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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