

---

# Transforming Growth Factor- $\beta$ 1 Promotes Matrix Metalloproteinase-9–Mediated Oral Cancer Invasion through Snail Expression

Limin Sun,<sup>1</sup> Michelle E. Diamond,<sup>1</sup> Adam J. Ottaviano,<sup>1</sup> Mathew J. Joseph,<sup>1</sup> Vijayalakshmi Ananthanarayan,<sup>4</sup> and Hidayatullah G. Munshi<sup>1,2,3</sup>

<sup>1</sup>Division of Hematology/Oncology, Department of Medicine, Feinberg School of Medicine, Northwestern University; <sup>2</sup>The Jesse Brown VA Medical Center; <sup>3</sup>The Robert H. Lurie Comprehensive Cancer Center of Northwestern University; and <sup>4</sup>Department of Pathology, University of Illinois at Chicago, Chicago, Illinois

## Abstract

Oral squamous cell carcinoma (OSCC), which is the most common malignancy of the oral cavity, is often associated with local and regional invasion. Increased expression of matrix metalloproteinase-9 (MMP-9) is correlated with invasive behavior of OSCC. Because transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1) is up-regulated in OSCC tumors, we examined the relationship between TGF- $\beta$ 1 signaling and MMP-9 in human OSCC specimens. Evaluation of human specimens showed that tumors with enhanced TGF- $\beta$ 1 signaling also showed increased MMP-9 expression. Because the transcription factor Snail has been determined to be a key mediator of TGF- $\beta$ 1 signaling, we evaluated the role of Snail in TGF- $\beta$ 1–mediated MMP-9 expression. Initially, we examined the extent to which TGF- $\beta$ 1 regulated Snail expression in oral keratinocytes and in OSCC cell lines. TGF- $\beta$ 1 enhanced Snail expression in a majority of the cell lines examined, with the largest induction of Snail detected in UMSSC1 cells. Interestingly, overexpression of Snail in UMSSC1 cells enhanced MMP-9 and tissue inhibitor of metalloproteinase-1 protein levels. Despite the increase in the tissue inhibitor of metalloproteinase-1 protein, there was a *net* increase in the pericellular proteolytic activity as shown by enhanced MMP-9–dependent Matrigel invasion. Moreover, Snail-specific siRNA blocked TGF- $\beta$ 1–induced MMP-9 expression and Matrigel invasion. In addition, Snail increased Ets-1 levels and Ets-1–specific siRNA blocked both Snail- and TGF- $\beta$ 1–mediated MMP-9 expression and Matrigel invasion. Thus, these data show that Snail functions as a molecular mediator of TGF- $\beta$ 1–regulated MMP-9 expression by increasing Ets-1 and thereby contributing to oral cancer progression. (Mol Cancer Res 2008;6(1):10–20)

Received 5/7/07; revised 7/25/07; accepted 8/20/07.

**Grant support:** National Cancer Institute grant K08CA94877, Merit grant award from the Department of Veterans Affairs, and the H Foundation (H.G. Munshi). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

**Requests for reprints:** Hidayatullah G. Munshi, Department of Medicine, Northwestern University Medical School, 303 East Superior Avenue, Lurie 3-117, Chicago, IL 60611. Phone: 312-503-2301; Fax: 312-503-0386. E-mail: h-munshi@northwestern.edu

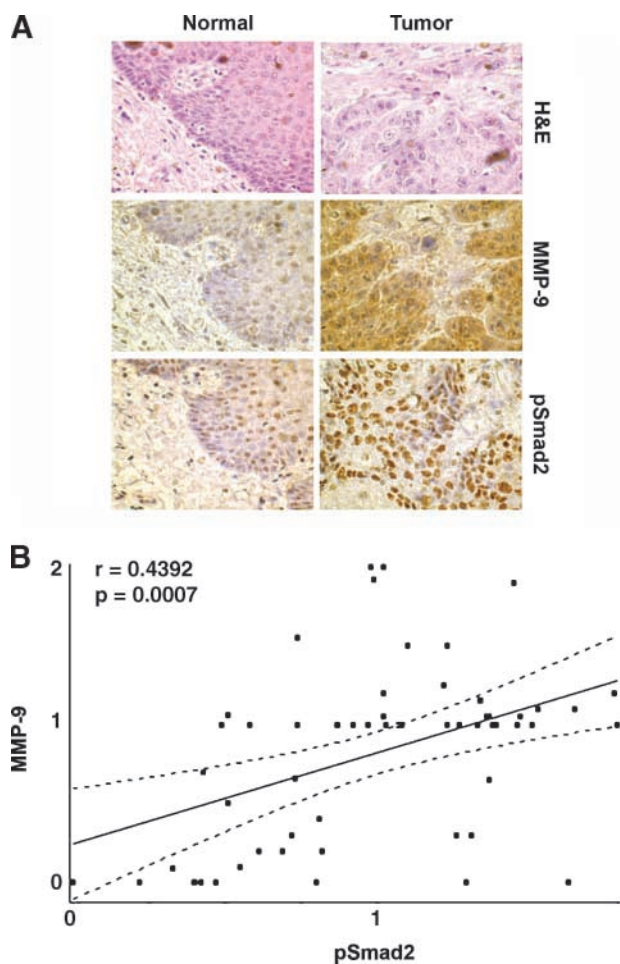
Copyright © 2008 American Association for Cancer Research. doi:10.1158/1541-7786.MCR-07-0208

## Introduction

The American Cancer Society estimates that there will be more than 20,000 new cases of cancer this year involving the oral cavity in the United States (1). Although the mortality due to oral cancer has been gradually declining since 1975, the 5-year survival rate for all patients with oral cancer is ~60%, with the 5-year rate dropping to ~30% for patients with metastatic oral cancer (1). Moreover, advanced oral squamous cell carcinoma (OSCC) is associated with high morbidity, and treatment is often complicated by disruption of speech and swallowing due to surgical resection (2, 3). This dismal outcome is attributed to the fact that OSCC is an aggressive and a highly invasive cancer, and the cellular and biochemical factors that underlie local, regional, and distant spread of the disease are poorly understood. OSCC progression is frequently associated with acquisition of a more invasive phenotype and with proteinase-dependent extracellular matrix degradation (4).

Invasive OSCC cells secrete matrix metalloproteinases (MMP; refs. 4-6), which are a large family of highly conserved metalloendopeptidases with activity directed against a variety of extracellular matrix substrates (7-9). Immunohistochemical studies have shown increased expression of MMPs, including MMP-9 (gelatinase B, 92-kDa type IV collagenase), in invasive and metastatic cases of human OSCC (4, 5). Moreover, increased expression of MMP-9 is associated with decreased staining of peritumoral extracellular matrix, suggesting that MMP-9 promotes matrix breakdown in human tumors (4, 5). In addition, animal studies have shown that blocking MMP-9 can decrease tumor cell invasion and metastasis (10). Because MMP-9 plays an important role in cancer progression, the regulation of MMP-9 expression and activity has been extensively studied (11-13).

Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1), which is elevated in human OSCC tumors compared with normal head and neck tissue (14), promotes tumorigenesis and increases metastasis (15, 16). Targeted ectopic expression of TGF- $\beta$ 1 in head and neck epithelia using transgenic mice results in severe hyperplasia of the oral cavity epithelia and facilitates tumor progression (14). Moreover, it has been shown in an orthotopic murine model of head and neck cancer that the metastatic tumors express higher levels of TGF- $\beta$ 1 compared with the initial primary tumor and that the metastatic tumors with increased TGF- $\beta$ 1 also have increased MMP-9 levels (17). Additionally, recent data indicate that TGF- $\beta$ 1 regulates



**FIGURE 1.** Analysis of MMP-9 and pSmad2 staining in previously untreated human oral samples. **A.** H&E, MMP-9, and pSmad2 staining in a normal oral tissue and in a moderately differentiated oral tumor specimen ( $\times 400$  magnification). **B.** The association between MMP-9 and pSmad2 was assessed using Pearson correlation coefficient.

expression of Snail family of transcription factors (18, 19), which have previously been shown to regulate MMP expression (20-22).

In the current study, evaluation of human OSCC specimens showed that tumors with enhanced TGF- $\beta$ 1 signaling also showed increased MMP-9 expression. Because Snail has been implicated as a key intermediary in TGF- $\beta$ 1 signaling, we specifically evaluated the role of Snail in TGF- $\beta$ 1-mediated MMP-9 expression and function. TGF- $\beta$ 1 promoted Snail expression in both immortalized oral keratinocytes and OSCC cells. Using a green fluorescent protein (GFP)-tagged Snail construct to further examine the role of Snail in OSCC invasion, we report that Snail overexpression suppressed E-cadherin, enhanced MMP-9 and tissue inhibitor of metalloproteinases (TIMP)-1, and promoted MMP-9-dependent matrix invasion. Similar results were obtained following TGF- $\beta$ 1 treatment, whereas MMP-9 expression and invasion-promoting activity were blocked by Snail-specific siRNA. Furthermore, Snail regulation of the transcription factor Ets-1 was shown, and that Ets-1-specific siRNA blocked both Snail-

and TGF- $\beta$ 1-mediated MMP-9 expression and cellular invasion. Together, these data support a model wherein Snail functions as a molecular mediator of TGF- $\beta$ 1-regulated MMP-9 expression in OSCC cells at the transcriptional level via an Ets-1-dependent mechanism, thereby contributing to oral cancer progression.

**Results**

*Analysis of MMP-9 and Phospho-Smad2 in Human OSCC Tumors*

Using microarrayed human tissue, we examined the relationship between expression of MMP-9 and phospho-Smad2 (pSmad2; as a marker of TGF- $\beta$ 1 signaling) in previously untreated human oral samples. A representative example of H&E, MMP-9, and pSmad2 staining in normal oral tissue and moderately differentiated OSCC is shown in Fig. 1A. Quantification of MMP-9 and pSmad2 staining showed a statistically significant overall increase in MMP-9 and pSmad2 expression in the tumor specimens compared with the normal oral tissue (Table 1). In addition, tumor areas with enhanced pSmad2 also showed increased MMP-9 expression with a Pearson correlation coefficient of  $r = 0.44$ ,  $P = 0.0007$  (Fig. 1B).

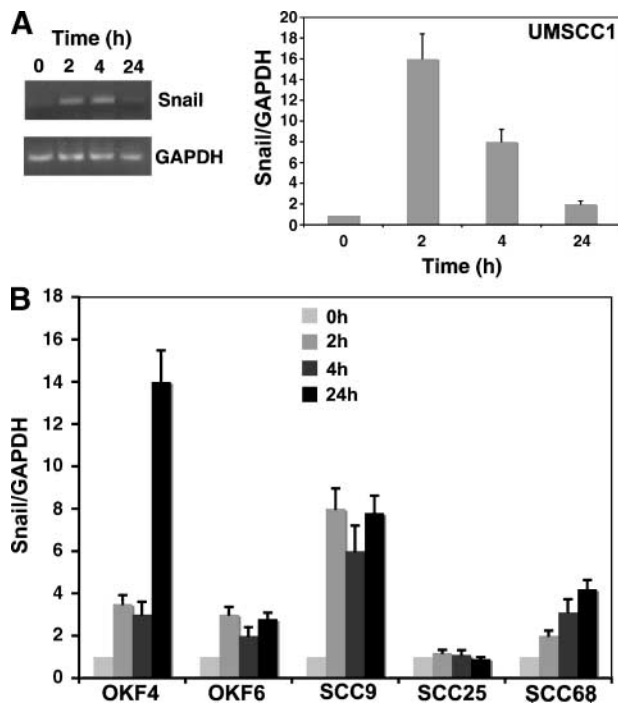
*TGF- $\beta$ 1 Promotes Snail Expression in OSCC Cells*

Recent reports have shown that Snail can function as a molecular mediator of TGF- $\beta$ 1 signaling (18, 19). Thus, we examined the specific role of Snail in TGF- $\beta$ 1-mediated MMP-9 expression in oral cancer cells. UMSCC1 cells were treated with TGF- $\beta$ 1 for varying periods of time (0, 2, 4, and 24 h), and reverse transcription-PCR (RT-PCR) was done using One-Step RT-PCR kit for Snail and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Fig. 2A). Treatment of UMSCC1 cells with TGF- $\beta$ 1 transiently enhanced Snail expression. Using real-time PCR, the changes in Snail and GAPDH messages were also quantified and relative gene expression determined using the comparative  $C_t$  method. As shown in Fig. 2A (right), TGF- $\beta$ 1 enhanced Snail message 16-fold at 2 h, which declined to 2-fold at 24 h. Subsequently, the effect of TGF- $\beta$ 1 on Snail expression was also quantified in

**Table 1. Immunohistochemical Expression of MMP-9 and pSmad2 in Previously Untreated Human Oral Samples**

Histology	n	0	0.01-1.00	1.01-2.00	Mean intensity	P
<b>MMP-9</b>						
Normal	10	5	5	0	0.12 $\pm$ 0.15	
Well differentiated	10	0	1	9	1.29 $\pm$ 0.36	<0.0001
Moderately differentiated	28	1	21	6	0.94 $\pm$ 0.44	<0.0001
Poorly differentiated	6	0	1	5	1.05 $\pm$ 0.57	0.01
<b>pSmad2</b>						
Normal	10	1	8	1	0.62 $\pm$ 0.35	
Well differentiated	10	0	3	7	1.20 $\pm$ 0.36	0.002
Moderately differentiated	28	0	12	16	1.06 $\pm$ 0.41	0.004
Poorly differentiated	6	0	2	4	0.92 $\pm$ 0.36	0.13

NOTE: Immunohistochemical expression of MMP-9 and pSmad2 was quantified by determining the percentages of tumor cells that were positive at each of the intensity scores: 0 (absent staining), 1+ (weak staining), and 2+ (strong staining). The sum of these scores gave a final score for each of the cores: 0, 0.01-1.00, or 1.01-2.00. The P values were calculated using unpaired t test comparing each of the tumor grades with the normal oral tissue samples.



**FIGURE 2.** TGF- $\beta$ 1 promotes Snail expression in OSCC cells. **A.** Serum-starved UMSSC1 cells were treated with 10 ng/mL TGF- $\beta$ 1 for varying periods of time, and RT-PCR for Snail and GAPDH was done with primers described in Materials and Methods. Relative expression of Snail and GAPDH was determined by real-time PCR, quantified, and normalized to untreated sample, arbitrarily set at 1.0. **B.** OKF4, OKF6, SCC9, SCC25, and SCC68 cells were treated with TGF- $\beta$ 1 for varying periods of time, and relative expression of Snail and GAPDH was determined by real-time PCR, quantified, and normalized to untreated sample, arbitrarily set at 1.0.

additional OSCC cell lines by real-time PCR (Fig. 2B). TGF- $\beta$ 1 did not modulate Snail expression in SCC25 cells; however, TGF- $\beta$ 1 significantly enhanced Snail message in the remaining four cell lines with varying kinetics and magnitude.

#### *Snail Increases MMP-9 and TIMP-1 without Affecting MT1-MMP or TIMP-2*

To determine if Snail can regulate MMP expression in OSCC cells, we generated stable UMSSC1 cell lines expressing GFP (UMSSC1-C) or GFP-tagged Snail (UMSSC1-Sn). As shown in Fig. 3A, real-time PCR showed that Snail was overexpressed by 70-fold in UMSSC1-Sn cells compared with UMSSC1-C cells. Consistent with the published reports (23, 24), Snail overexpression in UMSSC1 cells suppressed E-cadherin expression by 50% at the message level (Fig. 3A). To examine the changes at the protein level, equal amounts of cell lysates from UMSSC1-C and UMSSC1-Sn cells were probed with anti-GFP antibody. As shown in Fig. 3A, GFP and GFP-tagged Snail proteins were detected in these stable cell lines; however, the amount of GFP-tagged Snail protein was relatively decreased in UMSSC1-Sn cells compared with the amount of GFP present in the control UMSSC1-C cells. This is in agreement with reports showing that the Snail protein undergoes ubiquitination followed by degradation (25, 26). Despite the relatively short half-life of GFP-tagged Snail protein, Snail overexpression decreased E-cadherin at the

protein level (Fig. 3A), in agreement with the real-time PCR data. In contrast to the effect of Snail on E-cadherin, there was no significant change in the levels of N-cadherin, vimentin, or fibronectin in UMSSC1-Sn cells compared with the UMSSC1-C cells (data not shown). However, Snail expression resulted in a more scattered appearance of UMSSC1 cells as indicated by phalloidin staining (Fig. 3B).

To examine the effect of Snail on MMP and TIMP expression, equal numbers of UMSSC1-C and UMSSC1-Sn cells were counted using a Z1 Coulter particle counter, plated overnight in serum-containing media, serum starved for 8 h, and then allowed to condition the media for additional 24 h. The total RNA was isolated and the relative MMP-9 and TIMP-1 message levels were quantified by real-time PCR. Snail expression increased MMP-9 message level by 5-fold; however, no change in the TIMP-1 message level was observed (Fig. 3C). Importantly, when the stable cell lines overexpressed Snail by 15- to 30-fold, there remained a significant 2.5- to 3-fold increase in the MMP-9 mRNA levels. Thus, when Snail is expressed at levels comparable to those induced by TGF- $\beta$ 1 treatment, enhanced MMP-9 mRNA expression remains apparent in the UMSSC1 cells. In addition, the conditioned media were analyzed for MMP-9 protein expression by gelatin zymography and for TIMP-1 protein by Western blotting. Snail overexpression increased MMP-9 protein level; however, in contrast to the effect on TIMP-1 message level, Snail expression also increased TIMP-1 protein level (Fig. 3C).

Because it has been suggested that Snail can also regulate MT1-MMP expression (21, 22), we examined the effect of Snail overexpression on MT1-MMP at the message level by real-time PCR and at the protein level by Western blot analysis. Additionally, we examined the effect of Snail overexpression on TIMP-2, an endogenous inhibitor of MT1-MMP activity. Snail overexpression in UMSSC1 cells did not affect the message levels for MT1-MMP or TIMP-2 (Fig. 3D). No change in total MT1-MMP protein expression or cell-surface localization of MT1-MMP was detected (Fig. 3D). In addition, there was no difference in the levels of TIMP-2 protein present in the conditioned media of UMSSC1-C and UMSSC1-Sn cells (Fig. 3D).

We also examined the effect of Snail on the expression levels of MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-13, MMP-15, and MMP-16 by real-time RT-PCR. As shown in Fig. 3E, Snail expression increased MMP-7 mRNA levels while decreasing MMP-1 and MMP-3 mRNA levels in UMSSC1 cells. Snail had minimal or no effect on the mRNA levels of MMP-2, MMP-8, MMP-13, MMP-15, and MMP-16 (Fig. 3E).

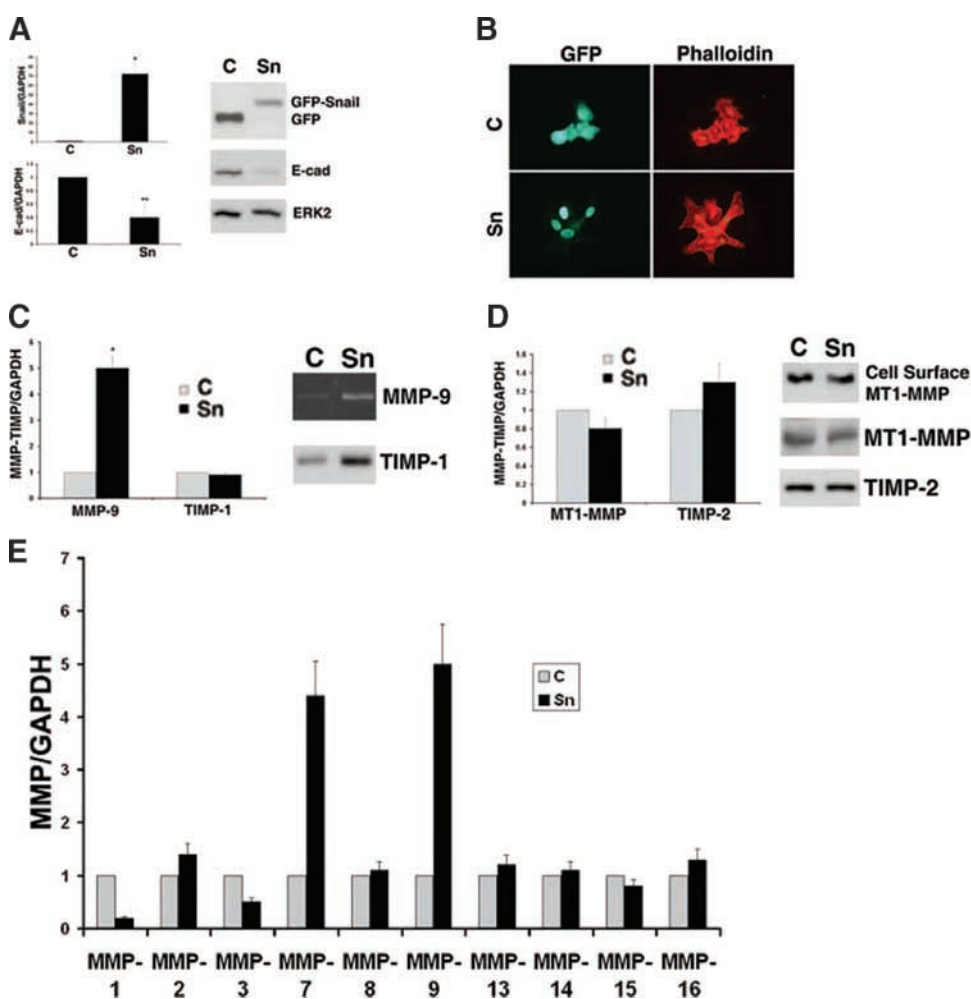
#### *Snail Promotes MMP-9-Dependent Matrigel Invasion*

To examine the functional consequence of increased MMP-9 levels with Snail expression, the ability of UMSSC1 cells to invade a protein rich matrix-like barrier was examined. UMSSC1-C and UMSSC1-Sn cells were plated onto transwell chambers coated with Matrigel and allowed to invade for 40 h. In addition, the protease-dependent component of invasion was examined in the presence of a broad-spectrum pharmacologic MMP inhibitor (GM6001), an endogenous inhibitor of MMP activity (TIMP-1), and a function-blocking anti-MMP-9 antibody. Although Snail increased both MMP-9 and TIMP-1 levels (Fig. 3C), a significant increase in invasive activity was

evident in Snail transfectants, indicating a net increase in pericellular proteolytic activity (Fig. 4A). The enhanced invasion was blocked by GM6001, showing a requirement for metalloproteinase activity (Fig. 4B). To examine the specific contribution of MMP-9 in cellular invasion, the ability of an endogenous inhibitor of MMP-9 activity (TIMP-1) and the function-blocking MMP-9 antibody to block invasion was examined. As shown in Fig. 4C, cellular invasion was blocked by TIMP-1 and by function-blocking anti-MMP-9 antibody, supporting a key role for MMP-9 in Snail-induced invasion. In contrast to the increased MMP-dependent invasion, there was no significant change in the motility of UMSCC1-Sn cells compared with the UMSCC1-C cells (data not shown).

*Snail-Specific siRNA Blocks TGF-β1-Mediated MMP-9 Expression and Invasion*

Our data indicate that TGF-β1 promotes expression of Snail, which can also function to regulate MMP-9 expression in UMSCC1 cells. To provide additional evidence of a key role for Snail in this pathway, Snail expression was blocked with siRNA (26) or control siRNA with no binding affinity for any known human sequence. The UMSCC1 cells were transfected with 100 nmol of control siRNA or Snail siRNA using Amaxa kit, allowed to recover overnight, serum starved for 24 h, and treated with TGF-β1 for 3 h to examine the effect on Snail expression or for 24 h to examine the effect on MMP-TIMP expression. As shown in Fig. 5A, transfection of



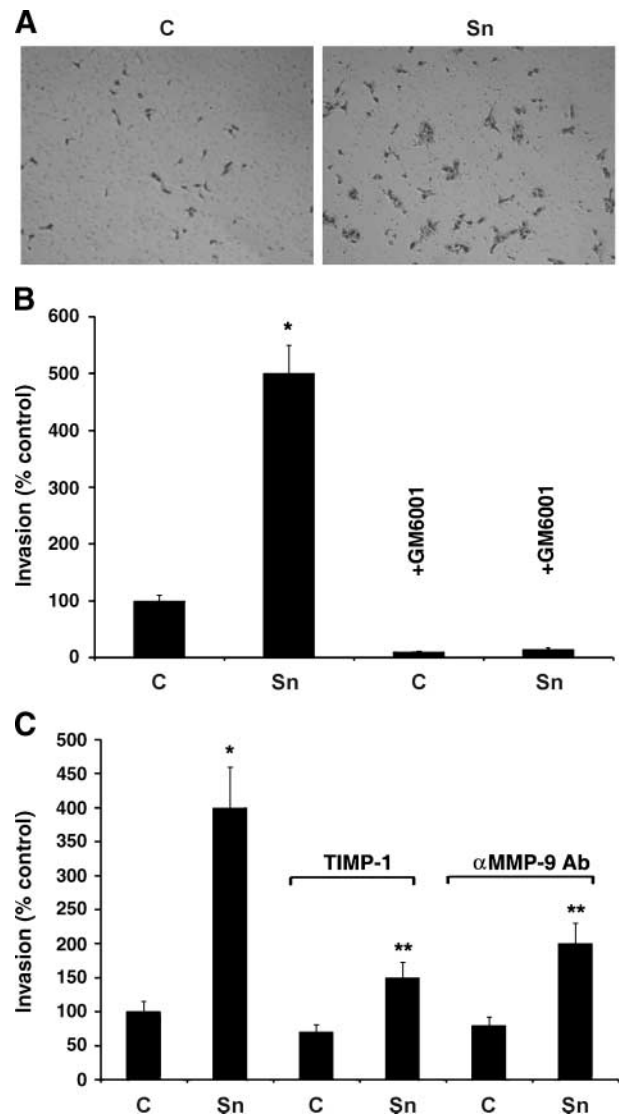
**FIGURE 3.** Snail increases MMP-9 and TIMP-1 without affecting MT1-MMP or TIMP-2. **A.** UMSCC1 cells overexpressing GFP (C) or GFP-tagged Snail (Sn) were generated as described in Materials and Methods. Relative expression of Snail, E-cadherin, and GAPDH mRNA was determined by real-time PCR, quantified, and normalized to UMSCC1-C cells, arbitrarily set at 1.0. Equal amounts of cell lysates from UMSCC1-C and UMSCC1-Sn cells were analyzed for GFP, E-cadherin, and total ERK2 (loading control) by Western blotting. \*,  $P < 0.005$ ; \*\*,  $P < 0.05$ , versus UMSCC1-C cells. **B.** UMSCC1-C and UMSCC1-Sn cells were plated on glass coverslip and stained with phalloidin to label actin. The cells were then examined for GFP expression and phalloidin staining using Zeiss fluorescence microscope. **C** and **D.** Equal numbers of UMSCC1-C and UMSCC1-Sn cells were plated in six-well tissue culture plates, serum starved overnight, and further allowed to condition the serum-free media for an additional 24 h. Relative expression of MMP-9, MT1-MMP, TIMP-1, TIMP-2, and GAPDH was determined by real-time PCR, quantified, and normalized to UMSCC1-C cells, arbitrarily set at 1.0. MMP-9 levels were analyzed by gelatin zymography, TIMP-1 and TIMP-2 levels in the conditioned media, and whole-cell and cell-surface MT1-MMP expression were analyzed by Western blotting as described in Materials and Methods. \*,  $P < 0.005$ , versus UMSCC1-C cells. Representative results of at least three independent experiments. **E.** Relative expression of the indicated MMPs and GAPDH was determined by real-time PCR, quantified, and normalized to UMSCC1-C cells, arbitrarily set at 1.0. Representative results of at least two independent experiments.

Snail siRNA significantly blocked TGF- $\beta$ 1-induced Snail expression without affecting the basal Snail mRNA levels, whereas transfection of control siRNA had no effect. To examine the effect of Snail down-regulation on TGF- $\beta$ 1-induced MMP-9 expression, the conditioned media and total RNA from samples treated with TGF- $\beta$ 1 for 24 h were analyzed for MMP-9 expression by gelatin zymography and real-time PCR, respectively. Gelatin zymography showed that TGF- $\beta$ 1-induced MMP-9 expression was blocked by Snail siRNA (Fig. 5B). This was confirmed by quantitative PCR analysis as TGF- $\beta$ 1 increased MMP-9 message by 60-fold in control samples, whereas down-regulation of Snail abrogated MMP-9 induction by TGF- $\beta$ 1 (Fig. 5B). In contrast to the effect of TGF- $\beta$ 1 on MMP-9 expression, TGF- $\beta$ 1 did not induce TIMP-1 message or affected the basal TIMP-1 message levels in the control siRNA- and Snail siRNA-transfected UMSSC1 cells (data not shown).

To determine whether Snail contributes to TGF- $\beta$ 1-stimulated invasion of UMSSC1 cells, we first examined the contribution of MMP-9 to TGF- $\beta$ 1-stimulated invasion. UMSSC1 cells were plated in invasion chambers coated with Matrigel and the effect on invasion was examined in the presence of function-blocking anti-MMP-9 antibody. As shown in Fig. 5C, TGF- $\beta$ 1 promoted Matrigel invasion, which was significantly reduced in the presence of function-blocking anti-MMP-9 antibody (Fig. 5C). Moreover, TGF- $\beta$ 1-mediated Matrigel invasion was completely blocked with the MMP inhibitor GM6001 (data not shown). To examine the contribution of Snail to this process, Snail expression was blocked with siRNA as detailed above, and then equal numbers of control siRNA- and Snail siRNA-transfected UMSSC1 cells were added to transwell inserts coated with Matrigel and allowed to invade in the presence or absence of TGF- $\beta$ 1. TGF- $\beta$ 1 promoted a 4-fold increase in Matrigel invasion of control UMSSC1 cells, and this effect was significantly reduced in UMSSC1 cells transfected with Snail siRNA (Fig. 5D). Similarly, blocking Snail expression in SCC68 cells with siRNA abrogated TGF- $\beta$ 1-induced MMP-9 expression and Matrigel invasion (Fig. 5E).

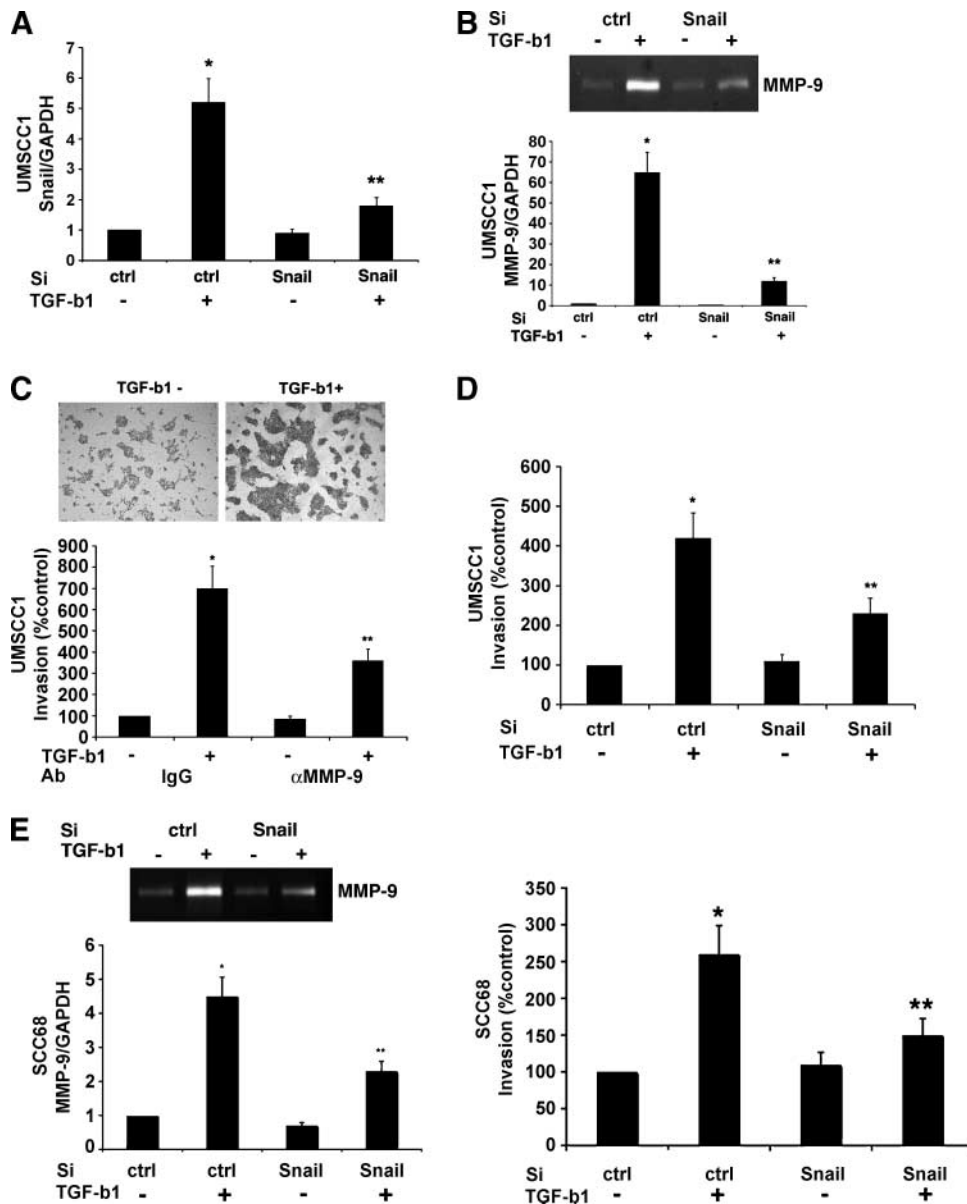
#### *Ets-1 Regulates Snail- and TGF- $\beta$ 1-Mediated MMP-9 Expression and Matrigel Invasion*

The MMP-9 promoter is regulated by a number of transcription factors including Ets-1 (27). To determine whether Snail alters MMP-9 levels via regulation of Ets-1, Ets-1 levels in wild-type and Snail-overexpressing UMSSC1 cells were compared. UMSSC1-Sn cells have increased levels of Ets-1 protein (Fig. 6A) and message levels as determined by real-time PCR (data not shown). To examine the potential role of Ets-1 in Snail-mediated MMP-9 expression, Ets-1 levels were down-regulated using Ets-1-specific siRNA (28), successfully blocking Ets-1 expression at the protein level and Ets-1 message by >90% (Fig. 6A). However, Ets-1 siRNA did not affect Snail message level (Fig. 6A), indicating that Ets-1 functions downstream of Snail. We then examined if Ets-1 was involved in Snail-mediated MMP-9 expression in UMSSC1 cells by transfecting Ets-1 or control siRNA followed by analysis of MMP-9 expression by gelatin zymography and real-time



**FIGURE 4.** Snail promotes MMP-9-dependent Matrigel invasion by UMSSC1 cells. **A.** Equal numbers of UMSSC1-C and UMSSC1-Sn cells were plated onto Matrigel-coated BD transwell chambers and allowed to invade for 40 h. Noninvading cells were removed from the upper chamber, filters were fixed and stained, and the invasive cells were photographed using Nikon camera. **B.** Matrigel invasion by UMSSC1-C and UMSSC1-Sn cells was examined in the absence or presence of a broad-spectrum MMP inhibitor GM6001 (12.5  $\mu$ mol/L), and the invading cells were enumerated using ocular micrometer. Columns, mean of four different experiments; bars, SE. \*,  $P < 0.005$ , versus UMSSC1-C cells. **C.** Matrigel invasion by UMSSC1-C and UMSSC1-Sn cells was examined in the absence or presence of TIMP-1 (10  $\mu$ mol/L) and in the presence or absence of function-blocking MMP-9 antibody (15  $\mu$ g/mL). Invading cells were then enumerated using ocular micrometer. Columns, mean of three different experiments; bars, SE. \*,  $P < 0.005$ , versus UMSSC1-C cells. \*\*,  $P < 0.05$ , versus control UMSSC1-Sn cells.

PCR. As shown in Fig. 6B (top), Ets-1 siRNA blocked Snail-induced MMP-9 expression as determined by zymography. Similarly, there was only a 3-fold increase in MMP-9 message in response to Snail in the UMSSC1-Sn cells transfected with Ets-1 compared with the 10-fold increase in MMP-9 message in UMSSC1-Sn cells transfected with control siRNA. Consistent with the decrease in



**FIGURE 5.** Snail siRNA blocks TGF- $\beta$ 1-mediated MMP-9 expression and Matrigel invasion. UMSSC1 cells were transfected with control siRNA or Snail-specific siRNA and treated with TGF- $\beta$ 1 for either 3 or 24 h. **A.** Relative expression of Snail and GAPDH was determined for the 3-h samples using real-time PCR, quantified, and normalized to untreated control siRNA transfected cells, set at 1.0. \*,  $P < 0.05$ , versus ctrlSi,TGF- $\beta$ 1(-) samples. \*\*,  $P < 0.05$ , versus ctrlSi,TGF- $\beta$ 1(+) samples. **B.** The 24-h samples were analyzed for MMP-9 expression by gelatin zymography and real-time PCR. \*,  $P < 0.005$ , versus ctrlSi,TGF- $\beta$ 1(-) samples, set at 1.0. \*\*,  $P < 0.05$ , versus ctrlSi,TGF- $\beta$ 1(+) samples. Representative results of at least three different experiments. **C.** UMSSC1 were plated onto Matrigel-coated BD transwell chambers, allowed to invade for 40 h in the presence or absence of TGF- $\beta$ 1, and invasive cells photographed using Nikon camera. TGF- $\beta$ 1-mediated Matrigel invasion by UMSSC1 cells was examined in the presence or absence of function-blocking MMP-9 antibody (15  $\mu$ g/mL). Columns, mean of three different experiments; bars, SE. \*,  $P < 0.05$ , versus TGF- $\beta$ 1(-) samples. \*\*,  $P < 0.05$ , versus IgG, TGF- $\beta$ 1(+) samples. **D.** UMSSC1 cells transfected with control siRNA or Snail-specific siRNA were allowed to invade Matrigel-coated BD transwell chambers for 40 h in the presence or absence of TGF- $\beta$ 1. Columns, mean of three different experiments; bars, SE. \*,  $P < 0.05$ , versus ctrlSi,TGF- $\beta$ 1(-) samples. \*\*,  $P < 0.05$ , versus ctrlSi,TGF- $\beta$ 1(+) samples. **E.** SCC68 cells were transfected with control siRNA or Snail-specific siRNA and treated with TGF- $\beta$ 1 for 24 h, and then analyzed for MMP-9 expression by gelatin zymography and real-time PCR. SCC68 cells transfected with control siRNA or Snail-specific siRNA were allowed to invade Matrigel-coated BD transwell chambers for 40 h in the presence or absence of TGF- $\beta$ 1. \*,  $P < 0.005$ , versus ctrlSi,TGF- $\beta$ 1(-) samples, set at 1.0. \*\*,  $P < 0.05$ , versus ctrlSi,TGF- $\beta$ 1(+) samples. Representative results of three different experiments.

MMP-9 expression, Ets-1 siRNA also significantly blocked Snail-induced invasion by 60% (data not shown), showing that Snail promotes MMP-9 expression in UMSSC1 cells through Ets-1.

Because our data show that TGF- $\beta$ 1-mediated MMP-9 expression involves Snail (Fig. 5) and that Snail increases

MMP-9 via Ets-1, we also examined if Ets-1 is involved in TGF- $\beta$ 1-mediated MMP-9 expression. UMSSC1 cells were transfected with 100 nmol of control siRNA or Ets-1 siRNA, serum starved, and treated with TGF- $\beta$ 1 for 24 h. The conditioned media and cell lysates were collected and examined for MMP-9 expression. As shown in Fig. 6C, TGF- $\beta$ 1

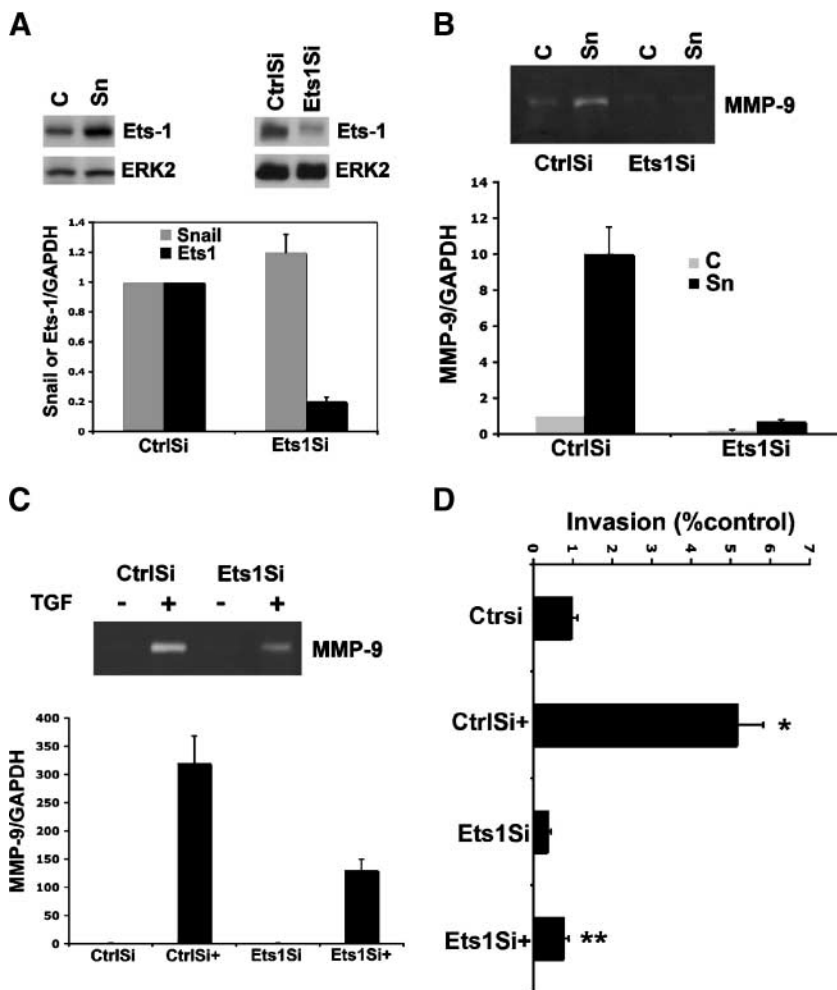
increased MMP-9 expression by 320-fold in the presence of control siRNA, whereas the increase was more modest (130-fold) in the presence of Ets-1 siRNA, supporting a role for Ets-1 in TGF- $\beta$ 1-mediated MMP-9 expression. In addition, TGF- $\beta$ 1 promoted a 5-fold increase in Matrigel invasion of control UMSSC1 and this effect was significantly reduced in UMSSC1 cells transfected with Ets-1 siRNA (Fig. 6D), suggesting a regulatory contribution of Ets-1 to TGF- $\beta$ 1-stimulated invasion.

## Discussion

The Snail family of transcription factors is one of the key regulators of epithelial to mesenchymal transition in normal development and during tumor progression (29-31). Correlative studies have shown a direct relationship between Snail expression and an invasive phenotype in a variety of cancers (32-34); consequently, Snail expression is under stringent regulation. One of the factors that have been shown to regulate Snail expression is TGF- $\beta$ 1. TGF- $\beta$ 1 treatment of Madin-Darby canine kidney cells was previously shown to trigger epithelial to mesenchymal transition by enhancing Snail expression (18). Data in this report show that TGF- $\beta$ 1 can also induce Snail expression in OSCC cells and thereby

regulate OSCC cellular behavior. In contrast to the effect of Snail in Madin-Darby canine kidney cells, Snail did not regulate vimentin or fibronectin in UMSSC1 cells (data not shown); however, decreased E-cadherin levels and cytoskeletal rearrangement indicate that Snail induced partial epithelial to mesenchymal transition. Furthermore, basal motility of UMSSC1 cells was unaffected by Snail, whereas MMP-9-dependent cellular invasion was significantly enhanced. Snail functions as a molecular mediator of TGF- $\beta$ 1 to promote invasion via enhanced MMP expression, as supported by loss of MMP-9 expression and invasive capacity using Snail siRNA.

Snail expression in UMSSC1 cells is sufficient to promote MMP-9 expression, in agreement with a recently published report showing that Snail expression in Madin-Darby canine kidney cells increased MMP-9 levels (35). Increased extracellular signal-regulated kinase (ERK)-1/2 and phosphatidylinositol 3-kinase signaling were implicated in pathways leading to Snail-enhanced MMP-9 promoter activity in Madin-Darby canine kidney cells, which was blocked with the mitogen-activated protein kinase/ERK kinase inhibitor U0126 and the phosphatidylinositol 3-kinase inhibitor wortmanin (35). In the current study, although treatment of control and Snail-expressing UMSSC1 cells with these inhibitors decreased basal MMP-9 levels, the Snail-induced increase in MMP-9 expression was



**FIGURE 6.** Ets-1 regulates Snail- and TGF- $\beta$ 1-mediated MMP-9 expression and Matrigel invasion. **A.** Cell lysates from equal numbers of UMSSC1-C and UMSSC1-Sn cells were analyzed for Ets-1 and ERK2 by Western blotting (top left). UMSSC1 cells were transfected with control siRNA (*ctrlSi*) or Ets-1-specific siRNA (*Ets1Si*), and cell lysates analyzed for Ets-1 and ERK2 by Western blotting (top right) and for Ets-1 and Snail expression by real-time PCR (bottom). **B.** Equal numbers of UMSSC1-C and UMSSC1-Sn cells were transfected with control siRNA or Ets-1-specific siRNA, and MMP-9 expression was determined by gelatin zymography and real-time PCR. **C.** UMSSC1 cells were transfected with control siRNA or Ets-1-specific siRNA and treated with TGF- $\beta$ 1 for 24 h, and MMP-9 expression was determined by gelatin zymography and real-time PCR. **D.** UMSSC1 cells were transfected with control siRNA or Ets-1-specific siRNA and then added to Matrigel-coated BD transwell chambers and allowed to invade for 40 h in the presence or absence of TGF- $\beta$ 1. Invading cells were then enumerated using ocular micrometer. Columns, mean of three different experiments; bars, SE. \*,  $P < 0.05$ , versus *ctrlSi*, TGF- $\beta$ 1(-) samples. \*\*,  $P < 0.05$ , versus *ctrlSi*, TGF- $\beta$ 1(+) samples.

unaffected (data not shown). Moreover, careful examination of the data by Jorda et al. (35) showed that these inhibitors also blocked basal MMP-9 levels in Madin-Darby canine kidney cells whereas Snail-mediated induction of MMP-9 was not significantly affected, suggesting that additional regulatory pathways participate in Snail-mediated MMP-9 expression.

Snail-induced MMP-9 expression in UMSCC1 cells is mediated by increases in Ets-1 levels, consistent with a recent report showing that Snail expression in A431 epithelial cells can enhance Ets-1 (36) and that Ets-1 siRNA blocked MMP-9 expression. Moreover, our data show that Snail-mediated Matrigel invasion was also blocked with Ets-1-specific siRNA. Furthermore, TGF- $\beta$ 1-induced MMP-9 expression and Matrigel invasion were both blocked with Ets-1 siRNA. Together with our results showing that TGF- $\beta$ 1-induced MMP-9 expression and invasion can be blocked with Snail siRNA, these data strongly suggest that Ets-1 functions downstream of Snail to regulate TGF- $\beta$ 1-mediated cellular behavior in OSCC cells. Importantly, recent reports have shown that Ets-1 is involved in tumor development and progression. Overexpression of Ets-1 in HeLa cells resulted in anchorage-independent growth and Matrigel invasion (37). Conversely, expression of Ets-1 antisense in an invasive melanoma Mel Im cell line blocked Matrigel invasion (38). Moreover, Ets-1 expression has been shown to correlate with a higher tumor stage and increased lymph node metastasis in human OSCC specimens (39).

Although stable transfection of liver cancer cell lines with Snail promoted expression of MT1-MMP (21, 22), Snail did not regulate MT1-MMP expression in UMSCC1 cells as shown herein and in epidermoid A431 cells (20). Moreover, Snail did not regulate expression of MT2-MMP (MMP-15) or MT3-MMP (MMP-16) in UMSCC1 cells. Similarly, although Snail up-regulated MMP-2 levels in liver cancer cell lines and in epidermoid A431 cells (21, 36), Snail expression did not affect MMP-2 mRNA expression in UMSCC1 cells. UMSCC1 cells do not express MMP-2 protein as determined by gelatin zymography (40, 41); in agreement with our real-time RT-PCR data, Snail did not increase MMP-2 protein levels as assessed by gelatin zymography (data not shown). Consistent with our findings in UMSCC1 cells, Snail did not affect expression of MMP-2 in the mouse squamous cell carcinoma HaCa4 cell line (42). Interestingly, although Snail did not regulate TIMP-1 at the message level in UMSCC1 cells, there was increased TIMP-1 protein, suggesting that Snail can also regulate gene expression at the posttranscriptional level. Despite the increase in TIMP-1 protein level, Snail expression enhanced Matrigel invasion, which was prevented using MMP-9 function-blocking antibody, showing that the invasion was indeed due to a net increase in MMP-9 proteolytic activity.

Interestingly, a recent report showed that MMPs also promote epithelial to mesenchymal transition by inducing Snail expression. Treatment of mammary epithelial cells with MMP-9 was reported to induce Snail expression with consequent down-regulation of E-cadherin (43). Conversely, our data show that Snail can in turn regulate MMP-9 expression in OSCC cells, providing evidence of reciprocal interactions between Snail and MMP-9 regulation. Moreover, we also show a novel mechanism by which TGF- $\beta$ 1 regulates MMP-9 expression in OSCC cells via enhanced Snail expression. As MMP-9 has

been shown to be important for OSCC invasion, these data identify Snail as a key factor that promotes OSCC progression, not only by regulating EMT but also by enhancing MMP expression downstream of TGF- $\beta$ 1 signaling.

## Materials and Methods

### Materials

TGF- $\beta$ 1, peroxidase-conjugated secondary antibodies, and the MT1-MMP antibody directed against the hinge region were purchased from Sigma. DMEM, Ham's F-12, and keratinocyte-SFM were purchased from Life Technologies. Purified human TIMP-1 protein, rabbit polyclonal TIMP-1 and TIMP-2 antibodies, and the broad spectrum MMP inhibitor GM6001 were purchased from Chemicon. Function-blocking anti-MMP-9 monoclonal antibody (Ab-1) was from Calbiochem. Anti-ERK2 and anti-Ets-1 antibodies were purchased from Santa Cruz Biotechnology. SuperSignal enhanced chemiluminescence reagent, EZ-Link Sulfo-NHS-LC-Biotin, and UltraLink immobilized streptavidin gel were obtained from Pierce. Microcon-10 microconcentrators and polyvinylidene difluoride membranes were purchased from Millipore. A nucleofector electroporation kit specifically designed for keratinocytes was obtained from Amaxa. Protease inhibitor mixture was purchased from Roche Diagnostics. BD Biocoat Matrigel Invasion Chambers were from BD Biosciences.

### Immunohistochemistry

Oral cancer microarrays were obtained from US Biomax and consisted of 54 tissue cores of normal oral tissue or previously untreated squamous cell carcinomas measuring 1.5 mm in diameter and 5  $\mu$ m in thickness. The slides were H&E stained or stained for MMP-9 and pSmad2 by the Pathology Core Facility of the Robert H. Lurie Comprehensive Cancer Center at Northwestern University. The microarray tissue specimen grades were verified by a pathologist (V.A.) and included 10 normal oral tissues and 10 well-differentiated, 28 moderately differentiated, and 6 poorly differentiated squamous cell carcinomas. Immunohistochemical staining with antibodies for MMP-9 (NeoMarkers; 1:200 dilution) and pSmad2 (Cell Signaling; 1:200 dilution) was done according to standard procedures. Analysis of tissue sections was done by light microscopy by a pathologist (V.A.) and a total of 100 tumor cells in each core (six fields) were examined at  $\times$ 40 magnification. Because many of the tumors showed nonhomogeneous staining, the percentages of tumor cells (excluding stromal cells) that were positive at each of the intensity scores [0 (absent staining), 1+ (weak staining), and 2+ (strong staining)] were recorded and the sum of these scores gave a final score for each of the cores: 0, 0-0.99, or 1-1.99. The *P* values were calculated using unpaired *t* test comparing the scores of each of the tumor grades with the normal oral tissue samples. The association between MMP-9 and pSmad2 expression in the tumor samples was assessed using Pearson correlation coefficient test. Statistical analyses were done using GraphPad Instat 3.

### Cell Cultures

SCC9, SCC25, SCC68, and UMSCC1 cells were derived from squamous cell carcinoma of the oral cavity. SCC9 and SCC25 cells were obtained from American Type Culture

Collection (44), whereas UMSSC1 cells were generously provided by Dr. E. Lengyel (University of Chicago, Chicago, IL; refs. 40, 41). SCC68 cells and tert-immortalized normal oral keratinocytes (OKF4 and OKF6 cells) were kindly provided by Dr. J. Rheinwald (Brigham and Women's Hospital, Harvard Institutes of Medicine, Boston, MA; ref. 45). SCC9, SCC25, and UMSSC1 cells were routinely maintained in DMEM and Ham's F-12 medium (1:1) containing 10% FCS and supplemented with 100 units/mL penicillin and 100 µg/mL streptomycin. OKF4, OKF6, and SCC68 cells were maintained in keratinocyte-SFM supplemented with 100 units/mL penicillin, 100 µg/mL streptomycin, 25 µg/mL bovine pituitary extract (supplied with the medium), 0.2 ng/mL epidermal growth factor, and 0.31 mmol/L CaCl<sub>2</sub>.

#### Cloning of GFP-Tagged Snail

The complete coding sequence of Snail was cloned out of human fetal kidney Marathon ready cDNA library (Clontech) according to the manufacturer's instructions using the following set of primers: forward, 5'-ATGCCGCGCTCTTTCCTCGT-CAGGAAGCTC-3'; reverse, 5'-TCAGCGAGGGCCCCCT-GAGCAGCCCCGACTC-3'. The *Snail* gene was then subcloned into eukaryotic expression vector pEGFP-C1 to obtain GFP-tagged Snail, with the GFP tag at the NH<sub>2</sub> terminus of Snail. All plasmids were verified by DNA sequencing.

#### Generation of UMSSC1-C and UMSSC1-Sn Cells

UMSSC1 cells were stably transfected with pEGFP-C1 empty vector or pEGFP-C1 vector containing full-length Snail using nucleofector Amaxa kit according to the manufacturer's instructions. Transfected cells resistant to 0.85 mg/mL G418 were then sorted for GFP expression by fluorescence-activated cell sorting analysis to obtain GFP-expressing control UMSSC1-C cells and GFP-tagged Snail-expressing UMSSC1-Sn cells. The stable cell lines were then routinely maintained in DMEM and Ham's F-12 medium (1:1) containing 10% FCS and supplemented with 100 units/mL penicillin, 100 µg/mL streptomycin, and 0.65 mg/mL G418.

UMSSC1-C and UMSSC1-Sn cells were plated overnight on glass coverslips, washed with PBS, fixed with 3.7% formaldehyde solution for 10 min, blocked with 1% bovine serum albumin for 20 min, permeabilized with 0.1% Triton X-100 for 3 min, and stained with Alexa Fluor 594 phalloidin; after which the cells were washed, mounted, and observed using a Zeiss Axiovert 200 microscope.

#### Down-Regulation of Snail and Ets-1 Expression

Snail expression was transiently down-regulated using the following duplex siRNA directed against Snail: forward primer, 5'-AGGCCUUAACUGCAAUAAdTdT-3'; reverse primer, 5'-UAUUUGCAGUUGAAGGCCUdTdT-3' (26). UMSSC1 or SCC68 cells were transiently transfected with 100 nmol of Snail-specific siRNA or control siRNA using Amaxa nucleofector kit, allowed to recover overnight, serum starved for 24 h, and then treated with TGF-β1 to examine the effect on Snail expression or MMP-TIMP expression. Similarly, Ets-1 expression was transiently down-regulated using the

following duplex siRNA directed against Ets-1: forward primer, 5'-GGACAAGCCUGUCAUCCUdTdT-3'; reverse primer, 5'-AGGAAUGACAGGCUUGUCCdTdT-3'(28).

#### Analysis of MMP-9 and TIMP Expression

Gelatinase activity in 24-h serum-free conditioned medium was determined using SDS-PAGE gelatin zymography as previously described (46, 47). The conditioned media were concentrated 15- to 20-fold using Microcon-10 microconcentrators, boiled in Laemmli sample dilution buffer, analyzed for TIMP-1 and TIMP-2 by SDS-PAGE (15% gels), and immunoblotted with rabbit polyclonal antibodies (Chemicon; ref. 48).

#### Analysis of MT1-MMP Expression

Cell-surface MT1-MMP expression was analyzed by labeling cell-surface proteins with biotin and isolating the biotin-labeled proteins with streptavidin beads as previously described (48, 49). Aliquots of the whole-cell lysate from UMSSC1-C and UMSSC1-Sn cells were analyzed by SDS-PAGE (9% gels) and immunoblotted for MT1-MMP.

#### Analysis of Ets-1 Expression

Whole-cell lysates from UMSSC1-C and UMSSC1-Sn cells were analyzed by SDS-PAGE (9% gels) and immunoblotted for Ets-1.

#### RT-PCR

Total RNA was isolated using RNeasy Mini kit (Qiagen) according to the manufacturer's specifications and quantified by spectrophotometric measurement. Primer pairs for human Snail and human GAPDH were as follows: Snail, 5'-AATCGGAAGCCTAACTACAAG-3' (forward) and 5'-AGGAAGAGACTGAAGTAGAG-3' (reverse); GAPDH, 5'-CGGAGTCAACGGATTGGTCTGAT-3' (forward) and 5'-AGCCTTCTCCATGGTGGTGAAGAC-3' (reverse; refs. 48, 50). The RT-PCR reactions were done using the One-step RT-PCR kit (Invitrogen) and the PCR products were visualized by UV transillumination of 1.5% agarose gels stained with ethidium bromide.

#### Real-time PCR

Reverse transcription of RNA to cDNA was done using GeneAmp RNA PCR kit (Applied Biosystems). Quantitative gene expression was done for Snail, Ets-1, E-cadherin, MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-13, MMP-14 (MT1-MMP), MMP-15 (MT2-MMP), MMP-16 (MT3-MMP), TIMP-1, TIMP-2, and GAPDH with gene-specific probes (Applied Biosystems) using TaqMan Universal PCR Master Mix and the 7500 Fast Real-time PCR System (Applied Biosystems). The data were then quantified with the comparative C<sub>t</sub> method for relative gene expression (51).

#### Analysis of Matrigel Invasion

Invasive activity was quantified using BD Biocoat Matrigel invasion chambers (8-µm pore size; refs. 46, 47). UMSSC1-C and UMSSC1-Sn cells (2 × 10<sup>5</sup>) were counted using Z1 Coulter Counter and added to the upper chamber in 500 µL of

serum-free medium, and 500  $\mu$ L of serum-containing medium were added to the lower well to promote invasion. In selected experiments, the proteinase dependence of invasion was determined by quantifying invasion in the presence of the broad-spectrum MMP inhibitor GM6001, TIMP-1, or function-blocking anti-MMP-9 antibody. In additional experiments, the effect of TGF- $\beta$ 1 on invasion was determined by adding TGF- $\beta$ 1 in the lower chambers. Nonmigrating cells after 40 h were removed from the upper chamber with a cotton swab, filters were fixed and stained with Diff-Quik stain, and migrating cells adherent to the underside of the filter were enumerated using an ocular micrometer and by counting a minimum of five high-power fields. Data are expressed as relative migration (number of cells per field).

The role of Snail in TGF- $\beta$ 1-mediated Matrigel invasion was examined by transiently transfecting UMSSC1 or SCC68 cells with Snail-specific siRNA or control siRNA using Amaxa nucleofector kit, allowing the cells to recover overnight, and then serum starving for additional 24 h. The cells were then trypsinized, counted using a Z1 Coulter Counter, and then equal numbers of Snail-specific siRNA- and control siRNA-transfected cells were plated in the upper chamber and allowed to invade for 40 h in the presence or absence of TGF- $\beta$ 1. The noninvasive cells were removed from the upper chamber using cotton swabs, fixed, stained, and quantified as described above. Similarly, the role of Ets-1 in TGF- $\beta$ 1-mediated Matrigel invasion was examined by transiently transfecting UMSSC1 cells with Ets-1-specific siRNA or control siRNA.

## Acknowledgments

We thank Dr. M. Sharon Stack for her support and encouragement throughout this project.

## References

- Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ. Cancer statistics, 2007. *CA Cancer J Clin* 2007;57:43–66.
- Forastiere A, Koch W, Trotti A, Sidransky D. Head and neck cancer. *N Engl J Med* 2001;345:1890–900.
- Neville BW, Day TA. Oral cancer and precancerous lesions. *CA Cancer J Clin* 2002;52:195–215.
- Kurahara S, Shinohara M, Ikebe T, et al. Expression of MMPS, MT-MMP, and TIMPs in squamous cell carcinoma of the oral cavity: correlations with tumor invasion and metastasis. *Head Neck* 1999;21:627–38.
- Hong SD, Hong SP, Lee JI, Lim CY. Expression of matrix metalloproteinase-2 and -9 in oral squamous cell carcinomas with regard to the metastatic potential. *Oral Oncol* 2000;36:207–13.
- Ikebe T, Shinohara M, Takeuchi H, et al. Gelatinolytic activity of matrix metalloproteinase in tumor tissues correlates with the invasiveness of oral cancer. *Clin Exp Metastasis* 1999;17:315–23.
- Sternlicht MD, Werb Z. How matrix metalloproteinases regulate cell behavior. *Annu Rev Cell Dev Biol* 2001;17:463–516.
- Overall CM, Kleinfeld O. Tumour microenvironment—opinion: validating matrix metalloproteinases as drug targets and anti-targets for cancer therapy. *Nat Rev Cancer* 2006;6:227–39.
- Munshi HG, Stack MS. Reciprocal interactions between adhesion receptor signaling and MMP regulation. *Cancer Metastasis Rev* 2006;25:45–56.
- Simon C, Nemecek AJ, Boyd D, et al. An orthotopic floor-of-mouth cancer model allows quantification of tumor invasion. *Laryngoscope* 1998;108:1686–91.
- Van den Steen PE, Dubois B, Nelissen I, Rudd PM, Dwek RA, Opdenakker G. Biochemistry and molecular biology of gelatinase B or matrix metalloproteinase-9 (MMP-9). *Crit Rev Biochem Mol Biol* 2002;37:375–536.
- Nair RR, Boyd DD. Expression cloning of novel regulators of 92 kDa type IV collagenase expression. *Biochem Soc Trans* 2005;33:1135–6.
- Kruger A, Arlt MJ, Gerg M, et al. Antimetastatic activity of a novel mechanism-based gelatinase inhibitor. *Cancer Res* 2005;65:3523–6.
- Lu SL, Reh D, Li AG, et al. Overexpression of transforming growth factor  $\beta$ 1 in head and neck epithelia results in inflammation, angiogenesis, and epithelial hyperproliferation. *Cancer Res* 2004;64:4405–10.
- Akhurst RJ, Derynck R. TGF- $\beta$  signaling in cancer—a double-edged sword. *Trends Cell Biol* 2001;11:S44–51.
- Shi Y, Massague J. Mechanisms of TGF- $\beta$  signaling from cell membrane to the nucleus. *Cell* 2003;113:685–700.
- Dasgupta S, Bhattacharya-Chatterjee M, O'Malley B W, Jr., Chatterjee SK. Tumor metastasis in an orthotopic murine model of head and neck cancer: possible role of TGF- $\beta$  1 secreted by the tumor cells. *J Cell Biochem* 2006;97:1036–51.
- Peinado H, Quintanilla M, Cano A. Transforming growth factor  $\beta$ -1 induces snail transcription factor in epithelial cell lines: mechanisms for epithelial mesenchymal transitions. *J Biol Chem* 2003;278:21113–23.
- Zavadil J, Bottinger EP. TGF- $\beta$  and epithelial-to-mesenchymal transitions. *Oncogene* 2005;24:5764–74.
- Yokoyama K, Kamata N, Fujimoto R, et al. Increased invasion and matrix metalloproteinase-2 expression by Snail-induced mesenchymal transition in squamous cell carcinomas. *Int J Oncol* 2003;22:891–8.
- Miyoshi A, Kitajima Y, Sumi K, et al. Snail and SIP1 increase cancer invasion by up-regulating MMP family in hepatocellular carcinoma cells. *Br J Cancer* 2004;90:1265–73.
- Miyoshi A, Kitajima Y, Kido S, et al. Snail accelerates cancer invasion by up-regulating MMP expression and is associated with poor prognosis of hepatocellular carcinoma. *Br J Cancer* 2005;92:252–8.
- Battle E, Sancho E, Franci C, et al. The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. *Nat Cell Biol* 2000;2:84–9.
- Cano A, Perez-Moreno MA, Rodrigo I, et al. The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nat Cell Biol* 2000;2:76–83.
- Zhou BP, Deng J, Xia W, et al. Dual regulation of Snail by GSK-3 $\beta$ -mediated phosphorylation in control of epithelial-mesenchymal transition. *Nat Cell Biol* 2004;6:931–40.
- Yook JI, Li XY, Ota I, Fearon ER, Weiss SJ. Wnt-dependent regulation of the E-cadherin repressor snail. *J Biol Chem* 2005;280:11740–8.
- Nakamura Y, Esnault S, Maeda T, Kelly EA, Malter JS, Jarjour NN. Ets-1 regulates TNF- $\alpha$ -induced matrix metalloproteinase-9 and tenascin expression in primary bronchial fibroblasts. *J Immunol* 2004;172:1945–52.
- Santiago FS, Khachigian LM. Ets-1 stimulates platelet-derived growth factor A-chain gene transcription and vascular smooth muscle cell growth via cooperative interactions with Sp1. *Circ Res* 2004;95:479–87.
- Peinado H, Marin F, Cubillo E, et al. Snail and E47 repressors of E-cadherin induce distinct invasive and angiogenic properties *in vivo*. *J Cell Sci* 2004;117:2827–39.
- Huber MA, Kraut N, Beug H. Molecular requirements for epithelial-mesenchymal transition during tumor progression. *Curr Opin Cell Biol* 2005;17:548–58.
- De Craene B, Gilbert B, Stove C, Bruyneel E, van Roy F, Bex G. The transcription factor snail induces tumor cell invasion through modulation of the epithelial cell differentiation program. *Cancer Res* 2005;65:6237–44.
- Sugimachi K, Tanaka S, Kameyama T, et al. Transcriptional repressor snail and progression of human hepatocellular carcinoma. *Clin Cancer Res* 2003;9:2657–64.
- Rosivatz E, Becker I, Specht K, et al. Differential expression of the epithelial-mesenchymal transition regulators snail, SIP1, and twist in gastric cancer. *Am J Pathol* 2002;161:1881–91.
- Moody SE, Perez D, Pan TC, et al. The transcriptional repressor Snail promotes mammary tumor recurrence. *Cancer Cell* 2005;8:197–209.
- Jorda M, Olmeda D, Vinyals A, et al. Up-regulation of MMP-9 in MDCK epithelial cell line in response to expression of the Snail transcription factor. *J Cell Sci* 2005;118:3371–85.
- Taki M, Verschuere K, Yokoyama K, Nagayama M, Kamata N. Involvement of Ets-1 transcription factor in inducing matrix metalloproteinase-2 expression by epithelial-mesenchymal transition in human squamous carcinoma cells. *Int J Oncol* 2006;28:487–96.
- Hahne JC, Okuducu AF, Kaminski A, Florin A, Soncin F, Wernert N. Ets-1 expression promotes epithelial cell transformation by inducing migration, invasion and anchorage-independent growth. *Oncogene* 2005;24:5384–8.

38. Rothhammer T, Hahne JC, Florin A, et al. The Ets-1 transcription factor is involved in the development and invasion of malignant melanoma. *Cell Mol Life Sci* 2004;61:118–28.
39. Pande P, Mathur M, Shukla NK, Ralhan R. Ets-1: a plausible marker of invasive potential and lymph node metastasis in human oral squamous cell carcinomas. *J Pathol* 1999;189:40–5.
40. Juarez J, Clayman G, Nakajima M, et al. Role and regulation of expression of 92-kDa type-IV collagenase (MMP-9) in 2 invasive squamous-cell-carcinoma cell lines of the oral cavity. *Int J Cancer* 1993;55:10–8.
41. Lengyel E, Gum R, Juarez J, et al. Induction of M(r) 92,000 type IV collagenase expression in a squamous cell carcinoma cell line by fibroblasts. *Cancer Res* 1995;55:963–7.
42. Olmeda D, Jorda M, Peinado H, Fabra A, Cano A. Snail silencing effectively suppresses tumour growth and invasiveness. *Oncogene* 2007;26:1862–74.
43. Radisky DC, Levy DD, Littlepage LE, et al. Rac1b and reactive oxygen species mediate MMP-3-induced EMT and genomic instability. *Nature* 2005;436:123–7.
44. Rheinwald JG, Beckett MA. Tumorigenic keratinocyte lines requiring anchorage and fibroblast support cultures from human squamous cell carcinomas. *Cancer Res* 1981;41:1657–63.
45. Dickson MA, Hahn WC, Ino Y, et al. Human keratinocytes that express hTERT and also bypass a p16(INK4a)-enforced mechanism that limits life span become immortal yet retain normal growth and differentiation characteristics. *Mol Cell Biol* 2000;20:1436–47.
46. Munshi HG, Ghosh S, Mukhopadhyay S, et al. Proteinase suppression by E-cadherin-mediated cell-cell attachment in premalignant oral keratinocytes. *J Biol Chem* 2002;277:38159–67.
47. Munshi HG, Stack MS. Analysis of matrix degradation. *Methods Cell Biol* 2002;69:195–205.
48. Munshi HG, Wu YI, Ariztia EV, Stack MS. Calcium regulation of matrix metalloproteinase-mediated migration in oral squamous cell carcinoma cells. *J Biol Chem* 2002;277:41480–8.
49. Munshi HG, Wu YI, Mukhopadhyay S, et al. Differential regulation of membrane type 1-matrix metalloproteinase activity by ERK 1/2- and p38 MAPK-modulated tissue inhibitor of metalloproteinases 2 expression controls transforming growth factor- $\beta$ 1-induced pericellular collagenolysis. *J Biol Chem* 2004;279:39042–50.
50. Tsutsumi S, Yanagawa T, Shimura T, Kuwano H, Raz A. Autocrine motility factor signaling enhances pancreatic cancer metastasis. *Clin Cancer Res* 2004;10:7775–84.
51. Ottaviano AJ, Sun L, Ananthanarayanan V, Munshi HG. Extracellular matrix-mediated membrane-type 1 matrix metalloproteinase expression in pancreatic ductal cells is regulated by transforming growth factor- $\beta$ 1. *Cancer Res* 2006;66:7032–40.