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Slug Is a Downstream Mediator of Transforming Growth Factor-β1-Induced Matrix Metalloproteinase-9 Expression and Invasion of Oral Cancer Cells

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ABSTRACT

Members of Snail family of transcription factors play an important role in oral cancer progression by inducing epithelial–mesenchymal transition, by promoting invasion and by increasing matrix metalloproteinase (MMP) expression. Although Snail (Snai1) is the best characterized and the most extensively studied member of this family, the role and regulation of Slug (Snai2) in oral cancer progression is less well understood. In this report, we show that transforming growth factor-β1 (TGF-β1) increases Slug levels in tert-immortalized oral keratinocytes and in malignant oral squamous cell carcinoma (OSCC) cells. Inhibiting ERK1/2 signaling, but not PI3-kinase signaling, blocked TGF-β1-induced Slug expression in the malignant UMSCC1 cells. To further examine the role of Slug in OSCC progression, we generated UMSCC1 cells with inducible expression of Slug protein. Induction of Slug in UMSCC1 cells did not repress E-cadherin levels or regulate individual movement of UMSCC1 cells. Instead, Slug enhanced cohort migration and Matrigel invasion by UMSCC1 cells. Slug increased MMP-9 levels and MMP-9-specific siRNA blocked Slug-induced Matrigel invasion. Interestingly, Slug-specific siRNA attenuated TGF-β1-induced MMP-9 expression and Matrigel invasion. These data demonstrate that TGF-β1 increases Slug via ERK1/2 signaling, and thereby contributes to OSCC progression. J. Cell. Biochem. 108: 726–736, 2009.

KEY WORDS: SLUG; MMP-9; ERK1/2; TGF-β1; MATRIGEL INVASION; COHORT MIGRATION

The American Cancer Society estimates that there will be greater than 20,000 new cases of cancer this year involving the oral cavity in the U.S. [Jemal et al., 2009]. Advanced oral squamous cell carcinoma (OSCC) is associated with high morbidity and diminished quality of life, with surgical resection frequently leading to disruption of speech and swallowing [Forastiere et al., 2001; Neville and Day, 2002]. The dismal outcome is attributed to the fact that OSCC is an aggressive and a highly invasive cancer. We had previously published that matrix metalloproteinases (MMPs), which are a large family of highly conserved metalloendopeptidases with activity directed against a variety of ECM substrates [Sternlicht and Werb, 2001; Munshi and Stack, 2006], contribute to OSCC invasion [Munshi et al., 2002a,b, 2004]. We also showed that the human OSCC tumors with increased MMP levels demonstrate increased transforming growth factor-β1 (TGF-β1) signaling [Sun et al., 2008].

TGF-β1, one of the key regulators of epithelial–mesenchymal transition (EMT) [Thiery, 2002, 2003], signals by binding to its receptor to promote phosphorylation of receptor-associated Smads (R-Smads) [Derynck and Zhang, 2003; Shi and Massague, 2003]. The R-Smads (Smad2 and Smad3) then bind to Smad4 and translocate to the nucleus to activate cellular responses, such as inducing MMP expression [Leivonen et al., 2002; Selvamurugan et al., 2004]. TGF-β1 also signals through non-Smad pathways to regulate gene

Abbreviations used: TGF-β1, transforming growth factor-β1; SCC, squamous cell cancer; OSCC, oral SCC; MMP, matrix metalloproteinase; siRNA, small interfering RNA; GFP, green fluorescent protein.

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expression. TGF-β1-mediated MMP-9 expression in HaCaT cells requires ERK1/2 [Zavadil et al., 2001], which has also been shown to mediate TGF-β1-induced EMT in thyroid cells [Grande et al., 2002].

The Snail family of transcription factors have been identified as key mediators of EMT [Nieto, 2002; Peinado et al., 2003]. Correlative studies have shown that there is an inverse relationship between E-cadherin and Snail (Snai1) expression in human samples [Come et al., 2006]. Interestingly, the cellular changes observed following overexpression of Snail family of transcription factors mimic cellular responses to TGF-β1 [Cicchini et al., 2006]. Also, TGF-β1-mediated Snail induction has been observed in a variety of cell types, including human OSCC cells [Sun et al., 2008]. Moreover, TGF-β1 and Snail have both been shown to enhance MMP expression. Snail can increase the levels of MMP-1, -2, -7, -9, and -14 [Yokoyama et al., 2003; Miyoshi et al., 2004; Sun et al., 2008], while TGF-β1 can increase MMP-2, -9, and -14 [Munshi et al., 2004]. We recently published that inhibiting Snail using siRNA attenuates TGF-β1-induced MMP-9 expression and Matrigel invasion by OSCC cells [Sun et al., 2008], thus, demonstrating that Snail is directly involved in regulating TGF-β1-dependent oral cancer progression.

Although the role of Snai1 (Snail) in cancer progression has been well studied [Barrallo-Gimeno and Nieto, 2005; Peinado et al., 2007], the role and regulation of Snai2 (Slug) in OSCC cells is less well understood. In this report, we show that TGF-β1 increases Slug levels in oral keratinocytes and OSCC cells and that inhibiting ERK1/2 signaling blocks TGF-β1-induced Slug expression. Although Slug did not repress E-cadherin levels or regulate individual movement of the malignant UMSCC1 cells, Slug enhanced cohort migration and MMP-9-dependent Matrigel invasion by UMSCC1 cells. Moreover, Slug-specific siRNA attenuated TGF-β1-induced MMP-9 expression and Matrigel invasion. These data demonstrate that TGF-β1 increases Slug via ERK1/2 signaling, and thereby contributes to OSCC progression.

MATERIALS AND METHODS

MATERIALS

TGF-β1, peroxidase-conjugated secondary antibodies, were purchased from Sigma (St Louis, MO). Dulbecco's modified Eagle medium (DMEM), Ham's F-12 and keratinocyte-SFM were purchased from Life Technologies (Grand Island, NY). Anti-ERK2 and anti-tubulin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), while anti-Slug antibody was purchased from Abcam (Cambridge, MA). A nucleofector electroporation kit specifically designed for keratinocytes was obtained from Amaxa (Gaithersburg, MD). MEK1/2 inhibitor U0126 and PI3-kinase inhibitor LY294002 were purchased from Calbiochem (Gibbstown, NJ). Protease inhibitor mixture was purchased from Roche Diagnostics (Indianapolis, IN). BD Biocoat Matrigel Invasion Chambers were from BD Biosciences (Medford, MA).

CELL CULTURES

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). These cells display normal keratin synthesis and can undergo stratified squamous epithelial differentiation [Dickson et al., 2000]. SCC25 and UMSCC1 cells were derived from squamous cell carcinoma of the oral cavity and are tumorigenic in nude mice. SCC25 cells were obtained from American Type Culture Collection [Rheinwald and Beckett, 1981], whereas UMSCC1 cells were generously provided by Dr. E. Lengyel (University of Chicago, Chicago, IL) [Lengyel et al., 1995]. SCC25 and UMSCC1 cells were routinely maintained in DMEM and Ham's F-12 medium (1:1) containing 10% fetal calf serum and supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. OKF4 and OKF6 cells were maintained in keratinocyte-SFM supplemented with 100 U/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 mM $CaCl_2$.

To examine the effect of TGF-β1 on gene expression, equal numbers of oral keratinocytes or OSCC cells were plated in serum-containing media, serum starved for 24 h, and then treated with 10 ng/ml TGF-β1 for varying periods of time.

CLONING OF GFP-TAGGED SLUG

The complete coding sequence of Slug 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 primer 5′-ATGCCGCGCTCCTTCCTGGTCAAGAAGCAT-3′, and reverse primer 5′-TCAGTGTGCTACACAGCAGCCAGATTCCTC-3′. The Slug gene was initially subcloned into eukaryotic expression vector pEGFP-C1 to obtain GFP-tagged Slug, with the GFP-tag at the N-terminus of Slug. Slug and GFP-tagged Slug were then subcloned into pRetroX-Tight-Pur vector. All plasmids were verified by DNA sequencing.

RETROVIRAL INFECTION OF UMSCC1 CELLS

To generate retroviral particles, GP2-293 packaging cells were transfected with pRetroX-Tight Pur vector and co-transfected with pVSV-G (Clontech) envelope vector according to the manufacturer's specifications (Clontech). The conditioned medium from the packaging cells containing the viral particles were filtered through a 0.45 μm cellulose acetate membrane and then added to UMSCC1 cells in the presence of polybrene 4 μg/ml.

GENERATION OF SLUG-INDUCIBLE UMSCC1 CELLS

UMSCC1 cells were infected with viral particles expressing pRetroX-Tet-On Advanced vector and stable cells resistant to 0.85 mg/ml of G418 were selected to create UM-tet cells. These cells were then infected with viral particles expressing pTight-GFP, pTight-GFPSlug, pTight-Slug, or control vector pTight-Luc and stable cell lines resistant to both G418 (0.85 mg/ml) and puromycin (1 μg/ml) were selected to generate UM-tet-GFP, UM-tet-GFPSlug, UM-tet-Slug, and UM-tet-Luc cells.

UM-tet-GFPSlug cells were plated overnight on glass coverslips, treated with doxycycline for 24 h, washed with phosphate-buffered saline, 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 DAPI and phalloidin

(actin), after which the cells were washed, mounted, and observed using a Zeiss Axiovert 200 microscope.

DOWNREGULATION OF SLUG, Ets-1, AND MMP-9 EXPRESSION

Slug expression was transiently downregulated using the following duplex siRNA directed against Slug: forward primer 5′-GCAUUUG-CAGACAGGUCAAdTdT-3′, reverse primer 5′-UUGAACUGUCUG-CAAAUGCdTdT-3′ [Tripathi et al., 2005]. UMSCC1 cells were transiently transfected with 100 nmol of Slug-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 Slug or MMP-9 expression. Similarly, MMP-9 was downregulated using Silencer Predesigned siRNA against MMP-9 (Cat #AM16708, Ambion), while Ets-1 using the following duplex siRNA (Ets1Si) forward primer 5′-GGACAAGCCUGUCAUUC-CUdTdT-3′ and reverse primer 5′-AGGAAUGACA GGCUU-GUCCdTdT-3′ [Santiago and Khachigian, 2004].

ANALYSIS OF MMP-9 EXPRESSION

Gelatinase activity in 24-h serum-free conditioned medium was determined using SDS–PAGE gelatin zymography as described previously [Munshi and Stack, 2002; Munshi et al., 2002a].

REAL-TIME PCR

Reverse transcription of RNA to cDNA was performed using GeneAmp RNA PCR kit (Applied Biosystems). Quantitative gene expression was performed for Slug, MMPs, 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_T method for relative gene expression [Schmittgen and Livak, 2008]. Briefly, C_T is defined as the PCR cycle at which the fluorescent signal crosses a particular threshold, with the numerical value of the C_T being inversely related to the amount of amplicon in the reaction. The data are then normalized to the C_T value of an internal control, for example, GAPDH, to obtain $\Delta C_T = C_T$ gene of interest $- C_T$ for GAPDH. The amount of gene of interest relative to internal control GAPDH is calculated as $2^{-\Delta C_T}$.

CELL SCATTERING AND COLLOIDAL GOLD ASSAY

Haptotactic motility was assessed, as described previously [Ottaviano et al., 2006], by plating 10^3 cells on matrices overlaid with colloidal gold. Cells were allowed to migrate for 18 h, and phagokinetic tracks (including circular clearings) were monitored by visual examination using a Zeiss microscope with dark-field illumination and photographed using Nikon camera. The relative motility was determined by quantifying the area generated by the tracks.

IN VITRO WOUND CLOSURE ASSAY

In vitro wound closure assay was performed as previously described [Hudson and McCawley, 1998; McCawley et al., 1998]. The cells were grown to confluence in 6-well tissue culture plates. The cells were pretreated with mitomycin (10 μg/ml) for 2 h to block proliferation and then cell-free area introduced by scratching with a pipette tip. The cellular debris was removed by extensive washing and then the cells were treated with doxycyline to induce Slug expression. Relative wound closure was determined by calculating the ratio of the surface area at 24 h and at the time of initial wounding.

ANALYSIS OF MATRIGEL INVASION

Invasive activity was quantified using BD Biocoat Matrigel invasion chambers (8-μm pore size). 2×10^5 UM-tet-Luc and UM-tet-Slug cells were counted using Z1 Coulter Counter, and added to the upper chamber in 500 μl of serum-free medium and 500 μl of serum-containing medium was added to the lower well to promote invasion in the presence or absence of doxycycline (2 μg/ml). The non-invasive cells were removed from the upper chamber 40 h later, the invasive cells were fixed and photographed, and the relative invasion was quantified. The role of MMP-9 in Slug-mediated Matrigel invasion was examined by transiently transfecting the cells with MMP-9-specific siRNA or control siRNA using Amaxa nucleofector kit. In selected experiments, the effect of TGF-β1 on invasion was determined by adding TGF-β1 in the lower chambers. The role of Slug in TGF-β1-mediated Matrigel invasion was examined by transiently transfecting UMSCC1 cells with Slug-specific siRNA or control siRNA using Amaxa nucleofector kit.

STATISTICAL ANALYSIS

Statistical analyses were done using GraphPad Instat 3 (San Diego, CA) and applying the one-way analysis of variance (ANOVA) method.

RESULTS

TGF-β1 PROMOTES SLUG EXPRESSION IN ORAL KERATINOCYTES AND OSCC CELLS

We recently published that TGF-β1 regulates Snail expression in OSCC cells to promote invasion [Sun et al., 2008]. Since the Snail-related protein Slug has also been shown to be involved in cancer progression [Nieto, 2002; Peinado et al., 2007], we initially examined the extent to which TGF-β1 regulated Slug levels in oral keratinocytes (OKF4 and OKF6 cells) and in OSCC cells (UMSCC1 and SCC25 cells) at the mRNA level by real-time PCR. As shown in Figure 1A, TGF-β1 increased Slug mRNA expression, with the increase most pronounced at 24 h. In addition, we examined the effect of TGF-β1 on Slug protein levels at 24 h by Western blotting. Consistent with the real-time data, TGF-β1 increased Slug protein levels in both oral keratinocytes and OSCC cells (Fig. 1B).

ERK1/2 REGULATES TGF-β1-INDUCED SLUG EXPRESSION

Snail expression in Mardin–Darby canine kidney cells was previously shown to involve both ERK1/2 and PI3-kinase signaling [Peinado et al., 2003]. Thus, we examined the role of ERK1/2 and PI3-kinase signaling pathways in mediating TGF-β1-induced Slug expression in OSCC cells. UMSCC1 cells were pretreated with the PI3-kinase inhibitor LY294002 or the MEK1/2 inhibitor U0126, and then treated with TGF-β1 for 24 h. As previously shown in Figure 1B, TGF-β1 increased Slug expression in UMSCC1 cells in the presence of vehicle control (DMSO) (Fig. 2A). Pretreatment with the PI3-kinase inhibitor did not block TGF-β1-mediated Slug

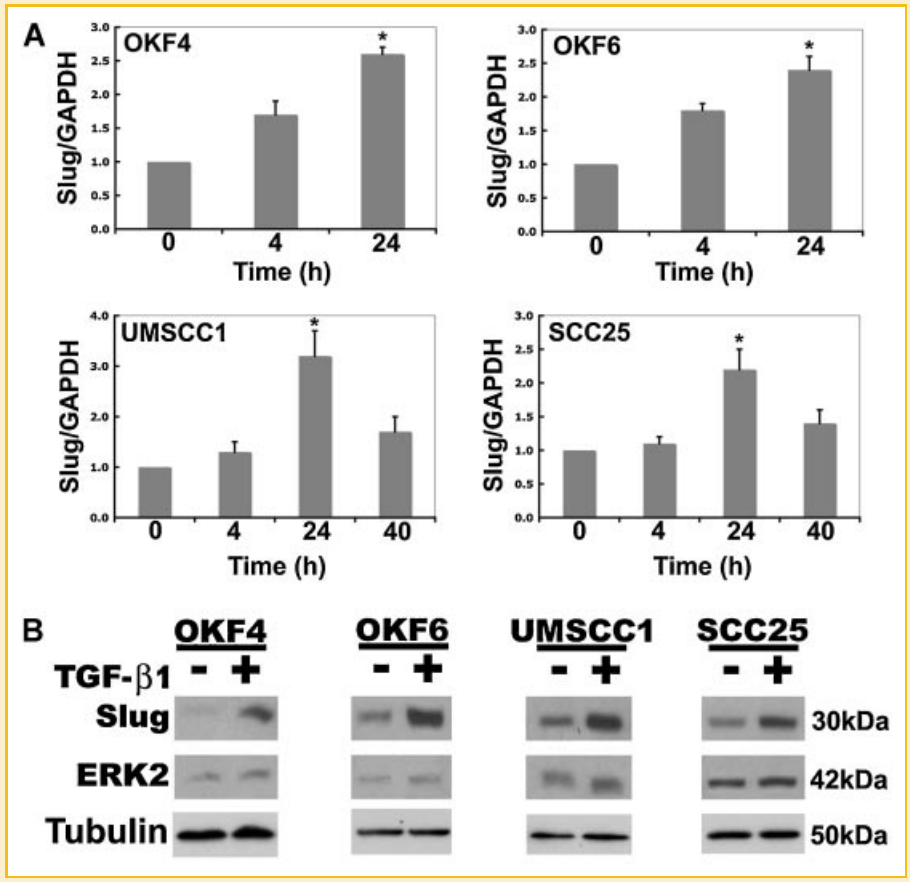

Fig. 1. TGF-β1 promotes Slug expression in oral keratinocytes and in oral SCC cells. Tert-immortalized oral keratinocyte cell lines (OKF4 and OKF6) and malignant oral SCC cell lines (UMSCC1 and SCC25) were treated with 10 ng/ml of TGF-β1 for varying periods of time. A: The relative expression of Slug and GAPDH was determined using real-time PCR, quantified and normalized to untreated sample, arbitrarily set at 1.0. *Significantly different from untreated sample with a $P < 0.05$. B: Equal amounts of cell lysates from the 24-h treated samples were analyzed for Slug, ERK2, and tubulin (loading control) by Western blotting. The results are representative of at least four independent experiments with error bars indicating SEM.

expression; however, the MEK1/2 inhibitor blocked TGF-β1-mediated Slug induction (Fig. 2A). In addition, the MEK1/2 inhibitor also blocked TGF-β1-induced Slug expression at the mRNA level (Fig. 2B), thus, indicating that TGF-β1 promotes Slug expression via the ERK1/2 signaling pathway in UMSCC1 cells.

Ets-1 has been shown to function downstream of ERK1/2 to regulate gene expression [Paumelle et al., 2002]. Additionally, we had previously published that Ets-1 can mediate TGF-β1-induced gene expression in UMSCC1 cells [Sun et al., 2008]. Thus, we examined the role of Ets-1 in mediating TGF-β1-induced Slug expression using siRNA against Ets-1. As shown in Figure 2C, Ets-1 siRNA failed to abrogate TGF-β1-induced Slug expression, indicating that Ets-1 is not involved downstream of ERK1/2 in mediating TGF-β1-induced Slug expression.

SLUG DOES NOT REGULATE E-CADHERIN LEVELS IN OSCC CELLS

To understand the role of Slug in OSCC progression, we generated UMSCC1 cell lines expressing GFP-tagged Slug protein using a tet-on system. As shown in Figure 3A, in the absence of doxycycline there was minimal GFP signal in the UM-GFP-Slug cells while with the addition of doxycycline there was robust expression of GFP-tagged Slug, with the majority of the staining detected in the nucleus. In addition to generating UMSCC1 cells expressing GFP-tagged Slug, we also created tet-on UMSCC1 cells expressing *untagged* Slug. As shown in Figure 3B, treatment of control UMSCC1-tet-luciferase (UM-Luc) cells with doxycycline did not affect Slug protein expression. In contrast, treatment of UMSCC1-tet-Slug (UM-Slug) cells with doxycycline resulted in a robust increase in Slug expression at both protein and mRNA levels (Fig. 3B,C). Overexpression of Slug in UMSCC1 cells also generated a lower molecular weight protein recognized by the anti-Slug antibody (Fig. 3B), suggesting that Slug may undergo degradation in OSCC cells. Interestingly, a recent report showed that Slug can undergo MDM2-mediated ubiquitination and degradation in H1299 lung cancer cells [Wang et al., 2009]. We also examined the effect of Slug on E-cadherin levels in OSCC cells. As shown in Figure 3B,C, Slug did not affect E-cadherin mRNA or protein levels in UMSCC1 cells.

SLUG DOES NOT REGULATE INDIVIDUAL CELL MOTILITY, BUT PROMOTES COHORT MIGRATION AND MATRIGEL INVASION

Since Slug expression is associated with tumor progression [Nieto, 2002; Peinado et al., 2007], we examined the effect of Slug on OSCC

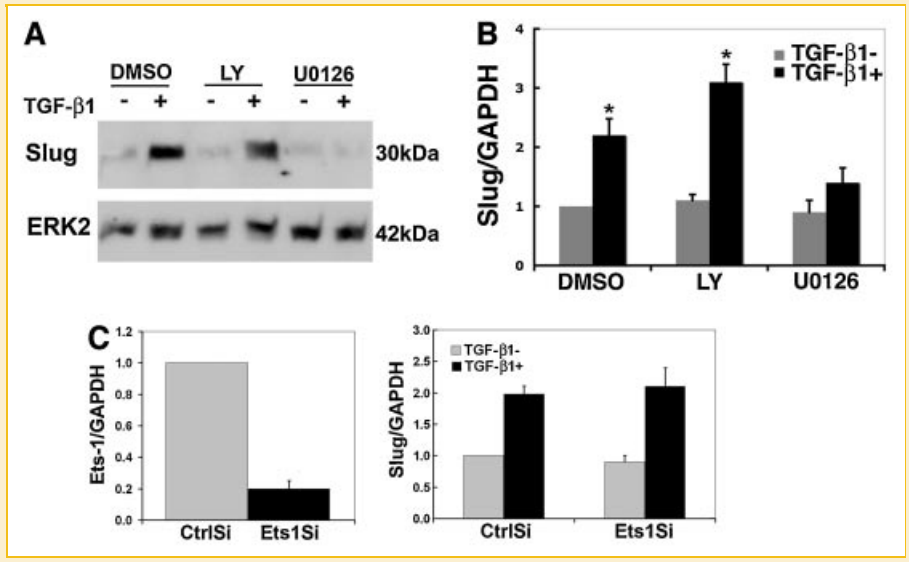

Fig. 2. ERK1/2 regulates TGF-β1-induced Slug expression. UMSCC1 cells were pretreated with vehicle control (DMSO), PI3-kinase inhibitor (LY, 10 μM), or MEK1/2 inhibitor (U0126, 10 μM) and then treated with TGF-β1 for 24 h. A: Equal amounts of cell lysates were analyzed for Slug and ERK2 (loading control) by Western blotting. B: The relative expression of Slug and GAPDH was determined using real-time PCR, quantified and normalized to untreated, vehicle control (DMSO) sample arbitrarily set at 1.0. *Significantly different from untreated vehicle sample with a $P < 0.05$. C: UMSCC1 cells were transfected with control siRNA (CtrlSi) or Ets-1-specific siRNA (Ets1Si) and treated with TGF-β1 for 24 h. The relative expression of Ets-1, Slug, and GAPDH was determined using real-time PCR, quantified and normalized to untreated, control siRNA-transfected cells. The results are representative of three independent experiments with error bars indicating SEM.

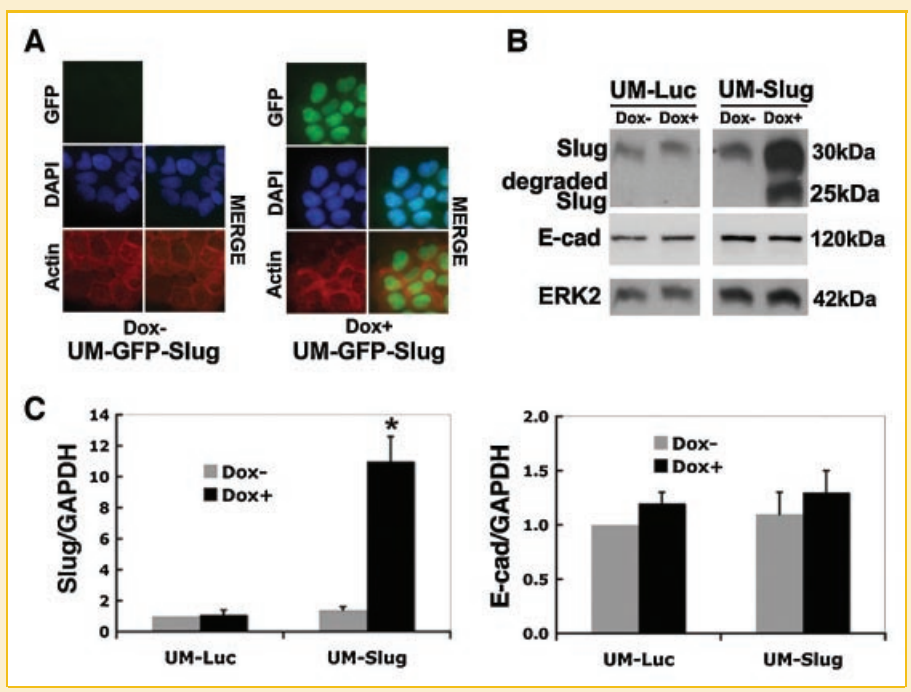

Fig. 3. Slug does not regulate E-cadherin levels in OSCC cells. A: UMSCC1 cells were transfected with pTet-on vector (Clontech) and co-transfected with pTight-GFPSlug, and cells resistant to both G418 (500 μg/ml) and puromycin (1 μg/ml) were selected to generate UM-GFP-Slug cell line. The UM-GFP-Slug cells were plated onto glass coverslips and GFP-Slug expression induced by treating the cells with doxycycline (2 μg/ml) for 8 h. The cells were then fixed, stained with DAPI (nucleus) and phalloidin (actin), and examined with a fluorescence microscope to determine relative expression of GFP-tagged Slug. "MERGE" combines GFP staining with DAPI or actin staining. B,C: UMSCC1 cells were transfected with pTet-on vector (Clontech) and co-transfected with either pTight-luciferase or pTight-Slug expressing untagged Slug, and stable cells lines resistant to both G418 (500 μg/ml) and puromycin (1 μg/ml) were selected to generate, respectively, UM-Luc and UM-Slug cell lines. The stable UM-Luc and UM-Slug cells were then plated and treated with doxycycline for 24 h. Equal amounts of cell lysates were analyzed for Slug, E-cadherin, and ERK2 (loading control) by Western blotting (B). The relative expression of Slug, E-cadherin, and GAPDH was determined using real-time PCR, quantified and normalized to untreated sample arbitrarily set at 1.0 (C). *Significantly different from untreated UM-Luc sample with a $P < 0.01$. The results are representative of three independent experiments with error bars indicating SEM.

cell migration using colloidal gold phagokinetic assay to measure individual cell movement and wound closure assay to measure cohort migration. As shown in Figure 4A,B, there was no significant difference in the phagokinetic tracks generated by doxycycline-treated UM-Slug cells compared to UM-Luc cells, suggesting that Slug does not regulate the movement of individual UMSCC1 cells. We next examined the effect of Slug on cohort or group migration using wound closure assay [McCawley et al., 1998]. As shown in Figure 4C,D, Slug expression enhanced the ability of UMSCC1 cells to close the wound by 1.7-fold, suggesting that Slug regulates cohort migration of UMSCC1 cells. We also examined the effect of Slug expression on the ability of UMSCC1 cells to invade through an artificial basement membrane [Munshi and Stack, 2002]. As shown in Figure 4E,F, expression of Slug in UMSCC1 cells resulted in an eightfold increase in Matrigel invasion. Together these data show that Slug promotes OSCC progression by increasing cohort migration and invasion.

SLUG INCREASES MMP-9 TO PROMOTE MATRIGEL INVASION

We had previously shown that Matrigel invasion by UMSCC1 cells requires MMP-9 [Sun et al., 2008], a key proteinase that is overexpressed in human OSCC tumors [Kurahara et al., 1999; Sorsa et al., 2004]. Thus, we examined the extent to which Slug increases MMP-9 levels in UMSCC1 cells. Equal numbers of UM-Luc and UM-Slug cells were plated in serum containing media, serum starved overnight and then treated with doxycycline for 24 h and the

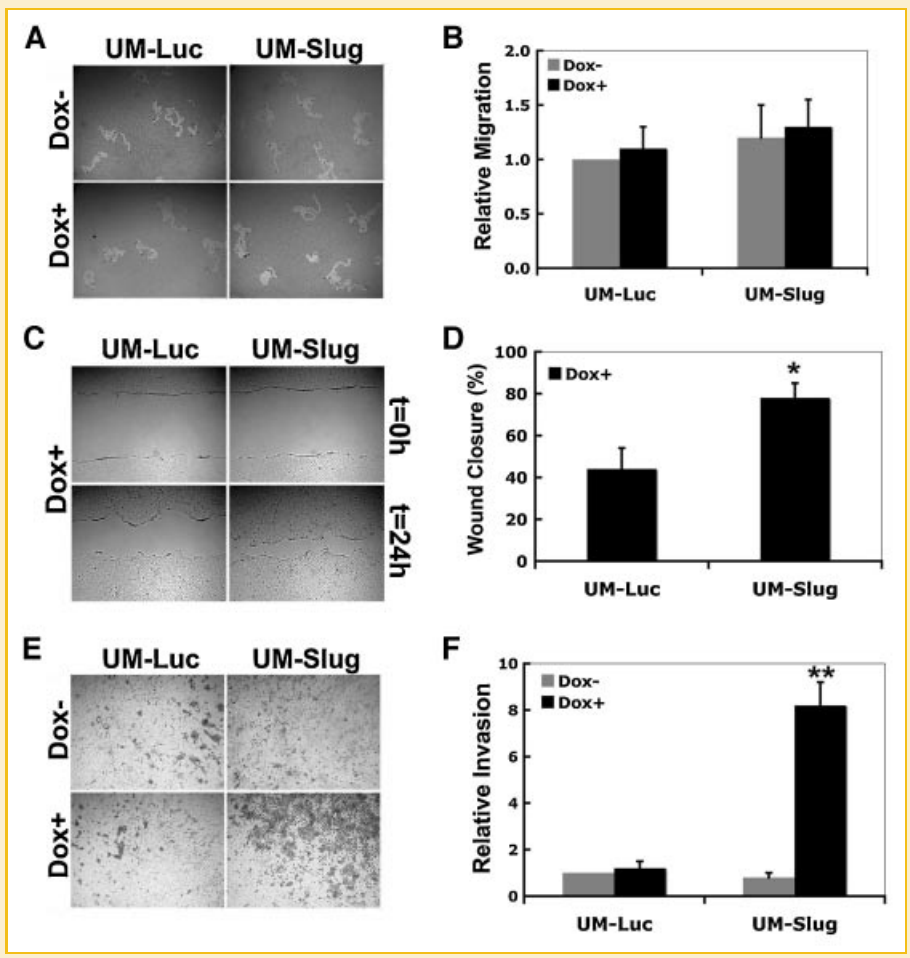

Fig. 4. Slug does not regulate individual cell motility, but promotes cohort migration and invasion. A,B: Individual cell migration by UM-Luc and UM-Slug cells in the presence or absence of doxycycline (2 μg/ml) was determined by plating 10^3 cells on thin-layer of type I collagen matrix overlaid with colloidal gold. Cells were allowed to migrate for 18 h, and phagokinetic tracks photographed using Nikon camera. The relative motility was determined by quantifying the area generated by the tracks. C,D: UM-Luc and UM-Slug cells were grown to confluence, pretreated with mitomycin for 2 h to block proliferation, and wounded with a pipette tip to create a uniform area of clearing. The cells were then allowed to close the wound in the presence of absence of doxycycline (2 μg/ml). Cell migration was photographed using a Nikon camera, and relative wound closure determined by quantifying the relative area of clearing still present at 24 h. *Significantly different from UM-Luc samples with a $P < 0.05$. E,F: Equal numbers of tet-inducible cells were plated onto Matrigel-coated BD transwell chambers, treated with doxycycline (2 μg/ml) and allowed to invade for 40 h. Non-invading cells were removed from the upper chamber, and filters were fixed and stained, the invasive cells were photographed using Nikon camera, and counted. **Significantly different from untreated UM-Slug cells with $P < 0.01$. The results are representative of at least three different experiments with error bars indicating SEM.

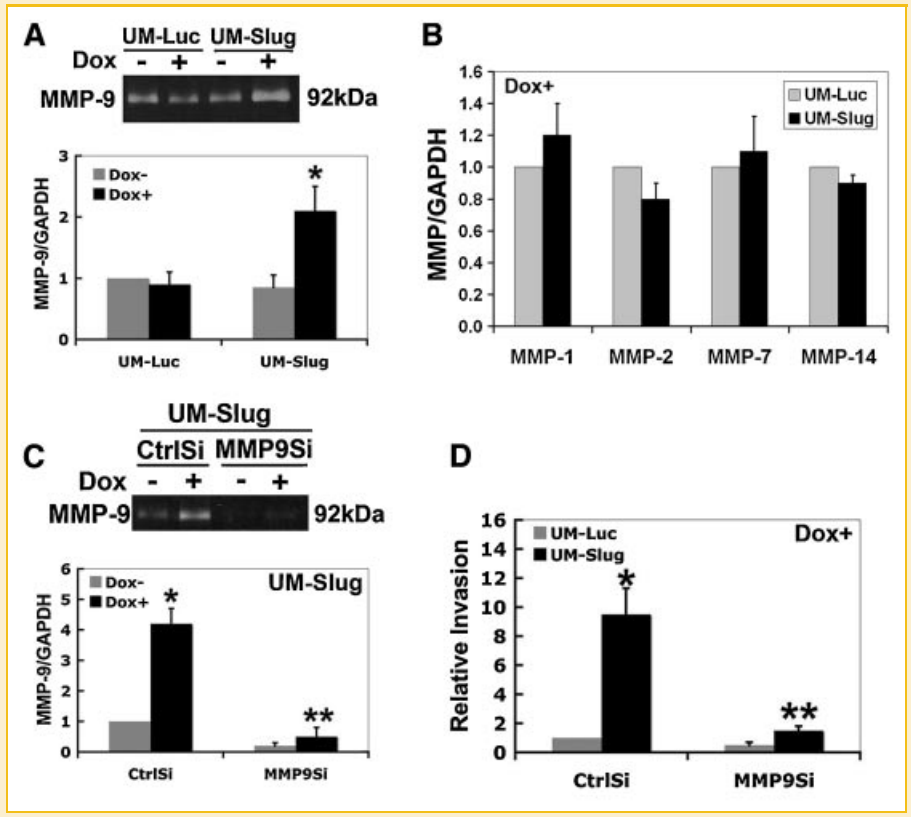

Fig. 5. Slug increases MMP-9 to promote Matrigel invasion. A: Equal numbers of tet-inducible UM-Luc and UM-Slug cells were plated in 6-well tissue culture plates, serum starved overnight, treated with doxycycline (2 μg/ml) and further allowed to condition the serum-free media for additional 24 h. MMP-9 expression was analyzed by gelatin zymography or by real-time PCR and normalized to the levels present in untreated UM-Luc cells, arbitrarily set at 1.0. *Significantly different from untreated UM-Luc samples with a $P < 0.05$. B: Equal numbers of tet-inducible UM-Luc and UM-Slug cells were plated in 6-well tissue culture plates, serum starved overnight, treated with doxycycline (2 μg/ml) for additional 24 h. MMP-1, -2, -7, and -14 expression were analyzed by real-time PCR and normalized to the levels present in UM-Luc cells. C: UM-Slug cells were transfected with control siRNA or MMP-9-specific siRNA, serum starved, treated with doxycycline, allowed to condition the media for 24 h, and MMP-9 expression analyzed by gelatin zymography and by real-time PCR. The relative mRNA expression was normalized to the levels present in the untreated, control siRNA-transfected cells. *Significantly different from Dox−, CtrlSi-transfected cells with a $P < 0.01$; **Significantly different from Dox+, CtrlSi-transfected cells with a $P < 0.01$. D: UM-Luc and UM-Slug cells were transfected with control siRNA or MMP-9-specific siRNA and plated onto Matrigel-coated BD transwell chambers, treated with doxycycline (2 μg/ml) and relative invasion quantified. *Significantly different from UM-Luc, CtrlSi-transfected cells with a $P < 0.01$; **Significantly different from UM-Slug, CtrlSi-transfected cells with a $P < 0.01$. The results are representative of three independent experiments with error bars indicating SEM.

conditioned media analyzed for MMP-9 protein levels by gelatin zymography and the lysates for MMP-9 mRNA levels by real-time PCR. As shown in Figure 5A, Slug increased MMP-9 at both the protein and mRNA levels. Since Snail can modulate the expression of MMP-1, -2, -7, and -14 [Yokoyama et al., 2003; Miyoshi et al., 2004; Sun et al., 2008], we also examined the effect of Slug on these particular MMPs by real-time PCR. As shown in Figure 5B, Slug did not regulate the expression of MMP-1, -2, -7, or -14 in UMSCC1 cells.

We next examined the role of MMP-9 in Slug-mediated invasion by knocking down MMP-9 using siRNA. Initially, we determined the effectiveness of the siRNA in blocking Slug-induced MMP-9 expression. As shown in Figure 5C, induction of Slug in the presence of control siRNA increased MMP-9 expression, while the MMP-9-specific siRNA blocked both basal and Slug-induced MMP-9 expression. We then examined the effect of knocking down MMP-9 on Slug-mediated invasion. As shown in Figure 5D, Slug increased Matrigel invasion when transfected with control siRNA. In contrast, transfection of MMP-9-specific siRNA significantly attenuated Slug-mediated invasion.

SLUG CONTRIBUTES TO TGF-β1-INDUCED MMP-9 EXPRESSION AND MATRIGEL INVASION

We had previously published that TGF-β1 promoted invasion of UMSCC1 cells by increasing MMP-9 levels [Sun et al., 2008]. Since we show that Slug increases MMP-9 levels (Fig. 5) and that TGF-β1 promotes Slug expression (Fig. 1), we therefore examined if Slug contributes to TGF-β1-induced MMP-9 expression and Matrigel invasion. As previously shown in Figure 1, TGF-β1 increased Slug levels by approximately twofold (Fig. 6A). Transfection of Slug siRNA blocked both basal and TGF-β1-induced Slug expression at both the protein and mRNA levels. TGF-β1 increased MMP-9 levels in the presence of control siRNA; however, the fold induction of MMP-9 in the presence Slug siRNA was decreased by ~50% (Fig. 6B), suggesting that Slug contributes to TGF-β1-induced MMP-9 expression. Finally, we examined the effect of knocking

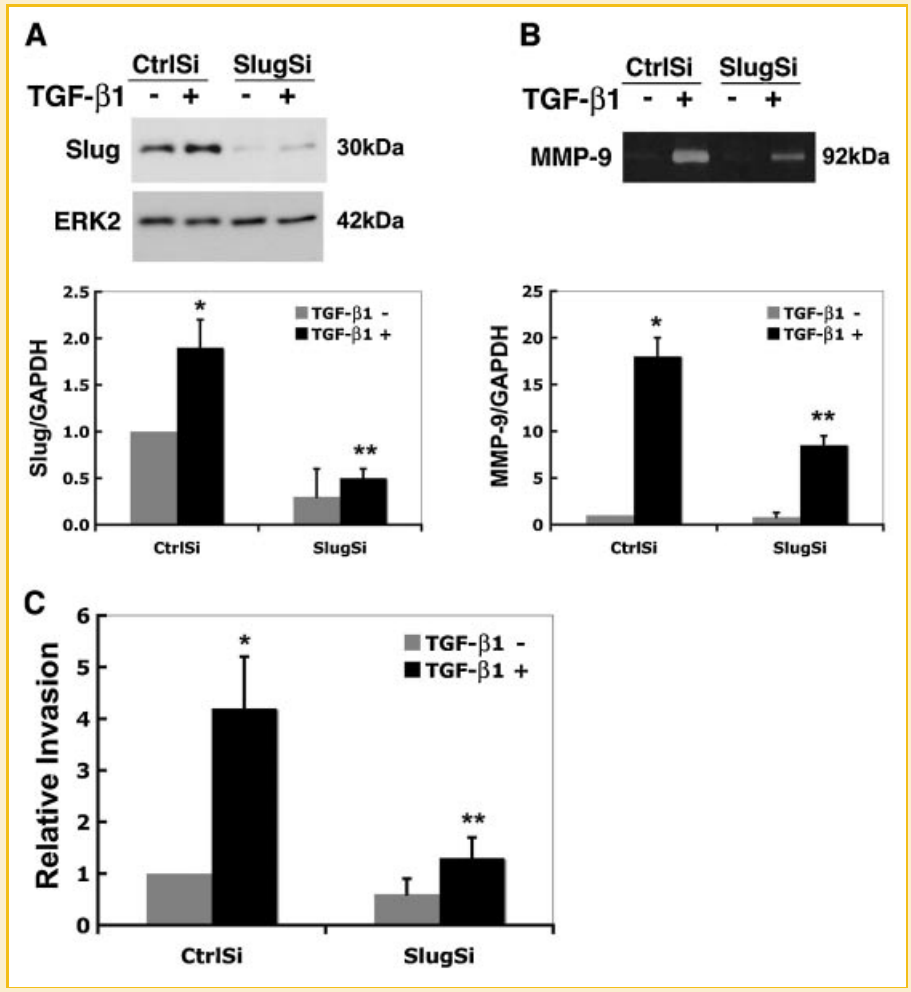

Fig. 6. Slug contributes to TGF-β1-induced MMP-9 expression and Matrigel invasion. UMSCC1 cells were transfected with control siRNA or Slug-specific siRNA, serum starved overnight, treated with TGF-β1 for 24 h, and allowed to condition the serum-free media for additional 24 h. A: Relative expression of Slug was determined at the protein level by Western blotting and at the mRNA level by real-time PCR. The relative mRNA expression was normalized to the levels present in the untreated, control siRNA-transfected cells. *Significantly different from TGF-β1−, CtrlSi-transfected cells with a $P < 0.05$; **Significantly different from TGF-β1+, CtrlSi-transfected cells with a $P < 0.01$. B: Samples analyzed for MMP-9 expression by gelatin zymography, and by real-time PCR. The relative mRNA expression was normalized to the levels present in the untreated, control siRNA-transfected cells. *Significantly different from TGF-β1−, CtrlSi-transfected cells with a $P < 0.01$; **Significantly different from TGF-β1+, CtrlSi-transfected cells with a $P < 0.05$. C: UMSCC1 cells were transfected with control siRNA or Slug-specific siRNA, added to Matrigel-coated BD transwell chambers and allowed to invade for 40 h in the presence or absence of TGF-β1. *Significantly different from TGF-β1−, CtrlSi-transfected cells with a $P < 0.05$; **Significantly different from TGF-β1+, CtrlSi-transfected cells with a $P < 0.05$. The results are representative of three different experiments with error bars indicating SEM.

down Slug on TGF-β1-mediated invasion. As shown in Figure 6C, TGF-β1 increased Matrigel invasion when transfected with control siRNA. In contrast, transfection of Slug-specific siRNA significantly attenuated basal and TGF-β1-induced invasion. Overall, these results demonstrate that Slug contributes to TGF-β1-mediated MMP-9 expression and invasion of OSCC cells.

DISCUSSION

Members of Snail family of transcription factors play an important role in cancer progression [Nieto, 2002; Barrallo-Gimeno and Nieto, 2005; Peinado et al., 2007]. Snail promotes invasion of breast [Olmeda et al., 2007], esophageal [Natsugoe et al., 2007], oral [Sun et al., 2008], and mouse skin cancer cells [Olmeda et al., 2008], and increased expression of Snail correlates with increased invasion in human breast and esophageal cancer samples [Natsugoe et al., 2007; Olmeda et al., 2007]. Snail promotes invasion, in part, by increasing MMP expression [Jorda et al., 2005]. We recently showed that Snail regulates MMP-9 to promote invasion of OSCC cells [Sun et al., 2008]. Although Slug has been associated with invasion and lymph node metastasis in human breast [Hajra et al., 2002] and esophageal cancers [Jethwa et al., 2008], the role of Slug in OSCC progression had not been heretofore examined. We show in this report that Slug can regulate invasion of OSCC cells and also contribute to TGF-β1-induced invasion.

Although TGF-β1 can increase the levels of both Snail and Slug [Thuault et al., 2006; Choi et al., 2007; Herfs et al., 2008; Medici

et al., 2008; Sun et al., 2008], these transcription factors are differentially regulated by TGF-β1 in OSCC cells. In contrast to the effect of TGF-β1 on Slug expression, TGF-β1 increased Snail with varying kinetics and magnitude in OSCC cells [Sun et al., 2008]. We previously showed that TGF-β1 increased Snail in OKF4, OKF6, and UMSCC1 cells, but not in SCC25 cells, and that maximal expression of Snail in UMSCC1 was at 2 h with gradual decline at 24 h. In contrast, TGF-β1 uniformly increased Slug in these four cell lines with very similar kinetics, resulting in maximal expression at 24 h. Interestingly, Snail and Slug expression during development also have different kinetics with Snail induced earlier than Slug during Xenopus neural crest development [Aybar et al., 2003]. Although Snail and Slug can regulate common pathways involved in tumor progression, our results suggest that Snail and Slug may target different sets of TGF-β1-induced genes in OSCC cells and may even have distinct function. We also show that TGF-β1 increases Slug in OSCC cells via ERK1/2-dependent pathways, and not via PI3-kinase signaling. Previously, it was shown that ERK1/2-mediated EGF-induced Slug expression in HaCaT cells [Arnoux et al., 2008], and also mediated ultraviolet radiation-induced Slug expression in SCC 12F cells [Hudson et al., 2007]. However, in agreement with TGF-β1 regulation of Snail in MDCK cells, Smad signaling did not mediate TGF-β1-induced Slug expression in OSCC cells (data not shown) [Peinado et al., 2003].

We had previously published that Snail increased MMP-9 in OSCC cells [Sun et al., 2008]. Since we were unable to successfully generate OSCC cell lines stably expressing Slug, we created tet-inducible UMSCC1 cell lines to examine the effect of Slug on MMP-9 expression and invasion. Slug increased MMP-9 to promote Matrigel invasion by OSCC cells, and siRNA against MMP-9 blocked Slug-mediated invasion. This is in contrast to the role of Slug in HaCa4 and CarB mouse keratinocyte cell lines where knocking down Slug failed to decrease MMP-9 expression [Olmeda et al., 2008]; however, knocking down Slug decreased collagen invasion by CarB cells [Olmeda et al., 2008]. In agreement with our data, blocking Slug in neuroblastoma cells also prevented Matrigel invasion [Vitali et al., 2008]. Although we show that Slug regulates MMP-9 expression and contributes to TGF-β1-induced MMP-9 expression and invasion in OSCC cells, the mechanism for Slug-induced MMP-9 expression is yet to be defined. It is not known whether the effect of Slug is mediated through its repressive SNAG domain or through its zinc-finger binding domain [Nieto, 2002]. It is also possible that Slug regulation of MMP-9 may be indirect through activation of additional signaling pathways and may not even involve Slug binding to the MMP-9 promoter. Previous reports had shown that there is a reciprocal relationship between MMPs and Snail, such that Snail can induce MMPs and that MMPs can also promote Snail expression [Radisky et al., 2005; Munshi and Stack, 2006]. However, it is not known whether MMPs can also induce Slug expression.

Although Slug and Snail appear to have redundant functions, it has been suggested that these two proteins may have distinct roles, as particularly noted during breast cancer progression. Slug-expressing breast tumors appear to invade as a cohesive group of cells, while Snail-expressing tumors show more individual invasion of cancerous cells into the surrounding stroma [Come et al., 2006]. Here, we also show that Slug enhances cohort migration of UMSCC1 cells without affecting individual cell movement. In support of cohort migration, Slug-expressing UMSCC1 cells also have preservation of E-cadherin levels. This is in contrast to the effect of Snail in UMSCC1 cells, in which Snail strongly represses E-cadherin expression [Sun et al., 2008]. Interestingly, blocking Snail function using a dominant negative mutant of Snail increased E-cadherin expression in MDA-MB-231 cells and caused a change in the migration of these cells from individual cell movement to cohort group migration [Fabre-Guillevin et al., 2008]. Genetic profiling of MDCK cells expressing Snail and Slug also show both common and distinct gene expression patterns between Snail- and Slug-expressing cells [Moreno-Bueno et al., 2006]. Interestingly, it was recently shown that Snail and Slug collaborate to promote tumor growth and metastasis when mouse skin cancer cells are injected into nude mice [Olmeda et al., 2008]. In this study, we add to the literature dissecting the overlapping and distinct roles of Snail and Slug in cancer progression and invasion by showing that Snail and Slug can both be regulated by TGF-β1 and that TGF-β1 can increase expression of Snail and/or Slug thereby increasing MMP-9 expression and promoting OSCC invasion.

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