Differential growth factor regulation of N-cadherin expression and motility in normal and malignant oral epithelium

Michelle E. Diamond[1,*], Limin Sun[1,2,*], Adam J. Ottaviano[1], Mathew J. Joseph[1] and Hidayatullah G. Munshi[1,2,3,‡]

[1]Division of Hematology/Oncology, Department of Medicine, Feinberg School of Medicine, Northwestern University, [2]The Jesse Brown VA Medical Center and [3]The Robert H. Lurie Comprehensive Cancer Center of Northwestern University, Chicago, IL 60611, USA

*These authors contributed equally to this work
‡Author for correspondence (e-mail: h-munshi@northwestern.edu)

Accepted 19 April 2008
Journal of Cell Science 121, 2197-2207 Published by The Company of Biologists 2008
doi:10.1242/jcs.021782

Summary

Aberrant expression of N-cadherin is associated with tumor progression in squamous cell carcinomas (SCCs). Consequently, we examined the regulation of N-cadherin by TGFβ1, an important mediator of keratinocyte and SCC function. N-cadherin expression was increased in oral SCC (OSCC) cell lines, regulating motility and correlating with TGFβ1 production. Moreover, in normal keratinocytes TGFβ1 increased expression of N-cadherin to regulate motility. TGFβ1-mediated N-cadherin expression in the oral keratinocytes was blocked using siRNA targeting Smads. Unexpectedly, we found that EGF blocked TGFβ1-mediated N-cadherin expression in oral keratinocytes and not in OSCC cells. Mechanistically, EGF enhanced Smad phosphorylation in the linker region, and attenuated TGFβ1-mediated phosphorylation of Smad at the C-terminus, localization of Smad to the nucleus as well as Smad-driven promoter activity exclusively in oral keratinocytes but not in OSCC cells. The effect of EGF on TGFβ1-mediated Smad-driven promoter activity and N-cadherin expression was reversed when activation of ERK1/2 was blocked. Although EGF and TGFβ1 independently promoted migration of both oral keratinocytes and OSCC cells, EGF decreased TGFβ1-mediated migration of oral keratinocytes but enhanced migration of OSCC cells. Together, these data support a model wherein EGF signaling has an important negative regulatory role on TGFβ1-mediated N-cadherin expression and motility in normal oral keratinocytes, and in which loss of this regulatory mechanism accompanies malignant transformation of the oral epithelium.

Key words: Motility, N-cadherin, TGFβ1, EGF, Smad, ERK1/2, Oral cancer

Introduction

The American Cancer Society estimates that there will be more than 20,000 new cases of squamous cell carcinoma (SCC) this year involving the oral cavity in the USA (Jemal et al., 2008), and that oral SCC (OSCC) will cause more deaths than any other oral disease (Forastiere et al., 2001; Neville and Day, 2002). Moreover, advanced OSCC is associated with high mortality, and treatment is often complicated by disruption of speech and swallowing due to surgical resection (Forastiere et al., 2001; Neville and Day, 2002). 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 (Forastiere et al., 2001; Neville and Day, 2002). However, a number of studies, including cDNA microarray analysis, have shown that changes in cadherin-mediated cell-cell adhesion are associated with OSCC progression and its poor outcome (Al Moustafa et al., 2002; Nagata et al., 2003).

Cadherins are a family of transmembrane proteins that mediate Ca^{2+}-dependent cell-cell adhesion (Angst et al., 2001; Gumbiner, 2005). Of the members of the cadherin family, epithelial-(E)-cadherin is distributed widely and is the most important intercellular adhesion molecule in epithelial cells (Angst et al., 2001; Gumbiner, 2005). Although E-cadherin is well-documented as an invasion suppressor for cancer cells (Behrens et al., 1992; Schipper et al., 1991), the role of anomalously expressed neural-(N)-cadherin in epithelial cells has only recently been defined more clearly (Hazan et al., 2004; Munshi and Stack, 2006; Wheelock et al., 2008). Unlike E-cadherin, N-cadherin is normally expressed in neuroectodermal- and mesodermal-derived tissues (Gumbiner, 2005; Hazan et al., 2004). However, in certain cancers aberrant expression of N-cadherin, rather than loss of E-cadherin, is associated with increased tumor progression (Hazan et al., 2004; Suyama et al., 2002). Moreover, inhibition of N-cadherin expression or function has been shown to block motility and invasion (De Wever et al., 2004; Maeda et al., 2005; Shintani et al., 2006; Watson-Hurst and Becker, 2006). Importantly, N-cadherin is being actively pursued as a therapeutic target, and inhibitors of N-cadherin are currently being evaluated in preclinical and clinical studies (Mariotti et al., 2007).

One of the key regulators of N-cadherin is transforming growth factor β1 (TGFβ1), which has an important role in epithelial tumor progression by acting both as a tumor suppressor at earlier stages of tumorigenesis and as a tumor promoter at later, more advanced stages of tumor formation (Akhurst and Derynck, 2001; Shi and Massague, 2003). For example, overexpression of TGFβ1 in mice mammary glands delayed the formation of primary breast tumors; however, the mice exhibited an increased number of lung metastases (Tang et al., 2003). TGFβ1 is also overexpressed in human OSCC tumors compared with normal head and neck tissue, and overexpression of TGFβ1 in murine oral cavity causes severe hyperplasia and the development of invasive OSCC tumors (Lu et al., 2004). It has been shown that TGFβ1 promotes invasion, in part by increasing N-cadherin levels, because small interfering RNA

(siRNA) directed against N-cadherin or function-blocking antibody blocked TGFβ1-mediated invasion and motility (De Wever et al., 2004). Although TGFβ1 can cooperate with other growth factors (including epidermal growth factor, EGF) to promote N-cadherin expression (Grande et al., 2002), the detailed mechanism by which TGFβ1 regulates N-cadherin expression in OSCC has not been examined previously .

Because of the importance of TGFβ1 and N-cadherin in cell motility, we examined the regulation of N-cadherin expression by TGFβ1 in two human oral keratinocyte cell lines and in four human OSCC cell lines. Under normal conditions, oral keratinocytes do not express N-cadherin. However N-cadherin was expressed in three out of four OSCC cell lines and its expression correlated with the production of TGFβ1. Treatment of oral keratinocytes with TGFβ1 increased N-cadherin expression through Smad signaling, resulting in N-cadherin regulation of TGFβ1-induced motility. Since EGF has been previously shown in pig thyrocytes to cooperate with TGFβ1 in the regulation of N-cadherin expression (Grande et al., 2002), we examined the potential for a coordinated regulation of N-cadherin expression by EGF and TGFβ1 in these oral cell lines. Although EGF did not affect N-cadherin expression in oral keratinocytes or in OSCC cells, EGF blocked TGFβ1-induced N-cadherin expression in the oral keratinocytes; however, no significant effects were observed in the malignant OSCC cells. Moreover, only in the oral keratinocytes EGF attenuated TGFβ1-induced phosphorylation of the Smad C-terminus, nuclear localization of Smad and Smad-driven promoter activity. Only in the oral keratinocytes, EGF also enhanced phosphorylation of Smad in the linker region through extracellular signal-regulated kinases 1 and 2 (ERK1/2). The effect of EGF on TGFβ1-mediated Smad-driven promoter activity and N-cadherin expression was reversed when ERK1/2 phosphorylation was blocked. Although independently EGF and TGFβ1 promoted migration of oral keratinocytes and OSCC cells, EGF decreased TGFβ1-driven migration of oral keratinocytes but enhanced TGFβ1-driven migration of OSCC cells. Together these data support a model wherein TGFβ1-mediated expression and motility of N-cadherin are regulated by EGF-ERK1/2-dependent phosphorylation of the Smad linker region in oral keratinocytes, and the data suggest that a loss of this regulatory mechanism accompanies malignant transformation of the oral epithelium.

Results

N-cadherin expression regulates migration of OSCC cells

N-cadherin has previously been shown to regulate the motility of breast and melanoma cells (Li et al., 2001; Nieman et al., 1999); however, the role and regulation of N-cadherin in OSCC has not been examined. To evaluate the role of N-cadherin in OSCC cells, we first determined the extent to which N-cadherin is expressed in a variety of cell lines derived from the oral cavity. We examined E- and N-cadherin expression in two oral keratinocyte cell lines (OKF4 and OKF6) and in four malignant OSCC cell lines (SCC9, SCC25, SCC68 and UMSCC1). As shown in Fig. 1A, OKF4, OKF6 and UMSCC1 cells did not demonstrate any N-cadherin protein expression, SCC68 cells showed minimal expression, whereas SCC9 and SCC25 cells showed strong N-cadherin protein expression under basal (unstimulated) conditions. By contrast, five out of six cell lines had basal E-cadherin expression, with the exception of SCC9 cells, which lacked E-cadherin. Consistent with the western blot analysis, real-time PCR showed a similar relative expression at the mRNA level (Fig. 1A, right panel).

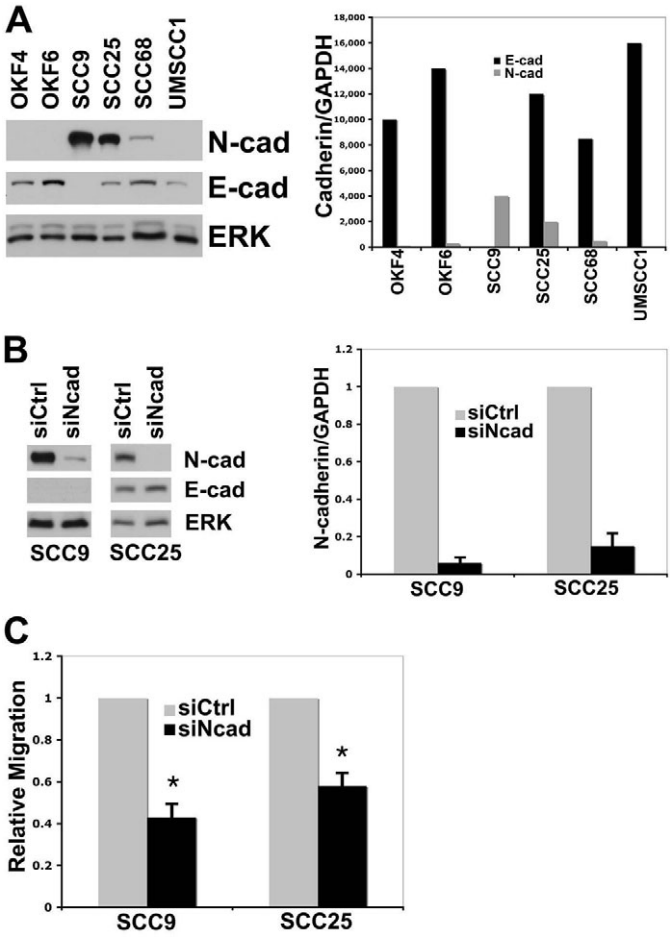

Fig. 1. N-cadherin expression regulates migration of OSCC cells. (A) Tert-immortalized oral keratinocytes (OKF4 and OKF6) and malignant OSCC cells (SCC9, SCC25, SCC68 and UMSCC1) were serum-starved overnight, and E- and N-cadherin expression was then determined by western blot analysis. Using real-time PCR the relative mRNA expression of E-cadherin, N-cadherin and GAPDH was determined, and normalized to the level of E-cadherin in SCC9 cells and the level of N-cadherin in OKF4 cells. (B) SCC9 and SCC25 cells were transfected with 100 nM of control siRNA (siCtrl) or 1:1 mixture of two N-cadherin-specific siRNAs at 50 nM each (siNcad); E-cadherin, N-cadherin and ERK protein levels were determined 72 hours later by western blot analysis. Using real-time PCR the relative expression of E-cadherin, N-cadherin and GAPDH was determined and normalized to siCtrl-transfected cells. (C) SCC9 and SCC25 cells were transfected with siCtrl or siNcad, and 48 hours following transfection cells were added to porous polycarbonate filters that had been coated with 5 μg type I collagen. Cells were allowed to migrate for 24 hours, nonmigratory cells were removed from the upper chamber and migrating cells counted. *P<0.05, significantly different from siCtrl-transfected cells. Results are representative of at least three independent experiments.

As N-cadherin expression has been mechanistically linked with altered cell motility (Li et al., 2001; Nieman et al., 1999), we examined the functional consequences of aberrant N-cadherin expression by downregulating N-cadherin using siRNA, and examining the effect on the motility of OSCC cells. N-cadherin expression was blocked in the two cell lines with increased levels under basal conditions. SCC9 and SCC25 cells were transfected with 100 nM of control siRNA (siCtrl) or 1:1 mixture of two N-cadherin-specific siRNAs at 50 nM each (siNcad), and the effect on protein and mRNA expression was determined at 72 hours. Transfection of siNcad significantly blocked N-cadherin expression

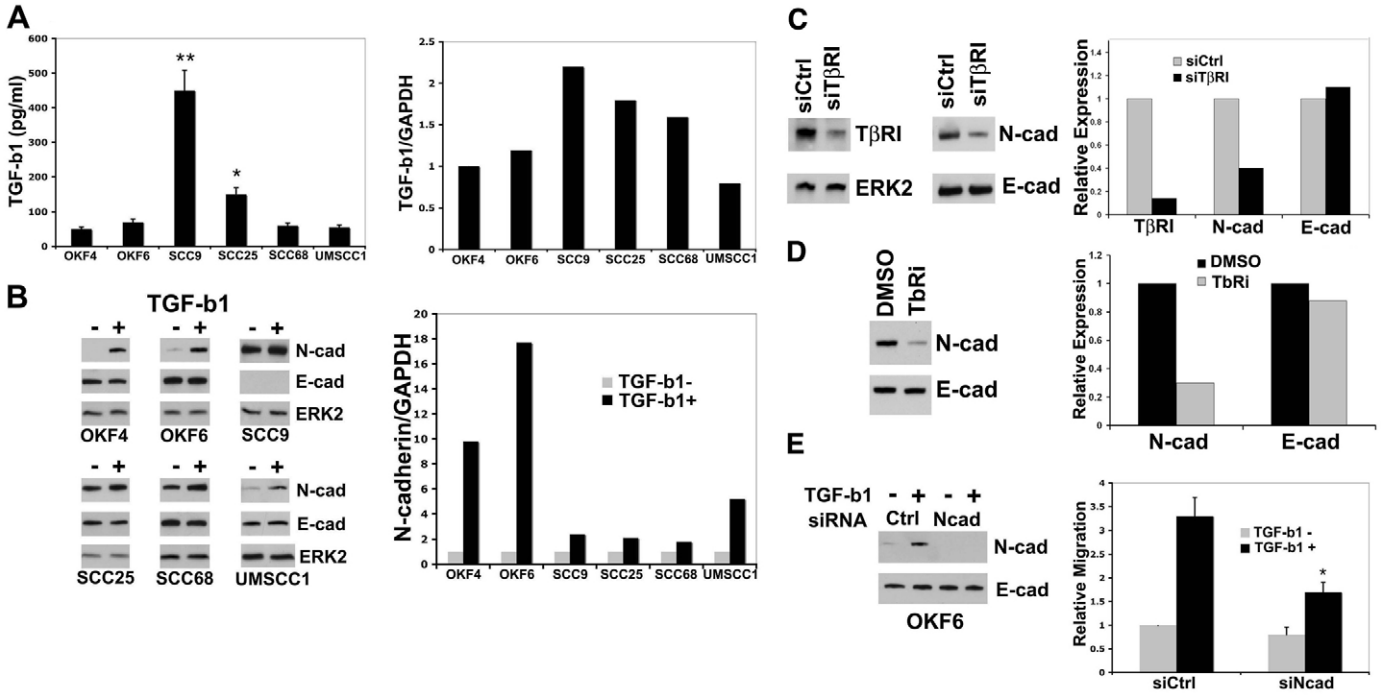

Fig. 2. TGFβ1 increases migration of oral keratinocytes by upregulating N-cadherin expression. (A) Equal numbers of cells of the different cell lines were plated, serum starved for 24 hours and then allowed to condition the medium for an additional 24 hours. The conditioned medium was analyzed for total TGFβ1 by ELISA, and cell lysates processed for TGFβ1 and GAPDH mRNA expression by using real-time PCR and normalized to the levels present in OKF4 cells. **P*<0.05, significantly different from the OKF4 levels; ***P*<0.005, significantly different from the OKF4 levels. (B) Oral keratinocytes and OSCC cells were serum-starved overnight, treated with TGFβ1 for 24 hours and the effect on E- and N-cadherin expression was determined by western blot analysis. Using real-time PCR, expression levels of E-cadherin, N-cadherin and GAPDH mRNA were determined and normalized to untreated samples. (C) SCC25 cells were transfected with 100 nM of control siRNA (siCtrl) or siRNA targeting TβRI (siTβRI); then TβRI, ERK2, N-cadherin and E-cadherin protein levels were determined 72 hours later by western blot analysis. Using real-time PCR, the relative expression of TβRI, E-cadherin, N-cadherin and GAPDH was determined and normalized to siCtrl-transfected cells. (D) SCC25 cells were serum starved overnight and then treated with DMSO (vehicle control) or with the TβRI inhibitor (TbRi, 10 μM) for 72 hours. N-cadherin and E-cadherin protein expression were determined by western blot analysis. N-cadherin, E-cadherin and GAPDH mRNA expression were determined by real time PCR and relative expression normalized to DMSO samples. The results are representative of 3 independent experiments. (E) OKF6 cells were transfected with 100 nM of siCtrl or 50 nM of siNcad1 and siNcad2 (siNcad), serum starved and treated with TGFβ1 for 24 hours. The cells were added to porous polycarbonate filters coated with type I collagen (5 μg), and allowed to migrate for 24 hours in the presence or absence of TGFβ1. Nonmigratory cells were removed from upper chamber, and migrating cells were counted. *, significantly different from the siCtrl, TGF-b1+ samples with *P*<0.05. The results are representative of at least three independent experiments.

without affecting E-cadherin levels (Fig. 1B) and also significantly blocked the migration of both SCC9 and SCC25 cells relative to cells transfected with siCtrl (Fig. 1C), which indicates that N-cadherin expression in OSCC cells contributes to the motility of oral cancer cells.

TGFβ1 increases the migration of oral keratinocytes by upregulating the expression of N-cadherin

Since TGFβ1 can regulate N-cadherin expression (De Wever et al., 2004; Maeda et al., 2005), we examined the relative TGFβ1 production in oral keratinocytes and in OSCC cells to provide a potential explanation for the differences in the levels of N-cadherin among the six cell lines. Equal numbers of cells from the six cell lines were plated, serum-starved for 24 hours and then allowed to condition their medium for additional 24 hours. The conditioned medium was analyzed for TGFβ1 protein by ELISA, while the cell lysates were analyzed for TGFβ1 mRNA by real-time PCR. As shown in Fig. 2A, SCC9 and SCC25 cells, which have increased N-cadherin expression (Fig. 1), have significantly higher levels of TGFβ1 in conditioned medium compared with those in the other cell lines. Moreover, these cell lines also had increased expression of TGFβ1 mRNA (Fig. 2A, right panel). These findings suggest that the differences in the levels of N-cadherin amongst the different cell lines are because of differences in the levels of TGFβ1 produced. To examine whether TGFβ1 regulates N-cadherin in these cell lines, the different cell lines were plated, serum-starved overnight and then treated with TGFβ1 for 24 hours. E- and N-cadherin expression was determined at protein and mRNA levels. As shown in Fig. 2B, TGFβ1 increased N-cadherin expression without affecting E-cadherin levels. Moreover, TGFβ1-induced N-cadherin expression at cell-cell borders did not affect E-cadherin distribution or cell surface expression (data not shown). The increase was most pronounced in the oral keratinocytes, which have very low basal N-cadherin expression, with a ten- to 20-fold increase in N-cadherin mRNA following TGFβ1 treatment. By contrast, the fold-induction of N-cadherin message was only two- to fivefold in the malignant OSCC cell lines. In addition, we examined the role of TGFβ1 in N-cadherin expression by downregulating or blocking TGFβ1 type I receptor (TGFBR1, hereafter referred to as TβRI), which is known to mediate TGFβ1 signaling (Akhurst and Derynck, 2001; Shi and Massague, 2003). SCC25 cells were transfected with 100 nM of siRNA targeting TβRI , allowed to recover overnight, and then maintained in serum-free medium for 72 hours. As shown in Fig. 2C, TβRI siRNA blocked expression of mRNA (and therefore TβRI protein levels), whereas siCtrl had no effect on the levels of TβRI mRNA in SCC25 cells. Moreover, transfection of

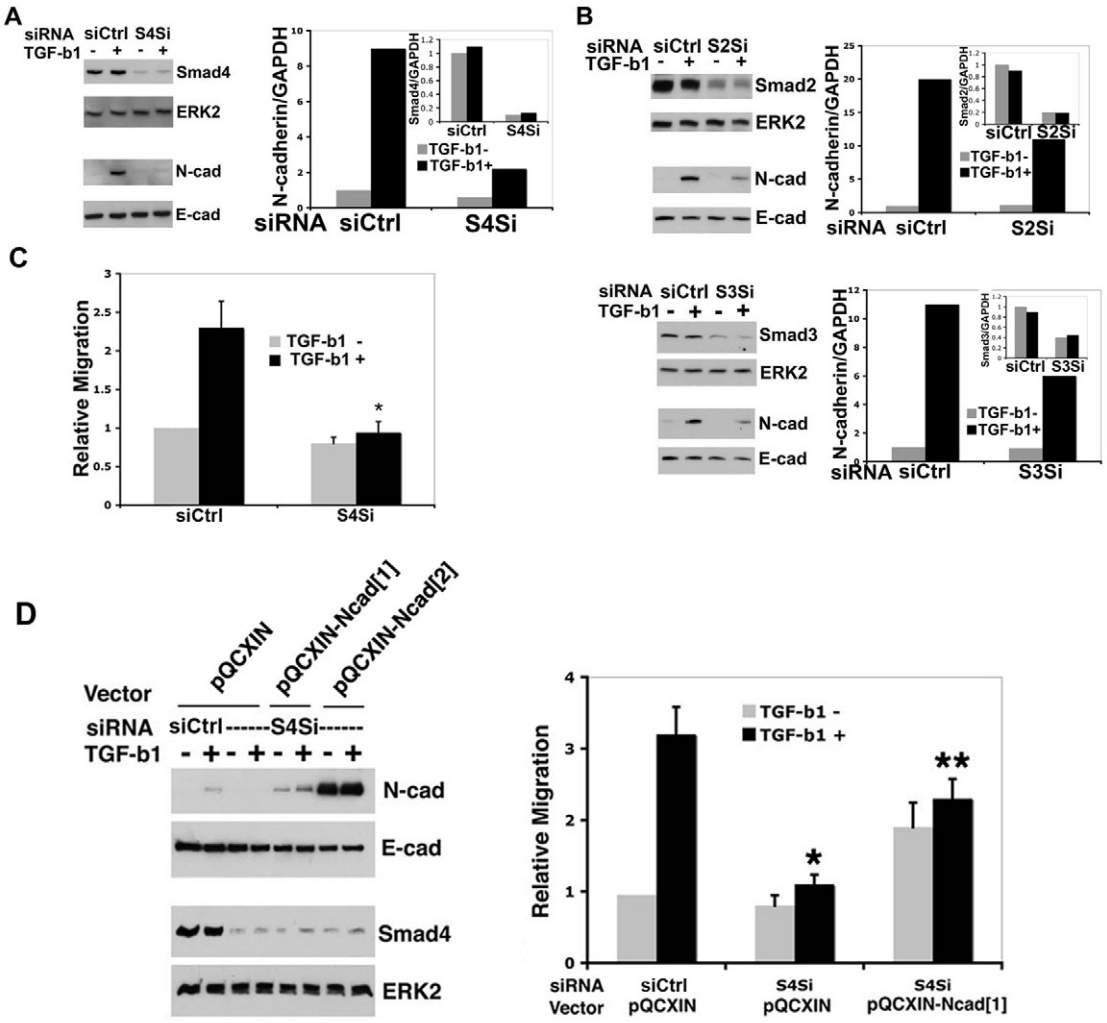

Fig. 3. Smad signaling regulates TGFβ1-mediated N-cadherin expression to promote migration. (A) OKF6 cells were transfected with 100 nM of control siRNA (siCtrl) or S4Si, allowed to recover overnight, serum starved and then treated with TGFβ1 for 24 hours. Smad4, ERK2, E- and N-cadherin protein levels were determined by western blotting. Using real-time PCR, the mRNA levels of Smad4, N-cadherin and GAPDH mRNA expression were determined and normalized to siCtrl TGF-b1 (–) samples. (B) OKF6 cells were transfected with 100 nM of siCtrl, S2Si or S3Si, and then treated with TGFβ1 for 24 hours. Smad2, Smad3, ERK2, E-cadherin and N-cadherin protein expression was determined by western blotting. Using real-time PCR the mRNA levels of Smad2, Smad3, N-cadherin and GAPDH were determined and normalized to siCtrl TGFβ1 (–) samples. (C) OKF6 cells were transfected with siCtrl or S4Si, serum starved and treated with TGFβ1 for 24 hours. The cells were then added to porous polycarbonate filters that had been coated with 5 μg type I collagen, and were allowed to migrate for an additional 24 hours in the presence or absence of TGFβ1. Nonmigratory cells were removed from upper chamber, and migrating cells were counted to determine migration relative to untreated siCtrl-transfected cells. *P<0.05, significantly different from the siCtrl, TGF-b1+ samples. The results are representative of at least three independent experiments. (D) OKF6 cells were transfected with siCtrl or S4Si, and 16 hours later the cells were infected as detailed in the Materials and Methods with viral particles containing the pQCXIN empty vector or pQCXIN-Ncad vector at two different concentrations for 8 hours, serum starved for 16 hours, and then treated with TGFβ1 for an additional 24 hours. N-cadherin, E-cadherin, Smad4 and ERK2 (loading control) were examined by western blotting. siRNA-transfected cells were infected with pQCXIN empty vector or pQCXIN-Ncad vector at the lower concentration and then added to porous polycarbonate filters that had been coated with 5 μg type I collagen, and allowed to migrate for 24 hours in the presence or absence of TGFβ1. Nonmigratory cells were removed from the upper chamber, and migrating cells were counted. *P<0.05, significantly different from the pQCXIN,siCtrl,TGF-b1(+) samples. **P<0.05, significantly different from pQCXIN,S4Si,TGF-b1(+) samples. The results are representative of at least two independent experiments.

TβRI siRNA blocked N-cadherin expression without affecting E-cadherin levels; and treatment of SCC25 cells with a highly specific small-molecule inhibitor (TbRi) that blocks TβRI kinase activity also blocked expression of N-cadherin (Fig. 2D).

We then examined the functional consequences of increased TGFβ1-mediated N-cadherin expression by determining the role of N-cadherin in TGFβ1-mediated migration of OKF6 cells. OKF6 cells were transfected with siCtrl or siNcad, serum starved and then treated with TGFβ1 for 24 hours. As shown in Fig. 2E, siNcad blocked TGFβ1-mediated N-cadherin expression and also significantly blocked TGFβ1-mediated migration of OKF6 cells. Overall, these results demonstrate that the differential production of TGFβ1 by oral keratinocytes and OSCC cells accounts for the differences in the N-cadherin among these cell lines, and TGFβ1 promotes N-cadherin expression to increase migration of oral keratinocytes.

Smad signaling regulates TGFβ1-mediated N-cadherin expression to promote migration of oral keratinocytes

TGFβ1 signals through its cell-surface receptors to increase phosphorylation of receptor-associated Smads (R-Smads), Smad2

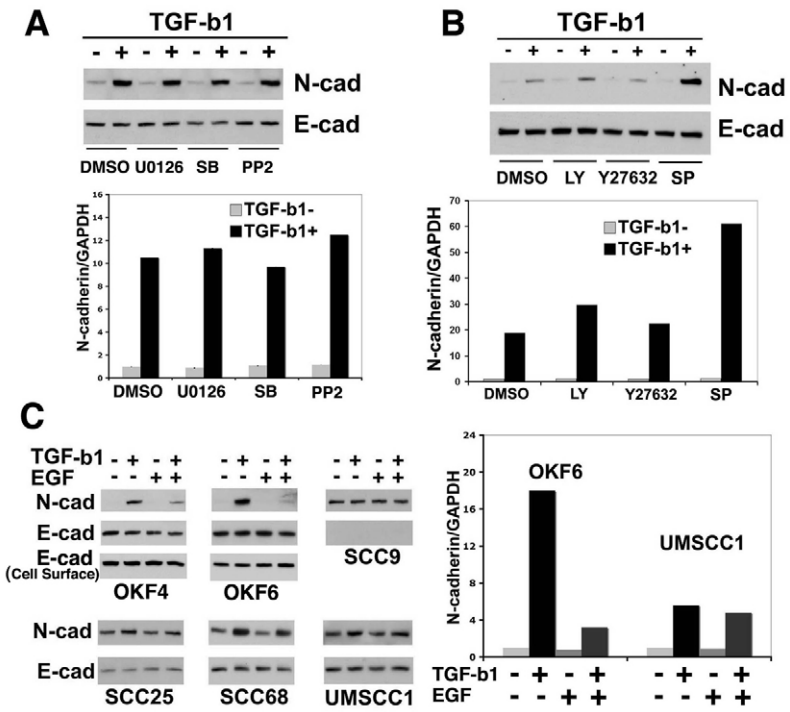

Fig. 4. TGFβ1-mediated N-cadherin expression does not involve ERK1/2, p38 MAPK, Src, PI3-kinase, Rho-associated kinase or Jun, but is blocked by EGF in oral keratinocytes. (A) OKF6 cells were serum starved overnight, pre-treated with DMSO (vehicle control), MEK1/2 inhibitor U0126 (10 μM), p38 MAPK inhibitor SB202190 (SB, 10 μM) or the Src inhibitor PP2 (5 μM) for 30 minutes, and then treated with TGFβ1 for 24 hours. N-cadherin and E-cadherin protein levels were determined by western blot analysis. N-cadherin and GAPDH mRNA expression were determined by real-time PCR and relative expression normalized to DMSO-treated TGF-b1 (–) samples. (B) OKF6 cells were serum starved overnight, pre-treated with DMSO (vehicle control), PI3-kinase inhibitor LY294002 (LY, 10 μM), Rho-associated kinase inhibitor Y27632 (10 μM) or the JNK inhibitor SP600125 (SP, 10 μM) for 30 minutes and then treated with TGFβ1 for 24 hours. N-cadherin and E-cadherin protein levels were determined by western blot analysis. N-cadherin and GAPDH mRNA expression were determined by real time PCR and relative expression normalized to DMSO-treated TGF-b1 (–) samples. The results are representative of three independent experiments. (C) OKF4, OKF6, SCC9, SCC25, SCC68 and UMSCC1 were serum starved overnight and then treated with 20 ng/ml EGF 30 minutes prior to treatment with TGFβ1 (10 ng/ml) for 24 hours. E- and N-cadherin protein expression was determined by western blot analysis. The relative levels of N-cadherin and GAPDH mRNA expression in OKF6 and UMSCC1 cells were determined by real-time PCR, and normalized to untreated samples. The results are representative of at least four independent experiments.

and Smad3 (Akhurst and Derynck, 2001; Shi and Massague, 2003). The R-Smads then bind to Smad4 and translocate to the nucleus to regulate expression of target genes (Akhurst and Derynck, 2001; Shi and Massague, 2003). Thus, we examined the role of Smad signaling in TGFβ1-mediated N-cadherin expression. Initially, we examined the role of Smad4 by downregulating its expression using siRNA and examining the effect on TGFβ1-mediated N-cadherin expression. OKF6 cells were transfected with 100 nM of siRNA targeting Smad4 (S4Si), allowed to recover overnight, serum starved for 8 hours and then treated with TGFβ1 for 24 hours. As shown in Fig. 3A Smad4 siRNA blocked protein and mRNA expression, whereas siCtrl had no effect on the levels of Smad4 in OKF6 cells. Moreover, transfection of S4Si blocked TGFβ1-mediated N-cadherin expression both at the protein and mRNA levels. Similarly, S4Si blocked TGFβ1-mediated N-cadherin expression in OKF4 cells (data not shown). In addition, we examined the effect of Smad2 and Smad3 on TGFβ1-mediated N-cadherin expression using siRNAs targeting Smad2 and Smad3. As shown in Fig. 3B (upper panels), siRNA targeting Smad2 (S2Si) blocked Smad2 at the protein and mRNA levels, and partially blocked TGFβ1-mediated N-cadherin expression in OKF6 cells. Similarly, siRNA targeting Smad3 (S3Si) partially blocked Smad3 at the protein and mRNA levels, and also attenuated TGFβ1-mediated N-cadherin expression (Fig. 3B, lower panels).

Since our results in Fig. 2E show that N-cadherin mediates keratinocyte migration and that the Smad signaling regulates N-cadherin expression, we determined the role of Smad signaling in TGFβ1-mediated migration of OKF6 cells. Since S4Siwas most effective in blocking TGFβ1-mediated N-cadherin expression, the specific role of Smad4 in TGFβ1-mediated migration was determined. OKF6 cells were transfected with siCtrl or S4Si, serum starved, treated with TGFβ1 for 24 hours and then added to transwell chambers for the migration assay. As shown in Fig. 3C, TGFβ1 promoted migration of OKF6 cells transfected with siCtrl, whereas transfection of S4Si significantly blocked TGFβ1-mediated migration of OKF6 cells. Similarly, transfection of S4Si also blocked TGFβ1-mediated migration of OKF4 cells (data not shown). Finally, to examine whether re-introduction of N-cadherin in OKF6 cells transfected with S4Si increased motility, OKF6 cells were transfected initially with siCtrl or S4Si, and 16 hours later were infected with viral particles containing pQCXIN empty vector or pQCXIN-Ncad vector at two different concentrations for 8 hours. Cells were then serum starved for 16 hours and treated with TGFβ1 for an additional 24 hours. As shown in Fig. 3D (left panel), transfection with S4Si blocked TGFβ1-mediated N-cadherin expression, and infection with viral particles containing pQCXIN-Ncad increased N-cadherin expression. Using the lower concentration of viral particles (which resulted in N-cadherin expression comparable with the levels induced following TGFβ1 treatment) we then examined the effect on motility. As previously shown in Fig. 3C, S4Si blocked TGFβ1-mediated motility in vector-infected cells (Fig. 3D, right panel). Importantly, re-expression of N-cadherin in OKF6 cells transfected with S4Si rescued motility (Fig. 3D, right panel).

TGFβ1-mediated N-cadherin expression does not involve ERK1/2, p38 MAPK, Src, PI3-kinase, Rho-associated kinase or Jun, but is blocked by EGF in oral keratinocytes

Since TGFβ1 can also signal through Smad-independent pathways (Akhurst and Derynck, 2001; Shi and Massague, 2003), we examined the role of ERK1/2, p38 mitogen-activated protein kinase (MAPK) and Src in TGFβ1-mediated N-cadherin. Initially, we examined the activation of these signaling pathways following TGFβ1 treatment. Although there was basal activation of all three signaling pathways, TGFβ1 increased phosphorylation of only p38 MAPK in OKF6 cells (data not shown). Because of persistent basal ERK1/2 and Src activity, we examined the role of all three signaling pathways in TGFβ1-mediated N-cadherin expression. OKF6 cells were pre-treated with the MAPK kinases 1 and 2 (MEK1/2) inhibitor U0126, the p38 MAPK inhibitor SB202190, or the Src inhibitor

PP2, and then treated with TGFβ1 for 24 hours. As shown in Fig. 4A, in OKF6 cells TGFβ1 enhanced N-cadherin expression, which was not blocked in the presence of U0126, SB202190 or PP2, suggesting that TGFβ1-mediated N-cadherin protein expression was not regulated by ERK1/2, p38 MAPK or Src in these cells. Moreover, real time PCR showed that these inhibitors did not block TGFβ1-mediated N-cadherin at the mRNA level (Fig. 4A, lower panel). Also these inhibitors failed to block TGFβ1-N-cadherin expression in OKF4, SCC68 and UMSCC1 cells (data not shown). In addition, we examined the role of PI3-kinase, Rho-associated kinase and Jun in TGFβ1-mediated N-cadherin expression. As shown in Fig. 4B, inhibitors of these signaling pathways also failed to block TGFβ1-mediated N-cadherin expression in OKF6 cells.

It has previously been shown in pig thyrocytes that EGF and TGFβ1 cooperate to regulate N-cadherin expression (Grande et al., 2002). Thus, we examined the potential for coordinate regulation of N-cadherin expression by treating cells with TGFβ1 alone, EGF alone or with both EGF and TGFβ1. As shown in Fig. 4C, EGF did not affect expression of N-cadherin in oral keratinocytes or in OSCC cell lines. Interestingly, EGF blocked TGFβ1-induced N-cadherin expression in OKF4 and OKF6 cells, whereas no significant effects were observed in malignant OSCC cells. In addition, EGF did not cooperate with TGFβ1 to modulate expression of E-cadherin or to regulate cell surface levels of E-cadherin in OKF4 and OKF6 cells (Fig. 4C). We also examined the effect of EGF on the TGFβ1-mediated N-cadherin expression at the mRNA level in OKF6 and UMSCC1 cells. As shown in Fig. 4C, EGF blocked TGFβ1-mediated N-cadherin expression in OKF6 cells, but did not affect N-cadherin expression in UMSCC1 cells. These data suggest that EGF blocks TGFβ1-mediated N-cadherin expression in oral keratinocytes, but this inhibitory effect of EGF on TGFβ1-mediated N-cadherin expression is absent in the malignant OSCC cells.

EGF attenuates TGFβ1-mediated Smad2 C-terminal phosphorylation and Smad-driven promoter activity, but does not attenuate TGFβ1-mediated expression of TGIF, SnoN or cSki

Since our results in Fig. 3 show that TGFβ1-mediated N-cadherin expression involves Smads, we examined the effect of EGF on TGFβ1-mediated Smad signaling. OKF6 cells were treated with TGFβ1 or pre-treated with EGF and then treated with TGFβ1, and the effect on Smad2 phosphorylation was determined using a phosphorylation-specific antibody. As shown in Fig. 5A, TGFβ1 transiently increased Smad2 phosphorylation, and EGF attenuated Smad2 phosphorylation at 3 hours and 5 hours, with the decrease more clearly demonstrated at 8 hours (Fig. 5B). In addition, we examined the effect of EGF on TGFβ1-induced nuclear translocation of Smad2. OKF6 cells were plated on glass coverslips, serum starved, treated with EGF for 30 minutes prior to treatment with TGFβ1 for the indicated times and then immunostained for Smad2. As shown in Fig. 5A, treatment with TGFβ1 enhanced nuclear translocation of Smad2, which was not affected by EGF treatment at 1 hour; however, EGF attenuated Smad2 nuclear translocation at 8 hours (Fig. 5B). These results suggest that EGF regulates TGFβ1-mediated Smad signaling by promoting Smad2 dephosphorylation and by attenuating the levels of Smad2 in the nucleus.

We also examined the effect of EGF on TGFβ1-mediated Smad4-driven transcription using a promoter-luciferase construct that contains four Smad-binding elements (SBE4) (Zawel et al., 1998). OKF6 cells were transfected with the SBE4 promoter-luciferase construct using the Amaxa nucleofector kit, allowed to recover overnight, serum starved for 8 hours, and then treated with EGF for 30 minutes prior to addition of TGFβ1 for an additional 24 hours. As shown in Fig. 5C, TGFβ1 increased Smad-driven promoter activity eightfold in OKF6 cells and, although EGF demonstrated a small but consistent increase in SBE4 luciferase activity, it significantly blocked TGFβ1-mediated increase in the promoter activity. In contrast to the effect of EGF on TGFβ1-mediated Smad-driven transcription in OKF6 cells, EGF had a minimal effect on Smad-driven transcription in UMSCC1 cells (data not shown). Since EGF can block Smad-mediated transcription by increasing the expression of co-repressors such as TGIF, Ski and SnoN (also known as SKIL) (Sun et al., 1999; Yang et al., 2005), we also examined the effect of EGF on the expression of these co-repressors. Although TGFβ1 increased the mRNA levels of these co-repressors, their expression was not affected by EGF in OKF6 cells (Fig. 5D).

EGF promotes phosphorylation of the linker region of Smad2 through ERK1/2 in oral keratinocytes

It has been shown previously that ERK1/2 can phosphorylate the linker regions of Smad2 and Smad3 to inhibit Smad signaling (Kretzschmar et al., 1997; Kretzschmar et al., 1999; Lo et al., 2001). We, therefore, examined the effect of EGF in promoting phosphorylation of the Smad2 linker region in oral keratinocytes. Initially, we examined the effect of EGF and TGFβ1 on activation of ERK1/2 in OKF6 cells. As shown in Fig. 6A, only EGF promoted ERK1/2 phosphorylation, and TGFβ1 did not modulate EGF-induced ERK1/2 phosphorylation. Moreover, the MEK1/2 inhibitor U0126 blocked EGF-mediated phosphorylation of ERK1/2 (Fig. 6B, upper panels). We then examined the effect of EGF on phosphorylation of the Smad2 linker region by using an antibody that specifically recognizes phosphorylated Ser245, Ser250 and Ser255 of Smad2. As shown in Fig. 6C (upper panels), treatment of OKF6 cells with EGF promoted phosphorylation within the Smad2 linker region, which was blocked in the presence of U0126. Although EGF promoted MEK1/2-dependent phosphorylation of ERK1/2 in UMSCC1 cells (Fig. 6B, lower panels), EGF did not enhance phosphorylation of Smad2 in the linker region in UMSCC1 cells (Fig. 6C, lower panels).

ERK1/2 mediates effect of EGF on TGFβ1-mediated Smad-driven promoter activity and N-cadherin expression by oral keratinocytes

We then examined the role of ERK1/2 signaling in the EGF-mediated downregulation of the Smad-driven promoter activity. OKF6 cells were transfected with SBE4 promoter-luciferase construct, serum-starved, and then pre-treated with dimethyl sulfoxide (DMSO; as control) or U0126 for 30 minutes prior to treatment with EGF and/or TGFβ1 for 24 hours. As shown previously in Fig. 5C, TGFβ1 increased the promoter activity of SBE4 promoter-luciferase construct, which was blocked with EGF in control DMSO-treated samples (Fig. 7A). Interestingly, pre-treatment with U0126 blocked the effect of EGF on TGFβ1-mediated Smad-driven promoter activity (Fig. 7A). Similarly, we examined the role of EGF-ERK1/2 signaling in modulating TGFβ1-mediated N-cadherin expression in the malignant OSCC cell line UMSCC1. TGFβ1 increased Smad-driven promoter activity; however, neither EGF nor U0126 had any effect on TGFβ1-mediated Smad-driven promoter activity (Fig. 7B). Overall, these

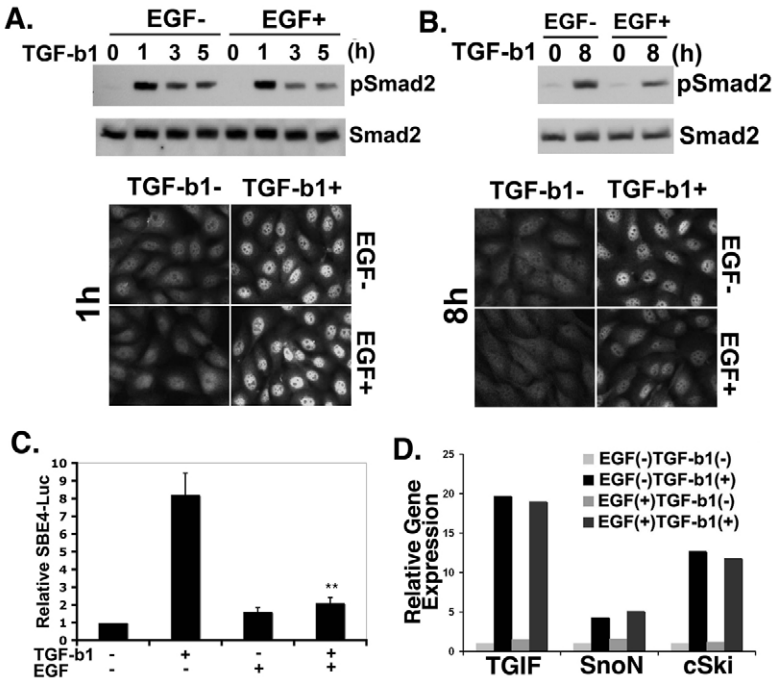

Fig. 5. EGF attenuates TGFβ1-mediated Smad2 C-terminal phosphorylation and Smad-driven promoter activity, but does not attenuate TGFβ1-mediated expression of TGIF, SnoN or cSki. (A,B) OKF6 cells were serum starved overnight and then pre-treated with EGF (20 ng/ml) for 30 minutes prior to the addition of TGFβ1 (10 ng/ml) for the indicated time points. The cell lysates were then probed for phosphorylated Smad2, and then stripped and re-probed for total Smad2. OKF6 cells were plated onto glass coverslips, serum starved overnight and then pre-treated with EGF (20 ng/ml) for 30 minutes, and then treated with TGFβ1 for 1-8 hours. The cells were then fixed in glutaraldehyde, permeabilized with Triton X-100, incubated with anti-Smad2 antibody followed by Alexa-Fluor-488-labeled secondary antibody. The immunofluorescence signal was detected using Zeiss microscope. (C) OKF6 cells were transfected with 1 μg of SBE-4 reporter luciferase construct, and then equal numbers of cells were plated overnight in 12-well tissue culture plates, serum starved for 8 hours and then pre-treated with EGF (20 ng/ml) for 30 minutes prior to treatment with TGFβ1 (10 ng/ml) for 24 hours. The cells were lysed, luciferase activity was quantified, normalized to untreated samples arbitrarily set at 1.0. $^{**}P<0.005$, significantly different from EGF-,TGF-b1+ samples with. The results are representative of at least four independent experiments. (D) OKF6 cells were serum starved overnight and then treated with 20 ng/ml EGF 30 minutes prior to treatment with TGFβ1 (10 ng/ml) for 24 hours. The relative levels of TGIF, SnoN, cSki and GAPDH mRNA expression were determined by real time PCR, and normalized to untreated samples. The results are representative of at least two independent experiments.

results suggest that EGF – through ERK1/2 signaling – blocks TGFβ1-mediated SBE4 promoter activity in oral keratinocytes but not in malignant cells.

We also examined the contribution of ERK1/2 in modulating the effect of EGF on TGFβ1-mediated N-cadherin expression. OKF6 cells were pre-treated with U0126 30 minutes before adding EGF. The cells were then treated with TGFβ1 for an additional 24 hours. As shown previously in Fig. 4A, in DMSO-treated control samples TGFβ1 increased N-cadherin expression, which was blocked with EGF (Fig. 8A). Interestingly, U0126 reversed the effect of EGF on TGFβ1-mediated N-cadherin expression without affecting E-cadherin levels. Similarly, U0126 reversed the effect of EGF on TGFβ1-mediated N-cadherin expression in OKF4 cells. In agreement with the changes in N-cadherin protein level, real-time PCR showed that U0126 reversed the changes at the mRNA level (Fig. 8B). These results indicate that the inhibitory effect of EGF on TGFβ1-mediated N-cadherin expression is mediated through the MEK1/2-ERK1/2 signaling pathway.

EGF attenuates TGFβ1-mediated migration of oral keratinocytes, but increases TGFβ1-mediated migration of OSCC cells

Since TGFβ1 promotes migration of oral keratinocytes through Smad-N-cadherin signaling (Figs 2 and 3) and because EGF attenuates Smad-dependent N-cadherin expression in oral keratinocytes (Fig. 8), we examined the effect of EGF on TGFβ1-mediated migration in OKF6 cells. As control, we also examined the effect of EGF on TGFβ1-mediated migration of UMSCC1 cells. OKF6 and UMSCC1 cells were plated in six-well plates, serum starved and treated with EGF and/or TGFβ1 for 24 hours. The cells were then plated onto transwell chambers that had been coated with type I collagen, for additional 24 hours in the presence or absence of TGFβ1 and/or EGF. As shown in Fig. 9, EGF increased migration of OKF6 and UMSCC1 cells two- and tenfold, respectively. Similarly, TGFβ1 increased migration of OKF6 and UMSCC1 cells six- and fourfold, respectively. Interestingly, EGF attenuated TGFβ1-mediated migration of OKF6 cells whereas promoting TGFβ1-induced migration of UMSCC1 cells.

Discussion

Cadherins have an important role in the behavior of keratinocytes and SCC cells (Al Moustafa et al., 2002; Munshi and Stack, 2006; Nagata et al., 2003). Both loss of E-cadherin and the increased expression of N-cadherin are associated with SCC progression (Behrens et al., 1992; Hazan et al., 2004; Islam et al., 1996; Munshi and Stack, 2006; Schipper et al., 1991). Our results show that N-cadherin is expressed in OSCC cells to regulate motility. Our results also show that TGFβ1 promotes Smad-mediated N-cadherin expression – without affecting E-cadherin levels or cellular localization – to regulate motility of oral keratinocytes. Interestingly, EGF blocked TGFβ1-mediated N-cadherin expression and motility by attenuating TGFβ1-mediated Smad signaling in oral keratinocytes but not in malignant OSCC cells.

TGFβ1 has previously been shown to increase expression of N-cadherin (Grande et al., 2002; Maeda et al., 2006); however, the mechanism by which TGFβ1 regulates N-cadherin in keratinocytes has not been clearly defined. Although ERK1/2 and Src were shown to be involved in TGFβ1-mediated N-cadherin expression in breast cancer cells and pig thyrocytes (Grande et al., 2002; Maeda et al., 2006), inhibitors of these signaling pathways did not affect TGFβ1-mediated N-cadherin expression in oral keratinocytes or in the OSCC cells. Moreover, inhibitors of p38 MAPK, PI3-kinase, Rho-associated kinase and Jun kinase also did not block TGFβ1-mediated N-cadherin expression. By contrast, inhibition of Smad expression using siRNAs clearly demonstrated the role of Smad2, Smad3 and Smad4 in the regulatory process. Moreover, EGF abrogated TGFβ1-mediated N-cadherin expression in oral keratinocytes cells by attenuating Smad-mediated signaling.

Growth factors such as EGF can negatively regulate TGFβ1 signaling at different steps in the Smad signaling pathway. EGF

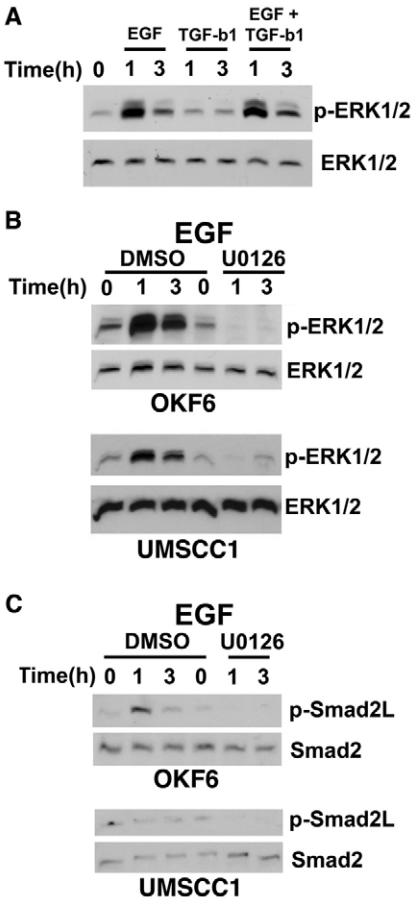

Fig. 6. EGF promotes phosphorylation of the linker region of Smad2 by ERK1/2 in oral keratinocytes. (A) OKF6 cells were serum starved overnight and then treated with EGF (20 ng/ml), TGFβ1 (10 ng/ml), or with EGF (20 ng/ml) for 30 minutes prior to the addition of TGFβ1 (10 ng/ml) for the indicated time points. The cell lysates were probed for phosphorylated ERK1/2, and then stripped and re-probed for total ERK1/2. (B) OKF6 or UMSCC1 cells were serum starved overnight and then pre-treated with DMSO (as vehicle control) or MEK1/2 inhibitor U0126 (10 μM) for 30 minutes prior to the addition of EGF (20 ng/ml) for the indicated time points. The cell lysates were probed for phosphorylated ERK1/2, and then stripped and re-probed for total ERK1/2. (C) OKF6 and UMSCC1 cells were serum starved overnight and then pre-treated with DMSO (vehicle control) or U0126 (10 μM) for 30 minutes prior to the addition of EGF (20 ng/ml) for the indicated time points. Cell lysates were probed for Smad2 phosphorylation in the linker region (p-Smad2L), and then stripped and re-probed for total Smad2. The results are representative of at least two independent experiments.

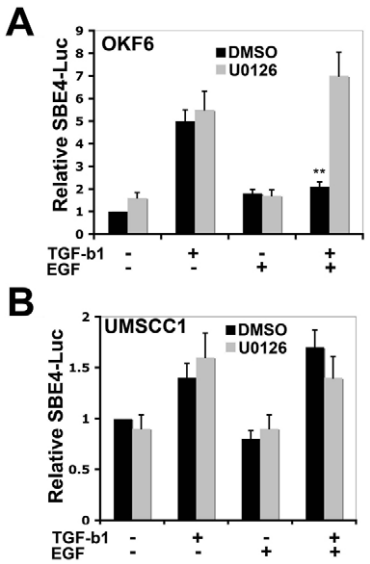

Fig. 7. ERK1/2 mediates the effect of EGF on TGFβ1-mediated Smad-driven promoter activity. OKF6 (A) and UMSCC1 (B) cells were transfected with 1 μg of SBE-4 luciferase construct, and equal numbers of cells were plated overnight, serum starved for 8 hours, and then treated with either DMSO or U0126 for 30 minutes prior to treatment with EGF (20 ng/ml). The samples were then treated with TGFβ1 (10 ng/ml) 30 minutes later for additional 24 hours. The samples were lysed, luciferase activity was quantified, and values normalized to DMSO-treated untreated samples arbitrarily set at 1.0. **P*<0.05, significantly different from the DMSO,EGF(–),TGF-b1(+) samples. The results are representative of at least three independent experiments.

has previously been shown to promote levels of inhibitory Smad7 to block TGFβ1-mediated phosphorylation of R-Smads (Nakao et al., 1997); however, no changes in the levels of Smad7 were detected following EGF treatment of OKF4 or OKF6 cells (data not shown). EGF signaling can also promote degradation of Smad4 to attenuate TGFβ1 signaling (Saha et al., 2001); however, no changes in Smad4 level were detected following EGF treatment of both OKF4 and OKF6 cells (data not shown). Moreover, EGF can block Smad-mediated transcription by increasing expression of co-repressors, such as TGIF, Ski and SnoN (Sun et al., 1999; Yang et al., 2005); however, no changes in the expression of these co-repressors were detected in OKF4 or OKF6 cells following EGF treatment.

Our results show that the MEK1/2 inhibitors block the effect of EGF, which indicates that the inhibitory effect of EGF on TGFβ1-mediated N-cadherin expression is mediated through ERK1/2. It has been shown previously that EGF signal via ERK1/2 to promote phosphorylation within the linker region of R-Smads to inhibit R-Smad activity (Kretzschmar et al., 1997; Kretzschmar et al., 1999; Lo et al., 2001). Interestingly, EGF enhanced Smad2 linker phosphorylation through ERK1/2 only in the oral keratinocytes and not in the OSCC cells. EGF can also block TGFβ1 signaling by preventing translocation of R-Smads to the nucleus (Kretzschmar et al., 1997; Kretzschmar et al., 1999; Lo et al., 2001). Note that the effect of EGF on TGFβ1-mediated Smad nuclear translocation is dependent on the concentration of TGFβ1 and on the particular cell line studied. EGF blocked Smad2/3 nuclear translocation in HaCat cells only when the TGFβ1 concentration was relatively low (Lo et al., 2001). Moreover, EGF had no effect on TGFβ1-mediated nuclear translocation of Smad2 in primary human cancer cells (Dunfield and Nachtigal, 2003). EGF did not block TGFβ1-mediated nuclear translocation of Smad2 in OKF6 cells at 1 hour, irrespective of the TGFβ1 treatment dose. However, there were decreased levels of both phosphorylated R-Smads and nuclear Smad2 in the EGF-treated samples at 8 hours. These data indicate that EGF by phosphorylating the linker region of R-Smads regulate the duration of TGFβ1 signaling, as it was recently shown to be the case with MAPKs regulating bone morphogenetic protein (BMP)-Smad1 signaling during embryonic pattern formation by phosphorylating the linker region and attenuating the duration of C-terminal Smad1 phosphorylation (Fuentealba et al., 2007). Alternatively, EGF may be promoting dephosphorylation of R-Smads and attenuating the levels of Smad protein in the nucleus by increasing expression and/or activity of phosphatases. Recently, it has been shown that dephosphorylation of the sites in the linker region of Smad2/3 is mediated by small C-terminal domain phosphatase 1, 2 and 3 (SCP1,

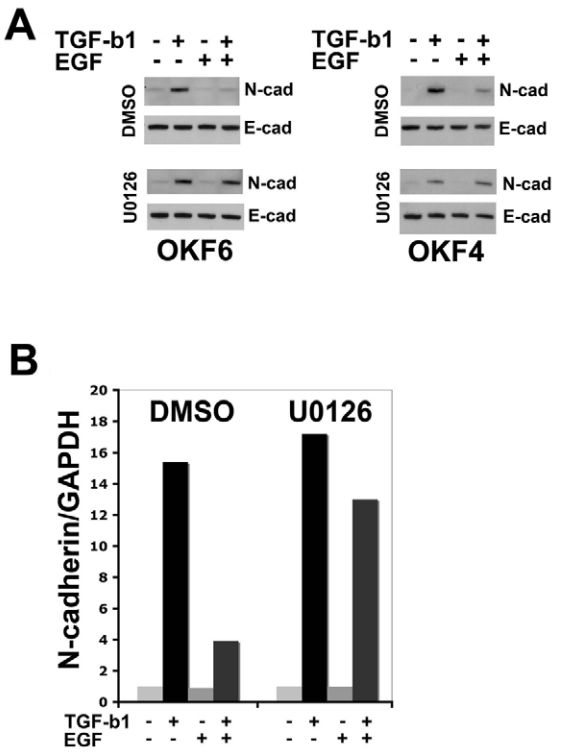

Fig. 8. MAPK/ERK1/2 inhibition reverses the effect of EGF on TGFβ1-mediated N-cadherin expression. Serum-starved OKF4 and OKF6 cells were pre-treated with DMSO (as vehicle control), or MEK1/2 inhibitor U0126 (10 μM) for 30 minutes prior to treatment with EGF (20 ng/ml). The samples were then treated 30 minutes later with TGFβ1 (10 ng/ml) for an additional 24 hours. (A) E- and N-cadherin protein levels were determined by western blot analysis. (B) N-cadherin and GAPDH mRNA levels were determined in OKF6 cells using real-time PCR, and normalized to DMSO,EGF(–),TGF-b1(–) samples arbitrarily set at 1.0. The results are representative of four independent experiments.

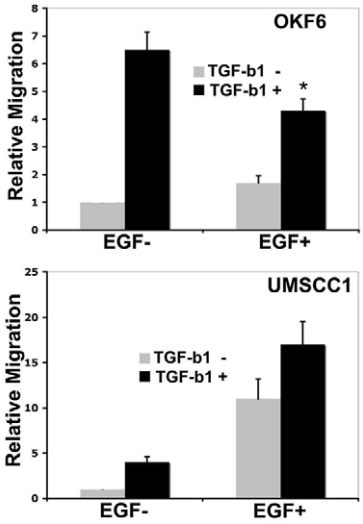

Fig. 9. EGF attenuates TGFβ1-mediated migration of oral keratinocytes, but increases TGFβ1-mediated migration of OSCC cells. Serum-starved OKF6 and UMSCC1 cells were untreated, treated with EGF (20 ng/ml) or TGFβ1 (10 ng/ml), or pre-treated with EGF (20 ng/ml) for 30 minutes and then treated with TGFβ1 for 24 hours. The cells were then added to transwell chambers that had been coated with type I collagen, for additional 24 hours in the presence or absence of TGFβ1 and/or EGF. Nonmigratory cells were removed from upper chamber, and migrating cells were counted. *P<0.05, significantly different from the EGF(–),TGF-b1(+) OKF6 cells. The results are representative of at least two independent experiments.

SCP2 and SCP3, respectively (Knockaert et al., 2006; Sapkota et al., 2006; Wrighton et al., 2006), whereas dephosphorylation of the C-terminus SxS site in Smad2 and Smad3 involves PPMIA (PP2Ca) (Lin et al., 2006; Schilling et al., 2006). It is important to determine the extent to which these phosphatases are involved downstream of EGF signaling to modulate Smad signaling in oral keratinocytes and also in OSCC cells.

Physiologically, R-Smads integrate diverse and opposing signals generated by growth factors and by members of TGF-β superfamily. Growth-factor-mediated activation of ERK1/2 blocks BMP-mediated differentiation of ectoderm to mesoderm during *Xenopus* development (Aubin et al., 2004; Massague, 2003). Also, hepatocyte growth factor (HGF) prevents, via ERK1/2, TGFβ1-mediated loss of E-cadherin in human proximal tubular epithelial cells (Yang et al., 2005). In addition, EGF blocks TGFβ1-mediated antiproliferative effects on primary human ovarian cells (Dunfield and Nachtigal, 2003). Overall, growth factor signaling in these particular examples abrogates the effect of TGFβ1 to maintain the cells in a more 'epithelial' state. Similarly, EGF, via ERK1/2, inhibits TGFβ1-mediated N-cadherin expression only in the oral keratinocytes, presumably, to keep these cells in a more 'epithelial' state. By contrast, this inhibitory effect is absent in malignant OSCC cells, suggesting that loss of this regulatory mechanism accompanies malignant transformation of the oral epithelium.

Materials and Methods

Materials

TGFβ1 and peroxidase-conjugated secondary antibodies were purchased from Sigma (St Louis, MO). Dulbecco's modified Eagle's medium (DMEM), Ham's F-12 and keratinocyte-SFM were obtained from Life Technologies (Grand Island, NY). Monoclonal anti-E-cadherin and anti-N-cadherin antibodies, rabbit anti-Smad2 antibody and EGF were purchased from Invitrogen (Carlsbad, CA). Rabbit anti-Smad4, anti-C-terminal phosphorylated Smad2 (at Ser 465 and Ser467), anti-linker region phosphorylated Smad2 (at Ser245, Ser250, Ser255) and anti-TGFβ1 type I receptor antibodies were obtained from Cell Signaling (Danvers, MA), whereas rabbit anti-Smad3 antibody was purchased from Abcam (Cambridge, MA). Alexa-Fluor-488-conjugated goat anti-mouse secondary antibody was purchased from Molecular Probes (Portland, OR). Rabbit anti-phosphorylated p38 MAPK, anti-p38 MAPK and anti-phosphorylated ERK1/2 antibodies were obtained from Cell Signaling (Danvers, MA), whereas anti-ERK2 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-phosphrylated Src (Tyr418) and anti-Src antibodies were obtained from Biosource (Camarillo, CA). The MEK inhibitor U0126, the p38 MAPK inhibitor SB202190, the Src kinase inhibitor PP2, the PI3-kinase inhibitor LY294002, the Rho-associated kinase inhibitor Y27632, the JNK inhibitor SP600125, and the TGFβ1 type I receptor inhibitor (TbRi; catalog no. 616451) were obtained from Calbiochem (LaJolla, CA). Supersignal enhanced chemiluminescence reagent, EZ-Link Sulfo-NHS-LC-Biotin and UltraLink immobilized streptavidin gel were obtained from Pierce (Rockford, IL). Polyvinylidene difluoride membranes were purchased from Millipore (Bedford, MA). A nucleofector electroporation kit specifically designed for keratinocytes was obtained from Amaxa (Gaithersburg, MD).

Cell culture

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 (ATCC) (Munshi et al., 2004; Rheinwald and Beckett, 1981), whereas UMSCC1 cells were generously provided by Ernst Lengyel (University of Chicago, IL) (Juarez et al., 1993; Lengyel et al., 1995). SCC68 cells and tert-immortalized normal oral keratinocytes (OKF4 and OKF6 cells) were kindly provided by James G. Rheinwald (Brigham and Women's Hospital, Harvard Institutes of Medicine, Boston, MA) (Dickson et al., 2000; Mukhopadhyay et al., 2004). SCC9, SCC25 and UMSCC1 cells were routinely maintained in DMEM and Ham's F-12 medium (1:1) supplemented with 10% fetal calf serum and 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 EGF, and 0.31 mM $CaCl_2$. The cells were plated in serum-containing medium, serum-starved overnight prior to treatment with EGF and/or TGFβ1. In additional experiments, inhibitors were added 30 minutes prior to treatment with EGF and/or TGFβ1.

Retroviral infection of OKF6 cells

Human N-cadherin cDNA (catalog no. SC119018, OriGene, Rockville, MD) was subcloned into the retroviral pQCXIN vector (Clontech, Mountain View, CA) and verified by DNA sequencing. To generate retroviral particles, GP2-293 packaging cells were transfected with pQCXIN vector or pQCXIN vector expressing human N-cadherin (pQCXIN-Ncad) and cotransfected 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 OKF6 cells in the presence of polybrene 4 μg/ml.

Migration assay

Migratory activity was quantified using transwell inserts (8 μm pore size) coated with type I collagen (5 μg) on the upper surface of the membrane (Munshi et al., 2004). Oral keratinocytes or OSCC cells (2×10^5) were added to the upper chamber in 500 μl of supplement-free or serum-free medium. In selected experiments, TGFβ1 (10 ng/ml) was added to the lower well that contained 500 μl of medium to promote migration. Nonmigratory cells were removed 24 hours later with a cotton swab from the upper chamber, filters were fixed and stained with Diff-Quik Stain, and migratory cells adherent to the underside of the filter were enumerated using an ocular micrometer and counting a minimum of five high-powered fields.

Analysis of E- and N-cadherin

Equal amounts of whole-cell lysates were analyzed for E- and N-cadherin protein expression by SDS-PAGE (9%). In addition, E- and N-cadherin mRNA levels were detected using real-time PCR. Briefly, reverse transcription of RNA to cDNA was performed using GeneAmp RNA PCR kit (Applied Biosystems). Quantitative gene expression was performed for E-cadherin, N-cadherin 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 (Ottaviano et al., 2006).

Downregulation of N-cadherin and TGFβ1 type I receptor expression

N-cadherin expression was transiently downregulated using a 1:1 mixture of the following duplex siRNAs: siN-Cad1 5′-GGAGUCAGCAGAAGUUGAAdTdT-3′ and siN-Cad2 5′-CCGUGUCUGUUACAGUUAUdTdT-3′ (De Wever et al., 2004). SCC9, SCC25 or OKF6 cells were transiently transfected with 50 nM each of siNcad1 and siNcad2 or 100 nM of control siRNA (siCtrl), using the Amaxa nucleofector kit, allowed to recover overnight, and the effect on N-cadherin expression was determined 72 hours following transfection by western blotting and real-time PCR. Similarly, TGFβ1 type I receptor (TβRI) expression was transiently downregulated using a validated siRNA that targets TβRI purchased from Ambion (Austin, TX).

Downregulation of Smad2, Smad3 and Smad4 expression

Expression of Smad2 and Smad4 was transiently downregulated using the following duplex siRNAs: Smad4 siRNA (S4Si) forward (fw) 5′-UGAAGGACAUUCA-AUUCAAdTdT-3′ and reverse (rev) 5′-UUGAAUUGAAUGUCCUUCAdTdT-3′ (Deckers et al., 2006); Smad2 siRNA (S2Si) fw 5′-GUCCCAUGAAAA-GACUUAAdTdT-3′ and rev 5′-UUAAGUCUUUUCAUGGGAC-dTdT-3′ (Cucoranu et al., 2005). Smad3 expression was downregulated using Smad3-specific siRNA (S3Si; catalog no. CLG1108) purchased from Cellogenetics (Ijamsville, MD). OKF6 cells were transiently transfected with 100 nM of S4Si, S3Si, S2Si or siCtrl using the Amaxa nucleofector kit, allowed to recover overnight, serum-starved for 24 hours and then treated with TGFβ1 for 24 hours. Equal amounts of the samples were then examined for Smad2, Smad3 or Smad4 expression by western blotting or real-time PCR. In addition, equal amounts of the samples were analyzed for E- and N-cadherin expression at the protein and mRNA levels.

Analysis of C-terminal- and Smad2-linker-region phosphorylation and Smad2 immunofluorescence

OKF6 cells were serum starved overnight, pre-treated with EGF for 30 minutes, and then treated with TGFβ1 for different times. Equal amounts of the cell lysates were analyzed for Smad2 phosphorylation at the C-terminus and in the linker region by western blot analysis. The effect on Smad2 nuclear translocation was determined by plating OKF6 cells onto glass coverslips; cells were then serum starved, pre-treated with EGF for 30 minutes, after which they were treated with TGFβ1 for different times. Cells were fixed with 3.7% formaldehyde, permeabilized with 0.1% Triton X-100, incubated with anti-Smad2 antibody followed by Alexa-Fluor-488-conjugated secondary antibody. Cells were then mounted and observed using a Zeiss fluorescence microscope.

Smad luciferase activity

The pBV-Luc vector, which expresses four copies of the Smad-binding site (SBE4-luciferase reporter vector) was kindly provided by B. Vogelstein (Howard Hughes Medical Institute and Johns Hopkins University) (Zawel et al., 1998). OKF6 and UMSCC1 cells were transfected with 1 μg of SBE4 luciferase reporter vector, and equal numbers of cells were plated overnight in tissue culture plates. Cells were then serum starved for 8 hours, pre-treated with EGF , followed by treatment with with TGFβ1 for 24 hours. Cells were lysed and relative luciferase activity was determined by luminometry. The results were normalized to protein concentration and expressed relative to untreated control cells.

Statistical analysis

Statistical analyses were done using GraphPad Instat 3 (San Diego, CA).

This research was supported by grant K08CA94877 (H.G.M.) from the National Cancer Institute and a Merit grant award from the Department of Veterans Affairs (H.G.M.). H.G.M. gratefully acknowledges M. Sharon Stack for her support and encouragement throughout this project.

References

Akhurst, R. J. and Derynck, R. (2001). TGF-beta signaling in cancer-a double-edged sword. *Trends Cell Biol.* **11**, S44-S51.

Al Moustafa, A. E., Alaoui-Jamali, M. A., Batist, G., Hernandez-Perez, M., Serruya, C., Alpert, L., Black, M. J., Sladek, R. and Foulkes, W. D. (2002). Identification of genes associated with head and neck carcinogenesis by cDNA microarray comparison between matched primary normal epithelial and squamous carcinoma cells. *Oncogene* **21**, 2634-2640.

Angst, B. D., Marcozzi, C. and Magee, A. I. (2001). The cadherin superfamily: diversity in form and function. *J. Cell Sci.* **114**, 629-641.

Aubin, J., Davy, A. and Soriano, P. (2004). In vivo convergence of BMP and MAPK signaling pathways: impact of differential Smad1 phosphorylation on development and homeostasis. *Genes Dev.* **18**, 1482-1494.

Behrens, J., Frixen, U., Schipper, J., Weidner, M. and Birchmeier, W. (1992). Cell adhesion in invasion and metastasis. *Semin. Cell Biol.* **3**, 169-178.

Cucoranu, I., Clempus, R., Dikalova, A., Phelan, P. J., Ariyan, S., Dikalov, S. and Sorescu, D. (2005). NAD(P)H oxidase 4 mediates transforming growth factor-beta1-induced differentiation of cardiac fibroblasts into myofibroblasts. *Circ. Res.* **97**, 900-907.

De Wever, O., Westbroek, W., Verloes, A., Bloemen, N., Bracke, M., Gespach, C., Bruyneel, E. and Mareel, M. (2004). Critical role of N-cadherin in myofibroblast invasion and migration in vitro stimulated by colon-cancer-cell-derived TGF-beta or wounding. *J. Cell Sci.* **117**, 4691-4703.

Deckers, M., van Dinther, M., Buijs, J., Que, I., Lowik, C., van der Pluijm, G. and ten Dijke, P. (2006). The tumor suppressor Smad4 is required for transforming growth factor beta-induced epithelial to mesenchymal transition and bone metastasis of breast cancer cells. *Cancer Res.* **66**, 2202-2209.

Dickson, M. A., Hahn, W. C., Ino, Y., Ronfard, V., Wu, J. Y., Weinberg, R. A., Louis, D. N., Li, F. P. and Rheinwald, J. G. (2000). 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.* **20**, 1436-1447.

Dunfield, L. D. and Nachtigal, M. W. (2003). Inhibition of the antiproliferative effect of TGFbeta by EGF in primary human ovarian cancer cells. *Oncogene* **22**, 4745-4751.

Forastiere, A., Koch, W., Trotti, A. and Sidransky, D. (2001). Head and neck cancer. *N. Engl. J. Med.* **345**, 1890-1900.

Fuentealba, L. C., Eivers, E., Ikeda, A., Hurtado, C., Kuroda, H., Pera, E. M. and De Robertis, E. M. (2007). Integrating patterning signals: Wnt/GSK3 regulates the duration of the BMP/Smad1 signal. *Cell* **131**, 980-993.

Grande, M., Franzen, A., Karlsson, J. O., Ericson, L. E., Heldin, N. E. and Nilsson, M. (2002). Transforming growth factor-beta and epidermal growth factor synergistically stimulate epithelial to mesenchymal transition (EMT) through a MEK-dependent mechanism in primary cultured pig thyrocytes. *J. Cell Sci.* **115**, 4227-4236.

Gumbiner, B. M. (2005). Regulation of cadherin-mediated adhesion in morphogenesis. *Nat. Rev. Mol. Cell Biol.* **6**, 622-634.

Hazan, R. B., Qiao, R., Keren, R., Badano, I. and Suyama, K. (2004). Cadherin switch in tumor progression. *Ann. N. Y. Acad. Sci.* **1014**, 155-163.

Islam, S., Carey, T. E., Wolf, G. T., Wheelock, M. J. and Johnson, K. R. (1996). Expression of N-cadherin by human squamous carcinoma cells induces a scattered fibroblastic phenotype with disrupted cell-cell adhesion. *J. Cell Biol.* **135**, 1643-1654.

Jemal, A., Siegel, R., Ward, E., Hao, Y., Xu, J., Murray, T. and Thun, M. J. (2008). Cancer statistics, 2008. *CA Cancer J. Clin.* **58**, 71-96.

Juarez, J., Clayman, G., Nakajima, M., Tanabe, K. K., Saya, H., Nicolson, G. L. and Boyd, D. (1993). 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* **55**, 10-18.

Knockaert, M., Sapkota, G., Alarcon, C., Massague, J. and Brivanlou, A. H. (2006). Unique players in the BMP pathway: small C-terminal domain phosphatases dephosphorylate Smad1 to attenuate BMP signaling. *Proc. Natl. Acad. Sci. USA* **103**, 11940-11945.

Kretzschmar, M., Doody, J. and Massague, J. (1997). Opposing BMP and EGF signalling pathways converge on the TGF-beta family mediator Smad1. *Nature* **389**, 618-622.

Kretzschmar, M., Doody, J., Timokhina, I. and Massague, J. (1999). A mechanism of repression of TGFbeta/ Smad signaling by oncogenic Ras. *Genes Dev.* **13**, 804-816.

Lengyel, E., Gum, R., Juarez, J., Clayman, G., Seiki, M., Sato, H. and Boyd, D. (1995). Induction of M(r) 92,000 type IV collagenase expression in a squamous cell carcinoma cell line by fibroblasts. *Cancer Res.* **55**, 963-967.

Li, G., Satyamoorthy, K. and Herlyn, M. (2001). N-cadherin-mediated intercellular interactions promote survival and migration of melanoma cells. *Cancer Res.* **61**, 3819-3825.

Lin, X., Duan, X., Liang, Y. Y., Su, Y., Wrighton, K. H., Long, J., Hu, M., Davis, C. M., Wang, J., Brunicardi, F. C. et al. (2006). PPM1A functions as a Smad phosphatase to terminate TGFbeta signaling. *Cell* **125**, 915-928.

Lo, R. S., Wotton, D. and Massague, J. (2001). Epidermal growth factor signaling via Ras controls the Smad transcriptional co-repressor TGIF. *EMBO J.* **20**, 128-136.

Lu, S. L., Reh, D., Li, A. G., Woods, J., Corless, C. L., Kulesz-Martin, M. and Wang, X. J. (2004). Overexpression of transforming growth factor beta1 in head and neck epithelia results in inflammation, angiogenesis, and epithelial hyperproliferation. *Cancer Res.* **64**, 4405-4410.

Maeda, M., Johnson, K. R. and Wheelock, M. J. (2005). Cadherin switching: essential for behavioral but not morphological changes during an epithelium-to-mesenchyme transition. *J. Cell Sci.* **118**, 873-887.

Maeda, M., Shintani, Y., Wheelock, M. J. and Johnson, K. R. (2006). Src activation is not necessary for transforming growth factor (TGF)-beta-mediated epithelial to mesenchymal transitions (EMT) in mammary epithelial cells. PP1 directly inhibits TGF-beta receptors I and II. *J. Biol. Chem.* **281**, 59-68.

Mariotti, A., Perotti, A., Sessa, C. and Ruegg, C. (2007). N-cadherin as a therapeutic target in cancer. *Expert Opin. Investig. Drugs* **16**, 451-465.

Massague, J. (2003). Integration of Smad and MAPK pathways: a link and a linker revisited. *Genes Dev.* **17**, 2993-2997.

Mukhopadhyay, S., Munshi, H. G., Kambhampati, S., Sassano, A., Platanias, L. C. and Stack, M. S. (2004). Calcium-induced matrix metalloproteinase 9 gene expression is differentially regulated by ERK1/2 and p38 MAPK in oral keratinocytes and oral squamous cell carcinoma. *J. Biol. Chem.* **279**, 33139-33146.

Munshi, H. G. and Stack, M. S. (2006). Reciprocal interactions between adhesion receptor signaling and MMP regulation. *Cancer Metastasis Rev.* **25**, 45-56.

Munshi, H. G., Wu, Y. I., Mukhopadhyay, S., Ottaviano, A. J., Sassano, A., Koblinski, J. E., Platanias, L. C. and Stack, M. S. (2004). 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-beta1-induced pericellular collagenolysis. *J. Biol. Chem.* **279**, 39042-39050.

Nagata, M., Fujita, H., Ida, H., Hoshina, H., Inoue, T., Seki, Y., Ohnishi, M., Ohyama, T., Shingaki, S., Kaji, M. et al. (2003). Identification of potential biomarkers of lymph node metastasis in oral squamous cell carcinoma by cDNA microarray analysis. *Int. J. Cancer* **106**, 683-689.

Nakao, A., Afrakhte, M., Moren, A., Nakayama, T., Christian, J. L., Heuchel, R., Itoh, S., Kawabata, M., Heldin, N. E., Heldin, C. H. et al. (1997). Identification of Smad7, a TGFbeta-inducible antagonist of TGF-beta signalling. *Nature* **389**, 631-635.

Neville, B. W. and Day, T. A. (2002). Oral cancer and precancerous lesions. *CA Cancer J. Clin.* **52**, 195-215.

Nieman, M. T., Prudoff, R. S., Johnson, K. R. and Wheelock, M. J. (1999). N-cadherin promotes motility in human breast cancer cells regardless of their E-cadherin expression. *J. Cell Biol.* **147**, 631-644.

Ottaviano, A. J., Sun, L., Ananthanarayanan, V. and Munshi, H. G. (2006). Extracellular matrix-mediated membrane-type 1 matrix metalloproteinase expression in pancreatic ductal cells is regulated by transforming growth factor-beta1. *Cancer Res.* **66**, 7032-7040.

Rheinwald, J. G. and Beckett, M. A. (1981). Tumorigenic keratinocyte lines requiring anchorage and fibroblast support cultures from human squamous cell carcinomas. *Cancer Res.* **41**, 1657-1663.

Saha, D., Datta, P. K. and Beauchamp, R. D. (2001). Oncogenic ras represses transforming growth factor-beta /Smad signaling by degrading tumor suppressor Smad4. *J. Biol. Chem.* **276**, 29531-29537.

Sapkota, G., Knockaert, M., Alarcon, C., Montalvo, E., Brivanlou, A. H. and Massague, J. (2006). Dephosphorylation of the linker regions of Smad1 and Smad2/3 by small C-terminal domain phosphatases has distinct outcomes for bone morphogenetic protein and transforming growth factor-beta pathways. *J. Biol. Chem.* **281**, 40412-40419.

Schilling, S. H., Datto, M. B. and Wang, X. F. (2006). A phosphatase controls the fate of receptor-regulated Smads. *Cell* **125**, 838-840.

Schipper, J. H., Frixen, U. H., Behrens, J., Unger, A., Jahnke, K. and Birchmeier, W. (1991). E-cadherin expression in squamous cell carcinomas of head and neck: inverse correlation with tumor dedifferentiation and lymph node metastasis. *Cancer Res.* **51**, 6328-6337.

Shi, Y. and Massague, J. (2003). Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* **113**, 685-700.

Shintani, Y., Wheelock, M. J. and Johnson, K. R. (2006). Phosphoinositide-3 kinase-Rac1-c-Jun NH2-terminal kinase signaling mediates collagen I-induced cell scattering and up-regulation of N-cadherin expression in mouse mammary epithelial cells. *Mol. Biol. Cell* **17**, 2963-2975.

Sun, Y., Liu, X., Ng-Eaton, E., Lodish, H. F. and Weinberg, R. A. (1999). SnoN and Ski protooncoproteins are rapidly degraded in response to transforming growth factor beta signaling. *Proc. Natl. Acad. Sci. USA* **96**, 12442-12447.

Suyama, K., Shapiro, I., Guttman, M. and Hazan, R. B. (2002). A signaling pathway leading to metastasis is controlled by N-cadherin and the FGF receptor. *Cancer Cell* **2**, 301-314.

Tang, B., Vu, M., Booker, T., Santner, S. J., Miller, F. R., Anver, M. R. and Wakefield, L. M. (2003). TGF-beta switches from tumor suppressor to prometastatic factor in a model of breast cancer progression. *J. Clin. Invest.* **112**, 1116-1124.

Watson-Hurst, K. and Becker, D. (2006). The role of N-cadherin, MCAM and beta3 integrin in melanoma progression, proliferation, migration and invasion. *Cancer Biol. Ther.* **5**, 1375-1382.

Wheelock, M. J., Shintani, Y., Maeda, M., Fukumoto, Y. and Johnson, K. R. (2008). Cadherin switching. *J. Cell Sci.* **121**, 727-735.

Wrighton, K. H., Willis, D., Long, J., Liu, F., Lin, X. and Feng, X. H. (2006). Small C-terminal domain phosphatases dephosphorylate the regulatory linker regions of Smad2 and Smad3 to enhance transforming growth factor-beta signaling. *J. Biol. Chem.* **281**, 38365-38375.

Yang, J., Dai, C. and Liu, Y. (2005). A novel mechanism by which hepatocyte growth factor blocks tubular epithelial to mesenchymal transition. *J. Am. Soc. Nephrol.* **16**, 68-78.

Zawel, L., Dai, J. L., Buckhaults, P., Zhou, S., Kinzler, K. W., Vogelstein, B. and Kern, S. E. (1998). Human Smad3 and Smad4 are sequence-specific transcription activators. *Mol. Cell* **1**, 611-617.

Journal of Cell Science