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

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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 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 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 Ca2+-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 mouse 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...
Moreover, only in the oral keratinocytes EGF attenuated TGF-β1 cadherin expression in the oral keratinocytes; however, no change in the linker region through extracellular signal-regulated kinases when ERK1/2 phosphorylation was blocked. Although EGF has been previously shown in pig thyrocytes to cooperate with TGF-β1 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 TGF-β1 in these oral cell lines. Although EGF did not affect N-cadherin expression in oral keratinocytes or in OSCC cells, EGF blocked TGF-β1-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 TGF-β1-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 TGF-β1-mediated Smad-driven promoter activity and N-cadherin expression was reversed when ERK1/2 phosphorylation was blocked. Although independently EGF and TGF-β1 promoted migration of oral keratinocytes and OSCC cells, EGF decreased TGF-β1-driven migration of oral keratinocytes but enhanced TGF-β1-driven migration of OSCC cells. Together these data support a model wherein TGF-β1-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).

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); 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 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 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 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.
**TGFβ1 increases migration of oral keratinocytes by upregulating N-cadherin expression**

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 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.

Oral keratinocytes and OSCC cells were serum-starved overnight, treated with TGFβ1 for 24 hours and the effect on N-cadherin expression was determined by western blot analysis. Using real-time PCR, expression levels of N-cadherin, N-cadherin and GAPDH mRNA were determined and normalized to untreated samples. 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. As shown in Fig. 2A, SCC25 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**

As shown in Fig. 2B, TGFβ1 increased N-cadherin expression 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 in OSCC cells contributes to the motility of oral cancer cells.

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. As shown in Fig. 2A, SCC25 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.
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. (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. Nommigratory 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 pQXIN empty vector or pQXIN-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 pQXIN empty vector or pQXIN-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. Nommigratory cells were removed from the upper chamber, and migrating cells were counted. *P<0.05, significantly different from the pQXIN,siCtrl,TGF-b1(+) samples. **P<0.05, significantly different from pQXIN,S4Si,TGF-b1(+) samples. The results are representative of at least two independent experiments.
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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 S4Si was 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 TGFIF, 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 TGFIF, 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). Thus, we 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, pretreatment 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
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 UMCC1 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 by attenuating Smad-mediated signaling.

Growth factors such as EGF can negatively regulate TGFβ1 signaling at different steps in the Smad signaling pathway. EGF...
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,
Regulation of N-cadherin expression

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 Ser 467), anti-linker region phosphorylated Smad2 (at Ser 245, Ser 250, Ser 255) and anti-TGFβ1 type 1 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 α-p38 MAPK, anti-α-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-phosphorylated 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 W7 and the TGFβ1 type I receptor inhibitor (Biber; catalog no. 616451) were obtained from Calbiochem (La Jolla, 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 nucleic acid electrophoresis kit specifically designed for keratinocytes was obtained from Amaza (Gaithersburg, MD).

Cell culture

SCC9, SCC25, SCC68 and UM12C1 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 UM12C1 cells were generously provided by Ernst Lengyl (University of Chicago, IL) (Jurez et al., 1993; Lengyl 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; Mukhopadhay et al., 2004). SCC9, SCC25 and UM12C1 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 CaCl2). 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 pQXIN vector (Clontech, Mountain View, CA) and verified by DNA sequencing. To generate retroviral particles, GP2-293 packaging cells were transfected with pQXIN vector or pQXIN vector expressing human N-cadherin (pQXIN-Ncad) and cotransfected with pVSV-G (Clontech) envelope vector according to the manufacturer’s instructions (Clontech). The conditioned media 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 (2×10⁶) 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 adhere 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 Ready-to-Go® Universal PCR kit (Amersham Biosciences). 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 Ct 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'-GGAGUGCCAGAAGUGUAGAdTdT-3' and siN-Cad2 5'-CCGUGUCUGUCGACUGUAdTdT-3' (Decker et al., 2006); SCC9, SCC25 or OKFs 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 (TβR1) expression was transiently downregulated using a validated siRNA that targets TβR1 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 (5′GUGAGAGAAAUCUUAAU-3′ and reverse (rev) 5′UCUAAUGGAAAUUACUUGG-3′ (Decker et al., 2006); Smad2 siRNA (5′GUGAGAGAAAUCAUUAUCUUAUAAUUUACAUAGGUA-3′ (Cucurru et al., 2005). Smad3 expression was downregulated using Smad3-specific siRNA (5′GUGAGAGAAGAGGUAAUUAUUAUAAUUACAUAGGUA-3′, catalog no. CLG1108) purchased from CellGenetics (Irvine, MD). OKFs cells were transiently transfected with 100 nM of SBE4 luciferase reporter vector, and the luciferase activity was quantified with the comparative luciferase activity (TGFβ1 or untreated control cells). 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