Differential Regulation of Epithelial and Mesenchymal Markers by δ EF1 Proteins in Epithelial–Mesenchymal Transition Induced by TGF- β [□]

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Epithelial–mesenchymal transition (EMT), a crucial event in cancer progression and embryonic development, is induced by transforming growth factor (TGF)- β in mouse mammary NMuMG epithelial cells. Id proteins have previously been reported to inhibit major features of TGF- β -induced EMT. In this study, we show that expression of the δ EF1 family proteins, δ EF1 (ZEB1) and SIP1, is gradually increased by TGF- β with expression profiles reciprocal to that of E-cadherin. SIP1 and δ EF1 each dramatically down-regulated the transcription of E-cadherin in NMuMG cells through direct binding to the E-cadherin promoter. Silencing of the expression of both SIP1 and δ EF1, but not either alone, completely abolished TGF- β -induced E-cadherin repression. However, expression of mesenchymal markers, including fibronectin, N-cadherin, and vimentin, was not affected by knockdown of SIP1 and δ EF1. TGF- β -induced the expression of Ets1, which in turn activated δ EF1 promoter activity. Moreover, up-regulation of SIP1 and δ EF1 expression by TGF- β was suppressed by knockdown of Ets1 expression. In addition, Id2 suppressed the TGF- β - and Ets1-induced up-regulation of δ EF1. Taken together, these findings suggest that the δ EF1 family proteins, SIP1 and δ EF1, are necessary, but not sufficient, for TGF- β -induced EMT and that Ets1 induced by TGF- β may function as an upstream transcriptional regulator of SIP1 and δ EF1.

INTRODUCTION

Transforming growth factor (TGF)- β , a prototypical member of the TGF- β family, regulates a broad range of cellular responses, including cell proliferation, differentiation, adhesion, migration, and apoptosis (Bierie and Moses, 2006). TGF- β and related factors exhibit their pleiotropic effects through binding to transmembrane serine-threonine kinase receptors type I (T β R-I) and type II (T β R-II). On ligand-induced heteromeric complex formation between T β R-I and T β R-II, T β R-I is phosphorylated and activated by T β R-II kinase and mediates specific intracellular signaling through phosphorylation of receptor-regulated Smads (R-Smads). Phosphorylated R-Smads interact with co-Smad (Smad4) and translocate into the nucleus, where they regulate transcription of target genes in cooperation with various transcription factors and transcriptional coactivators or corepressors (Miyazawa *et al.*, 2002; Miyazono *et al.*, 2003; Shi and Massague, 2003).

TGF- β has potent antiproliferative effects on a wide variety of cells, including epithelial cells, endothelial cells, and hematopoietic cells, although under certain conditions it

promotes the proliferation of mesenchymal cells, including fibroblasts, chondrocytes, and osteoblasts. TGF- β also induces the deposition of extracellular matrix proteins. In early stages of tumorigenesis, TGF- β inhibits the growth of epithelial cells, and insensitivity to this growth-inhibitory effect is associated with progression of tumors (Akhurst and Derynck, 2001; Derynck *et al.*, 2001). Transgenic mice expressing a dominant-negative T β R-II in epidermis exhibit malignant conversion of epithelial cells and promotion of tumor formation (Gorska *et al.*, 2003). Resistance to the antiproliferative effects of TGF- β is observed in numerous types of cancer (Park *et al.*, 1994; Heldin *et al.*, 1997; Lu *et al.*, 2006). The refractoriness of many carcinomas to effects of TGF- β is due to mutations in or loss of expression of receptors for it and to mutations in R-Smads and Smad4. Increase in expression of some negative regulators of TGF- β signaling, e.g., Smad7, has also been reported in certain cancers (Kleeff *et al.*, 1999; Kim *et al.*, 2004). In contrast to its tumor-suppressive effects in the early stages of carcinogenesis, TGF- β also acts as a promoter of tumor cell invasion and metastasis in advanced stages of tumorigenesis (Bierie and Moses, 2006). TGF- β is often overexpressed in various tumor tissues, induces migration and invasion of cancer cells and facilitates immunosuppression, angiogenesis, and deposition of extracellular matrix proteins. Blockade of TGF- β signaling thus leads to suppression of tumor cell motility, intravasation, and metastasis (Muraoka *et al.*, 2002; Azuma *et al.*, 2005). Chronic exposure to TGF- β results in loss of TGF- β -mediated growth inhibition and marked changes in cell morphology (Caulin *et al.*, 1995; Portella *et al.*, 1998). One mechanism by which TGF- β induces formation of spindle cell carcinomas and promotes tumor cell motility and invasion involves the epithelial–mesenchymal transition (EMT; Zavadil and Bottinger, 2005).

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Abbreviations used: TGF- β , transforming growth factor- β ; HLH, helix-loop-helix; EMT, epithelial–mesenchymal transition; SIP1, Smad-interacting protein1; δ EF1 (ZEB1), delta-crystallin/E2-box factor 1.

During the process of embryonic development and that of wound healing and reorganization in adult tissues, epithelial cells may lose their epithelial polarity and acquire mesenchymal phenotypes (Lee *et al.*, 2006). The process of invasion of tumor cells involves the loss of cell–cell interaction together with acquisition of migratory properties and is often associated with EMT of cells. Formation of tight cell–cell adhesions is mainly dependent on the E-cadherin system in both embryonic and adult epithelial cells. Loss of E-cadherin-mediated cell–cell interaction is thus essential for the EMT that occurs during normal embryonic development as well as during invasion of tumor cells into adjacent connective tissues (Peinado *et al.*, 2004). Besides the loss of E-cadherin, EMT is characterized by the down-regulation of cytokeratins, up-regulation of mesenchymal markers including fibronectin, N-cadherin, and vimentin and acquisition of a fibroblast-like motile and invasive phenotype (Lee *et al.*, 2006; Thiery and Sleeman, 2006).

Recent studies on the molecular mechanisms by which expression of E-cadherin is repressed in epithelial cells have revealed that several transcription factors, including the zinc-finger factors Snail and Slug, the two-handed zinc-finger factors of δ EF1 family proteins (δ EF1/ZEB1 and SIP1), and the basic helix-loop-helix (bHLH) factors Twist and E12/E47, are involved in this process (Comijn *et al.*, 2001; Yang *et al.*, 2004; Moreno-Bueno *et al.*, 2006). These transcription factors repress expression of E-cadherin and elicit EMT when overexpressed in normal epithelial Madin-Darby canine kidney (MDCK) and Eph4 cells. In addition, when overexpressed in cancer cells, these factors induce EMT with the development of metastatic properties such as migration and invasion *in vitro* and *in vivo* (Barrallo-Gimeno and Nieto, 2005; Thiery and Sleeman, 2006). However, it is still uncertain how the expression of these transcriptional factors is regulated at the onset of EMT in cancer cells.

TGF- β was first described as an inducer of EMT during development (Miettinen *et al.*, 1994) and is now thought to promote metastasis through induction of EMT on the front-edge cells of invasive cancer. It has been reported that TGF- β induces the expression of Snail and SIP1 mRNAs in some epithelial cells (Comijn *et al.*, 2001; Nagata *et al.*, 2006). Snail is rapidly and transiently up-regulated through the TGF- β -Smad signaling pathway in mouse mammary epithelial NMuMG cells (Nagata *et al.*, 2006), whereas SIP1 is up-regulated at a later phase through unknown mechanisms. In addition to these transcription factors, a microarray screen of epithelial cells identified Id genes (inhibitors of differentiation or inhibitors of DNA binding) as early targets of TGF- β . Id proteins have an HLH domain but lack the basic DNA-binding domain. Through constitutive association with bHLH E12/E47 proteins, Id proteins maintain epithelial phenotypes by repressing the function of E12/E47, which acts as a repressor of E-cadherin. Down-regulation of Id2 by TGF- β thus relieves this inhibition, permitting conversion of epithelial cells to cells with mesenchymal phenotypes (Kondo *et al.*, 2004; Kowanzet *et al.*, 2004).

In the present study, we investigated the regulatory mechanisms by which TGF- β elicits EMT in NMuMG cells. We show that the δ EF1 family proteins, SIP1 and δ EF1, are required for TGF- β -induced E-cadherin repression, but not for the expression of mesenchymal markers. Ets1 functions as an upstream component of SIP1 and δ EF1 in TGF- β -mediated EMT; its expression is up-regulated by TGF- β , and it acts in cooperative manner with E47 to induce expression of SIP1 and δ EF1 genes, leading to EMT. SIP1 and δ EF1 are thus critical components of TGF- β -induced EMT in NMuMG cells, although they are not sufficient to induce mesenchymal phenotype in these cells.

MATERIALS AND METHODS

Cell Culture and Reagents

Mouse mammary epithelial NMuMG cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM supplemented with 4.5 g/l glucose, 10% fetal bovine serum (FBS), 10 μ g/ml insulin, 100 U/ml penicillin, and 100 μ g/ml streptomycin. MDCK epithelial cells were grown in DMEM in the presence of 10% FBS and antibiotics. pBabe- or pBabe-Id2-infected NMuMG cells were generated as described previously (Kondo *et al.*, 2004). All cells were grown in a 5% CO₂ atmosphere at 37°C. Recombinant human TGF- β 3 was purchased from R&D Systems (Minneapolis, MN).

DNA Construction

Mouse E-cadherin promoters with point mutations in E-boxes (termed E1, E2, E3, E21, E13, E23, and E213) were constructed using PCR-based mutagenesis on the mouse E-cadherin promoter (–178 to +92 base pairs) in pGL2 (Kondo *et al.*, 2004). Human δ EF1 promoter (–1107 to +55 base pairs from the translation start site) was cloned using PCR from genomic DNA of human keratinocyte HaCaT cells isolated by DNeasy (QIAGEN, Chatsworth, CA). Amplification was performed by high-fidelity Taq polymerase (LA-Taq, Takara, Kyoto, Japan) using oligonucleotides 5'-CAGAAATCCCAAACTTGTACC-3' (sense) and 5'-CTGCTTTCTGCGCTTACACCT-3' (antisense). The purified PCR fragment was first cloned into pCR2.1 vector with a TA-cloning kit (Invitrogen, Carlsbad, CA), confirmed by sequencing, and then recloned into pGL3 vector (Promega, Madison, WI). The mouse SIP1 and δ EF1 cDNAs were obtained from Dr. F. van Roy (Ghent University) and Dr. Y. Higashi (Osaka University), respectively. The human E47 and Ets1 cDNAs were kindly provided by Dr. C. Murre (University of California, San Diego) and Dr. N. Kamata (Hiroshima University), respectively. Adenoviral vectors encoding SIP1 or δ EF1 epitope-tagged with Flag at their N-termini were constructed using the ViraPower Adenoviral Gateway Expression System (Invitrogen). Adenoviruses were produced in transfected 293A cells and amplified in the same cells according to the manufacturer's protocol. Purification of adenoviruses was carried out using the Virakit for Adenovirus 5 and Recombinant Derivatives 4-Pack (Virapur, Carlsbad, CA).

Antibodies

Mouse monoclonal anti-Flag M2 and anti- α -tubulin antibodies were purchased from Sigma-Aldrich (St. Louis, MO), and mouse monoclonal anti-E-cadherin and anti-N-cadherin antibodies were from BD Transduction Laboratories (Lexington, KY). Rat monoclonal anti-E-cadherin antibody for immunostaining was from Zymed (San Francisco, CA). Goat polyclonal anti-fibronectin and anti- δ EF1 antibodies were from Calbiochem (La Jolla, CA) and Santa Cruz Biotechnology (Santa Cruz, CA; E-20, Lot no. K0702), respectively.

Transfection and Infection of DNA

Transient transfection into NMuMG cells and MDCK cells was performed using Fugene 6 reagent (Roche Molecular Biochemicals, Indianapolis, IN) and Lipofectamine 2000 reagent (Invitrogen), respectively, as recommended by the manufacturers. For infection of adenoviruses, NMuMG cells were plated at a density of 1.0×10^5 cells/well in eight-well Culture Slides (BD Falcon, Bedford, MA). After 8 h, cells were infected with each adenovirus for 1 h with gentle agitation, washed once with the same media, and incubated for another 24 h.

RNA Interference

Transfection of short interfering RNAs (siRNAs) was performed in 12-well tissue culture plates according to the protocol recommended for HiPerFect reagent (QIAGEN). Final concentrations of the siRNAs used were 5 nM, except for 10 nM for SIP1 siRNA. At 8 h after transfection, 1 ng/ml TGF- β was added to the media, and culture was continued for an additional 24 h. The target sequences of these siRNA duplexes were as follows: mouse SIP1 (GGAAAAACGUGGUGAACUA; B-Bridge, Sunnyvale, CA), mouse δ EF1 (Stealth RNAi MSS210696; Invitrogen), mouse E2A (Stealth RNAi MSS210711; Invitrogen), mouse Ets1 (Stealth RNAi MSS215651, MSS215652, and MSS215653; Invitrogen), and Negative Control (Stealth RNAi 12935-200; Invitrogen).

Immunoblotting

NMuMG cells were seeded at a density of 8.0×10^4 cells/well in 12-well tissue culture plates. After 12 h, cells were treated with 1 ng/ml TGF- β for 24 h. Cells were lysed in RIPA buffer solution (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM EDTA, 1 mM EGTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS). After measurement of protein concentrations with a BCA Protein Assay Kit (Pierce, Rockford, IL), equal amounts of total protein per lane were subjected to SDS gel-electrophoresis, followed by semidry transfer of the proteins to Fluoro Trans W membrane (Pall, Glen Cove, NY). Nonspecific binding of proteins to the membrane was blocked by incubation in TBS-T buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween-20) containing 5% skim milk. Immunodetection was performed with the ECL blotting system (Amersham, Piscataway, NJ).

Immunofluorescence Labeling

To allow direct fluorescence of the actin cytoskeleton, cells were fixed in 3.7% paraformaldehyde in phosphate-buffered saline (PBS), permeabilized with 0.2% Triton X-100 in PBS for 5 min at room temperature, and subsequently stained with 0.25 mM tetramethylrhodamine isothiocyanate (TRITC)-conjugated phalloidin (Sigma-Aldrich). Immunocytochemical analyses were carried out on eight-well Culture Slides. Cells were fixed in 1:1 acetone-methanol solution and incubated with antibodies diluted with Blocking One solution (Nacalai Tesque, Tokyo, Japan) for 1 h at room temperature. The cells were then incubated with secondary antibodies and TOTO3 (Invitrogen Molecular Probes, Eugene, OR) for 1 h. Fluorescence was examined by confocal laser scanning microscopy (Carl Zeiss, Thornwood, NY).

Extraction of RNAs and Quantitative RT-PCR

Total RNAs were extracted from NMuMG cells using the RNeasy Mini Kit (QIAGEN). First-strand cDNAs were generated by Oligo (dT) priming using Superscript III Reverse Transcriptase (Invitrogen) following the manufacturer's instructions. Quantitative RT-PCR analyses were performed using the ABI PRISM 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) and Power SYBR Green (Applied Biosciences, Foster City, CA).

Luciferase Assays

Cells were seeded in duplicate in 12-well tissue culture plates, followed by transient transfection with various combinations of promoter-reporter constructs and expression plasmids as required. Luciferase activity in cell lysates was determined with a dual luciferase reporter assay system (Promega) using a luminometer (AutoLumat LB953, EG&G Berthold, Natick, MA). Luciferase activity was normalized to sea-pansy luciferase activity of cotransfected pRL-TK plasmid (Promega).

Electrophoretic Mobility Shift Assay

The E-box2/1 WT probe covers the region from -84 to -73 of the mouse E-cadherin promoter. Double-stranded oligonucleotides were labeled with [γ - 32 P]ATP and T4 polynucleotide kinase. Preparation of nuclear extracts was performed as previously described (Kobayashi *et al.*, 2007). Briefly, cells were infected with Flag-tagged SIP1 adenovirus, washed with PBS, collected in buffer A (10 mM HEPES-KOH, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 50 U/ml aprotinin), and incubated on ice for 10 min. After centrifugation, the precipitate was washed with buffer A and suspended in buffer C (20 mM HEPES-KOH, pH 7.9, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 50 U/ml aprotinin). The suspension was incubated on ice for 20 min, and the supernatant obtained by centrifugation was used as a nuclear fraction. DNA-binding assay (20 μ l final volume) was carried out for 30 min at 4°C, with 5 μ g of NMuMG nuclear proteins, poly(dI-dC) [poly(deoxyinosinic-deoxycytidylic acid)], and 32 P-labeled double-stranded oligonucleotide in binding buffer (15 mM Tris-HCl, pH 7.5, 75 mM NaCl, 1.5 mM EDTA, 1.5 mM dithiothreitol, 7.5% glycerol, 0.3% Nonidet P-40, and 1 mg/ml bovine serum albumin). For supershift experiments, the extracts were incubated with anti-Flag M2 antibody and were loaded onto a 6% polyacrylamide gel prepared in 0.25 $\times$ TBE buffer. After electrophoresis, gels were dried, exposed to imaging plates, and analyzed with the BAS-5000 system (FujiFilm, Tokyo, Japan).

Cell Motility Assay

NMuMG cells transfected with siRNAs were seeded at 1.0×10^5 cells/well in 6-well tissue culture plates. After 12 h, wounds were incised by scratching the cell monolayers using 200- μ l pipette tips, and then 1 ng/ml TGF- β was added to the media. Photographs were taken under phase-contrast microscopy immediately after incision and after 24 h of ligand stimulation. The assays were independently performed in triplicate. The area of migrating cells was estimated by counting the number of pixels after the photographs had been converted to Photoshop data (Adobe, San Jose, CA).

RESULTS

TGF- β Promotes EMT

TGF- β has been reported to induce EMT in NMuMG cells (Miettinen *et al.*, 1994; Piek *et al.*, 1999; Kondo *et al.*, 2004). As previously reported, treatment with TGF- β dramatically altered the morphological phenotypes of NMuMG cells from cobblestone-like to spindle shapes (Figure 1A). It also induced actin fiber formation typical of transdifferentiation and elicited the so-called cadherin switch, i.e., down-regulation of E-cadherin and up-regulation of N-cadherin (Figure 1B). Immunoblotting of whole-cell lysates from NMuMG cells revealed that treatment with TGF- β for 24 h resulted in down-regulation of

E-cadherin expression with concomitant up-regulation of representative mesenchymal markers, i.e., N-cadherin and fibronectin (Figure 1C).

Analyses by semiquantitative RT-PCR revealed that levels of E-cadherin mRNA gradually decreased and reached a minimum level at 12 h after TGF- β treatment (Figure 1D). To determine whether certain transcription factors are regulated by TGF- β in a manner reciprocal to that of the reduction in E-cadherin expression, we examined the expression of SIP1, δ EF1, Snail, Twist, and E12/47 mRNAs after TGF- β treatment. SIP1 and δ EF1 mRNA levels were gradually increased by TGF- β , with profiles of expression reciprocal to that of E-cadherin (Figure 1D). Greater than 10-fold expression of Snail mRNA was induced by 1 h after TGF- β treatment (Figure 1E). Expression of Twist could not be detected, whereas that of E12/E47 was not altered after TGF- β treatment (data not shown and Kondo *et al.*, 2004). Expression of mRNA for Id2, a negative regulator of TGF- β -induced EMT (Kondo *et al.*, 2004; Kowanetz *et al.*, 2004), decreased rapidly by 4 h after treatment with TGF- β (Figure 1D). These findings demonstrated that, among these factors, increases in SIP1 and δ EF1 by TGF- β appear to strongly correlate with the reduction in E-cadherin in NMuMG cells.

SIP1 and δ EF1, But Not Snail, Repress E-Cadherin Expression

Because expression of SIP1, δ EF1, and Snail mRNAs was up-regulated by TGF- β in NMuMG cells, we next examined the effects of these factors on E-cadherin promoter activity. Transfection of ALK5TD, a constitutively active form of T β RI, and E47 repressed E-cadherin promoter activity in NMuMG cells (Figure 2A). Overexpression of either SIP1 or δ EF1 strongly repressed E-cadherin promoter activity to the level equal to that suppressed by E47 in NMuMG cells. Protein levels of transfected SIP1 determined by immunoblotting were much less than those of transfected E47 (data not shown). Snail and Slug each repressed E-cadherin promoter activity in MDCK cells (Figure 2A and data not shown). However, neither Snail, Slug, nor Twist exhibited significant effects on E-cadherin promoter activity in NMuMG cells, suggesting that Snail and Slug function in a cell context-dependent manner, as previously reported (Barrallo-Gimeno and Nieto, 2005).

SIP1 was originally identified as a Smad-interacting protein by yeast two-hybrid screening and was shown to contain the Smad-binding domain (SBD) at its N-terminus (Verschuere *et al.*, 1999). The degree of amino acid sequence similarity in the SBD between SIP1 and δ EF1 is ~40%, and interaction of δ EF1 with Smad2/3 was weaker than that of SIP1 (data not shown and Postigo, 2003), suggesting the possibility that the SBD is not necessary for repression of E-cadherin promoter activity. To test this, we prepared a SIP1 deletion mutant lacking SBD (SIP1- Δ SBD) and measured E-cadherin promoter activity after transient transfection of NMuMG cells. Similar to wild-type SIP1, the SIP1- Δ SBD mutant repressed E-cadherin promoter activity (Figure 2B). Binding of the wild-type SIP1 to Smad2/3 was observed only in the presence of TGF- β , whereas that of SIP1- Δ SBD was not observed even in the presence of TGF- β (data not shown). These findings suggest that the interactions with Smads through the SBD are not required for repression by SIP1 and δ EF1 of E-cadherin promoter activity.

Consistent with the results of E-cadherin reporter assays, overexpression of either SIP1 or δ EF1 by adenoviruses repressed the expression of E-cadherin mRNA as well as that of E-cadherin protein (Figure 2, C and D). These findings suggest that SIP1 and δ EF1 act as potent suppressors of E-cadherin transcription in NMuMG cells.

Figure 1. Induction of EMT by TGF- β in NMuMG cells. (A) Phase-contrast images of cells treated for 24 h without (a) or with 1 ng/ml TGF- β (b). (B) Immunofluorescence images of cells showing the localization and organization of the indicated markers after 24 h of treatment without (a–c) or with (d–f) 1 ng/ml TGF- β . Reorganization of the actin cytoskeleton determined by TRITC-phalloidin staining (a and d), E-cadherin staining (b and e), and N-cadherin staining (c and f) are shown. (C) Endogenous levels of E-cadherin, fibronectin, and N-cadherin proteins were detected by immunoblotting at 24 h after treatment with or without 1 ng/ml TGF- β . α -tubulin levels were monitored as a loading control for whole-cell extracts. (D and E) Induction of target genes, including E-cadherin, SIP1, δ EF1, Id2 (D), and Snail (E) was examined in cells treated with TGF- β for the indicated periods by semiquantitative RT-PCR analyses. Ratios of mRNA levels in TGF- β -treated cells to those in nontreated cells are shown. Values were normalized to the amount of house keeping GAPDH mRNA.

δ EF1 Family Proteins Bind to E-box1 and E-box2 in Mouse E-Cadherin Promoter

The E-cadherin promoter contains two E-boxes (E-box1 and E-box3) in humans and three E-boxes (E-box1, E-box2, and E-box3) in mice. To determine whether SIP1 and δ EF1 affect transcription of the mouse E-cadherin promoter through the E-boxes, we transfected SIP1 expression plasmid with a reporter plasmid driven by mouse E-cadherin core promoter (–178 to +92) in mouse NMuMG cells. SIP1 and δ EF1 induced an ~60% decrease in mouse E-cadherin promoter activity (Figure 3, B and C). To determine which E-boxes are involved in the repression induced by SIP1 and δ EF1, we mutated the E-boxes in the mouse E-cadherin promoter (Figure 3A). SIP1 and δ EF1 repressed the E-cadherin promoter activity of the single E-box point mutants (E1, E2, and E3) to extents similar to those of the wild-type E-cadherin promoter (Figure 3, B and C). Among three double E-box mutants of the mouse E-cadherin promoter, the E-box1/E-box3 mutant (E13) and E-box2/E-box3 mutant (E23) exhibited activity similar to the wild-type E-cadherin promoter. In

contrast, SIP1 and δ EF1 failed to repress transcription of the E-box2/E-box1 double mutant (E21), similar to the triple mutant of mouse E-cadherin promoter (E213, Figure 3, B and C).

To confirm these findings, electrophoretic mobility shift assay (EMSA) against radioisotope-labeled mouse E-cadherin probes was performed using nuclear extracts of NMuMG cells overexpressing SIP1 (Figure 3D). SIP1 bound to the wild-type E-box2/1 probe and gave rise to a specific band that was efficiently competed by the unlabeled wild-type probe but only weakly competed by excess ($\times 50$) unlabeled mutant probe. However, no binding of SIP1 protein could be detected in EMSA using double E-box2/1 mutant as a labeled probe. The specificity of binding of Flag-SIP1 to the E-box2/1 probe was confirmed by supershift assay using anti-Flag M2 antibody. Addition of anti-Flag mAb led to the disappearance of the SIP1-specific band and the appearance of a slowly migrating supershift complex (Figure 3D). Similar results were obtained with overexpression of Flag- δ EF1 (data not shown). These findings indicate that SIP1 and δ EF1 directly repress mouse E-cadherin promoter activity through interaction with E-box2

Figure 2. Down-regulation of E-cadherin expression by exogenous SIP1 or δ EF1. (A) NMuMG cells (left) were cotransfected with mouse E-cadherin promoter-reporter construct (E-cadherin-Luc.) in combination with 0.5 μ g of ALK5TD or 0.7 μ g of E47-, SIP1-, δ EF1-, Snail-, Slug-, and Twist-expressing plasmids. MDCK cells (right) were cotransfected with E-cadherin-Luc with 0.7 μ g of E47-, SIP1-, and Snail-expressing plasmids. At 24 h after transfection, the cells were harvested and assayed for luciferase activity. (B) NMuMG cells were transiently cotransfected with the E-cadherin promoter-reporter plasmid and with wild-type SIP1 (WT) or SIP1- Δ SBD (Δ SBD). Luciferase activity was determined as in A. (C) NMuMG cells infected with mock, Flag-SIP1, and Flag- δ EF1 adenoviruses were immunostained with anti-Flag M2 (second from left side) and anti-E-cadherin antibodies (second from right side) and stained with TOTO3 to detect nuclei (right side). (D) Levels of expression of E-cadherin mRNA in NMuMG cells infected with mock, SIP1, or δ EF1 adenoviruses were determined by quantitative RT-PCR. Values were normalized to GAPDH mRNA.

and E-box1 elements of the mouse E-cadherin promoter in NMuMG cells.

Double Knockdown of SIP1 and δ EF1 Blocks TGF- β -induced E-Cadherin Repression and Cell Migration
To determine whether SIP1 and δ EF1 are required for TGF- β -mediated repression of E-cadherin promoter activity, we used siRNAs directed against SIP1 and δ EF1 to reduce the expression of endogenous proteins. SIP1 or δ EF1 siRNAs were transfected into NMuMG cells, followed by stimulation of the cells with TGF- β . SIP1 and δ EF1 siRNAs each successfully knocked down the expression of corresponding endogenous mRNAs (Figure 4A). Down-regulation of δ EF1 protein expression was also confirmed by immunoblotting (see Figure 5B). In cells transfected with control siRNA, TGF- β induced about twofold expression of SIP1 and δ EF1 mRNAs at 24 h after stimulation and repressed the expression of E-cadherin within 24 h after stimulation (Figure 4A). In cells transfected with either SIP1 siRNA or δ EF1 siRNA alone, TGF- β -mediated E-cadherin repression was not or was only partially blocked. Interestingly, transfection of both SIP1 and δ EF1 siRNAs completely abolished TGF- β -induced E-cadherin repression at the mRNA level (Figure 4A). Immu-

nostaining of E-cadherin in NMuMG cells also revealed that the level of E-cadherin protein was not suppressed by TGF- β in cells transfected with both SIP1 and δ EF1 siRNAs (Figure 4B).

Next, we investigated whether functions of SIP1 and δ EF1 are required for TGF- β -induced migration and invasion by cell motility assay. In cells transfected with control siRNA, TGF- β accelerated wound closure (Figure 4C) and invasion (data not shown), possibly through E-cadherin-regulated cell-cell adhesion. These responses were partially impaired in cells transfected with both SIP1 and δ EF1 siRNAs (Figure 4C). These findings indicate that TGF- β requires both SIP1 and δ EF1 to repress E-cadherin expression and migratory behavior of NMuMG cells.

SIP1 and δ EF1 Are Not Involved in the Up-Regulation of Mesenchymal Markers by TGF- β

Because transfection with both SIP1 and δ EF1 siRNAs completely abolished down-regulation of E-cadherin in response to TGF- β , we examined whether other EMT markers, including fibronectin and N-cadherin, are affected by these siRNAs in NMuMG cells. Cells were transfected with both SIP1 and δ EF1 siRNAs. Nontransfected cells and those transfected with a control siRNA were used as controls. In the control cells,

Figure 3. E-box1 and E-box2 in mouse E-cadherin promoter are required for SIP1- and δ EF1-dependent repression of E-cadherin. (A) Schematic diagram of the mouse wild-type E-cadherin-luciferase construct (WT) and its mutants used in luciferase assays. (B and C) SIP1 or δ EF1 expression plasmids at concentrations of 0, 0.1, 0.25, and 0.5 μ g were cotransfected with mouse wild-type E-cadherin luciferase construct (WT) and its mutants in NMuMG cells. At 24 h after transfection, luciferase activity was determined as described in *Materials and Methods*. (D) Gel shift assay was performed with probes resembling the E-box2/1 of mouse E-cadherin gene (E-box2/1 WT) or its mutant (mut.). Nuclear extracts from NMuMG cells overexpressing Flag-SIP1 were incubated with the indicated probes. SIP1 binding to E-box2/1 was competed with a 50-fold molar excess of unlabeled wild-type or mutant probes. Black arrowhead indicates the position of the complex of SIP1. The supershifted complex (white arrowhead) is observed upon addition of the anti-Flag M2 antibody to the binding reaction.

TGF- β down-regulated the expression of E-cadherin and up-regulated those of fibronectin and N-cadherin at both the mRNA and protein levels (Figure 5, A and B). In contrast to the effect of knockdown of both SIP1 and δ EF1 on TGF- β -induced E-cadherin repression, cells transfected with SIP1 and δ EF1 siRNAs exhibited expression profiles of fibronectin and N-cadherin similar to those of the control cells (Figure 5, A and B). Moreover, examination of actin reorganization by TRITC-phalloidin staining in the control cells revealed dramatic actin fiber formation by TGF- β , which was also observed in cells transfected with both SIP1 and δ EF1 siRNAs (Figure 5C). These findings strongly suggest that SIP1 and δ EF1 are not involved in regulation of the expression of mesenchymal markers by TGF- β .

Activation of Smad Signaling and Subsequent De Novo Protein Synthesis Are Required for TGF- β -induced SIP1 Expression

To elucidate whether de novo protein synthesis is required for TGF- β -induced SIP1 and δ EF1 expression, RNAs were extracted from cells stimulated with TGF- β in the presence or absence of cycloheximide, an inhibitor of protein synthesis, and analyzed by semiquantitative RT-PCR. Because rapid increase in Smad7 mRNA by TGF- β has been reported as a direct effect of Smad signaling activated by TGF- β (Akiyoshi *et al.*, 2001), Smad7 was used as a negative control to evaluate the effect of cycloheximide. Snail mRNA was

up-regulated until 12 h by TGF- β (Figure 6 and see Figure 1E). Similar to the effect of cycloheximide on TGF- β -induced increase in Smad7, induction of Snail was not affected by cycloheximide, indicating that Snail is a direct target of the TGF- β -Smad pathway. In contrast, although TGF- β induced expression of SIP1 mRNA in the absence of cycloheximide, pretreatment of cells with cycloheximide blocked the TGF- β -mediated induction of SIP1 (Figure 6) and δ EF1 (data not shown). The up-regulation of SIP1 and δ EF1 mRNAs may thus be an indirect transcriptional response and require de novo protein synthesis by TGF- β .

Increase in *Ets1* by TGF- β Is Involved in Expression of SIP1 and δ EF1

To examine the molecular mechanisms by which δ EF1 and SIP1 are transcriptionally activated through de novo-synthesized proteins in response to TGF- β , we investigated TGF- β -regulated genes from our microarray data for human HaCaT keratinocyte cells (Akiyoshi *et al.*, 2001) and from published data of NMuMG cells (Xie *et al.*, 2003). Expression of several representative genes was up-regulated by TGF- β in HaCaT cells; among such genes, we found that *Ets* family genes were induced by TGF- β , even in the presence of cycloheximide in HaCaT cells (Akiyoshi *et al.*, 2001). Expression of *Ets* family genes was also up-regulated in NMuMG cells (Xie *et al.*, 2003). Semiquantitative RT-PCR analyses using RNAs from NMuMG cells treated with TGF- β re-

Figure 4. Double knockdown of SIP1 and δ EF1 relieves TGF- β -induced E-cadherin regulation. (A) NMuMG cells transfected with SIP1 siRNA, δ EF1 siRNA, or both siRNAs (SIP1 + δ EF1) were stimulated with 1.0 ng/ml TGF- β for 24 h and examined by semiquantitative RT-PCR analyses for SIP1 (left), δ EF1 (center), and E-cadherin levels (right). Values were normalized to the amount of GAPDH mRNA. (B) NMuMG cells transfected without or with control (NC) or both SIP1 and δ EF1 (SIP1 + δ EF1) siRNAs were stimulated with 1.0 ng/ml TGF- β for 24 h and subjected to immunofluorescence staining for E-cadherin. (C) Migratory behaviors of knocked-down cells in the absence or presence of TGF- β were determined by a wound assay (left) and quantified as described in *Materials and Methods* (right). NC, control siRNA.

vealed that, among several Ets family proteins, including Ets1, Ets2, and ESE-1, only Ets1 was increased after TGF- β stimulation (Figure 7A), and that this increase was unaffected by cycloheximide pretreatment (data not shown).

To examine the roles of Ets1 in TGF- β -induced EMT, we determined the effect of Ets1 on E-cadherin promoter activity. Transfection of Ets1 in NMuMG cells resulted in dose-dependent repression of E-cadherin promoter activity (Figure 7B), suggesting that repression of E-cadherin by Ets1 may be mediated by SIP1 and/or δ EF1. To explore this possibility, δ EF1 promoter was cloned from genomic DNA of HaCaT cells. Computer analyses showed that sequence

similarity between the mouse and human δ EF1 promoter regions is ~65–70% in the -620 to +1 region of the mouse promoter (corresponding to -810 to +1 of the human promoter) and that these regions contain two potential binding sites for Ets1 and three binding sites for bHLH E47 proteins. Transient transfection of Ets1 into NMuMG cells induced a threefold increase in δ EF1 promoter activity (Figure 7C). siRNA directed against Ets1 successfully and specifically knocked down the expression of endogenous Ets1 without off-target effects on other Ets family genes (Figure 7D). In cells transfected with the Ets1 siRNA, TGF- β -induced expression of SIP1 and δ EF1 was inhibited compared with that in cells trans-

Figure 5. Changes in mesenchymal markers in SIP1 and δ EF1 knocked-down cells. (A) NMuMG cells transfected with both SIP1 and δ EF1 (SIP1+ δ EF1) siRNAs were stimulated with 2.5 ng/ml TGF- β for 24 h and examined by semiquantitative RT-PCR analyses for levels of E-cadherin (left), fibronectin (center), and N-cadherin (right). Values were normalized to the amount of GAPDH mRNA. (B) NMuMG cells transfected with control (NC) or SIP1 and δ EF1 (SIP1+ δ EF1) siRNAs were stimulated with TGF- β for 24 h and subjected to immunoblot analysis using anti- δ EF1, E-cadherin, fibronectin, and N-cadherin antibodies. α -tubulin levels were monitored as a loading control for whole-cell extracts. (C) After being transfected without or with control (NC) or both SIP1 and δ EF1 (SIP1+ δ EF1) siRNAs, cells were grown in the absence or presence of 1 ng/ml TGF- β , followed by direct staining of the actin cytoskeleton using TRITC-phalloidin. NC, control siRNA.

fected with control siRNA, suggesting that Ets1 may be involved in TGF- β -regulated induction of δ EF1 and SIP1 and that it may in turn repress E-cadherin transcription.

We and another group have previously reported that, in NMuMG cells, endogenous Id2 acts as a negative regulator of TGF- β -induced EMT and that overexpression of Id2 partially blocks the E-cadherin repression and EMT phenotype

Figure 6. Requirement of de novo protein synthesis for induction of SIP1 by TGF- β . NMuMG cells pretreated with 5 μ M of cycloheximide (CHX) for 1 h were stimulated with 1 ng/ml TGF- β for 12 h. RNAs were extracted and analyzed by semiquantitative RT-PCR to determine levels of endogenous Smad7, Snail, and SIP1. Values were normalized to the amount of GAPDH mRNA.

evoked by TGF- β (Kondo *et al.*, 2004; Kowanetz *et al.*, 2004). To investigate whether Id2 regulates the effect of Ets1 on up-regulation of δ EF1 and SIP1, we examined the induction of SIP1/ δ EF1 by TGF- β in NMuMG cells retrovirally infected with pBabe-mouse Id2. In cells infected with control pBabe vector, endogenous Id2 expression was down-regulated by TGF- β , and δ EF1 mRNA was increased by TGF- β (Figure 7E), similar to the noninfected NMuMG cells (see Figure 4A). In Id2-infected NMuMG cells, high levels of endogenous Id2 mRNA were maintained and were not affected by TGF- β . TGF- β failed to up-regulate the expression of δ EF1 (Figure 7D) and SIP1 (data not shown) in these cells, despite the finding that TGF- β could increase the expression of Ets1 (data not shown). Supporting these observations, the δ EF1 promoter activity induced by Ets1 was enhanced by cotransfection with constitutively active T β R-I (ALK5TD), which was partially inhibited by cotransfection with Id2 (Figure 7C). Taken together, these findings suggest that Id2 may regulate the function of Ets1 to modulate the transcription of SIP1 and δ EF1 without alteration of the transcription of Ets1. Ets1-regulated δ EF1/SIP1 transcription may thus be of great importance for TGF- β -induced EMT in NMuMG cells.

DISCUSSION

In the present study, we investigated the roles of transcriptional repressors for E-cadherin in regulation of TGF- β -induced EMT in NMuMG cells and found that the δ EF1 family proteins, δ EF1 and SIP1, are required for TGF- β -

Figure 7. Involvement of Ets1 in TGF- β -induced δ EF1 family gene expression. (A) RNAs were extracted from NMuMG cells treated with 1 ng/ml TGF- β for the indicated periods and analyzed for expression of Ets mRNA by semiquantitative RT-PCR. Ratios of the Ets1 mRNA level in TGF- β -treated cells to those in nontreated cells are shown. Values were normalized to the amount of GAPDH mRNA. (B) Cells were cotransfected with mouse E-cadherin promoter-luciferase plasmid (E-cadherin-Luc) and 0.1 or 0.3 μ g of Ets1 plasmid, incubated for 24 h and measured for luciferase activity. (C) Cells were cotransfected with δ EF1 promoter-luciferase plasmid (δ EF1-Luc), and the indicated plasmids were incubated for 24 h and measured for luciferase activity. Id2 expression plasmids were used at concentrations of 0.1 μ g (+) and 0.5 μ g (++). (D) Cells transfected with siRNA against mouse Ets1 were incubated for 8 h, treated with 1 ng/ml TGF- β for another 24 h, and then analyzed by semiquantitative RT-PCR to determine levels of endogenous Ets1 (left), SIP1 (center), and δ EF1 (right). Values were normalized to the amount of housekeeping β -glucuronidase mRNA. NC, control siRNA. (E) NMuMG cells infected with pBabe or pBabe-Id2 were stimulated with 1 ng/ml TGF- β . After 12 h, levels of Id2 and endogenous δ EF1 were measured by semiquantitative RT-PCR. Values were normalized to the amount of β -glucuronidase mRNA.

induced repression of E-cadherin but not for up-regulation of mesenchymal markers. We also found that TGF- β -induced expression of Ets1 promoted transcription of SIP1 and δ EF1 mRNAs through potential cooperation with E12/E47 bHLH transcription factors.

δEF1 Family Proteins Are Essential for Repression of E-Cadherin Expression by TGF-β

Various transcription factors have been reported to be involved in EMT (Barrallo-Gimeno and Nieto, 2005). Among such factors, expression of SIP1 and δ EF1 mRNAs was gradually increased by TGF- β in NMuMG cells with expression profiles reciprocal to that of E-cadherin (Figure 1D). Although SIP1 and δ EF1 (ZEB1) have been reported to play opposing roles in TGF- β and BMP signaling (Postigo, 2003),

we have found that both SIP1 and δ EF1 repress the expression of E-cadherin and are essential for TGF- β -induced EMT in NMuMG cells. In agreement with our findings, it has been reported that overexpression of SIP1 in the absence of TGF- β fully suppressed E-cadherin promoter activity in NME cells, a line of cells derived from NMuMG cells (Comijn *et al.*, 2001) and that overexpression of δ EF1 also repressed E-cadherin expression in epithelial cells (van Grunsven *et al.*, 2003).

SIP1 was originally identified as a Smad-interacting protein by yeast two-hybrid screening and was shown to contain the SBD at its N-terminus (Verschuere *et al.*, 1999). SIP1 interacts with R-Smads through the SBD in a TGF- β -dependent manner. In contrast, δ EF1 interacts with Smads only weakly even in the presence of TGF- β (data not shown and Postigo, 2003), possibly due to the low degree of sequence

similarity in the SBD between δ EF1 and SIP1. Although δ EF1 and SIP1 have been reported to regulate the promoter activity of p3TP-lux and p21-lux in TGF- β -dependent manner (Postigo, 2003; Postigo *et al.*, 2003) and Comijin *et al.* (2001) reported that a SIP1 deletion mutant lacking SBD failed to repress the transcription of human E-cadherin in MCF7 cells, the present findings revealed that interaction of Smads with SIP1 is not required for repression of transcription of E-cadherin. These findings indicate that SIP1 and δ EF1 regulate mouse E-cadherin transcription independently of interaction with Smads in NMuMG cells.

Comijin *et al.* (2001) also reported that the human E-cadherin promoter contains E-box1 and E-box3, but lacks E-box2, and that SIP1 suppresses transcription of human E-cadherin promoter activity in human MCF7 cells through E-box1 and E-box3. However, in mouse NMuMG cells, E-box1 and E-box2 were sufficient for the repression of mouse E-cadherin transcription by SIP1 and δ EF1, and E-box3 was not required for this repression (Figure 3). Using chromatin immunoprecipitation assays, we have found that overexpressed δ EF1 and SIP1 bound to E-box2/1 elements of mouse E-cadherin promoter (data not shown). Although the reason for this difference is unclear, it is possible that SIP1 and δ EF1 act on E-box3 through interaction with Smads and that E-box2, which is located close to E-box1 in the mouse E-cadherin promoter, may compensate for the function of E-box3. Further studies using mouse and human E-cadherin promoters and using chromatin immunoprecipitation assays by endogenous δ EF1, SIP1, and Snail are required in the future.

Snail Is Not Involved in the Repression of E-Cadherin Expression in NMuMG Cells

Snail has been shown to be induced by TGF- β and to play pivotal roles in E-cadherin repression and EMT in various cells *in vitro* and *in vivo* (Barrallo-Gimeno and Nieto, 2005). Although Snail mRNA expression was strongly induced by TGF- β in NMuMG cells (Figure 1E), we were not able to determine Snail protein levels because of the low efficiency of several commercially available antibodies to Snail (our unpublished data). Overexpression of Snail failed to affect the phenotypes of NMuMG cells and to regulate the expression of EMT markers, including E-cadherin, N-cadherin, fibronectin, and vimentin (Figure 2A and Supplementary Figure 1, D and E). Experiments using siRNA also demonstrated that TGF- β efficiently induced EMT markers in NMuMG cells transfected with Snail siRNA (Supplementary Figure 1, A–C). Snail has recently been reported to be activated during EMT through various mechanisms, including up-regulation by HMGA2, phosphorylation by GSK3 β , activation by lysyl oxidase-like 2, and induction of nuclear localization by zinc transporter LIV1 (Yamashita *et al.*, 2004; Zhou *et al.*, 2004; Peinado *et al.*, 2005; Thuault *et al.*, 2006). The present findings, however, suggest that Snail is not the primary mediator of TGF- β -mediated EMT in NMuMG cells and that it may require other molecules to induce EMT in these cells.

Ets1 and Id Proteins Act as Upstream Factors for SIP1 and δ EF1

Ets1 has been shown to be a direct target of TGF- β -Smad signaling in some cells (Akiyoshi *et al.*, 2001; del Valle-Perez *et al.*, 2004). In the present study, we also observed that TGF- β up-regulated the expression of Ets1, which was not affected by cycloheximide treatment (data not shown). Because cycloheximide treatment blocked TGF- β -induced expression of SIP1 and δ EF1 mRNAs, Ets1 is a direct target, and SIP1 and δ EF1 are indirect targets, of TGF- β -Smads pathways in NMuMG cells. The promoter-reporter assay we

performed revealed that Ets1 activated the activity of δ EF1 promoter and suppressed the activity of E-cadherin promoter (Figure 7, B and C). Knock-down of Ets1 decreased the mRNA levels of endogenous δ EF1 or SIP1 (Figure 7D). Interestingly, in Ets1 siRNA-transfected cells, expression of Ets1 mRNA was remarkably suppressed in the presence and absence of TGF- β , whereas mRNA levels of SIP1 and δ EF1 in the absence of TGF- β were similar to those in control siRNA-transfected cells (Figure 7D). This may have been due to redundant effects of other Ets family transcription factors; we observed that expression of Ets2 and ESE1, a breast cancer-specific Ets protein, was up-regulated in NMuMG cells when Ets1 expression was knocked down by Ets1-specific siRNA (our unpublished data).

Previous studies reported that the transcription activity of Ets1 is regulated through phosphorylation by certain serine-threonine kinases, including ERK1/2 (Tootle and Rebay, 2005). Recently, importance of the cross-talk between Ras and TGF- β signaling pathways has been reported, although roles of Ras signaling on TGF- β signaling appear to be context-dependent (Gotzmann *et al.*, 2002; Peinado *et al.*, 2003). Smad1 is phosphorylated by Ras-activated ERK MAP kinases and degraded by Smurf1 (Sapkota *et al.*, 2007), whereas Smad2/3 are irregularly translocated to the nucleus in certain cancer cells transfected with active Ras (Yamagata *et al.*, 2005; Oft *et al.*, 2002). Interestingly, it was reported that TGF- β -induced EMT was found only in the presence of active Ras (Gotzmann *et al.*, 2002), suggesting that Ets1 would be a key molecule for the cross-talk between TGF- β and Ras signaling pathways. In the present study, we demonstrated that, although overexpression of Ets1 accelerated δ EF1 promoter activity, coexpression with constitutively active T β R-I (ALK5TD) further enhanced the δ EF1 promoter activity induced by Ets1 (Figure 7C). These findings suggest that Ets1 is activated through posttranslational modifications, including phosphorylation, by TGF- β in NMuMG cells.

We and another group have previously reported that HLH transcriptional inhibitor Id proteins inhibit TGF- β -induced E-cadherin repression through constitutive association with E12/E47 bHLH transcriptional factors in NMuMG cells (Kondo *et al.*, 2004; Kowanetz *et al.*, 2004). Thus, down-regulation of Id proteins by TGF- β abolishes this inhibition, leading to EMT in NMuMG cells. Consistent with these findings, when Id2 is overexpressed in NMuMG cells, TGF- β -induced E-cadherin repression and δ EF1 induction were partially impaired (Figure 7E and Kondo *et al.*, 2004). Previous studies reported that Id proteins bind directly to Ets1 through the conserved HLH domain *in vitro* (Yates *et al.*, 1999), and we have shown here that Ets1-induced activation of the δ EF1 promoter was partially suppressed by overexpression of Id2 (Figure 7C). These findings suggest inhibitory effects of Id proteins on Ets1 in TGF- β -induced EMT. In addition, Ets1 activity on δ EF1 promoter was partially repressed by transfection of E12/E47 siRNA (Supplementary Figure 2, A and B), indicating that Ets1 may act in cooperative manner with E47 to regulate expression of δ EF1. However, it is currently unknown whether Ets1 directly interacts with E47 or binds with it indirectly through other molecule(s) in TGF- β -induced EMT (Dang *et al.*, 1998). Taken together, these findings suggest that collaboration of Ets1 with E47 may regulate the expression of SIP1 and δ EF1, which may occur in a cell- and promoter-context manner and that down-regulation of Id protein expression by TGF- β may thus enhance this cooperation in inducing expression of δ EF1 family proteins, leading to EMT.

SIP1 and δ EF1 Regulate the Expression of E-Cadherin, But Not That of Mesenchymal Markers

The findings of the present study suggest that transcriptional regulation of δ EF1 family proteins is required for regulation of E-cadherin during TGF- β -induced EMT and that Ets1 may act as an inducer of δ EF1 family genes in collaboration with E47. However, the molecular mechanisms by which Id proteins inhibit the effects of Ets1 on induction of ZEB and SIP1 in NMuMG cells remain to be elucidated. In the process of EMT, the transcriptional program of epithelial cells shifts toward that of mesenchymal cells. Various transcription factors have been suggested to play key roles in this process. In TGF- β -induced EMT, Smad signaling has been reported to be essential for EMT (Piek *et al.*, 1999), whereas certain signals involved in EMT are transmitted via non-Smad pathways. In the present study, we showed that, in addition to SIP1 (Comijn *et al.*, 2001), δ EF1 plays a key role in TGF- β -induced EMT in NMuMG cells. Moreover, several non-Smad pathways, including the RhoA (Bhowmick *et al.*, 2001) and p160^{ROCK} pathways (Ozdamar *et al.*, 2005), have been reported to transduce signals for TGF- β -induced EMT, and expression of fibronectin occurs independently of Smad activation (Hoccar *et al.*, 1999). Although SIP1 and δ EF1 are transcription factors of critical importance to the induction of EMT, they appear to regulate only certain subsets of EMT markers. EMT may thus be induced by an array of signals activated by TGF- β as well as by other cytokines. The mechanism of induction of mesenchymal markers during TGF- β -induced EMT will require further determination in the future.

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