Involvement of Ets-1 transcription factor in inducing matrix metalloproteinase-2 expression by epithelial-mesenchymal transition in human squamous carcinoma cells

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Abstract. Epithelial-mesenchymal transition (EMT) is a crucial event in cancer progression. We previously reported that EMT up-regulates matrix metalloproteinase-2 (MMP-2) expression in squamous cell carcinoma (SCC) cells. In this study, we showed that Tet Off-induced expression of Snail or SIP1, and treatment with TGF-ß1 induced EMT in terms of down-regulation of E-cadherin, and up-regulation of vimentin and MMP-2 expression with morphological changes. In SCC cells, SIP1 expression was induced by Snail and TGF-ß1, but Snail expression was not induced by SIP1 or TGF-ß1. However, expression of Snail but not SIP1 was strongly increased by TGF-ß1 in highly invasive SCC cells with mesenchymal phenotypes. Analysis of the MMP-2 promoter revealed that an Ets-1 binding site, located between position -1255 and -1248 relative to the transcriptional start site, was critical for the activation by Snail, SIP1 and TGF-ß1 in SCC cells. Induced expression of Snail and SIP1 resulted in the increased expression of Ets-1 and DNA-binding activities of nuclear proteins to the Ets-1-binding site and strong Ets-1 expression was detected in highly invasive SCC cells. Furthermore, overexpression of Ets-1 induced the promoter-activation and expression of MMP-2 without EMT. These results indicate that EMT induces Ets-1 expression, which activates the MMP-2 promoter, but Ets-1 by itself has no activity to induce EMT in SCC cells.

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Introduction

The loss of epithelial characteristics and acquisition of a mesenchymal phenotype are important events in the progression towards more invasive and metastatic cancerous cells. This transformation is referred to as epithelial-mesenchymal transition (EMT), which was originally found to occur during embryonic development including gastrulation and neuro-epithelium formation (1,2).

E-cadherin is an adhesion molecule of epithelial cells whose expression is frequently down-regulated in invasive cancers. Genes encoding transrepressors of E-cadherin, through binding to E-box sequences in the E-cadherin promoter, have been reported to closely associate with EMT. Snail and Zeb families of zinc-finger proteins and basic helix-loop-helix transcription factors including E12/E47 (3) and Twist (4) have been identified as repressors of E-cadherin and triggers of EMT. An inverse correlation between Snail and E-cadherin expression has been reported in many types of cancer including squamous cell carcinoma (SCC) (5-8). Overexpression of Snail results in the dramatic down-regulation of E-cadherin, conversion to a fibroblastic phenotype, and acquisition of more invasive properties (9-12). The Zeb zinc-finger homeobox family includes Zeb-1/δEF1 and Zeb-2/Smad-interacting protein 1 (SIP1) (13). Increased expression of SIP1 in invasive cancers without expression of E-cadherin has been also reported (14,15). SIP1 is expressed in response to TGF-ß1 and binds to Smads, the TGF-ß1 signaling proteins (16-18). TGF-ß1 also induces EMT in several epithelial cell types (19-21). Different effects of TGF-ß1 on the expression of the Snail or SIP1 gene have been reported and the correlation of Snail with SIP1 expression is still not clear (14,22-24).

Besides E-cadherin, other epithelial cell-specific molecules are repressed by EMT, such as desmoplakins (9) cytokeratin 18 and MUC1 (23), claudin, occludin, α and ß-catenin (25-27). The up-regulation of mesenchymal markers including vimentin and fibronectin (9,28) has also been reported. We previously

reported that the expression of Wnt-4 was repressed and that of Wnt-5a was induced by EMT (29). Furthermore, we showed that MMP-2, a matrix-degrading enzyme, the expression of which has been reported to be increased in many types of invasive cancers (30,31), was induced by EMT in SCC cells (12). Both down- and up-regulated proteins may contribute to the acquisition of more invasive properties of cancer cells through loss of cell adhesion and increased capabilities for matrix degradation and migration. However, the functions and control mechanism of the individual proteins, especially the mechanism of the up-regulation, which may not be due to repression of the E-box, should be clarified. In this report, we studied the expression of Snail and SIP1 in SCC cells with both epithelial and mesenchymal phenotypes and the effects of TGF-ß1 on the expression of these genes. Furthermore, we studied the mechanism of up-regulation of MMP-2 expression and identified Ets-1 as a new effector of EMT, existing downstream of SIP1, Snail or TGF-ß1.

Materials and methods

Cells and cell culture. The human vulval epithelial cell line A431 was obtained from the Japanese Collection of Research Bioresources. The human oral SCC cell lines, OM-1, HOC719-PE, HOC719-NE, TSU and HOC313 have been reported previously (5,12,29,32,33). HOC719-PE and HOC719-NE cells were isolated from HOC719 cells expressing E-cadherin heterogeneously (5). Stable Snail-overexpressing cells, A431SNA1, A431SNA2, OM-1SNA1 and OM-1SNA2, and their control pcDNA3-transfected cells, A431pcD1, A431pcD2, OM-1pcD1 and OM-1pcD2, were described previously (12,29). All cells were cultured at 37˚C in a humidified atmosphere of 5% CO_2 in air and maintained with DMEM (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Sigma).

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Total RNA was isolated from the cells using TRIzol (Invitrogen, Carlsbad, CA, USA). RT-PCR analysis was performed as described previously (5,12,29). The RNA samples were first treated with deoxyribonuclease I (Invitrogen) and converted into cDNA using random hexamer primers and reverse transcriptase (Invitrogen). PCR consisting of 30 cycles of denaturing at 94˚C for 30 sec, annealing for 30 sec and extension at 72˚C for 1 min was carried out using PCR Master (Boehringer Mannheim GmbH, Germany). Amplified products were analyzed on 1.8% agarose gels. The product size, annealing temperature, and primer sequences were: SIP1, 466 bp, 58˚C, 5'-CTCCAGGAGTAATACTCCT TCTCC-3' (forward), 5'-TAGGAAGCTCATCTGATCCAG TCC-3' (reverse). Ets-1, 395 bp, 58˚C, 5'-GCCTATCCAGA ATCCCGCTATAC-3' (forward), 5'-CGCTGCAGGCTGTT GAAAGATGA-3' (reverse). The other primers were described previously (12,29). All of the primers were obtained from Hokkaido System Science Co., Ltd. (Sapporo, Japan).

Western blot analysis. Western blot analysis was performed as previously described (12,29). Cell lysate was denatured at 100˚C for 5 min, separated by SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane (Bio-Rad, Richmond, CA, USA). The membranes were incubated with a blocking buffer consisting of 5% skimmed milk and 1% BSA in TBST (137 mM NaCl, 2.68 mM KCl, 0.1% Tween-20, and 25 mM Tris-HCl, pH 7.5) for 3 h, with the primary antibody for one night at 4˚C, and with secondary antibody in TBST. The signals were detected using an enhanced chemiluminescence (ECL) system (Amersham, Piscataway, NJ, USA) and photographed with an ECL Mini-camera (Amersham). Primary antibodies used were: rat monoclonal anti-HA, clone 3F10 (Roche, Mannheim, Germany), mouse monoclonal anti-Myc, clone 9B11 (Cell Signaling Technology, Beverly, MA, USA), rabbit polyclonal anti-c-Ets-1 (Active Motif, Carlsbad, CA, USA), rabbit polyclonal anti-MMP-2 (Biomol, Plymouth Meeting, PA, USA), mouse monoclonal anti-V5 (Invitrogen) and HECD-1. Secondary antibodies were anti-rat IgG (Sigma), anti-mouse IgG (Dako) and anti-rabbit IgG (Dako).

Vector construction and transfection. An inducible Snail expression vector, pTRE2-SNA, was constructed by transferring the Snail cDNA from pCDNA3-mm snail-HA (10,12), a gift from Dr A.G. De Herreros (Universitat Pompeu Fabra, Barcelona, Spain), to a BamHI/NotI-digested pTRE2 vector (Clontech, Palo Alto, CA, USA). Inducible expression vectors for mouse full-length SIP1 (pTREHyg-SIP1) and the mutant SIP1 (pTREHyg-SIP1/MT) with mutated zinc finger clusters at both the N- and C-terminus and lacking DNA-binding activity (14) were constructed in pTREHyg (Clontech). A431 cells were transfected with a regulator plasmid, pTet-Off (Clontech), using Tfx-20 (Promega) according to the manufacturer's directions. Sixty G418-resistant clones were isolated using cloning rings (Iwaki, Tokyo, Japan). Each clone was transiently transfected with pTRE2-Luc (Clontech) and screened for doxycyline-dependent induction of luciferase activity with a low background. One of the clones, A431-Tet Off-26, was used for further isolation of the cells which doxycyclin-dependently expressed the transfected genes. A431-Tet SNA cells were isolated from A431-Tet Off-26 cells by co-transfection with pTRE2-SNA and pTK-Hyg (Clontech) and selection with 400 μg/ml of hygromycin B (Invitrogen). The control A431-Tet TRE cells were similarly isolated with pTRE2 and pTK-Hyg. A431-Tet SIP1 and A431-Tet SIP1/MT cells were obtained by transfection with pTREHyg-SIP1 and pTREHyg-SIP1/MT, respectively. These cells were cultured in the presence of 1 μg/ml of doxycycline to prevent expression of the transfected genes, which was induced by withdrawing doxycycline from the culture medium. To construct a human ets-1 expression vector, pCDNA6/V5 Ets-1, full-length Ets-1 cDNA was amplified by RT-PCR from mRNA of human gingival fibroblasts and cloned into a KpnI/ApaI-digested pcDNA6/V5 vector (Invitrogen). The sequences of the PCR primers used were 5'-TCTAGGTACCATGAAGGCGGCCGTCGATCT-3' (forward) and 5'-AAGTGGGCCCTCGTCGGCATCTGGCT TGA-3' (reverse). PCR products were confirmed by sequence analysis using an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). A431 cells were transfected with pCDNA6/V5 Ets-1 or pCDNA6/V5 and stable transfectants were selected with 20 μg/ml of blasticidin (Invitrogen).

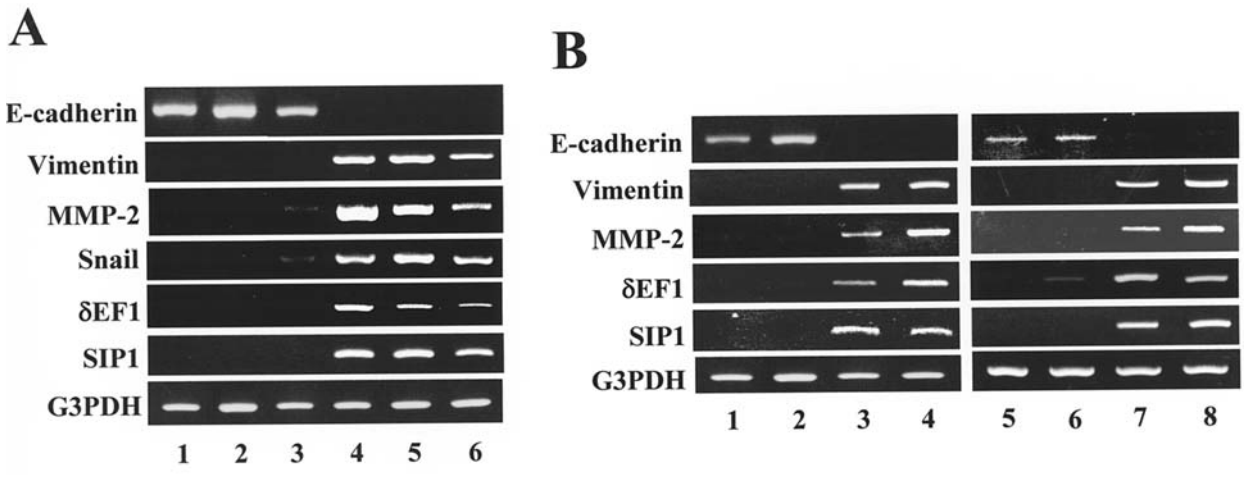

Figure 1. Induction of EMT by Snail overexpression in SCC cells. (A), Gene expression examined by RT-PCR in six human SCC cell lines. lane 1, A431; lane 2, OM-1; lane 3, HOC719-PE; lane 4, HOC719-NE; lane 5, HOC313; lane 6, TSU cells. (B), Gene expression in Snail-overexpressing and control clones. Lane 1, A431pcD1; lane 2, A431pcD2; lane 3, A431SNA1; lane 4, A4341SNA2; lane 5, OM-1pcD1; lane 6, OM-1pcD2; lane 7, OM-1SNA1; lane 8, OM-1SNA2.

Luciferase analysis. Human MMP-2 promoter fragments from -1714, -1630, -1255, -1248, -960 and -411 to +22 relative to the transcriptional start site were amplified by PCR from genomic DNA of normal human fibroblasts. The primer sequences used were (-1714): 5'-CAAGGTACCTCCCAAG AGGGTCCTTTAAACTG-3'; (-1630): 5'-ATCAGGTACCG AAGCCCACTGAGACCCAAGCCG-3'; (-1271): 5'-ACGT GGTACCAGAAGTCACTTCTTCCAGGAAGCC-3'; (-1255): 5'-CCGAGGTACCAGGAAGCCTTCCTTGATTGTCTT TA-3'; (-1248): 5'-GGCAGGTACCCTTCCTTGATTGTCTT TACTAGTTTAGGGGC-3'; (-960): 5'-TGGCACGCGTGG GTGCTTCCTTTAACATGCTAATG-3'; (-411): 5'-CATTC CTACGCGTTCCTGACCCCAGGGAGT-3'; (-211): 5'-CTC TAACGCGTGGCCCCTGACTGCTCTATTTC-3'; (reverse,+22): 5'-CAACCTCGAGCCACCGCCTGAGGA AGTCTG-3'.

PCR products were confirmed by sequence analysis. PCR fragments of the MMP-2 promoter were digested with KpnI or MluI and XhoI and inserted into the KpnI or MluI/XhoI-digested pGL2-Basic Vector (Promega). A431, A431-TetSNA or A431-TetSIP1 cells were co-transfected with 4 μg of the reporter vector containing the MMP-2 promoter sequence and 1ng of pRL-CMV as an inner control using Lipofectamine 2000 (Invitrogen). In the study of the effects of Ets-1 on the MMP-2 promoter, A431 cells were further co-transfected with 2 μg of pcDNA6/V5Ets-1 or pcDNA6. After 48 h of transfection, cells were lysed with passive lysis buffer and the promoter activity was measured using a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol.

Electrophoretic mobility shift analysis. Extraction of the nuclear proteins was performed as described (34). Cells were homogenized in 0.4 ml of 20 mM HEPES (pH 7.9) containing 0.4 M NaCl, 1 mM EDTA, 1.5 mM $MgCl_2$, 20% glycerol, 10 mM NaF, 1 mM Na_3VO_4, 0.2 mM DTT, 20 mM ß-glycerophospate, 0.5 mM PMSF, 60 μg/ml aprotinin and 2 μg/ml leupeptin. The samples were incubated on ice for 15 min, then centrifuged at 15,000 rpm for 10 min at 4˚C. The resulting supernatants were collected and the protein concentration was determined. Synthetic 20-bp oligonucleotides with the MMP-2 promoter sequence from -1261 to -1241 relative to the transcriptional start site containing the Ets-1-binding sequence (5'-TTCTTCCAGGAAGCCTTCCT-3') and its complementary sequence were heated for 10 min at 95˚C with subsequent cooling to room temperature over 6 h. The probe was end-labeled with [γ-^{32}P]-ATP using T4 polynucleotide kinase (Amersham). The nuclear protein (10 μg) was incubated with 10 fmol of the radiolabeled probe at room temperature for 30 min in binding buffer [20 mM HEPES (pH 7.9), 20% glycerol, 0.1 M NaCl, and 0.2 M EDTA] containing 0.2 mM PMSF, 0.5 mM DTT, 300 μg/ml acetylated BSA, and 2 μg of poly (dI-dC) in a total volume of 20 μl. The sample was electrophoresed on a non-denaturing 4% polyacrylamide gel, in a buffer containing 67 mM Tris-HCl, 10 mM EDTA, and 33 mM sodium acetate. The gel was run at 150 V for 2 h, dried and subjected to autoradiography. The anti-Ets-1 antibody (1 μl) was added to the reaction mixture for the supershift experiments. The sequence of the mutant oligonucleotide used in the competition assay was 5'-TTCTT CCATTAAGCCTTCCT-3'.

Results

Different genes expressed in SCC cells with epithelial and mesenchymal phenotypes. We previously reported that A431, OM-1 and HOC719-PE cells exhibited a cuboidal morphology whereas HOC719-NE, HOC313 and TSU cells showed a spindle-like morphology and suggested that these cells have acquired EMT (12,29). RT-PCR analysis revealed decreased expression of E-cadherin and strong expression of vimentin, MMP-2, Snail and δEF1 in HOC719-NE, HOC313 and TSU cells compared to A431, OM-1 and HOC719-PE cells (Fig. 1A). We found that SIP1 was also strongly expressed in these cells. An inverse correlation in these genes was similarly observed between the control and Snail-overexpressing clones of both A431 and OM-1 cells (Fig. 1B).

Change of gene expression with induction of Snail expression. We established A431-Tet SNA cells, which showed a doxycyclin-dependent expression of Snail, and the control

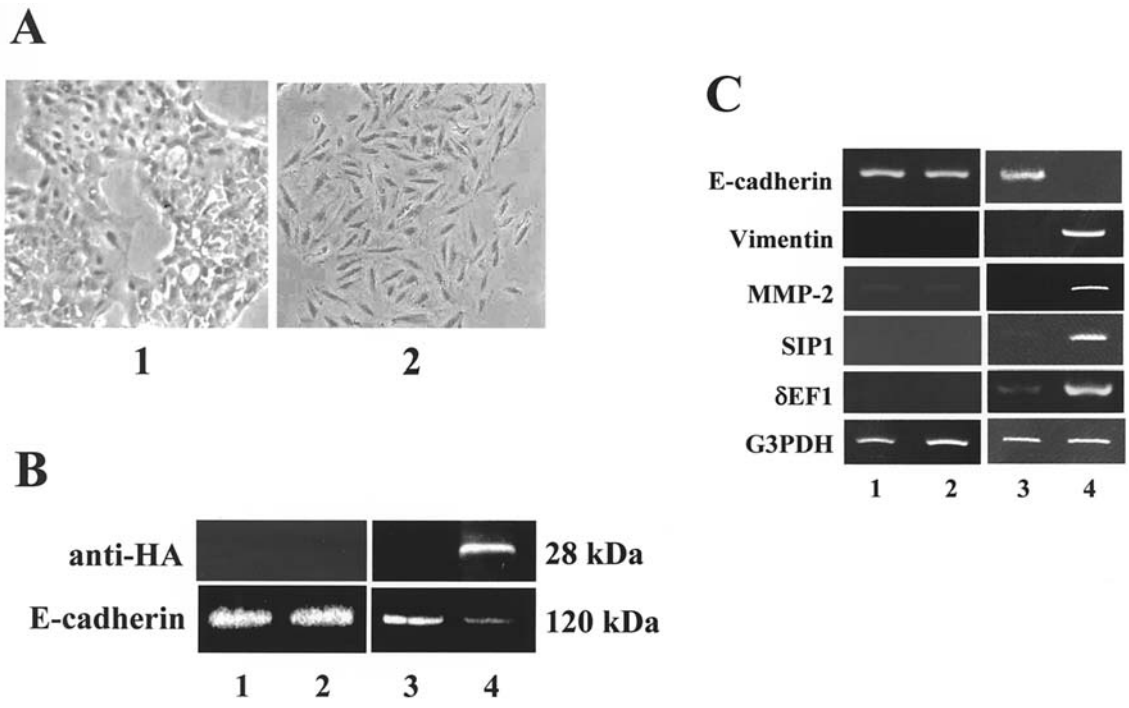

Figure 2. Effects of inducible Snail expression in A431 cells. (A), A431-Tet SNA cells cultured in the presence (1) or absence (2) of doxycycline. Photographs were taken under a phase-contrast microscope at a magnification x100. (B), Expression of Snail and E-cadherin proteins analyzed by Western blotting. A431-Tet SNA (lanes 3, 4) and control A431-Tet TRE cells (lanes 1, 2) were cultured with (lanes 1, 3) or without (lanes 2, 4) doxycycline. (C), Gene expression in A431-Tet SNA (lanes 3, 4) and A431-Tet TRE cells (lanes 1, 2) cultured with (lanes 1, 3) or without (lanes 2, 4) doxycycline.

A431-Tet TRE cells. The expression of Snail was induced by withdrawing doxycycline from the culture medium. After 7 days, A431-TetSNA cells changed from a cuboidal to a spindle-shaped morphology with loss of the cell-cell contacts (Fig. 2A). Western blotting using anti-HA antibody which recognized the HA epitope attached to the C-terminus of the Snail protein demonstrated the induction of Snail expression. Decreased expression of E-cadherin protein was detected by both Western blotting in these cells (Fig. 2B). RT-PCR analysis demonstrated the down-regulation of E-cadherin expression, and up-regulation of vimentin, MMP-2, δEF1 and SIP-1 mRNA expression in A431-Tet SNA cells, but not in control A431-Tet TRE cells (Fig. 2C).

Effects of SIP1 on expression of the EMT-associated genes. We also established A431 clones in which wild-type or mutated SIP-1 lacking the DNA-binding activity was expressed. A431-Tet SIP1 cells cultured in the absence of doxycycline had an enlarged intercellular space, although the spindle shape was less significant than that of Snail-expressing cells (Fig. 3A). The expression of both wild-type and mutated SIP1 protein was confirmed by Western blotting using anti-Myc antibody recognizing the Myc epitope at the C-terminus of the SIP1 proteins (Fig. 3B). The change of cell shape was not observed in A431-Tet SIP1/MT cells in which the mutated SIP1 protein was expressed (data not shown). By RT-PCR analysis, down-regulation of E-cadherin expression, and up-regulation of vimentin and MMP-2 expression were detected accompanied by the induction of SIP1 protein in A431-Tet SIP1, but not in A431-Tet SIP1/MT cells (Fig. 3C). However, although the expression of both SIP1 and δEF1 was up-regulated by Snail (Fig. 2C), we found that neither Snail nor δEF1 expression was induced by SIP1 (Fig. 3C).

Effects of TGF-ß1 on EMT in SCC cells. To study the effect of TGF-ß1, SCC cells were treated with 2 ng/ml of TGF-ß1 for 72 h. A431 cells showed a morphological change to a more spindle-like shape with reduced cell-cell contact (Fig. 4A). RT-PCR analysis showed that the expression of E-cadherin mRNA was down-regulated, and that of vimentin and MMP-2 was increased by TGF-ß1 in both A431 and OM-1 cells (Fig. 4B). However, TGF-ß1 had different effects on the expression of Snail and SIP1 in SCC cells with the epithelial or mesenchymal phenotype. In A431 cells, increased expression of SIP1 was detected but Snail expression was not induced by TGF-ß1. In contrast, the expression of SIP1 was constant, but the expression of Snail was strongly increased by TGF-ß1 in HOC313 cells (Fig. 4C).

Activation of MMP-2 promoter by Snail, SIP1 and TGF-ß1 through an Ets-1-binding site. To study the regulatory mechanism of the expression of MMP-2 by EMT, we constructed reporter vectors containing MMP-2 promoter sequences (Fig. 5A). A431-Tet SNA and A431-Tet SIP1 cells were transfected with these vectors and cultured for 2 days in the presence or absence of doxycycline. Luciferase analysis demonstrated the activation of promoter fragments containing sequences up to -1714, -1630, -1271 and -1255 upon induction of Snail expression in A431-Tet SNA cells. However, an abrupt decrease in activation was detected containing promoter fragments -1255 and -1248 (Fig. 5B). Similarly, a strong activation of fragments -1714, -1630, -1271 and -1255, but

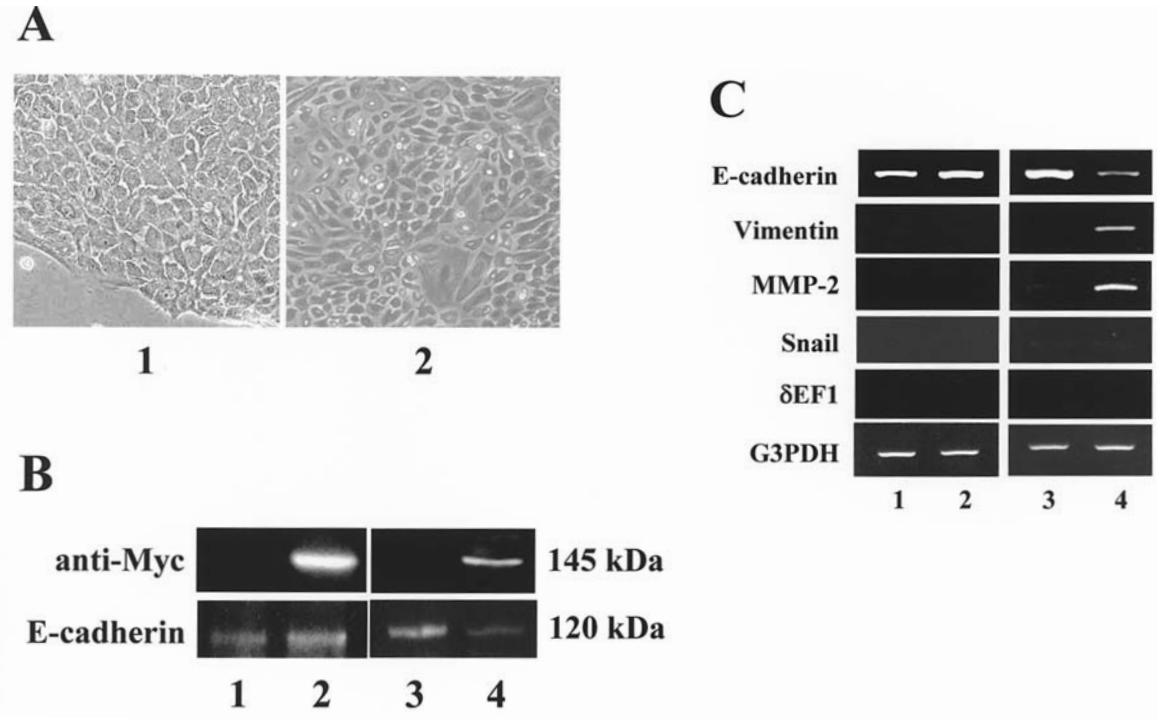

Figure 3. Effects of inducible SIP1 expression in A431 cells. (A), A431-Tet SIP1 cells cultured in the presence (1) or absence (2) of doxycycline. Photographs were taken under a phase-contrast microscope at a magnification x100. (B), Expression of SIP1 and E-cadherin proteins analyzed by Western blotting. A431-Tet SIP1 (lanes 3, 4) and A431-Tet SIP1/MT cells (lanes 1, 2) were cultured with (lanes 1, 3) or without (lanes 2, 4) doxycycline. (C), Gene expression in A431-Tet SIP1/MT (lanes 1, 2) and A431-Tet SIP1 cells (lanes 3, 4) cultured with (lanes 1, 3) or without (lanes 2, 4) doxycycline.

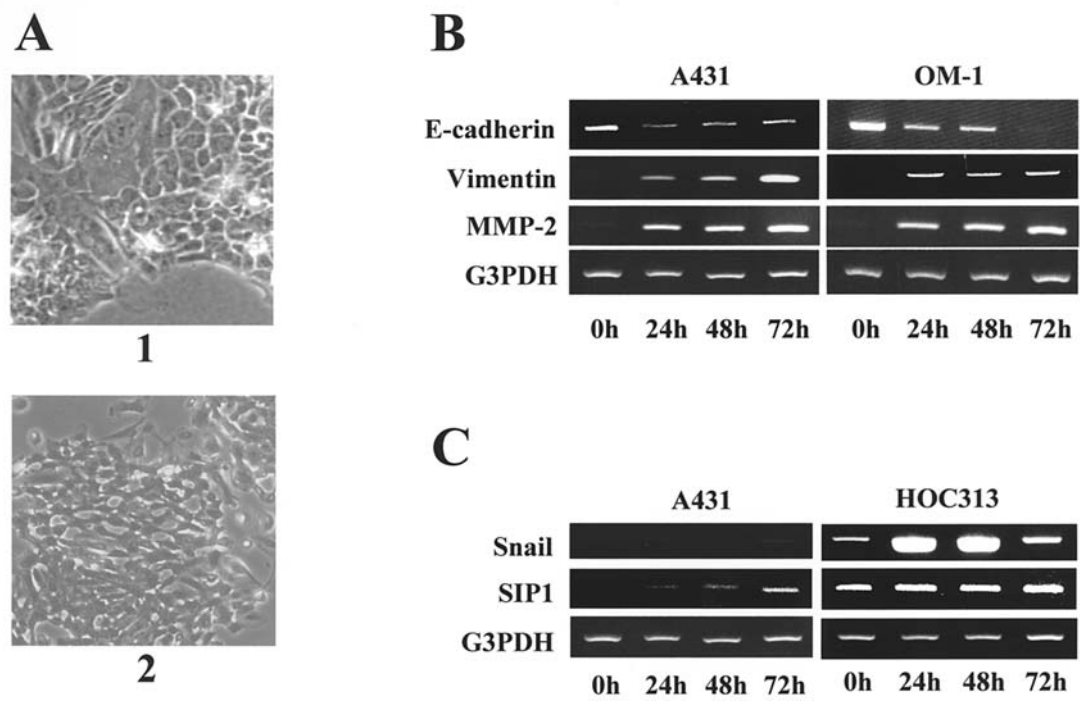

Figure 4. Effects of TGF-ß1 on EMT in SCC cells. (A), Morphology of A431 cells cultured in the absence (1) or presence (2) of TGF-ß1 for 72 h. Photographs were taken under a phase-contrast microscope at a magnification of x100. (B), Gene expression in A431 and OM-1 cells cultured with TGF-ß1. (C), Effects of TGF-ß1 on gene expression of Snail and SIP1 in SCC cells.

the loss of activation of -1248, -960, -411 and -211 of the MMP-2 promoter was observed in A431-Tet SIP1 cells (Fig. 5C). Furthermore, TGF-ß1 induced strong promoter activity of fragments up to -1255 bp, but not less than -1248 bp, of MMP-2 promoter (Fig. 5D). These results indicated that an Ets-1-binding sequence, located between -1255 and -1248

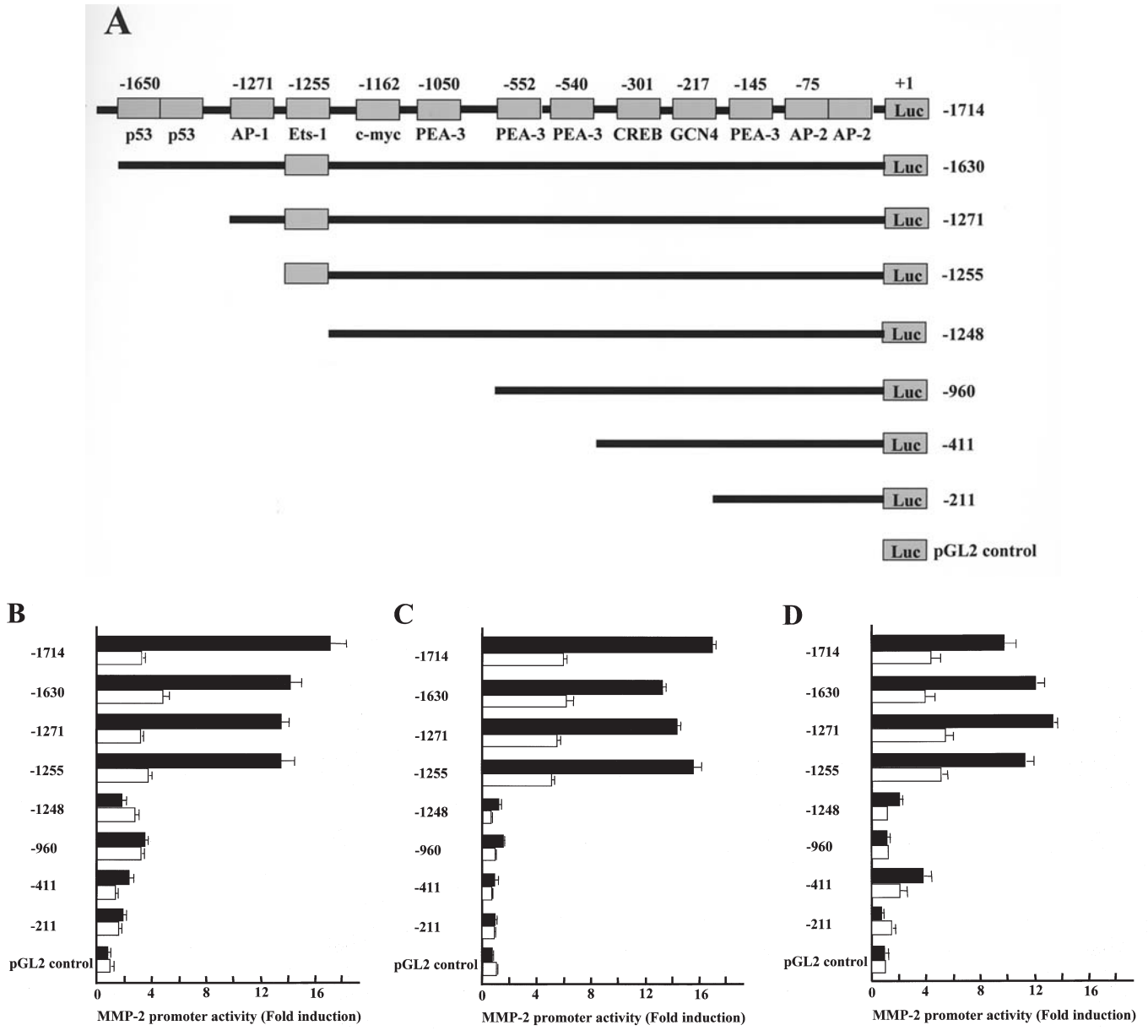

Figure 5. Activation of MMP-2 promoter by Snail, SIP1 and TGF-ß1. (A), Structures of MMP-2 promoter harboring the 5' deletion constructs. (B), MMP-2 promoter activity in A431-Tet SNA cells. Cells were cultured in the presence (□) or absence (■) of doxycycline for 48 h. (C), MMP-2 promoter activity in A431-Tet SIP1 cells. Cells were cultured in the presence (□) or absence (■) of doxycycline for 48 h. (D), Effect of TGF-ß1 on MMP-2 promoter activity in A431 cells. Cells were cultured in the presence (□) or absence (■) of TGF-ß1 for 48 h. Luciferase assay was performed as described in Materials and methods. Fold-increase over control cells transfected with the pGL2 vector and the mean ± SD (n=3) are shown. Data shown are representative of at least three experiments.

relative to the transcriptional start site (Fig. 5A), was responsible for the activation of the MMP-2 promoter induced by Snail, SIP1 and TGF-ß1 in SCC cells.

Up-regulation of Ets-1 expression by EMT in SCC cells. Western blotting using an anti-Ets-1 antibody demonstrated increased Ets-1 protein expression in both A431-Tet SNA and A431-Tet SIP1 cells cultured in the absence of doxycycline (Fig. 6A). RT-PCR analysis revealed an up-regulation of Ets-1 mRNA expression in A431 cells treated with TGF-ß1 (Fig. 6B). At RT-PCR analysis, strong mRNA expression of Ets-1 was detected in HOC719-PE, HOC313, TSU cells (Fig. 6C), however there were no relationships between other Ets genes expressions and EMT in SCC cells (data not shown). Up-regulation of Ets-1 expression was detected accompanied with induction of Snail and SIP1 in SCC cells (Fig. 6D).

Induction of Ets-1 binding to the MMP-2 promoter by Snail and SIP1. We next studied the DNA-binding activities of nuclear proteins extracted from A431-Tet SNA and A431-Tet SIP1 cells cultured in the presence or absence of doxycycline. A double-stranded oligonucleotide with 20-bp of the MMP-2 promoter sequence from -1261 to -1241 relative to the transcriptional start site was ^{32}P-radiolabeled and incubated with the nuclear extracts. Although the nuclear protein extracted from A431-Tet SNA cells cultured in the presence of doxycycline showed weak binding to the target DNA, binding was clearly enhanced in the cells cultured in the absence of doxy-

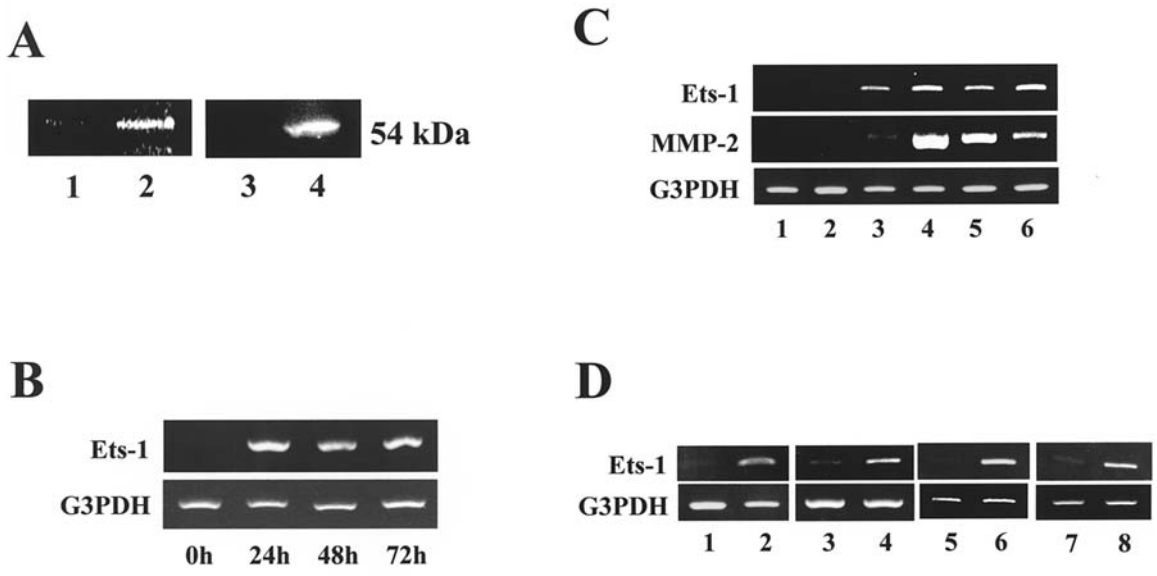

Figure 6. Induction of Ets-1 expression by Snail, SIP1 and TGF-ß1. (A), Expression of Ets-1 protein in A431-Tet SNA (lanes 1, 2) and A431-Tet SIP1 (lanes 3, 4) analyzed by Western blotting. Cells were cultured in the presence (lanes 1, 3) or absence (lanes 2, 4) of doxycycline. (B), Gene expression of Ets-1 examined by RT-PCR in A431 cells cultured in the presence of TGF-ß1. (C), Expression of Ets-1 and MMP-2 analyzed by RT-PCR in SCC cells. Lane 1, A431; lane 2, OM-1; lane 3, HOC719-PE; lane 4, HOC719-NE; lane 5, HOC313; lane 6, TSU cells. (D), Expression of Ets-1 in Snail or SIP1 overexpressing (lane 2, A431SNA1; lane 4, OM-1SNA1; lane 6, A431-Tet SNA without doxycycline; lane 8, A431-Tet SIP1 without doxycycline) and control cells (lane 1, A431pcD1; lane 3, OM-1pcD1; lane 5, A431-Tet SNA with doxycycline; lane 7, A431-Tet SIP1 with doxycycline).

cycline (Fig. 7A, lanes 1 and 2). This binding was sequence-specific because it was inhibited by the addition of a 30-fold excess of unlabeled oligonucleotide (Fig. 7A, lanes 4 and 5). Incubation with the anti-Ets-1 antibody resulted in a shifted band (Fig. 7A, lane 3), indicating that the DNA-protein complex contained the Ets-1 protein. Similarly, the nuclear extract from A431-TetSIP1 cells cultured in the absence of doxycycline showed strong DNA-binding activity which was inhibited by addition of an excess of unlabeled oligonucleotide containing a wild-type, but not mutant, Ets-1-binding sequence. The DNA-protein complex was also partially shifted by the anti-Ets-1 antibody (Fig. 7B).

Ets-1 activates the MMP-2 promoter without induction of EMT. To study the effect of Ets-1 on the expression of MMP-2, an expression vector for Ets-1 was constructed and transiently transfected into A431 cells with the reporter vectors containing MMP-2 promoter sequences. Luciferase analysis demonstrated a strong activation of the MMP-2 promoter from -1255 to +22 on transfection of the Ets-1-expression vector. A significant decrease in the activation by the Ets-1 expression was observed with a fragment from -1248 to +22 of the promoter (Fig. 8A). A stable Ets-1-over-expressing clone (A431Ets1) and a control clone (A431-pcDV5) were isolated. Western blotting using anti-Ets-1 and anti-V5 antibody demonstrated strong expression of the transfected Ets-1 protein in A431Ets1, but not A431pcDV5 cells (Fig. 8B). Increased protein expression of MMP-2 in A431Ets1 cells was clearly detected using anti-MMP-2 antibody. RT-PCR analysis also showed the increased expression of MMP-2 mRNA in A431Ets1 cells (Fig. 8C, lane 2) compared to A431pcDV5 cells (lane 1). Furthermore, MMP-1, -3, -7, -9 expressions were up-regulated. However, no morphological change was seen in A431Ets1 cells (data not shown). No up-regulation of vimentin expression was detected

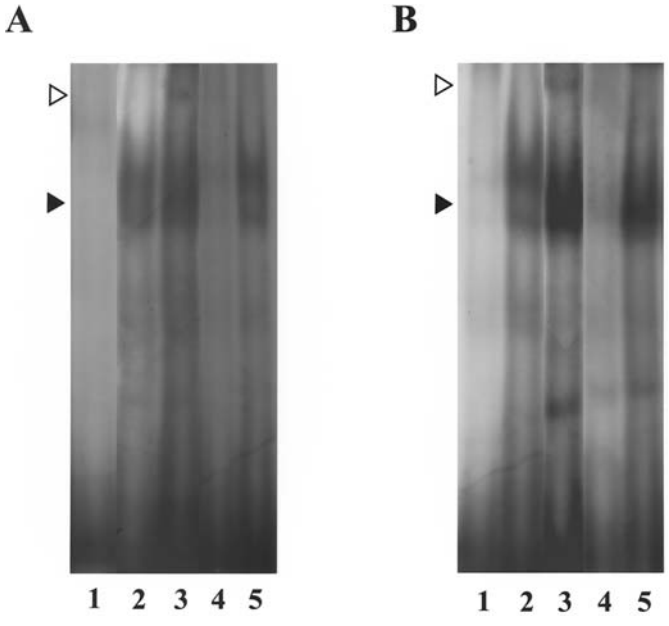

Figure 7. Induction of Ets-1 binding to the MMP-2 promoter by Snail and SIP1. Nuclear extracts from A431-Tet SNA (A) and A431-Tet SIP1 (B) cells were incubated with a ^{32}P-labeled double-stranded oligonucleotide with the MMP-2 promoter sequence. Cells were cultured in the presence (lane 1) or absence (lanes 2-5) of doxycycline. The nucleoprotein complexes were incubated with the anti-Ets-1 antibody (lane 3), a 30-fold excess of cold oligonucleotide (lane 4) or a cold oligonucleotide with a partially mutated sequence at the Ets-1 site (lane 5) as described in Materials and methods. Specific DNA-binding protein complexes are depicted by block arrowheads, and are supershifted with anti-Ets-1 antibody (open arrowhead).

but increased rather than decreased expression of E-cadherin was observed in A431Ets1 cells, indicating that the overexpression of Ets-1 did not induce EMT in A431 cells (Fig. 8C).

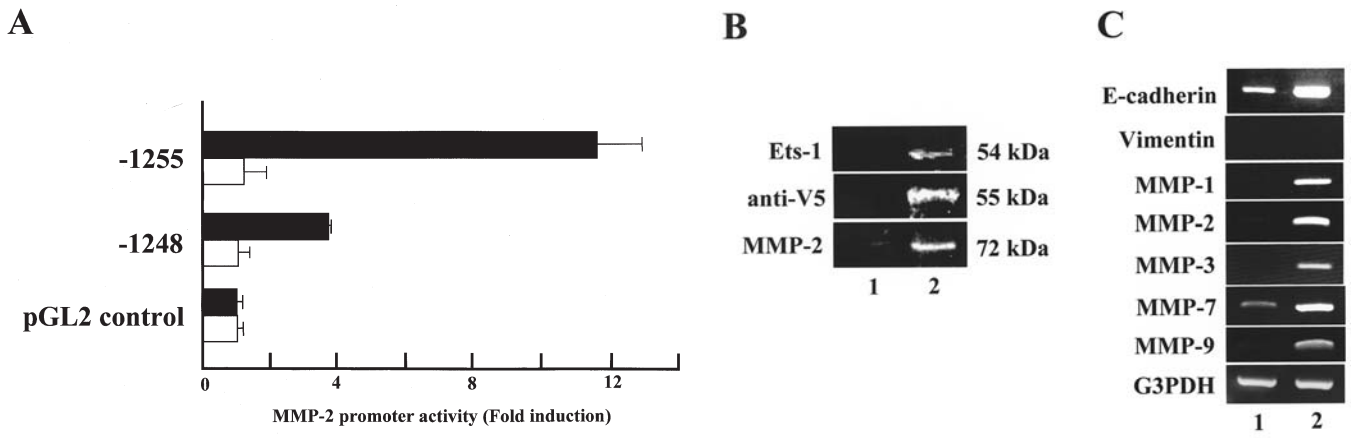

Figure 8. Induction of MMP-2 expression and promoter activity by Ets-1. (A), A431 cells were cotransfected with MMP-2 promoter constructs and pcDNA6/V5Ets-1 (■) or control pcDNA6/V5 (□). After 48-h incubation, luciferase analysis was performed. Fold-increase over control cells transfected with the pGL2 vector and the mean ± SD (n=3) are shown. Data shown are representative of three experiments. (B), A431 cells were transfected with pcDNA6/V5Ets-1 (lane 2) or control pcDNA6/V5 (lane 1) and stable clones were isolated. The expression of Ets-1 and MMP-2 proteins was analyzed by Western blotting. (C), Expression of EMT-associated genes and MMPs in Ets1-overexpressing (lane 2, A431Ets1) and control (lane 1, A431pcDV5) cells examined by RT-PCR.

Discussion

In this study, we found strong expression of Snail, SIP-1 and δEF1 in SCC cells with a mesenchymal phenotype, but not in those with an epithelial phenotype. In stable Snail-overexpressing clones of A431 and OM-1 cells, increased levels of SIP1 and δEF1 were also detected. Tet Off-induced expression of Snail or SIP1, and treatment with TGF-ß1, resulted in EMT in A431 cells, in terms of the down-regulation of E-cadherin and up-regulation of vimentin and MMP-2 expressions accompanied by changes of cell shape. The expression of Snail resulted in increased levels of SIP1 and δEF1, whereas the expression of SIP1 did not induce Snail or δEF1 expression. TGF-ß1 induced the expression of SIP1 but not Snail in A431 and OM-1 cells, while it strongly increased the expression of Snail without affecting the levels of SIP1 in the cells with a mesenchymal phenotype.

Although TGF-ß1 induces EMT in several epithelial cells (19-21), it has different effects on the expression of the Snail and SIP1 genes. Peinado *et al* reported that TGF-ß1 induced EMT with the expression of Snail in MDCK cells which was dependent on the MAPK signaling pathway (24). In contrast, induction of EMT and SIP-1 expression, but not Snail expression, by TGF-ß1 has been reported in mouse mammary cells (14) and in hepatocyte cell lines (22). Furthermore, no clear correlation between Snail and SIP1 expression has been reported; Snail mRNA levels were not influenced by SIP1 (14) and Snail induced the expression of δEF1 but not SIP1 (23). These results, taken together, indicated that the effects of TGF-ß1 on Snail and SIP1 expression are dependent on the epithelial or mesenchymal phenotype of SCC cells.

We next studied the mechanism of the up-regulation of MMP-2 expression accompanying EMT. Studies have shown a positive correlation between increased activity of MMP-2 and invasion and metastasis by cancer cells (12,30,31). We also previously reported strong activity and gene expression of MMP-2 in SCC cells with a mesenchymal phenotype. Furthermore, both the expression and activity of MMP-2 were induced by Snail in SCC cells (12). Similarly, MMP-2 mRNA expression was induced by EMT in mouse mammary cells (35). In the present study, we found that the expression of MMP-2 was induced by not only Snail but also SIP1 and TGF-ß1. Luciferase analysis using reporter vectors containing MMP-2 promoter sequences revealed that an Ets-1-binding site, located between -1255 and -1248 relative to the transcriptional start site, was the response sequence for the activation by Snail, SIP1 and TGF-ß1. Although our previous study using shorter MMP-2 promoter sequences and the transient expression of Snail suggested that a region between -411 and -262 was necessary for about a 2-fold induction (12), the present study using 1714 bp of the MMP-2 promoter sequence and inducible expression vectors clearly indicated that the Ets-1 site was responsible for a more than 10-fold induction of the promoter activity.

We further demonstrated that the expression of Ets-1 was induced by all of Snail, SIP1 and TGF-ß in A431 cells and strong expression of Ets-1 and MMP-2 was detected in SCC cells with a mesenchymal phenotype. Nuclear proteins extracted from the cells with Tet Off-induced expression of Snail or SIP1 showed increased DNA-binding activity to the Ets-1 site. Furthermore, the MMP-2 promoter activity was strongly induced by a transient expression of Ets-1, and increased protein expression of MMP-2 was detected in the stable Ets-1-overexpressing cells. These results indicated a cascade mechanism of MMP-2 expression; Snail, SIP1 and TGF-ß1 induced the expression of Ets-1 accompanying EMT, and the Ets-1 bound to and activated the MMP-2 promoter, and finally induced the expression of MMP-2.

Ets proteins constitute a family of transcription factors implicated in the regulation of several matrix-degrading proteinases (36-39). Ets-1 has been reported to activate the expression of MMP-1, -3, and -9 and urokinase-type plasminogen activator (uPA) via the Ets-1-binding sites in the promoters of these genes (40-42). Increased expression of Ets-1 was reported in several invasive cancers (43-45). In the

present study, the expression of MMP-1, -3 and -9 was also induced by overexpression of Ets-1 in A431 cells. However, there was no clear correlation between the expression levels of MMP-9 and the status of the epithelial or mesenchymal phenotype of SCC cells (5). Furthermore, the expression levels of MMP-1 and MMP-3 did not associate with the epithelial or mesenchymal phenotype of these cells (data not shown).

Among the several transcription factors which have been reported to activate the MMP-2 promoter (46-50), Reisdorff *et al* found that a specific Ets-1-binding site, located between -1053 and -1004 relative to the transcriptional start site, was responsible for the constitutively strong expression of MMP-2 in rat glomerular mesangial cells (49). They also demonstrated an increased expression of MMP-2 protein caused by a transient expression of Ets-1 in these cells. Recently, Ito *et al* reported that prostaglandin E2 enhanced the invasiveness of pancreatic cancer through an Ets-1-dependent induction of MMP-2 expression (51). Although Ets-1-binding site exists in the promoter sequences of several MMP genes, a TATA-box and NF-κB regulatory element, which are present in the promoters of MMP-1, -3 and -9, are absent in the MMP-2 promoter (52-54). These results, taken together with ours, suggest that the control mechanism for MMP-2 expression accompanying EMT is different from that for the other MMPs, which might be influenced by other transcription factors including other members of the Ets family.

However, it is worth noting that the up-regulation of vimentin and a change of cell morphology were not induced by the overexpression of Ets-1. Furthermore, increased, rather than decreased, level of E-cadherin expression was detected in the Ets-1-overexpressing cells. These results indicate that Ets-1 functions as one of the effectors of EMT. Although Ets-1 by itself has no activity to induce EMT, it enhances the malignancy of SCC cells through the up-regulation of MMP-2 expression in the course of EMT. EMT may simultaneously activate independent signal pathways affecting each other. Further characterization of the signal pathways of EMT will provide important targets for drug discovery, which should lead to new therapeutic approaches for the treatment of highly invasive and metastatic cancers.

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