J. Biochem. 2013;153(3):303–315 doi:10.1093/jb/mvs144

JB THE JOURNAL OF BIOCHEMISTRY

Transforming growth factor-β1 induces epithelial–mesenchymal transition and integrin α3β1-mediated cell migration of HSC-4 human squamous cell carcinoma cells through Slug

Received November 9, 2012; accepted November 26, 2012; published online December 17, 2012

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We investigated whether transforming growth factor (TGF)-β1 promoted epithelial–mesenchymal transition (EMT) and migration of human oral squamous cell carcinoma (hOSCC) cells. Among 6 hOSCC cell lines investigated, Smad2 phosphorylation and TGF-β target genes expression were most clearly upregulated following TGF-β1 stimulation in HSC-4 cells, indicating that HSC-4 cells were the most responsive to TGF-β1. In addition, the expression levels of the mesenchymal markers N-cadherin and vimentin were most clearly induced in HSC-4 cells among the hOSCC cell lines by TGF-β1 stimulation. Interestingly, E-cadherin and β-catenin at the cell surface were internalized in HSC-4 cells stimulated with TGF-β1. In addition, the expression levels of the EMT-related transcription factor Slug was significantly upregulated on TGF-β1 stimulation. Moreover, the downregulation of Slug by RNA interference clearly inhibited the TGF-β1-induced expression of mesenchymal marker and the migration of HSC-4 cells. Proteomics analysis also revealed that the expression levels of integrin α3β1-targeted proteins were upregulated in TGF-β1-stimulated HSC-4 cells. Neutral antibodies against integrin α3 and β1, as well as a focal adhesion kinase (FAK) inhibitor, clearly suppressed TGF-β1-induced cell migration. These results suggest that the EMT and integrin α3β1/FAK pathway–mediated migration of TGF-β1-stimulated HSC-4 hOSCC cells is positively controlled by Slug.

Keywords: EMT/integrin α3β1/migration/squamous cell carcinoma/TGF-β.

Abbreviations: BCA, bicinchoninic acid; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; EMT, epithelial–mesenchymal transition; FAK, focal adhesion kinase; FBS, fetal bovine serum; hOSCC, human oral squamous cell carcinoma; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MEM, Eagle's minimum essential medium; PAI-1, plasminogen activator inhibitor-1; PBS, phosphate-buffered saline; qRT–PCR, quantitative real-time reverse transcriptase polymerase chain reaction; SD, standard deviation; RIPA, radio-immunoprecipitation assay; R-Smad, receptor-regulated Smad; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; siRNA, small interfering RNA; TGF-β, transforming growth factor-β; TSP-1, thrombospondine-1; TβR-I, TGF-β receptor type I; TβR-II, TGF-β receptor type II; uPA, urokinase-type plasminogen activator; uPAR, urokinase-type plasminogen activator receptor; ZEB, zinc finger E-box–binding homeobox; α-SMA, α-smooth muscle actin; βig-h3, TGF-β–inducible gene-h3.

It is well known that transforming growth factor (TGF)-β inhibits the growth of epithelial cells (*1*, *2*). In addition, TGF-β generally induces the secretion of extracellular matrix (ECM) proteins from epithelial cells (*3*). TGF-β exerts multiple effects by binding to transmembrane serine/threonine kinases, TGF-β receptor type I (TβR-I) and TGF-β receptor type II (TβR-II). TβR-I is phosphorylated and activated by TβR-II kinase upon ligand-induced heteromeric complex formation between TβR-I and TβR-II and mediates specific intracellular signal pathways through the phosphorylation of receptor-regulated Smads (R-Smads). Phosphorylated R-Smads associate with Smad4 and translocate to the nucleus where they control the transcription of target genes in cooperation with other transcription factors and transcriptional co-activators or co-repressors (*4*, *5*). In cancer, TGF-β is associated with malignant transformation and the aggravation of cancer through the induction of the epithelial–mesenchymal transition (EMT) (reviewed in *6*, *7*). TGF-β signalling pathway plays critical and dual roles in the progression of human cancer. TGF-β acts as a tumour suppressor at the early stage of tumour progression. In contrast, TGF-β also promotes tumour progression, for example tumour cell invasion, dissemination and immune evasion (*8*). Therefore, the functional outcome of the TGF-β-induced response is strongly dependent on the type of cancer cells (*3*).

EMT is a process that allows a polarized epithelial cell to assume a mesenchymal cell phenotype, which is

characterized by enhanced motility and invasiveness (*9*). In addition, EMT also causes the disruption of cell–cell adherence, loss of apico–basal polarity, matrix remodelling and increased motility and invasiveness, thereby promotes tumour metastasis (*8*, *10*). The tumour microenvironment plays an important role in facilitating cancer metastasis and may induce EMT in tumour cells: the large number of inflammatory cells infiltrating the tumour site, hypoxia existing in a large area of the tumour and the many stem cells present in the tumour microenvironment, such as cancer and mesenchymal stem cells, may induce EMT in tumour cells (*10*). EMT is characterized by the loss of epithelial markers such as E-cadherin, the downregulation of cytokeratins, the upregulation of mesenchymal markers such as N-cadherin and vimentin and the acquisition of a fibroblast-like motile and invasive phenotype (*11*). In addition, disruption of E-cadherin-mediated adherens junctions causes the translocation of β-catenin from the cell membrane into the cytoplasm. Then, β-catenin enters the nucleus and transactivates genes via the canonical Wnt signalling pathway (*12–14*). Thus, Wnt/β-catenin activation is an integral part of EMT (*15*, *16*). Nuclear localization of β-catenin is predominant in human oral squamous cell carcinoma (hOSCC) cells at the invasive front (*17*). On the other hand, various signalling pathways are involved in EMT, including TGF-β, NF-κB, Notch and others (*10*, *18*). It is known that many transcriptional factors participate in EMT (*19*, *20*), for example Snail (*21*), Slug (*22*), Twist (*23*) and FOXC2 (*24*). Recently, it was reported that several EMT-promoting transcriptional factors such as Snail, zinc finger E-box–binding homeobox (ZEB) 1 and ZEB2 act as cofactors of Smads (*25*). The interaction between Smads and EMT-promoting transcriptional factors results in the formation of EMT-promoting Smad complexes that engage in both repressing epithelial genes and activating mesenchymal genes.

Conversely, several ECM proteins are expressed during TGF-β-induced EMT (*19*). Thrombospondine-1 (TSP-1) is an adhesive glycoprotein that mediates cell-to-cell and cell-to-matrix interactions (*26*, *27*). The plasminogen activation system is negatively regulated by plasminogen activator inhibitor-1 (PAI-1; also known as SERPINE1), which inhibits the activity of urokinase-type plasminogen activator (uPA) by binding to it (*28*). TGF-β-inducible gene-h3 (βig-h3; also known as TGFBI) is an ECM protein identified as a major TGF-β-responsive gene (*29*). Fibronectin is also recognized as a TGF-β-inducible ECM protein during EMT (*30*). Recent advanced studies in tumour biology revealed that complex interactions of tumour cells with their adjacent microenvironment involving the ECM are necessary for the expression of various mechanisms involved in tumour development and progression (reviewed in *31*). Thus, the interaction between tumour cells and the ECM seems to control most aspects of tumorigenesis, such as EMT, along with the subsequent tumour cell migration.

Various hOSCC cell lines have been used as models to study aspects of tumorigenesis such as EMT or migration of oral cancer *in vitro*, whereas only a few studies have examined how TGF-β affects the EMT of hOSCC cells (*32–34*). In particular, the molecular mechanisms underlying the TGF-β-induced EMT or EMT-associated hOSCC cell migration remain to be clarified. Here, we evaluated Smad2 phosphorylation and the expression levels of TGF-β target genes such as Smad7, fibronectin and PAI-1 following TGF-β1 stimulation in various hOSCC cells to identify TGF-β1-responsive hOSCC cells. Then, to examine whether TGF-β1 responsiveness is closely related to the EMT of hOSCC cells, we evaluated the expression of epithelial and mesenchymal cell markers in hOSCC cells, as well as EMT-associated cell migration, after TGF-β1 stimulation. In addition, we investigated the type of transcriptional factors that cause TGF-β1-induced EMT. We also investigated the molecular mechanisms underlying EMT-associated cell migration. This is the first report to demonstrate the molecular mechanisms underlying TGF-β1-induced EMT and the EMT-associated migration of hOSCC cells.

Materials and Methods

Materials

Cultured cell lines were obtained from the Human Science Resource Cell Bank (Osaka, Japan). Recombinant human TGF-β1 was purchased from PEPROTECH (Rocky Hill, NJ, USA). The TβRI kinase inhibitor SB431542 and focal adhesion kinase (FAK) inhibitor I (1,2,4,5-benzenetetramine, 4HCl) were provided by Merck-Millipore (Frankfurt, Germany). Protease inhibitor cocktail for use with mammalian cell and tissue extracts and phosphatase inhibitor cocktail 1 and 2 were purchased from Sigma-Aldrich (St. Louis, MO, USA). All of the other reagents were of analytical grade.

Cell culture

All human squamous cell carcinoma cell lines were grown at 37°C and 5% CO_2. HSC-2 and HSC-4 cells were cultured in Eagle's minimum essential medium (MEM; Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Gibco BRL, Rockville, MD, USA). SAS cells were cultured in RPMI1640 medium (Gibco BRL) supplemented with 10% FBS. HO-1-N1 and OSC-19 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 medium (1:1; Gibco BRL) with 10% FBS. HSC-3 cells were cultured in DMEM (Gibco BRL) containing 10% FBS. The culture medium was removed and replaced with serum-free medium 24 h before the TGF-β1-stimulated experiments. In the experiments pertaining to the production of ECM proteins, 1.0×10^4 hOSCC cells were cultured in 200 μL medium without serum containing 10 ng/mL TGF-β1 for 48 or 120 h in 96-well tissue culture plates. The conditioned media were harvested and then concentrated by ultrafiltration with Microcon-10 filters (cutoff, 10 kDa; Merck). The obtained media were treated with an equal volume of sample buffer (Laemmli 2 × concentrate; Sigma-Aldrich). The samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

Western blot analysis

For the experiments on Smad phosphorylation, 3.0×10^6 cells were lysed in radio-immunoprecipitation assay (RIPA) buffer (50 mM Tris–HCl [pH 7.2], 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS) containing a protease and phosphatase inhibitor cocktail (Sigma-Aldrich). The protein content of the samples was measured using the bicinchoninic acid (BCA) reagent (Pierce, Rockford, IL, USA). For the preparation of cell lysates to examine marker proteins, 1.0×10^6 cells were cultured in a six-well plate in serum-free MEM with or without 10 ng/mL TGF-β1 for the indicated times. The cells were dissolved in SDS sample buffer containing a protease and phosphatase inhibitor cocktail (Sigma-Aldrich). Acrylamide gels of 7.5 or 12.5% (ATTO Co., Tokyo, Japan) for SDS–PAGE were used for protein separation, and the proteins were subsequently transferred onto polyvinylidene

difluoride membranes (Millipore, Bedford, MA, USA). The membranes were probed with the primary antibodies anti-Smad2/3 mouse antibody (BD Transduction Laboratories, Franklin Lakes, NJ, USA) and anti-phospho-Smad2 (Ser465/467) (Cell Signaling, Beverly, MA, USA), while an anti-β-actin antibody (clone C4; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as a loading control in the Smad phosphorylation experiments (Fig. 1A). Anti-fibronectin (P1H11; Santa Cruz), anti-PAI-1 (H-135; Santa Cruz), anti-βig-h3 (R&D Systems, Minneapolis, MN, USA) and anti-TSP-1 (N-20; Santa Cruz) antibodies were used for the detection of extracellular proteins (Figs 1C and 6B). Anti-E-cadherin (clone 36; BD Transduction Laboratories), anti-vimentin (sc-6260; Santa Cruz), anti-cytokeratin18 (sc-6259; Santa Cruz) and anti-*N*-cadherin (8C11; Santa Cruz) antibodies were used for the detection of marker proteins (Fig. 3A). Anti-Slug (A-7; Santa Cruz) and anti-lamin B (C-20; Santa Cruz) antibodies were also used for western blot analysis (Fig. 4C). The blots were incubated with alkaline phosphatase–conjugated secondary antibody, and signals were detected using an alkaline phosphatase substrate kit (BCIP/NBT Substrate Kit; Vector Laboratories Inc., Burlingame, CA, USA). For the evaluation of Smad2 phosphorylation, the proteins were detected using the appropriate horseradish peroxidase–conjugated secondary antibodies and an Amersham ECL™ Prime Western Blotting Detection Reagent (GE Healthcare, Buckinghamshire, UK).

Quantitative real-time RT–PCR

For total RNA preparation, 1.0×10^6 cells were cultured in 6-cm dishes. Total RNA was isolated using the ISOGEN reagent (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. We reverse transcribed 4 μg total RNA to first-strand cDNA using the poly-A primer provided in the RT–PCR System Kit (Takara Bio Inc., Shiga, Japan). Quantitative real-time reverse transcriptase polymerase chain reaction (qRT–PCR) was performed on a Thermal Cycler Dice Real Time System (Takara Bio) using SYBR Premix Ex Taq II (Takara Bio) with gene-specific primers (listed in Table I). The mRNA expression levels of the target genes were normalized to that of the endogenous reference gene β-actin and were shown in terms of fold increase or decrease relative to the level of the control sample.

Suppression of gene expression by small interfering RNAs

The sense sequence of human Slug small interfering RNA (siRNA; MISSION siRNA, Sigma-Aldrich) is 5′-GCAUUUGCAGACAGG UCAATT-3′. Logarithmically growing cells were seeded at a density of 1×10^5 cells per 6-cm dish and transfected with 10 nM siRNA using Lipofectamine RNAiMAX (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Forty-eight hours after transfection, cells were stimulated using 10 ng/mL TGF-β1 and then used for qRT–PCR analysis of vimentin gene expression or the wound healing assay as described below. Stealth™ RNAi Negative Control High GC Duplex (Life Technologies), which does not possess significant homology to vertebrate gene sequences, was used as a negative control. Suppression of gene expression by siRNA was evaluated by qRT–PCR and western blotting analyses for the targeted molecules.

Confocal microscopy

Cells plated on eight-well chamber slides were incubated at 37°C for 24 h and then stimulated with 10 ng/mL TGF-β1 for a further 48 h. Some cells were treated with 10 μM SB431542 at 30 min before TGF-β1 treatment. The slides were fixed with 4% paraformaldehyde at room temperature for 15 min. Epithelial and mesenchymal marker proteins (Fig. 3B) were detected using the following method. The cells were treated with the following specific antibodies (1:200): anti-E-cadherin (clone 36), anti-cytokeratin18 (sc-6259), anti-N-cadherin (clone 32; BD Transduction Laboratories), anti-vimentin (sc-6260), anti-β-catenin (#9587; Cell Signaling) and anti-α-smooth muscle actin (α-SMA; Abcam Ltd, Cambridge, UK) for 90 min. After the cells were rinsed with phosphate-buffered saline (PBS), they were stained with Alexa Fluor® 488 goat anti-mouse or anti-rabbit antibodies (1:1000; Life Technologies) for 1 h at room temperature and then with 4′-6-diamidino-2-phenylindole (DAPI; 1:500, Sigma-Aldrich) for 10 min. The slides were washed and observed using confocal microscopy (C1 si, Nikon, Tokyo, Japan). Cytoskeletal proteins (Fig. 5C) were observed using the following method. After rinsing, cells were stained with Alexa Fluor® 488-conjugated phalloidin (Life Technologies, Grand Island, NY, USA) and an anti-vinculin antibody (clone hVIN-1; Sigma-Aldrich) for 1 h at room temperature and then with DAPI (1:500; Sigma-Aldrich) for 10 min. Then, the slides were washed with PBS and observed as described above.

Wound healing assay

The cells were seeded at a density of 5.0×10^5 cells into each well of six-well plates. First, the cells were transfected with Slug siRNA as described above. Then, the cells were treated with 10 ng/mL TGF-β1 under serum-free conditions with or without reagents such as SB431542, anti-integrin β1 antibody (clone 6S6; Merck), anti-integrin α3 antibody (clone P1B5; Merck) or FAK inhibitor I. These reagents were added to the culture 30 min before TGF-β1 stimulation. Then, the cells were incubated in a serum-free medium for 48 h before wounding. Scratch wounds of 20 mm in length were made by a scratch with the plane head of a 10-μL white micropipette tip. The pictures of the wounds were taken at 0 min and 12 h after wounding. The cells were observed using a 10 × objective on an Olympus photomicroscope (Olympus Corporation, Tokyo, Japan) equipped with a fluorescence detector.

Table I. Sequence of primers for qRT–PCR.

Target mRNA	Oligonucleotide sequence (5′-3′)	Predicted size (bp)
E-Cadherin	(F) TACACTGCCCAGGAGCCAGA (R) TGGCACCAGTGTCCGGATTA	103
N-Cadherin	(F) CGAATGGATGAAAGACCCATCC (R) GCCACTGCCTTCATAGTCAAACACT	171
Cytokeratin18	(F) AGGAGTATGAGGCCCTGCTGAA (R) TTGCATGGAGTTGCTGCTGTC	128
Integrin α3	(F) CAACCGGACTGGTGCTGTGTA (R) GCTGGCCACAGTCACTCCAA	136
Integrin β1	(F) TGCGAGTGTGGTGTCTGTAA (R) AGGCTCTGCACTGAACACAT	118
Slug	(F) TGTTGCAGTGAGGGCAAGAA (R) GACCCTGGTTGCTTCAAGGA	158
Smad7	(F) TGCAACCCCCATCACCTTAG (R) GACAGTCTGCAGTTGGTTTGAGA	100
Vimentin	(F) GGTGGACCAGCTAACCAACGA (R) TCAAGGTCAAGACGTGCCAGA	183
β-Actin	(F) GGAGATTACTGCCCTGGCTCCTA (R) GACTCATCGTACTCCTGCTTGCTG	89

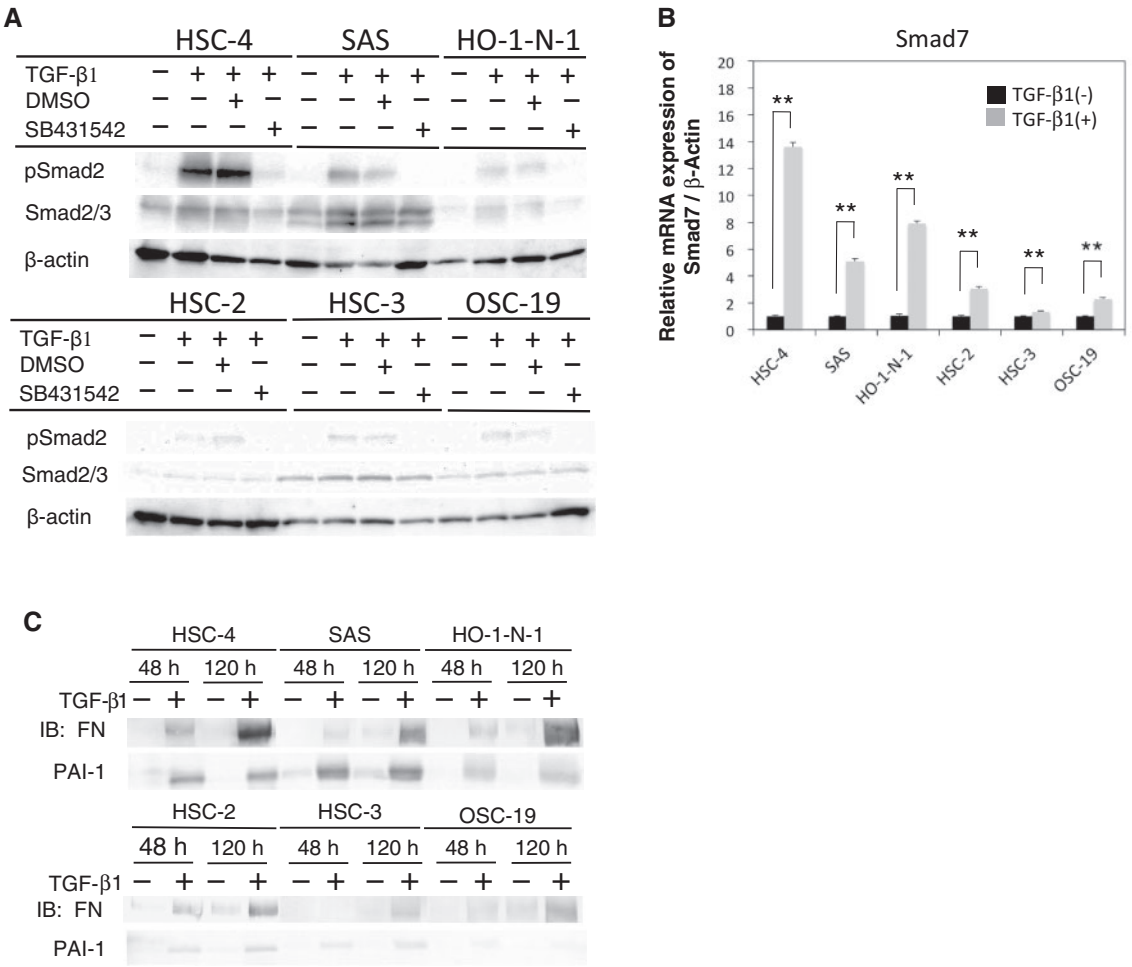

Fig. 1 Identification of TGF-β-responsive hOSCC cell lines. (**A**) Phosphorylation of Smad2 activated by TGF-β1 in six hOSCC cell lines was examined using western blotting. Whole-cell extracts were prepared from cells treated with 10 ng/mL TGF-β1. Some cells were treated with dimethyl sulfoxide (vehicle) or 10 μM SB431542 at 60 min before TGF-β1 treatment. Twenty micrograms of protein sample were loaded into each well. The level of β-actin was monitored as a loading control. (**B**) Smad7 mRNA expression in hOSCC cells treated with (gray bars) or without (black bars) 10 ng/mL TGF-β1 for 1 h was analyzed by qRT–PCR. The values have been normalized to the β-actin mRNA level. Data are the mean ± SD of quadruplicate experiments (**$P < 0.01$). (**C**) The production of extracellular matrix proteins from hOSCC cell lines stimulated with TGF-β1 was examined using western blotting. We cultured 1.0×10^4 cells in 200 μL medium without serum containing 10 ng/mL TGF-β1 for 48 or 120 h in a 96-well dish. The conditioned media were harvested, and 12.5 μL of each sample was treated with SDS sample buffer and electrophoresed on 7.5% SDS-polyacrylamide gels followed by western blotting with anti-fibronectin (FN) and anti-PAI-1 antibodies.

All photographs are representatives of three independent experiments.

Cell migration assay with a Boyden chamber

Boyden chamber migration assays were performed as follows. First, the cells were transfected with Slug siRNA as described above. Then, the cells were treated with 10 ng/mL TGF-β1 under serum-free conditions with or without reagents such as SB431542, anti-integrin β1 antibody (clone 6S6) or anti-integrin α3 antibody (clone P1B5) for 72 h. Subsequently, the cells were plated at a density of 1.0×10^5 cells in the upper chamber of a Boyden chamber apparatus in serum-free media with or without SB431542 or the neutralizing antibodies described above and were allowed to migrate into medium containing 10% FBS in the lower chamber for 24 h at 37°C. Following the 24 h incubation, the filter was fixed in 4% paraformaldehyde and stained with hematoxylin–eosin (Sigma-Aldrich) for 16 h. Cells that had migrated onto the underside of the membrane were counted in nine fields on the membrane. The values are average of triplicate experiments. The migration of the control was set to 100%, and all other datasets were measured against this value. The level of significance was determined using the Student's *t*-test.

Mass spectrometry analysis

SDS–PAGE was carried out on a gel that contained a 10–20% acrylamide gradient gel (ATTO Co., Tokyo, Japan). Protein bands were stained with Flamingo fluorescent gel stain (BIO-RAD, Hercules, CA, USA). The bands were cut out from the gel and washed three times with a 100 mM ammonium carbonate/acetonitrile (1:1 v/v) solution with shaking. Protein thiol groups in the gels were reduced and then alkylated by iodoacetamide. Digestion of proteins in the gel pieces was carried out using 10 μg/mL trypsin (Promega, Madison, WI, USA) for 12–16 h at 37°C. The liquid was collected, and the resulting peptides were recovered after two extractions with a solution containing 50% acetonitrile/2.5% formic acid. The digestion mixture was subjected to a reversed-phase column (Zorbax 300 S-C18, 3.5 μm, 150 × 0.3 mm; Agilent, Santa Clara, CA, USA) by capillary high-performance liquid chromatography (Agilent 1100 System; Agilent). The peptides were eluted with a gradient of 10–65% acetonitrile in 0.1% formic acid. Ion-trap tandem mass spectrometry was carried out on an HCT ultra si (Bruker Daltonics, Bremen, Germany), according to the manufacturer's instructions. Protein sequence database searches were performed with Mascot (Matrix Science, Boston, MA, USA) using the MS/MS peptide ions.

Statistical analysis

All experiments were performed at least three times independently. The results are expressed as the mean ± standard deviation (SD). Data were analyzed using the unpaired Student's *t*-test. In all

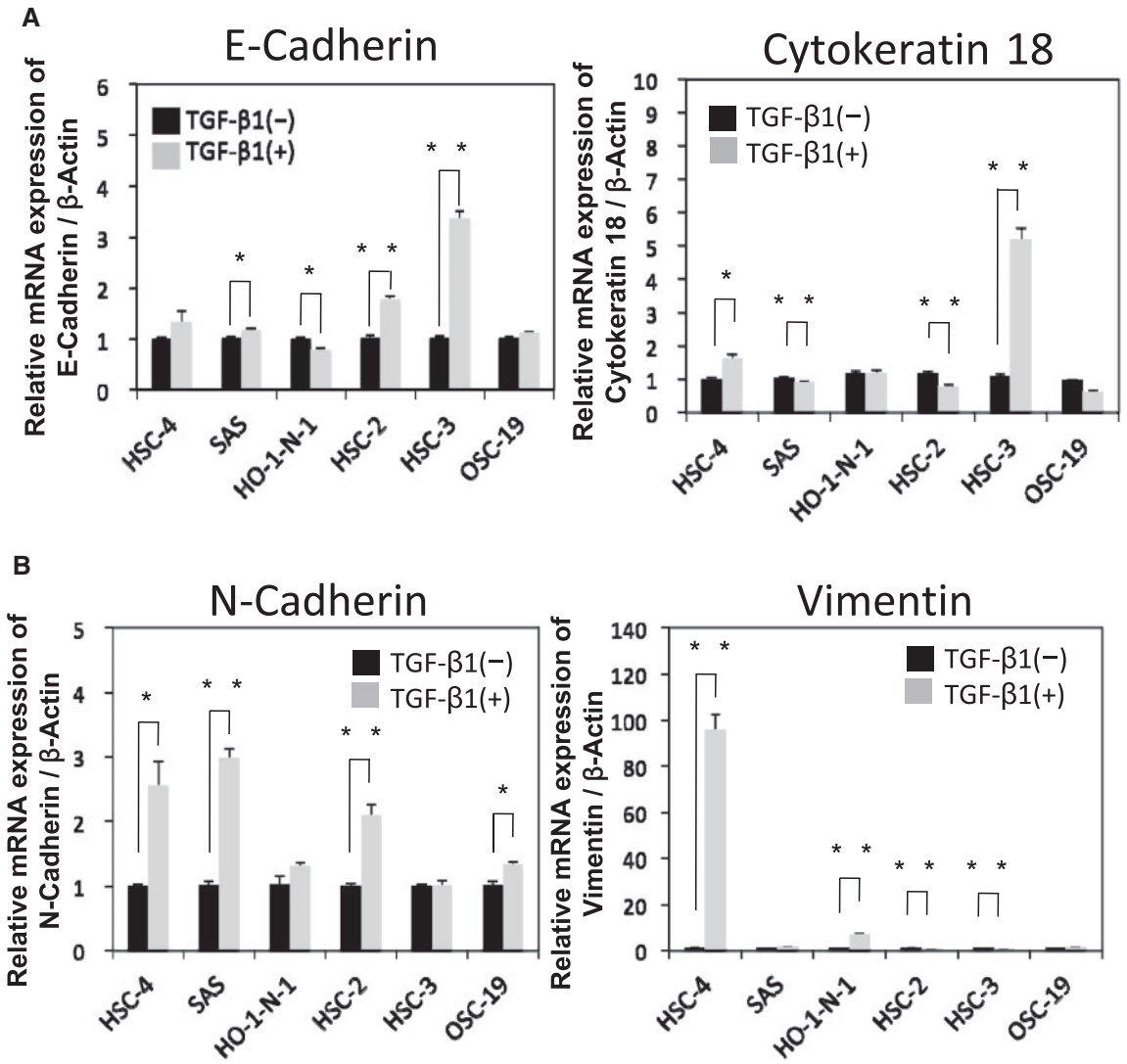

Fig. 2 Effects of TGF-β1 on the expression levels of EMT-related markers in TGF-β-stimulated hOSCC cell lines. The mRNA expression levels of EMT-related markers in hOSCC cell lines after treatment with TGF-β1 in serum-free medium were analyzed using qRT–PCR. The primers for qRT–PCR are described in Table I. The cells were stimulated with (gray bars) or without (black bars) 10 ng/mL TGF-β1 for 48 h. The expression levels of (A) E-cadherin (left) and anti-cytokeratin18 (right) as epithelial markers and (B) N-cadherin (left) and vimentin (right) as mesenchymal markers were examined. The values have been normalized to the β-actin mRNA levels. Data are the mean ± SD of quadruplicate experiments (*$P<0.05$; **$P<0.01$).

statistical analyses, a $P<0.05$ was considered statistically significant, and all P values are two-sided.

Results

Identification of TGF-β-responsive hOSCC cell lines

To identify TGF-β-responsive hOSCC cell lines among HO-1-N-1, HSC-2, HSC-3, HSC-4, SAS and OSC-19, we examined Smad2 phosphorylation and the expression levels of TGF-β target genes such as *Smad7* (*35*), *fibronectin* (*36*) and *PAI-1* (*37*) in various hOSCC cells after TGF-β1 stimulation. As shown in Fig. 1A, phosphorylation of Smad2 was highly upregulated in HSC-4 cells after TGF-β1 stimulation and moderately upregulated in HO-1-N-1 and SAS cells. In addition, TGF-β1-induced Smad2 phosphorylation in HSC-4 and SAS cells was clearly suppressed by the TGF-β receptor inhibitor SB431542. The mRNA expression of Smad7 was highly upregulated in HSC-4 after TGF-β1 stimulation and moderately upregulated in HO-1-N-1 and SAS cells (Fig. 1B). The protein expression of fibronectin was highly upregulated in HSC-4 cells after TGF-β1 stimulation and moderately upregulated in HO-1-N-1 and SAS cells (Fig. 1C). In addition, the protein expression of PAI-1 was clearly upregulated in HSC-4 and SAS cells (Fig. 1C) and moderately upregulated in HO-1-N-1 cells. These results suggest that among the hOSCC cell lines tested, HO-1-N-1, HSC-4 and SAS cells are TGF-β1-responsive, whereas HSC-2, HSC-3 and OSC-19 cells are poorly responsive to TGF-β1. Moreover, among three types of TGF-β1-responsive hOSCC cells, HSC-4 cells were the most responsive to TGF-β1 stimulation (Fig. 1A–C). In addition, the upregulation of TGF-β target gene expression levels by TGF-β1 stimulation in HSC-4 cells were suppressed by SB431542 (data not shown).

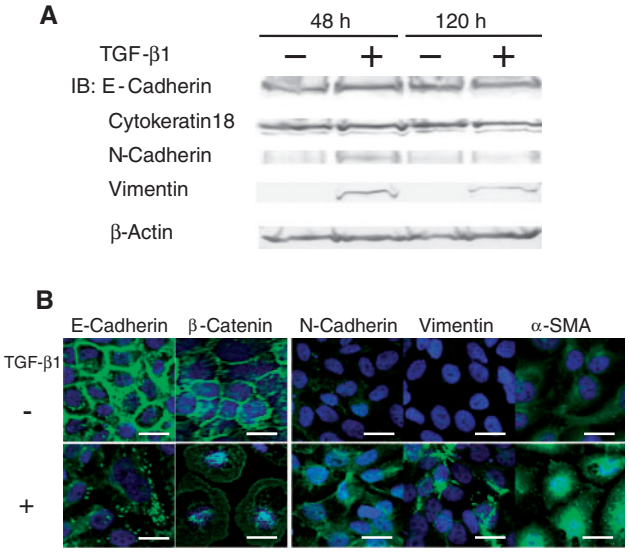

Fig. 3 Expression and localization of EMT-related marker proteins in TGF-β1-treated HSC-4 cells. (**A**) The expression of EMT-related marker proteins was examined using western blotting. HSC-4 cells were treated with 10 ng/mL TGF-β1, or left untreated, under serum-free condition for 48 or 120 h. Cell lysates were electrophoresed and immunoblotted using anti-E-cadherin and anti-cytokeratin18 antibodies to detect epithelial markers and anti-N-cadherin and anti-vimentin antibodies to detect mesenchymal markers. The levels of β-actin were monitored as a loading control. (**B**) The localization of EMT-related marker proteins was observed using confocal microscopy. The cells were cultured with or without 10 ng/mL TGF-β1 under serum-free conditions for 48 h. These cells were immunostained with the indicated antibodies, stained with DAPI to detect nuclei, and observed using confocal microscopy, as described in Materials and Methods. The scale bars represent 25 μm.

Effects of TGF-β1 on the expression of EMT-related markers in TGF-β-stimulated hOSCC cells

To obtain insights into the relationship between TGF-β1-responsiveness and TGF-β1-induced EMT progression in hOSCC cells, we investigated the effects of TGF-β1 on the expression of EMT-associated genes in TGF-β1-responsive (HSC-4, HO-1-N-1 and SAS) and poorly responsive (HSC-2, HSC-3 and OSC-19) hOSCC cells. qRT–PCR analyses revealed that the downregulation of definitive epithelial cell markers E-cadherin and cytokeratin18 was not observed in all the hOSCC cells (Fig. 2A). In contrast, the definitive mesenchymal marker N-cadherin was strongly upregulated in both HSC-4 and SAS cells and moderately in HSC-2 cells (Fig. 2B, left panel). In addition, the definitive mesenchymal marker vimentin was strongly upregulated in HSC-4 cells and moderately in HO-1-N-1 cells (Fig. 2B, right panel). Thus, among TGF-β1-responsive cell lines, mesenchymal markers were most strongly upregulated after TGF-β1 stimulation in HSC-4 cells (Fig. 2B). In addition, the upregulation of mesenchymal markers in TGF-β1-stimulated HSC-4 cells was suppressed by SB431542 (data not shown).

Expression and localization of EMT-related marker proteins in TGF-β1-treated HSC-4 cells

We examined the protein levels of EMT markers in HSC-4 cells stimulated with TGF-β1. Western blotting analysis revealed that the expression levels of N-cadherin and vimentin were significantly increased in HSC-4 cells at 48 h after TGF-β1 treatment and weakly at 120 h (Fig. 3A). However, the expression levels of E-cadherin and cytokeratin18 were hardly affected by TGF-β (Fig. 3A). Interestingly, immunofluorescence analysis revealed that E-cadherin and β-catenin at the cell surface were internalized in HSC-4 cells stimulated with TGF-β1 (Fig. 3B). Immunofluorescence analysis also clearly revealed that the mesenchymal markers α-SMA, N-cadherin and vimentin were expressed in HSC-4 cells with TGF-β treatment, but not in cells that did not receive TGF-β treatment (Fig. 3B).

Regulation of TGF-β1-induced EMT of HSC-4 cells by Slug

Various transcription factors are related to EMT in different cell types (*19–23*); therefore, we investigated the mRNA expression levels of EMT-related transcription factors in HSC-4 cells using qRT–PCR. Slug expression level was significantly upregulated at 24 h after TGF-β1 stimulation (Fig. 4A). In addition, the TGF-β1-induced upregulation of Slug expression was suppressed by SB431542 (data not shown). However, no change was observed in the expression levels of Twist1/2, ZEB1/ZEB2 and FOXC2 at 6 h, or ZEB1 at 48 h, after TGF-β1 stimulation (data not shown). These data suggest that Slug is important EMT-related transcription factor in HSC-4 cells stimulated with TGF-β1. To investigate how these transcriptional factors affect the EMT of HSC-4 cells, HSC-4 cells treated with Slug siRNA were stimulated with TGF-β1. The expression of Slug was lower in HSC-4 cells treated with Slug siRNA than in those treated with control siRNA at both the mRNA and protein levels (Fig. 4B and C). qRT–PCR analysis revealed that Slug siRNA significantly suppressed the TGF-β1-induced expression of vimentin mRNA in HSC-4 cells compared with that of control siRNA (Fig. 4D). In contrast, Slug siRNA did not affect the expression of N-cadherin mRNA in HSC-4 cells (Supplementary Fig. S1).

Regulation of EMT-related cell migration of HSC-4 cells by Slug

Migration ability, which is related to tumour invasion and metastasis, following TGF-β1 stimulation of HSC-4 cells was examined using a wound healing assay. The cell migration ability of HSC-4 cells was increased following TGF-β1 stimulation but was inhibited by SB431542 or Slug siRNA in the wound healing assay (Fig. 5A). This inhibition of the cell migration ability of TGF-β1-stimulated HSC-4 cells by SB431542 and Slug siRNA was also observed in the migration chamber assay (Fig. 5B). In general, cell adhesion plays essential roles in cell movement: The generation of focal adhesion sites is necessary for the stable adhesion of the lamellipodium or filopodium in migrating cells (reviewed in *38*). At focal adhesion sites, vinculin is involved in mechanocoupling between integrins bound to the underlying ECM and the actin cytoskeleton (*39–41*). In TGF-β1-stimulated HSC-4 cells, many focal adhesion contacts were detected as

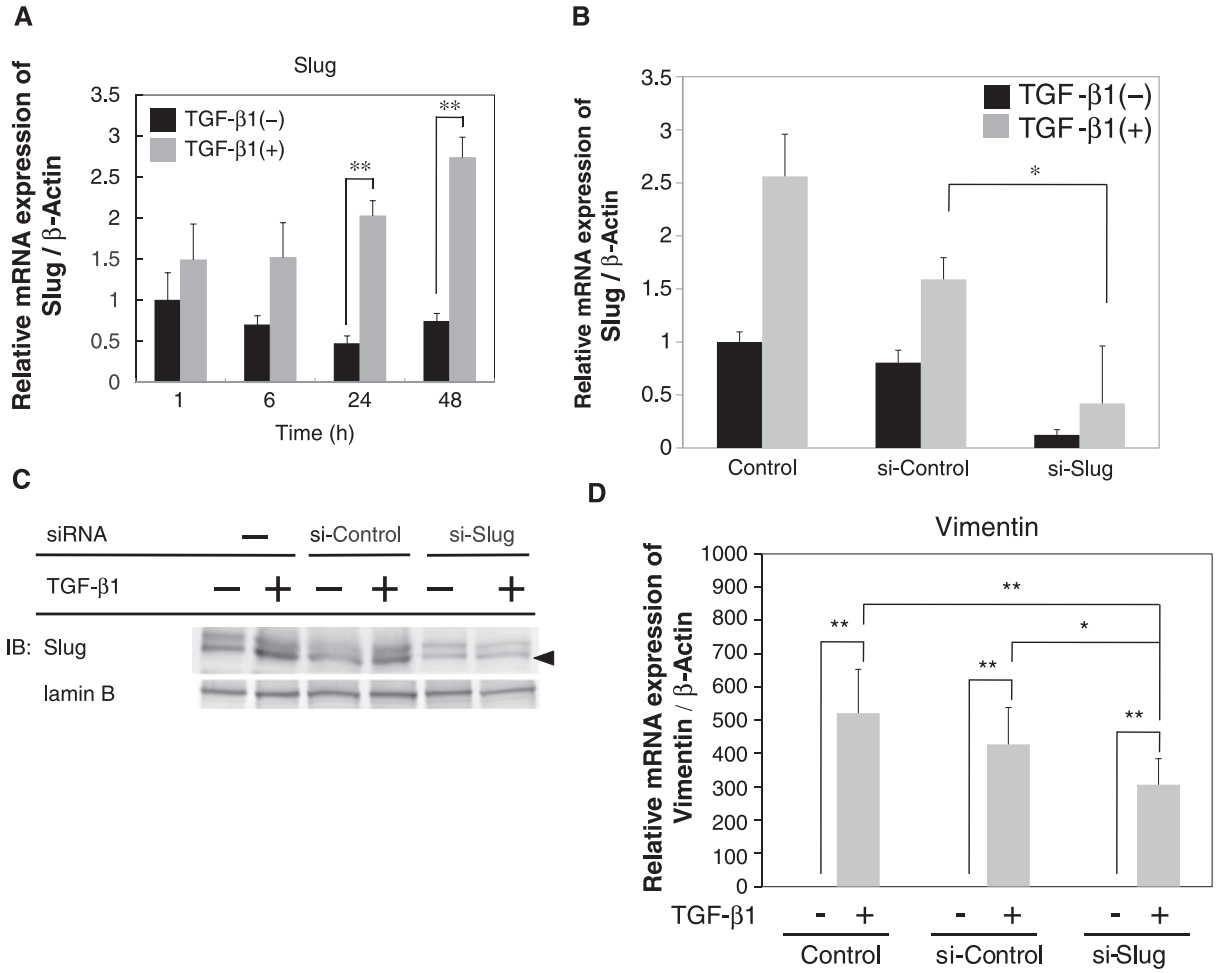

Fig. 4 Role of the EMT-related transcription factor Slug on the TGF-β1-induced EMT of HSC-4 cells. (**A**) mRNA expression level of EMT-related transcription factor Slug in HSC-4 cells after treatment with TGF-β1 in serum-free medium was analyzed using qRT–PCR. The primers used for qRT–PCR are described in Table I. The cells were cultured with (gray bars) or without (black bars) 10 ng/mL TGF-β1 for up to 48 h for Slug. (**B**) The cells were transfected with Slug siRNA (si-Slug) or negative control siRNA (si-Control) as described in the Materials and Methods. The expression of Slug mRNA was examined by qRT–PCR. The values have been normalized to the β-actin mRNA levels. Data are the mean ± SD of quadruplicate experiments (*$P<0.05$; **$P<0.01$). (**C**) For the expression of Slug at the protein level, the nuclear fractions were obtained by ProteoExtract® Subcellular Proteome Extraction Kit (Merck) 72 h after TGF-β1 stimulation. The expression levels of Slug in the nuclei were determined by western blotting. The position of Slug was indicated by arrowhead. Lamin B was used as the loading control. (**D**) The expression level of vimentin mRNA in HSC-4 cells stimulated with or without 10 ng/mL TGF-β1 for 48 h following siRNA treatment for 48 h was evaluated by qRT–PCR. The values have been normalized to the β-actin mRNA levels. Data are the mean ± SD for experiments in triplicate (*$P<0.05$; **$P<0.01$).

punctate spots of vinculin around the lamellipodium-like structure (Fig. 5C, arrowheads; red punctate). Interestingly, some spots of vinculin were seen to colocalize with actin stress fibers, which were prevalent around at the lamellipodium-like structure (Fig. 5C, arrows; yellow).

Identification of ECM proteins secreted from TGF-β1-treated HSC-4 cells

Our next aim was to elucidate the molecular mechanisms underlying the TGF-β-induced migration of HSC-4 cells, by identifying extracellular protein molecules secreted by HSC-4 cells that played a role in TGF-β-induced cell migration. We identified extracellular proteins from TGF-β1-treated HSC-4 cells using SDS–PAGE (Fig. 6A). Some of the detected protein bands were enriched at 120 h of TGF-β1 stimulation, in comparison with unstimulated HSC-4 cells. These proteins were digested with trypsin, and the peptide fragments were analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The mass data were analyzed using Mascot software against a protein database to identify the proteins (Table II). The expression levels of fibronectin, PAI-1, βig-h3 and TSP-1 were upregulated after TGF-β1 stimulation. These results were confirmed by western blotting using anti-fibronectin, anti-PAI-1 (Fig. 1C), anti-βig-h3 and anti-TSP-1 antibodies (Fig. 6B).

Roles of integrin and FAK on cell migration of TGF-β1-treated HSC-4 cells

The TGF-β1-induced extracellular proteins, TSP-1, βig-h3, fibronectin and uPA receptor (uPAR), a related molecule to PAI-1, are integrin α3β1-targeted molecules (*42–45*). Therefore, we examined how integrin α3β1 on the surface of HSC-4 cells affected TGF-β-induced cell migration. The effects of blocking

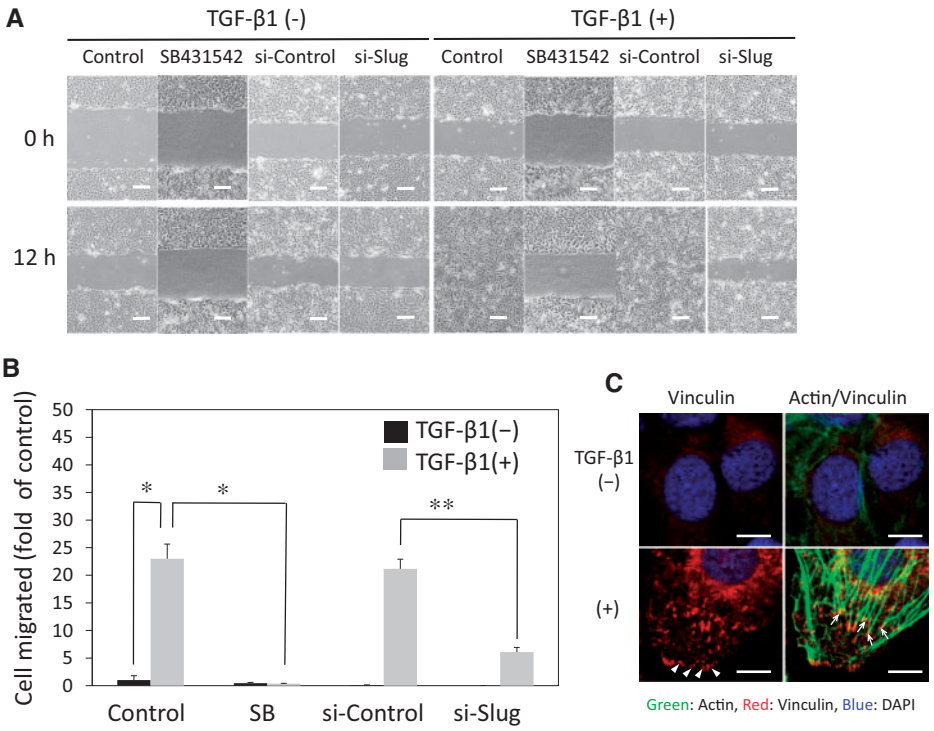

Fig. 5 Roles of the EMT-related transcription factor Slug on the TGF-β1-induced migration and intracellular distribution of cell adhesion-related molecules of HSC-4 cells. (**A**) HSC-4 cells were allowed to migrate in the wound healing assay described in Materials and Methods. First, the cells were cultured with or without siRNA treatment for 48 h. Then, cells were treated with 10 ng/mL TGF-β1 for 48 h in the absence or presence of 10 μM SB431542. After wounding, the cells were incubated for the indicated times and the pictures of cells appearance were taken using phase-contrast microscopy. (**B**) The migration ability of the cells was assayed using a Boyden chamber as described in Materials and Methods. Data are the mean ± SD of experiments conducted in three dishes for each time point (*$P<0.05$; **$P<0.01$). (**C**) Cells cultured with or without 10 ng/mL TGF-β1 under serum-free conditions were incubated for 48 h. These cells were immunostained with anti-vinculin, stained with Alexa Fluor® 488-conjugated phalloidin and DAPI and observed using confocal microscopy. The scale bars represent 10 μm.

antibodies against integrin α3 and β1 on the TGF-β-stimulated migratory activity of HSC-4 cells were examined using the wound healing assay (Fig. 7A) and the migration chamber assay (Fig. 7B). TGF-β-stimulated migratory activity was clearly suppressed by blocking antibodies against integrins α3 and β1 (Fig. 7A and B). We also investigated the expression of integrins α3 and β1 in TGF-β-stimulated HSC-4 cells by qRT–PCR (Fig. 7C and D). The expression levels of both integrin α3 and β1 increased significantly at 72 or 96 h after TGF-β1 stimulation. In contrast, integrin expression was not upregulated within 48 h. As FAK is an important regulator of cell migration and signal transduction from integrins, we evaluated the effect of FAK inhibitor I on the TGF-β-induced migration of HSC-4 cells. The TGF-β1-induced migration activity of HSC-4 cells was inhibited by 10 ng/mL FAK inhibitor I in the wound healing assay (Fig. 7E).

Discussion

TGF-β1 clearly induced Smad2 phosphorylation in HSC-4 and SAS cells (Fig. 1A). The expression levels of the TGF-β target genes *Smad7, fibronectin* and *PAI-1* were clearly upregulated in HSC-4, HO-1-N-1 and SAS cells after TGF-β1 stimulation (Fig. 1B and C). These results suggest that HO-1-N-1, HSC-4 and SAS cells were TGF-β1-responsive hOSCC cells. Conversely, the definitive mesenchymal marker N-cadherin was clearly upregulated in HSC-4 and SAS cells after TGF-β1 treatment (Fig. 2B, left). In addition, the definitive mesenchymal marker vimentin was clearly upregulated in HSC-4 and HO-1-N-1 cells after TGF-β1 treatment (Fig. 2B, right). These results suggest that responsiveness to TGF-β1 is closely and positively related to the induction of the mesenchymal phenotype in hOSCC cells. Moreover, HSC-4 cells seemed to be the most typical TGF-β-responsive hOSCC cells with mesenchymal phenotype expression (Figs 1, 2B and 3). Interestingly, E-cadherin and β-catenin at the cell surface were internalized in HSC-4 cells stimulated with TGF-β1 (Fig. 3B), whereas the expression level of E-cadherin was hardly affected (Fig. 3A). The internalization of E-cadherin during EMT progression has been reported previously (*46*, *47*). Moreover, Chen et al. reported that the TGF-β/Smad signal pathway is involved in E-cadherin and β-catenin internalization during TGF-β-induced EMT in human colon cancer cells or human prostate cancer cells (*48*). However, it remains to be clarified whether Smad2 mediates the TGF-β-induced internalization of E-cadherin and β-catenin in HSC-4 cells.

Previous reports demonstrated that TGF-β clearly induced EMT events involving repression of epithelial marker expression, induction of mesenchymal marker

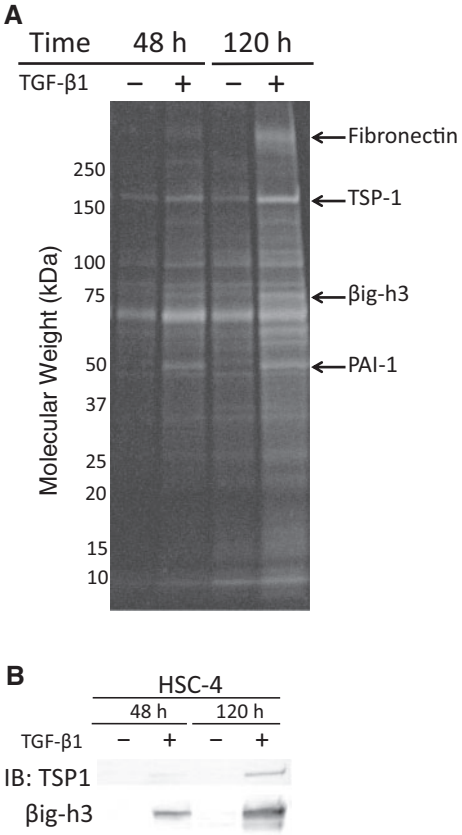

Fig. 6 Identification of enriched ECM proteins secreted from HSC-4 cells after TGF-β1 treatment. (**A**) HSC-4 cells (1.0×10^4 cells) treated with or without 10 ng/mL TGF-β1 were cultured under serum-free conditions for 48 or 120 h in a 96-well dish. Two hundred microlitres of the obtained conditioned media were concentrated by ultrafiltration using a Microcon-10 filter. The extracellular proteins from the TGF-β1-treated or non-treated cells were separated and detected by SDS–PAGE and stained with Flamingo fluorescent gel stain (BIO-RAD). The protein bands enriched by TGF-β1-treatment were cut out and then analyzed by LC-MS/MS. Proteomic analysis identified fibronectin, PAI-1, βig-h3 and TSP-1 as the indicated bands by arrows from the TGF-β1-stimulated cells. (**B**) Immunoblotting was performed using anti-βig-h3 and anti-TSP-1 antibodies as described in Materials and Methods.

Table II. TGF-β1-induced extracellular proteins identified by mass spectrometry.

Protein name	Mass (Da)	No. of matched peptides[a]	Mascot score	Sequence coverage (%)
Fibronectin	266,052	16	339	8
TSP-1	133,291	30	844	15
βig-h3	75,261	10	170	15
PAI-1	45,088	13	259	31

[a]Matched peptides include all peptides that differ by sequence, modification, or charge.

expression and the increased expression of EMT-related transcription factors such as Snail and Slug in hOSCC cells (*34*, *49*). However, the mechanism by which EMT-related transcription factors affect the EMT marker expression in hOSCC cells remained to be clarified. Here, we have demonstrated that the expression of Slug was increased in response to TGF-β1 (Fig. 4A). To investigate whether Slug affected the expression levels of the TGF-β1-induced mesenchymal markers N-cadherin and vimentin, Slug siRNA was added to the HSC-4 cell culture before TGF-β1 stimulation. Slug downregulation significantly suppressed TGF-β1-induced vimentin expression in HSC-4 cells (Fig. 4B–D) but did not affect TGF-β1-induced *N*-cadherin expression (Supplementary Fig. S1). These data suggested that Slug contributes to the expression of mesenchymal marker genes during the EMT of HSC-4 cells. Conversely, the Snail mRNA expression transiently increased after the TGF-β1 stimulation (data not shown). The function of Snail in TGF-β1-stimulated HSC-4 cells is now under investigation in our laboratory.

It has been widely recognized that cell migration is an EMT-related event (*50*, *51*), which is related to tumour invasion and metastasis. It has been reported that the EMT-related transcription factor Slug enhances the migration activity of corneal epithelial cells (*52*). However, the effect of Slug on the EMT-related migration of hOSCC cells remained to be clarified. In this study, wound healing and migration chamber assays demonstrated that Slug downregulation by Slug siRNA clearly suppressed the TGF-β1-induced migration ability of HSC-4 cells (Fig. 5A and B). In general, cell adhesion plays essential roles in cell movement: The generation of focal adhesion sites is necessary for stable adhesion of the lamellipodium or filopodium in migrating cells (reviewed in *38*). In TGF-β1-stimulated HSC-4 cells, many focal adhesion contacts were detected as punctate spots of vinculin, and actin stress fibers were observed around the lamellipodium-like structure (Fig. 5C). Thus, punctate vinculin seemed to play a positive role in the TGF-β1-induced migration of HSC-4 cells.

Next, we attempted to identify secreted extracellular proteins generated by TGF-β1 treatment by directly analyzing the proteins in the culture medium using LC-MS/MS. We found that the expression levels of fibronectin, PAI-1, βig-h3, and TSP-1 were enriched after TGF-β1 stimulation (Fig. 6A). These results were confirmed by western blotting using anti-fibronectin, anti-PAI-1 (Fig. 1C), anti-βig-h3 and anti-TSP-1 antibodies (Fig. 6B). The N-terminal domain of TSP-1 binds to integrin α3β1 on the surface of endothelial cells to induce their proliferation and angiogenesis (*42*). PAI-1-related molecule uPAR forms a complex with integrin α3β1 and induces epithelial cell migration (*45*). βig-h3 has multiple cell adhesion motifs that can mediate interactions with a variety of cell types via integrin α3β1 (*43*). Integrin α3β1 on human melanoma cells binds to fibronectin, thereby increasing the invasive capacity of the cells (*53*). Therefore, we expected that the increased expression of fibronectin, PAI-1, TSP-1 and βig-h3 affected the migration ability of HSC-4 cells following TGF-β1 stimulation through integrin α3β1. Blocking antibodies against integrin α3 or β1 clearly suppressed the TGF-β-stimulated migratory activity of HSC-4 cells

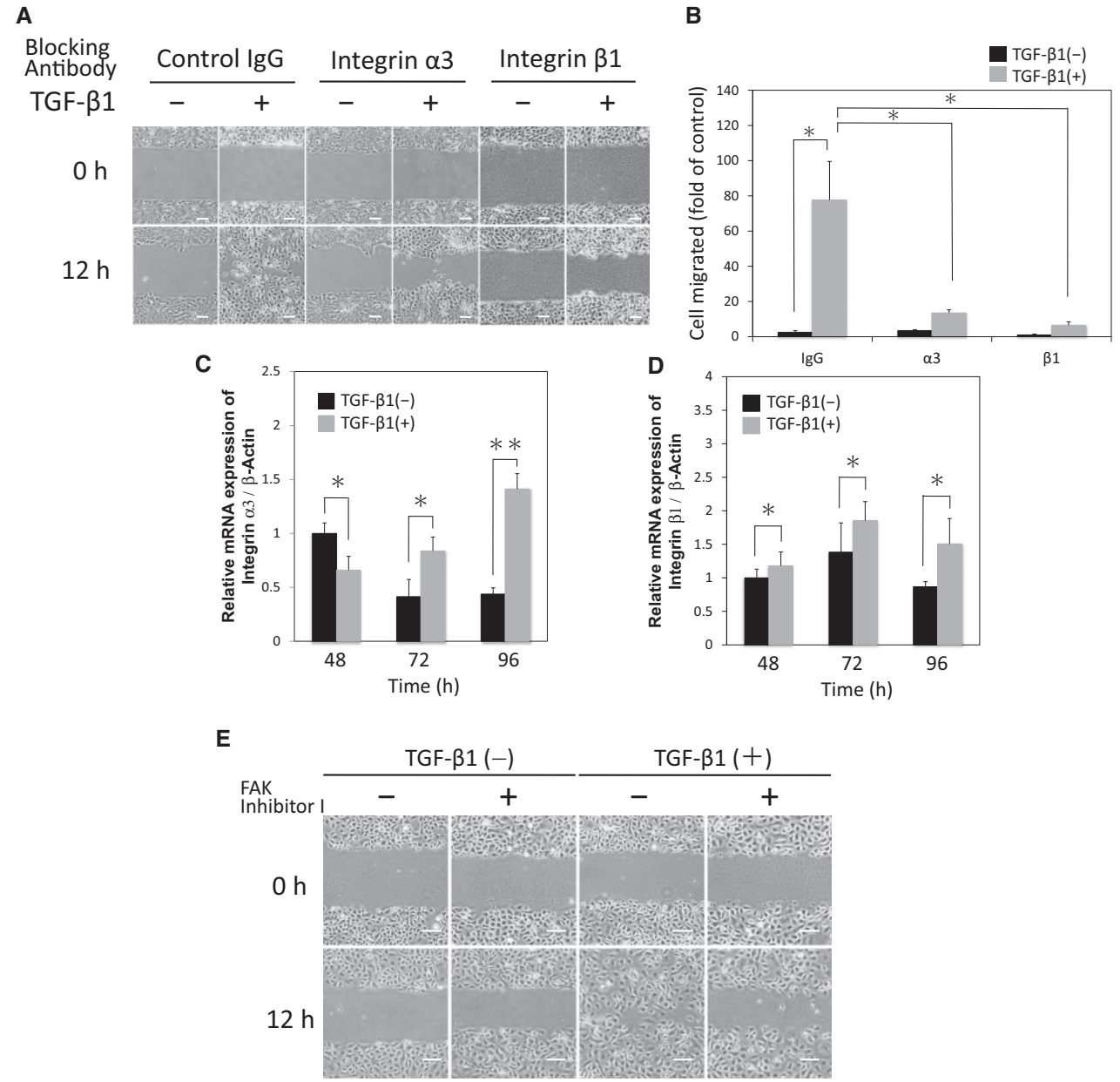

Fig. 7 Role of the integrin α3β1/FAK pathway in the migration of TGF-β1-treated HSC-4 cells. (**A**) HSC-4 cells were allowed to migrate in a wound healing assay. Before wounding, the cells were cultured with or without 10 ng/mL TGF-β1 for 48 h in the presence of control IgG, integrin α3 - or β1-blocking antibodies (10 μg/mL). Control IgG means 10 μg/mL mouse IgG from normal mouse serum, which was used instead of a blocking antibody as a control. The appearance of cells migration was observed by phase-contrast microscopy at indicated times after the wounding. (**B**) The migration ability of the cells cultured with or without 10 ng/mL TGF-β1 in the presence of control IgG, integrin α3- and β1-blocking antibodies (10 μg/mL) was assayed using a Boyden chamber as described in the Materials and Methods. Data are the mean ± SD of experiments in three dishes for each time point (*$P<0.05$; **$P<0.01$) (**C** and **D**). The time course of integrin α3 and β1 mRNA expression in the cells after treatment with 10 ng/mL TGF-β1 was analyzed using qRT–PCR. The primers for qRT–PCR are described in Table I. The expression levels of (C) integrin α3 and (D) integrin β1 were examined at the indicated time points. The values have been normalized to the β-actin mRNA levels. Data are the mean ± SD of triplicate experiments (*$P<0.05$; **$P<0.01$). (**E**) The effect of a FAK inhibitor on the TGF-β1-induced cell migration was examined using a wound healing assay. Before wounding, the cells were cultured with or without 10 ng/mL TGF-β1 for 48 h. Some cells were pretreated with 10 μM FAK inhibitor I at 30 min before the TGF-β1 stimulation. After wounding, the cells were incubated for indicated times and the appearance of cells migration was observed using phase-contrast microscopy.

(Fig. 7A and B). Thus, TGF-β1-induced migration activity in HSC-4 cells was dependent on integrin α3β1. Interestingly, integrin α3 and β1 expression levels were upregulated in HSC-4 cells at 72 and 96 h after TGF-β stimulation (Fig. 7C and D), suggesting that the TGF-β-induced expression of integrin α3β1 might play positive roles in cell migration. Conversely, some of the TGF-β1-induced extracellular proteins might also play positive roles in cell migration. We investigated whether fibronectin played a positive role in HSC-4 cell migration after TGF-β1 stimulation. However, a blocking antibody against fibronectin or the fibronectin-related peptide GRGDSP, which blocks fibronectin, did not affect the migration of

TGF-β1-stimulated HSC-4 cells (data not shown). These results suggest that ECM components other than fibronectin contribute to migration.

FAK is an important regulator of cell migration, which is required for the invasion and metastasis of cancer cells (*54*, *55*). In general, FAK is activated by integrins and functions as a positive regulator of cell migration (*55*): Activated FAK associates with regulatory proteins that promote the activation of Rho family proteins, which promote cell migration (*56*). As shown in Fig. 7E, the TGF-β-induced migration of HSC-4 cells was inhibited by FAK inhibitor I. These results suggest that TGF-β-induced migration activity was transduced by the integrin α3β1/FAK signal pathway.

In summary, we have demonstrated that the TGF-β1-induced EMT and EMT-related migration of HSC-4 hOSCC cells are positively controlled by Slug and the integrin α3β1/FAK pathway. This is the first report investigating the molecular mechanisms underlying TGF-β1-induced EMT and EMT-related migration in hOSCC cells.

Supplementary Data

Supplementary data are available at *JB Online*.

Acknowledgements

The authors are grateful to Ms. Mariko Yoshida in the Division of Cellular Biosignal Sciences for assistance with the cell culture of hOSCC cell lines and western blot analysis.

Funding

Grant-in-aid for Scientific Research (22791935 to N.O., 22791936 to N.T., 23592896 to A.I., 19791370 to N.C. and 22592076 to M.K.) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; the Open Research Project, High-tech Research project from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; Grant-in-aid for Strategic Medical Science Research Center from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (2010-2014) and a grant from the Keiryokai Research Foundation (100 to N.C., 2008 and 106 to A.I., 2009).

Conflict of interest

None declared.

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