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TGF-β-induced mesenchymal transition of MS-1 endothelial cells requires Smad-dependent cooperative activation of Rho signals and MRTF-A

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Endothelial–mesenchymal transition (EndMT) plays important roles in various physiological and pathological processes. While signals mediated by transforming growth factor (TGF)-β have been implicated in EndMT, the molecular mechanisms underlying it remain to be fully elucidated. Here, we examined the effects of TGF-β signals on the EndMT of mouse pancreatic microvascular endothelial cells (MS-1). By addition of TGF-β2, MS-1 cells underwent mesenchymal transition characterized by re-organization of actin stress fibre and increased expression of various mesenchymal markers such as α-smooth muscle actin (α-SMA) through activation of Rho signals. Whereas activation of Rho signals via TGF-β-induced non-Smad signals has been implicated in epithelial–mesenchymal transition (EMT), we found that Arhgef5, a guanine nucleotide exchange factor, is induced by Smad signals and contributes to the TGF-β2-induced α-SMA expression in MS-1 cells. We also found that TGF-β2 induces the expression of myocardin-related transcription factor-A (MRTF-A) in a Smad-dependent fashion and its nuclear accumulation in MS-1 cells and that MRTF-A is required and sufficient for TGF-β2-induced α-SMA expression. These results indicate that activation of Smad signals by TGF-β2 have dual effects on the activation of Rho signals and MRTF-A leading to the mesenchymal transition of MS-1 endothelial cells.

Keywords: TGF-β2/Rho/MRTF-A/EndMT/α-SMA.

Abbreviations: ALK, activin receptor-like kinase; EMT, epithelial–mesenchymal transition; EndMT, endothelial–mesenchymal transition; FCS, fetal calf serum; GEF, guanine nucleotide exchange factor; MMP2, matrix metalloproteinase 2; PECAM1, platelet-endothelial cell adhesion molecule-1; R-Smad, receptor-regulated Smad; α-SMA, α-smooth muscle actin; TGF-β, transforming growth factor-β.

Epithelial–mesenchymal transition (EMT) converts polarized epithelial cells to motile mesenchymal cells (*1*), and plays important roles in gastrulation and cancer cell invasion (*2*). During EMT, epithelial markers including E-cadherin are down-regulated and the mesenchymal markers such as α-smooth muscle actin (α-SMA) and fibronectin are up-regulated. EMT is regulated by signalling pathways mediated by multiple cytokines such as Wnt, Notch and transforming growth factor (TGF)-β (*3*, *4*).

Members of the TGF-β family bind to two different types of serine/threonine kinase receptors. Upon ligand binding, the constitutively active type II receptor kinase phosphorylates the type I receptor which, in turn, activates the downstream signal transduction cascades, including Smad pathways. TGF-βs signal through the type I receptor known as activin receptor-like kinase (ALK)-5. The activated type I receptor phosphorylate receptor-regulated Smad proteins (R-Smads). Smad2 and 3 transduce signals as R-Smads for TGF-βs (*5*). Once activated, R-Smads complex with the common mediator Smad4 (Co-Smad), and translocate to the nucleus, where Smad complexes regulate transcription of target genes. In addition, TGF-β has been shown to activate diverse non-Smad parallel downstream pathways, such as extracellular signal regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK) and p38 MAP kinase (*6*, *7*).

Small Rho GTPases are activated by TGF-β and play important roles in TGF-β-induced actin cytoskeleton reorganization (*8*). Rho GTPases have been implicated in many cellular processes, including actin and microtubule cytoskeleton organization, cell adhesion, cell division and motility (*9*). Rho proteins cycle constantly between GTP-bound active forms and GDP-bound inactive forms, and this process is positively and negatively regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs), respectively. Activation of Rho GTPases leads to activation of downstream effectors including Rho-associated coiled-coil containing protein kinase (ROCK) and mDia. TGF-β rapidly activates various Rho GTPases including RhoA, RhoB, Rac and Cdc42 leading to short-term actin cytoskeleton remodelling via the ROCK1/LIMK/cofilin pathway in epithelial cells. This activation is mediated by non-Smad pathways including p38 MAP kinase (*10*) and phosphatidylinositol 3-kinase pathways (*11*). TGF-β also activates Rho GTPases via Smad-dependent induction of Rho GEFs such as Net1 (*12*) and GEF-H1 (*13*).

Several lines of evidence have shown that multiple transcription factors are involved in TGF-β-induced EMT. TGF-β signals induce the expression of Snail, SIP-1 and δEF-1 during EMT of mammary epithelial cells (*14*, *15*). Among them, Snail, a zinc finger-containing transcription factor, represses E-cadherin expression and induces EMT when over-expressed in epithelial cells (*16*, *17*). Knockout mice deficient for Snail gene die at gastrulation as they fail to undergo a complete EMT process, forming an abnormal mesodermal layer that retains E-cadherin expression (*18*).

Morita and colleagues (*19*) reported that myocardin-related transcription factors (MRTFs; also known as MAL and MKL) play critical roles in TGF-β-induced EMT. In epithelial cells, TGF-β triggers the nuclear translocation of MRTFs via activation of the Rho signalling pathway (*20–22*), leading to formation of a transcriptional complex of MRTFs and Smad3 which activates the Slug transcription. MRTFs also induce the expression of α-SMA in cooperation with serum response factor (SRF), leading to reorganization of the α-SMA containing actin cytoskeleton.

Differentiated endothelial cells have been shown to differentiate into mesenchymal cells *in vivo* (*23*). During heart development, cardiogenic mesodermal cells give rise to two types of heart cells, *i.e.* myocardial and endocardial cells. Endocardial cells acquire endothelial markers, such as VE cadherin and platelet-endothelial cell adhesion molecule-1 (PECAM1). A population of endocardial/endothelial cells in atrio-ventricular (AV) canal transit to the mesenchymal heart cushion cells, which resembles EMT. These cells further form the mesenchymal region of cardiac septa and valves (*24*, *25*). During endothelial–mesenchymal transition (EndMT) in heart development, expression of VE cadherin is down-regulated while that of α-SMA is up-regulated. This phenomenon was genetically documented by Zeisberg and colleagues (*26*) using the *Tie2-Cre*; *R26R-lox-STOP-lox-lacZ* transgenic mice, in which all cells of endothelial origin are irreversibly labelled with LacZ (β-galactosidase) expression. They found that the mesenchymal heart cushion cells express β-galactosidase, suggesting that these cells are formed through EndMT.

Roles of the TGF-β family in cardiovascular development have been implicated by the findings that knockout mice deficient in various TGF-β family signalling components exhibit defects in cardiovascular systems (*27–29*). Especially, TGF-β2-deficient mice have multiple defects in AV cushion formation, suggesting its roles in EndMT of endocardiac tissues (*30*, *31*). Furthermore, various *in vitro* studies have shown that TGF-βs induce differentiation of vascular endothelial cells into mesenchymal cells (*32–37*). However, molecular mechanisms that govern TGF-β-induced EndMT remain to be largely unknown.

Here, we found that TGF-β family members induce the EndMT of mouse pancreatic microvascular endothelial cells (MS-1). This mesenchymal transition of MS-1 cells by TGF-β requires cooperative activation of Rho signals and MRTF-A leading to the transcriptional activation of mesenchymal marker genes by MRTF-A. However, this TGF-β/Rho/MRTF signalling axis in EndMT of MS-1 cells is partly different from that observed in EMT.

Material and Methods

Cells and cell culture

MS-1 cells were obtained from American Type Culture Collection, and maintained in Minimum Essential Medium-α (MEM-α) medium (Invitrogen) supplemented with 10% fetal calf serum (FCS), 50 U/ml penicillin and 50 μg/ml streptomycin. TGF-β1, 2, 3 (R&D: 1 ng/ml), Y27632 (Sigma-Aldrich: 10 μM) and C3 exoenzyme (Cytoskeleton: 2 μg/ml) were used in each experiment.

RNA interference and oligonucleotides

siRNAs were introduced into cells as described previously (*38*, *39*). The target sequences of siRNAs for mouse Snail, Smad4, Arhgef5 and MRTF-A were:

5′-CGGAAGATCTTCAACTGCAAATATT-3′,
5′-GCAAAGGAGTGCAGTTGGAATGTAA-3′,
5′-CCCTATCATAGATCCTTCCTCTGAA-3′ and
5′-CCATCACCCATGGACACCTCTGAAT-3′,

respectively. Control siRNAs were obtained from Invitrogen.

Lentiviral production and infection

A lentiviral vector encoding constitutively active MRTF-A mutant (ca-MRTF-A) was generated by Gateway technology (Invitrogen) (*19*, *40*). Ca-MRTF-A cDNA was subcloned into the pENTR vector, and subsequently transferred into the pCSII-EF-RfA lentiviral expression vector by the LR recombination reaction (Invitrogen). Empty pENTR vector was used to prepare control lentiviral vector. 293FT cells were co-transfected with the expression plasmids and packaging plasmids (pCMV-VSV-G-RSV-Rev and pCAG-HIVgp) using Lipofectamine 2000 (Invitrogen). The viral supernatants were collected 72 h after transfection. For viral infection, 5.0×10^4 MS-1 cells/well in 6-well tissue culture plates were infected with lentiviral particles.

Immunohistochemistry and Western blot analysis

Immunohistochemistry of culture cells was performed as described previously (*41*, *42*) using monoclonal antibodies to VE cadherin (BV13: eBioscience) and α-SMA (1A4: Sigma-Aldrich). F-actin was stained by fixing the MS-1 cells that were treated with or without TGF-β2 for 24 h in 4% paraformaldehyde and 0.2% Triton X-100, followed by incubation with phalloidin–tetramethylrhodamine B isothio cyanate, from Amanita phalloides (Sigma-Aldrich). Stained cells were photographed using a confocal microscope (model LSM510 META; Carl Zeiss MicroImaging Inc.) with 40× (Plan-Neofluar 40×/1.3) objectives, and LSM Image Browser. All images were taken at room temperature. Western blot analyses were performed as described previously (*43*) using antibodies to α-SMA (Sigma-Aldrich), MRTF-A (abcam) and α-tubulin (Sigma-Aldrich).

Pull-down assay of GTP-bound Rho-GTPases

MS-1 cells (6.0×10^7) were used for each assay. Rho activation assay was performed as described previously (*44*) by using Rho activation assay kits (Millipore).

RNA isolation and RT-PCR

Total RNA was prepared with RNeasy reagent (QIAGEN) and reverse transcribed by random priming and a Superscript first strand synthesis kit (Invitrogen). Quantitative RT–PCR analysis was performed using the GeneAmp 5700 Sequence Detection System and Step One Plus Real-Time PCR System (Applied Biosystems). All expression data were normalized to those for GAPDH. The primer sequences are available online as indicated in Supplementary Table S1.

Collagen gel contraction assay

Type I collagen gel was prepared using an 8:1:1 ratio of cold collagen solution (Cellmatrix I-P; Nitta Gelatin, Osaka, Japan), 10 × concentrated MEM medium (Invitrogen) and collagen dilution buffer containing 0.05 N NaOH, 2.2% $NaHCO_3$ and 200 mM HEPES pH 7.4. MS-1 cell suspensions (1.0×10^6 cells/200 μl) were mixed in 800 μl of the collagen gel solution. A measure of 1 ml of the mixture was added to each well of 12-well culture plates and allowed to solidify at 37°C for 30 min. After solidification, 1 ml of MEM-α medium containing 10% FCS, 50 U/ml penicillin and 50 μg/ml streptomycin was overlaid to float the gel. The floating gels were incubated at 37°C in 5% CO_2 for 2 days. The gel surface area was quantified based on pixel number using ImageJ (US National Institutes of Health). The relative changes in the surface area are shown as the ratio of the original surface area.

Luciferase assay

Construction of luciferase reporter plasmids was performed according to a previous report (*45*). Briefly, the −724/+51 sequence of the α-SMA gene, containing the promoter sequence, exon 1 and 7 bp of intron 1, was subcloned into the pGL4 basic vector containing the luciferase gene (Promega). By site-directed mutagenesis, wild-type CCTTGTTTGG (CArG A) and CCCTATATGG (CArG B) sequences were replaced by CCTTGAATTC and CCCGAATTC G sequences, respectively. MS-1 cells were seeded in 24-well plates and then transiently transfected with promoter–reporter constructs with or without expression plasmids encoding ca-MRTF-A. Cell lysates were then prepared, and luciferase activities in the lysates were measured with the Dual-Luciferase reporter system (Promega) using a luminometer (MicroLumat Plus, Berthold). Values were normalized to Renilla luciferase activity driven by thymidine kinase promoter.

Statistical analyses

Results were compared by Student's *t*-test. Differences were considered significant when $P < 0.05$. All statistical tests were two-sided.

Results

TGF-β2 induces differentiation of MS-1 endothelial cells into α-SMA-expressing cells

In order to examine the roles of TGF-β signals in the mesenchymal transition of endothelial cells, we treated mouse pancreatic microvascular endothelial cells (MS-1) with TGF-β2, which appears to be the physiologically most relevant TGF-β isoform for EndMT during heart cushion development (*46*). MS-1 cells contact with neighbouring cells, and exhibit cobblestone-like structures (Fig. 1A). However, when they were treated with 1 ng/ml TGF-β2, they lost the cell–cell contact and became spindle shaped (Fig. 1A).

Next, we investigated the effect of TGF-β2 on cytoskeletal structure of MS-1 cells. As shown in Fig. 1B, F-actin was stained on marginal region of control MS-1 cells, whereas TGF-β2-treated MS-1 cells have thicker stress fibres in central region which are parallel to long axis of cells.

Since expression of endothelial and mesenchymal markers decreases and increases, respectively, during the mesenchymal transition of endothelial cells, we examined the changes of these hallmark markers of EndMT. MS-1 cells were treated with 1 ng/ml TGF-β2 for 72 h, followed by staining for an endothelial marker VE cadherin and a mesenchymal marker α-SMA. As shown Fig. 1C, treatment of MS-1 cells with TGF-β2 decreased the expression of VE-cadherin protein and increased the expression of α-SMA. In order to further examine how TGF-β2 induces α-SMA expression, we studied the kinetics of the α-SMA expression in MS-1 cells treated with TGF-β2. As shown in Fig. 1D, the expression of α-SMA mRNA became significantly higher than control at 48 h and continued to increase until 96 h. This was confirmed at 48 and 72 h after the treatment by western blot analysis (Fig. 1E).

Mammalian TGF-β includes three isoforms, TGF-β1, 2 and 3. We examined the effects of other TGF-β isoforms on the α-SMA expression in MS-1 cells. As shown in Supplementary Fig. S1, when the equivalent levels of PAI-1 expression were induced by TGF-β1, 2 and 3 in MS-1 cells, the α-SMA expression was also induced at equivalent levels, suggesting that all three TGF-β isoforms are able to induce the α-SMA expression.

As one of the most characteristic features of mesenchymal cells is their ability to reorganize extracellular matrix, this property was determined by a collagen gel contraction assay (*47*). MS-1 cells pre-treated with TGF-β2 were suspended in a collagen type I gel. After the collagen had solidified, the gel was detached from the sides and bottoms of the dishes and floated in media containing the ligand for 48 h. There was no significant degradation of the collagen gel in the control cells, but the volume of the collagen gel was reduced by ~70% in cells treated with TGF-β2 (Fig. 1F). Taken together, we concluded that MS-1 endothelial cells undergo EndMT by the stimulation of TGF-β2.

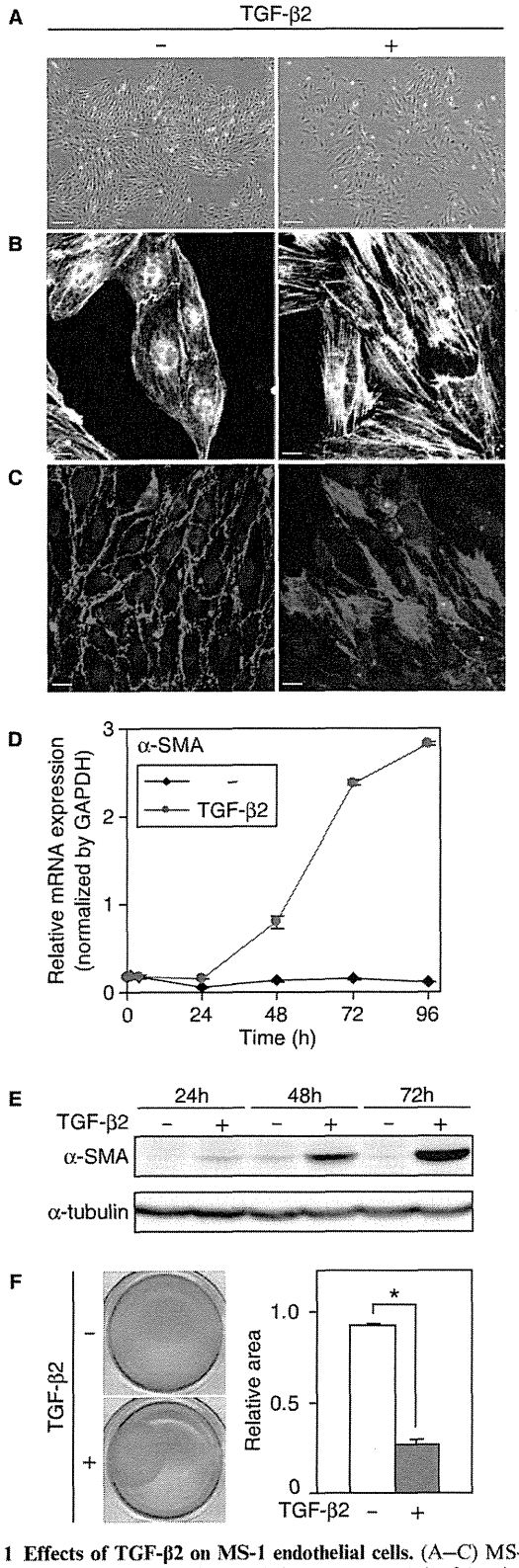

Fig. 1 Effects of TGF-β2 on MS-1 endothelial cells. (A–C) MS-1 cells were cultured in the absence (−) or presence (+) of 1 ng/ml of TGF-β2, followed by observation of morphology (A), fluorescence immunostaining for F-actin (white) and nuclei (blue) (B), and fluorescence immunostaining for VE cadherin (green), α-SMA (red) and nuclei (blue) (C). Scale bars: 200 μm (A) and 20 μm (B and C). (D and E) MS-1 cells were treated with TGF-β2 for various time periods, followed by quantitative RT–PCR analysis for α-SMA expression (D) and Western blot analysis for the expression of α-SMA (top) and α-tubulin (bottom) (E). Error bars represent standard deviations. (F) MS-1 cells were preincubated with or without TGF-β2 for 72 h, and then mixed in collagen matrices. After solidification, the mixtures were released from the culture dishes and incubated for additional 2 days. Experiments were performed in duplicate, and representative results are shown. $^*P<0.05$.

Activation of Rho signal by TGF-β2 is necessary for EndMT

Expressions of various EMT-related transcription factors including Snail, SIP-1 and δEF-1 have been shown to be induced by TGF-β during EMT. We previously reported that Snail is necessary for the TGF-β2-induced mesenchymal differentiation of mouse embryonic stem cell-derived endothelial cells (MESEC) (*48*). In order to examine the possibility that such EMT-related transcription factors are involved in TGF-β2-induced mesenchymal transition of MS-1 endothelial cells, we studied the effects of TGF-β2 on their expression. TGF-β2 induced the Snail expression while it did not induce the expression of Slug, SIP-1 and δEF-1 (Supplementary Fig. S2A–D). However, in contrast to MESEC, the knockdown of Snail expression in MS-1 cells did not suppress the TGF-β2-induced α-SMA expression (Supplementary Fig. S2E and F), suggesting that Snail is not involved in the TGF-β2-induced mesenchymal transition of MS-1 endothelial cells.

Since the activation of Rho signals by TGF-β has been implicated in EMT (*49*, *50*), we examined whether Rho signals are involved in the TGF-β2-induced EndMT. First, we examined whether TGF-β2 activates RhoA in MS-1 cells. When MS-1 cells were treated with TGF-β2, the amount of GTP-bound form of RhoA was increased (Fig. 2A). We next examined whether activation of Rho signals are necessary for TGF-β2-induced EndMT. The actions of the Rho GTPases are mediated by the multiple groups of downstream effectors including ROCK and mDia. Treatment of MS-1 cells with Y27632, an inhibitor of ROCK, inhibited the induction of actin reorganization, which was observed by phalloidin staining (Fig. 2B). Y27632 and C3 exoenzyme, an inhibitor of Rho GTPases, also abrogated the induction of α-SMA expression by TGF-β2 at both mRNA (Fig. 2C) and protein levels (Fig. 2D). These results suggest that activation of Rho signal is required for the TGF-β2-induced mesenchymal transition of MS-1 cells.

Expression of mesenchymal markers is induced by TGF-β2 in Rho-dependent and independent fashions

We next examined which mesenchymal markers are induced by TGF-β2 in MS-1 cells. TGF-β2 induced the expression of various other mesenchymal markers including SM22α (Fig. 3A), fibronectin 1 (Fig. 3B) and matrix metalloproteinase 2 (MMP2) (Fig. 3C) in MS-1 cells at 72 h after treatment. However, some of the fibroblast markers such as FSP-1 were not induced (data not shown).

Since TGF-β2-induced α-SMA expression was abrogated by Y27632, we next questioned whether expression of all of the mesenchymal markers requires

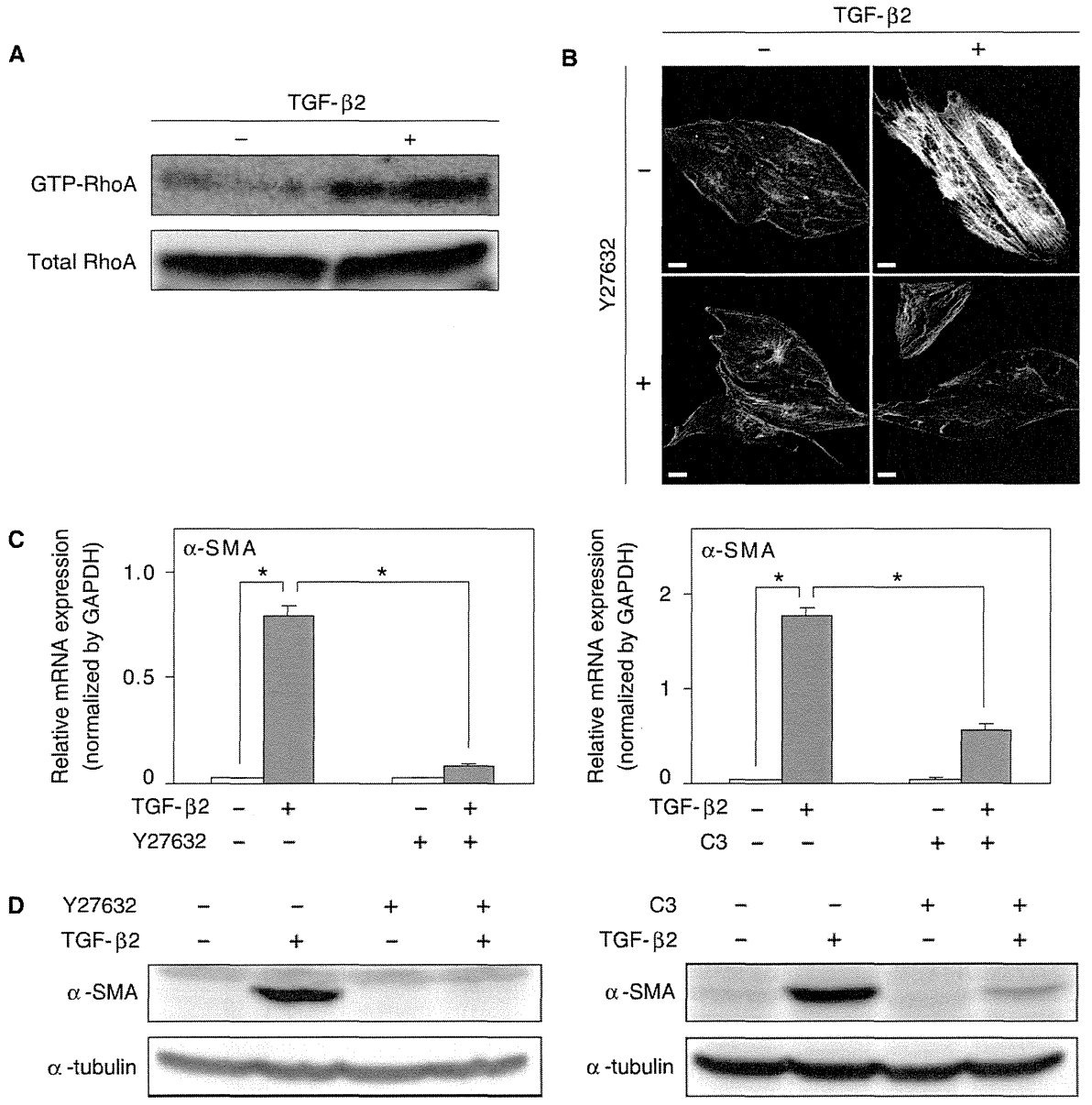

Fig. 2 Roles of Rho signals in the TGF-β2-induced mesenchymal transition of MS-1 cells. (A) MS-1 cells were cultured in the absence (–) or presence (+) of 1 ng/ml of TGF-β2 for 24 h, and were subjected to RhoA activation assays. The levels of GTP-bound RhoA (top) and total RhoA (bottom: internal control) are shown. (B) MS-1 cells were treated with TGF-β2 in the absence (–) or presence (+) of Y27632, a ROCK inhibitor, followed by fluorescence immunostaining for F-actin (white) and nuclei (blue). (C and D) MS-1 cells were treated with TGF-β2 in the absence (–) or presence (+) of Y27632 (left) and C3 exoenzyme (C3, right), an inhibitor of Rho GTPases for 72 h, followed by quantitative RT–PCR analysis for α-SMA expression (C) and Western blot analysis for the expression of α-SMA (top) and α-tubulin (bottom) (D). Scale bars: 20 μm. Error bars represent standard deviations. $^*P<0.05$.

the activation of Rho signals. When MS-1 cells were treated with Y27632, TGF-β2 failed to induce the expression of SM22α (Fig. 3A), while the induction of fibronectin 1 (Fig. 3B) and MMP2 (Fig. 3C) was not affected by Y27632. Treatment of MS-1 cells with C3 exoenzyme also elicited similar inhibitory effects to those of Y27632 on the expression of various mesenchymal markers (Supplementary Fig. S3). These results suggest that TGF-β2 induces the expression of a group of mesenchymal markers such as α-SMA and SM22α in a Rho-dependent fashion while Rho signals are not required for TGF-β2-induced expression of other mesenchymal markers including fibronectin 1 and MMP2.

Silencing of Smad4 abolishes the induction of mesenchymal markers by TGF-β2

Previous reports have shown that TGF-β-induced activation of non-Smad pathway is involved in the activation of Rho signals leading to EMT (*49*, *50*). We examined the involvement of Smad signals in the TGF-β2-induced expression of various mesenchymal markers in MS-1 cells. When Smad4 expression was decreased by siRNA (Fig. 4A), TGF-β2 failed to

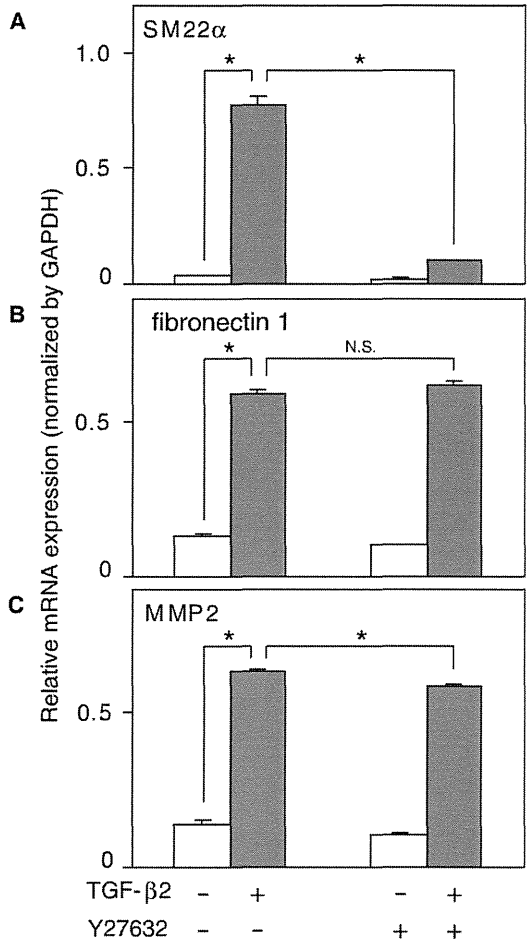

Fig. 3 Effects of Y27632 on the TGF-β2-induced expression of mesenchymal markers in MS-1 cells. MS-1 cells were treated with TGF-β2 in the absence (−) or presence (+) of Y27632, a ROCK inhibitor, for 72 h, followed by quantitative RT-PCR analysis for the expression of SM22α (A), fibronectin 1 (B) and MMP2 (C). Error bars represent standard deviations. $^*P<0.05$; N.S., not significant.

induce the expression of PAI-1 (Fig. 4B). To our interest, TGF-β2 was not capable of inducing the expression of α-SMA (Fig. 4C) and SM22α (Fig. 4D), whose expression was dependent on Rho signals, as well as fibronectin 1 (Fig. 4E) and MMP2 (Fig. 4F), when Smad4 expression was knocked down. These results suggest that Rho-dependent induction of mesenchymal markers by TGF-β2 is dependent on Smad pathways.

TGF-β2-induced expression of Arhgef5 is dependent on Smad pathways and is important for the α-SMA expression induced by TGF-β2

We next questioned whether TGF-β2-induced expression of mesenchymal markers is directly regulated by Smad signals. As shown in Fig. 1D, α-SMA expression started to be elevated at 48 h after treatment of TGF-β2. When we examined the kinetics of TGF-β2-induced expression of other mesenchymal markers, we found that SM22α also became induced at 72 h after treatment of TGF-β2 (Supplementary Fig. S4A), while the expression of fibronectin 1 (Supplementary

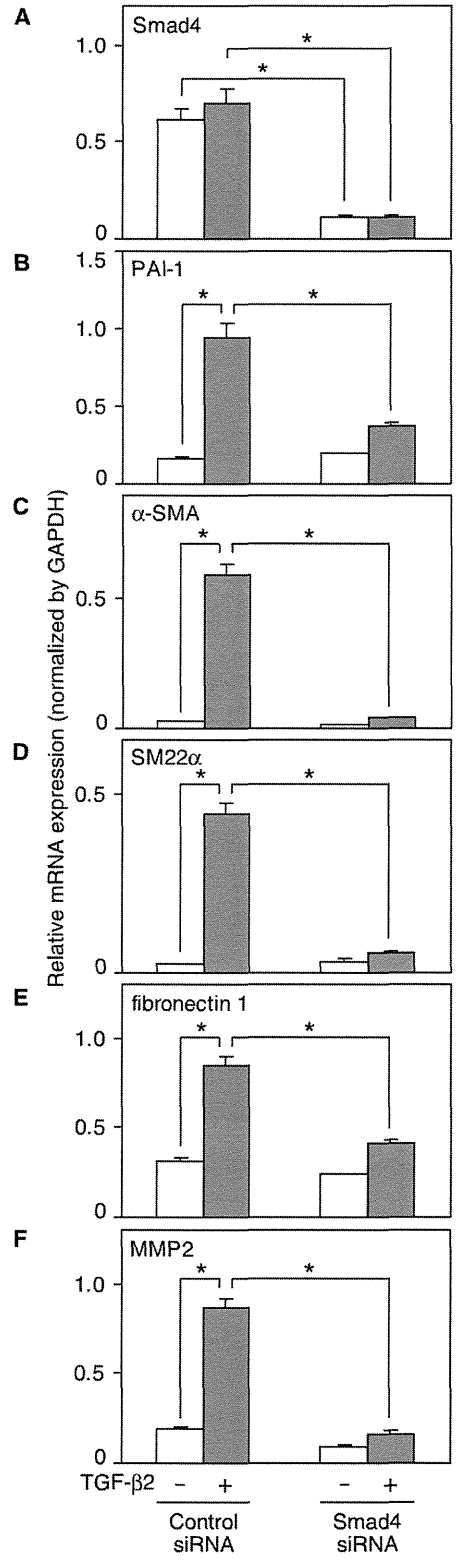

Fig. 4 Roles of Smad4 in the TGF-β2-induced expression of mesenchymal markers in MS-1 cells. MS-1 cells transfected with control siRNA or siRNA for Smad4 were cultured in the absence (−) or presence (+) of 1 ng/ml of TGF-β2 for 72 h, followed by quantitative RT–PCR analysis for the expression of Smad4 (A), PAI-1 (B), α-SMA (C), SM22α (D), fibronectin 1 (E) and MMP2 (F). Error bars represent standard deviations. $^*P<0.05$.

Fig. S4B) and MMP2 (Supplementary Fig. S4C) was significantly induced at 24 h after treatment, suggesting that α-SMA and SM22α, whose expression is dependent on TGF-β2-activated Rho signals, are indirectly regulated by Smad signals.

Previous reports have shown that TGF-β activates Rho signals not only by non-Smad pathways but by Smad pathways, which induce the expression of GEFs including Net1 (*12*) and GEF-H1 (*13*). Based on Smad dependency of mesenchymal marker induction by Rho signals, we examined the effects of TGF-β2 on the expression of various GEFs and the involvement of Smad signals by knocking down Smad4 expression (Fig. 5A and Supplementary Fig. S5). We found that TGF-β2 increased the expression of Arhgef5 (also known as Tim1), a member of the Dbl family of GEFs (*51*), in MS-1 cells (Fig. 5B) to a higher extent than those of Net1 (Supplementary Fig. S5A) and GEF-H1 (Supplementary Fig. S5B). Furthermore, we confirmed that the TGF-β2-induced expression of Arhgef5 is dependent on Smad4 by knocking down the Smad4 expression (Fig. 5B).

We next examined the causal relationship between TGF-β2-induced Arhgef5 expression and TGF-β2-induced α-SMA expression in MS-1. When Arhgef5 expression was knocked down by siRNA in MS-1 cells (Fig. 5C), the effect of TGF-β2 on the α-SMA expression in MS-1 cells was partially but decreased (Fig. 5D). These results suggest that Arhgef5 expression induced by Smad4-dependent TGF-β2 signals is involved in α-SMA induction in TGF-β2-treated MS-1 cells.

TGF-β2 induces the expression and nuclear accumulation of MRTF-A protein

Rho signals activate multiple cellular events to induce EMT. A previous report showed that Rho activity induces the nuclear translocation of MRTFs, myocardin family proteins, leading to the complex formation with SRF on the CArG box in the actin cytoskeletal genes including α-SMA and with Smad3 on the GCCG box in Slug promoter in MDCK epithelial cells (*19*). These dual functions of Rho-MRTF pathways are important for the TGF-β-induced EMT.

We first examined whether TGF-β2 changes the level of nuclear accumulation of MRTF-A protein in MS-1 cells. As shown in Fig. 6A, to our surprise, TGF-β2 increased the level not only of nuclear accumulated MRTF-A protein but also of total MRTF-A protein, suggesting that TGF-β2 regulates the expression of MRTF-A protein in addition to its nuclear transport. Furthermore, we found that Y27632 suppressed the effects of TGF-β2 on the nuclear accumulation of MRTF-A protein but not on its expression (Fig. 6A), suggesting that Rho signals play important roles not in the MRTF-A expression but in its nuclear transport. In order to examine whether the TGF-β2-induced increase in MRTF-A expression is regulated transcriptionally, we carried out RT-PCR analysis, and found that TGF-β2 increases the level of MRTF-A mRNA (Fig. 6B). It is noteworthy that this increase in MRTF-A

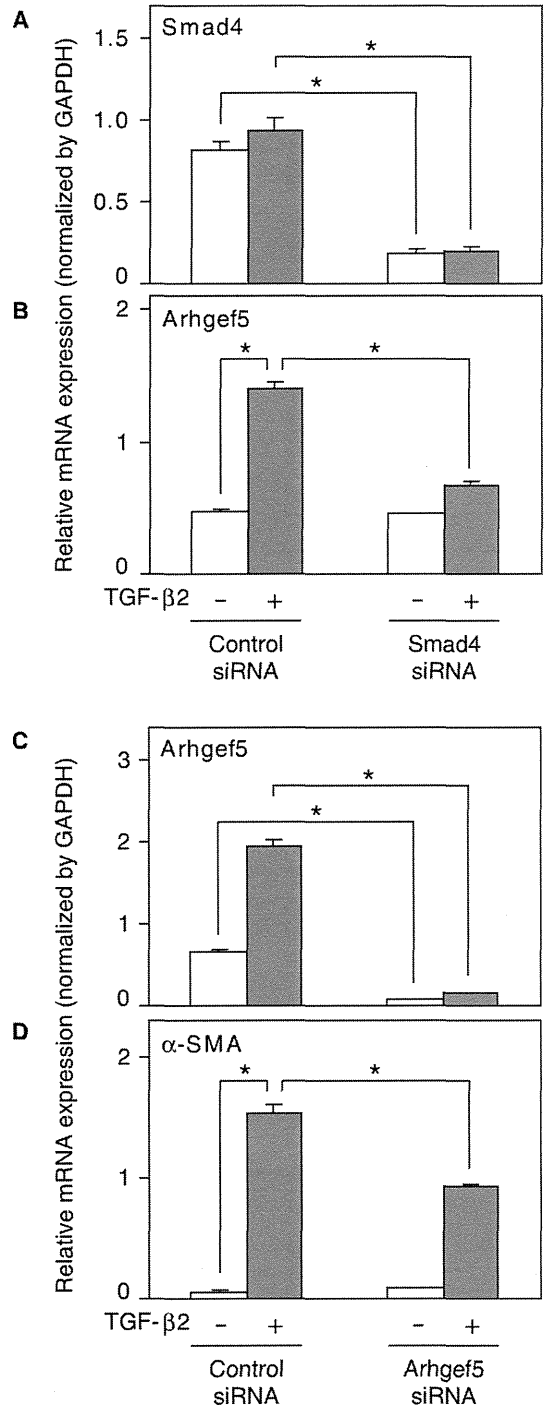

Fig. 5 Induction of Arhgef5 by TGF-β2 and its contribution to the TGF-β2-induced α-SMA expression in MS-1 endothelial cells. (A and B) MS-1 cells transfected with control siRNA or siRNA for Smad4 were cultured in the absence (−) or presence (+) of 1 ng/ml of TGF-β2 for 72 h, followed by quantitative RT–PCR analysis for the expression of Smad4 (A) and Arhgef5 (B). (C and D) MS-1 cells transfected with control siRNA or siRNA for Arhgef5 were cultured in the absence (−) or presence (+) of 1 ng/ml of TGF-β2 for 72 h, followed by quantitative RT–PCR analysis for the expression of Arhgef5 (C) and α-SMA (D). Error bars represent standard deviations. $^{*}P<0.05$.

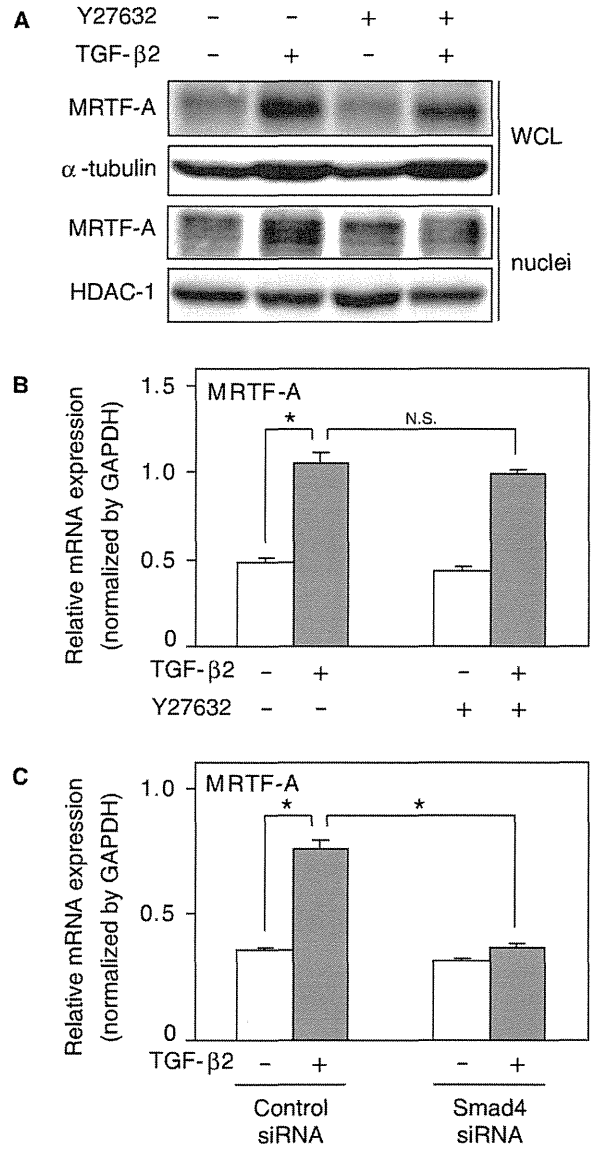

Fig. 6 Effects of TGF-β2 and Rho signals in the expression and nuclear localization of MRTF-A in MS-1 endothelial cells. (A and B) MS-1 cells were treated with TGF-β2 in the absence (−) or presence (+) of Y27632 for 72 h, followed by Western blot analysis for the expression of MRTF-A in whole-cell lysates (WCL) (top panel) and in the nuclei (third panel) and α-tubulin (second panel) and HDAC-1 (bottom panel) (A), and quantitative RT-PCR analysis for the expression of MRTF-A transcripts (B). (C) MS-1 cells transfected with control siRNA or siRNA for Smad4 were cultured in the absence (−) or presence (+) of 1 ng/ml of TGF-β2 for 72 h, followed by quantitative RT–PCR analysis for the expression of MRTF-A. Error bars represent standard deviations. $^*P<0.05$; N.S., not significant.

mRNA by TGF-β2 was not inhibited by Y27632 (Fig. 6B) or C3 exoenzyme (Supplementary Fig. S3D) but was inhibited by knocking down Smad4 expression (Fig. 6C). These results suggest that TGF-β2 induces MRTF-A expression through Smad signals rather than Rho signal, which is involved in the nuclear translocation of MRTF-A in MS-1 cells.

MRTF-A is necessary and sufficient for TGF-β2-induced α-SMA expression in MS-1 endothelial cells

We next examined whether the TGF-β2-induced MRTF-A activation is involved in the α-SMA upregulation in MS-1 cells. When the endogenous level of MRTF-A expression was decreased by siRNA (Fig. 7A), TGF-β2 failed to induce the α-SMA expression in MS-1 cells (Fig. 7B), suggesting that MRTF-A is necessary for the TGF-β2-induced mesenchymal transition of MS-1 cells.

We next studied the effect of gain-of-function of MRTF-A on the expression of α-SMA. When constitutively active (ca) MRTF-A was introduced to MS-1 cells by lentivirus, the expression of α-SMA in MS-1 cells increased in the absence of TGF-β2 (Fig. 7C), suggesting that activation of MRTF-A can mimic the effect of TGF-β2 on the α-SMA expression (*19*).

In order to further dissect the molecular mechanisms underlying the transcriptional regulation of α-SMA gene by MRTF-A, we used the promoter region of α-SMA gene. As previously reported (*45*), the 724 bp fragment of 5′-flanking region of α-SMA gene consists of two CArG boxes that contribute to the MRTF-A-mediated transcription of α-SMA gene (Fig. 7D). When MS-1 cells were transfected with a luciferase reporter construct containing the 724 bp promoter fragment in the absence or presence of ca-MRTF-A, the reporter activity was enhanced by ca-MRTF-A (Fig. 7E). α-SMA transcription is regulated via interaction of multiple transcription factors with distinct *cis*-elements in the promoter. When point mutations were introduced to the two CArG elements, the effects of ca-MRTF-A on the α-SMA promoter were abolished (Fig. 7D and E). These results suggest that TGF-β2 induces the α-SMA expression in MS-1 cells by inducing the expression of MRTF-A which binds to the CArG elements in the α-SMA promoter.

Discussion

In the present study, we illustrated a novel mechanism by which TGF-β2 induces the mesenchymal transition of MS-1 endothelial cells (Fig. 8). TGF-β2 induces the expression of Arhgef5, a GEF, in a Smad-dependent fashion, and activates Rho signals. TGF-β2 also enhances the expression of MRTF-A by Smad signals, and its translocation to nucleus, which is required and sufficient for TGF-β2-induced expression of α-SMA. These results highlighted novel molecular mechanisms by which TGF-β2 activates Rho signals and MRTF-A in order to induce the mesenchymal transition of MS-1 endothelial cells.

In epithelial cells, TGF-β-induced activation of Rho signals is involved in mesenchymal transition (*8*). While many reports have shown that TGF-β activates Rho signals via non-Smad signalling pathways, recent studies including the present study have shown that Smad signalling pathways induce the expression of Rho GEFs, which in turn activates Rho GTPases (*12*, *13*). While Arhgef5 has been implicated in many biological events including changes in cell shape and cytoskeletal organization (*52*), immature dendritic cell migration (*53*) and podosome formation (*54*), the

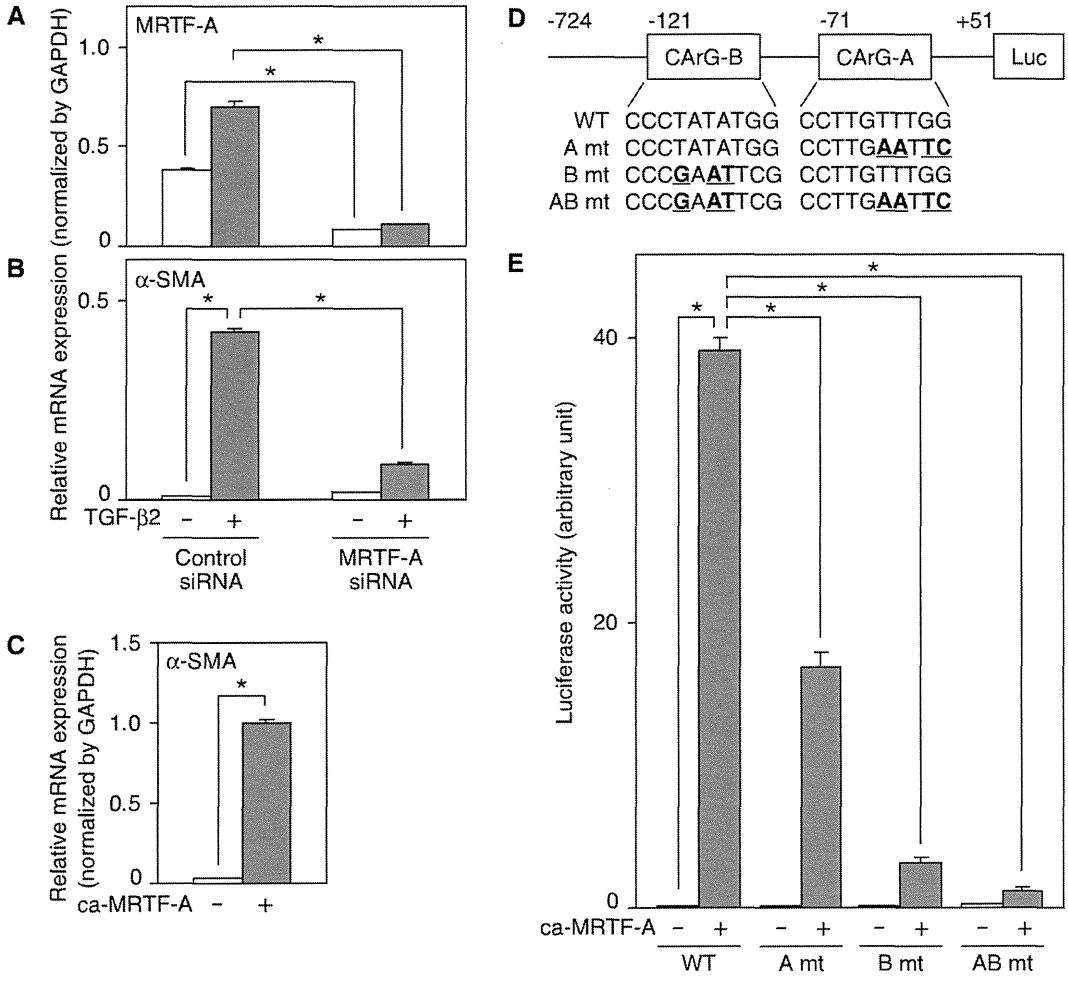

Fig. 7 Roles of MRTF-A in the TGF-β2-induced α-SMA expression in MS-1 endothelial cells. (A and B) MS-1 cells transfected with control siRNA or siRNA for MRTF-A were cultured in the absence (−) or presence (+) of 1 ng/ml of TGF-β2 for 72 h, followed by quantitative RT–PCR analysis for the expression of MRTF-A (A) and α-SMA (B). (C) MS-1 cells were infected with lentivirus encoding control (−) or constitutively active MRTF-A (+) and were subjected to quantitative RT–PCR analysis for the expression of α-SMA. (D and E) Effect of MRTF-A on the α-SMA promoter activity. (D) Schematic representation of mouse α-SMA promoter construct (WT) and its site-mutated series (mt). The indicated numbers present nucleic acid positions from the transcription initiation site. The mutated nucleotides are underlined. (E) MS-1 cells were transfected with the site-mutated reporter constructs along with expression construct encoding ca-MRTF-A (+) or control (−), followed by measurement of luciferase activity. Error bars represent standard deviations. $^{*}P<0.05$.

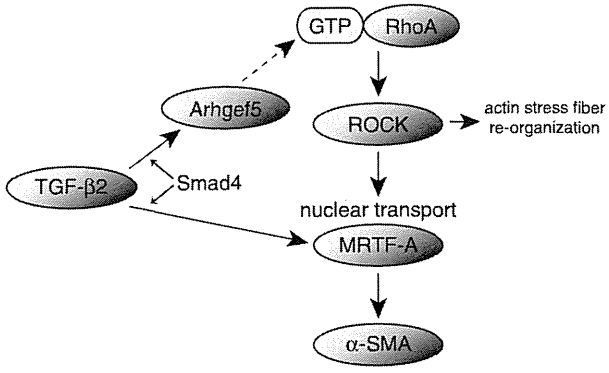

Fig. 8 Scheme of Smad-dependent activation of Rho signals and MRTF-A during TGF-β2-induced mesenchymal transition of MS-1 endothelial cells. TGF-β2 induces the expression of Arhgef5, a GEF, and activates Rho signals. TGF-β2 also enhances the expression of MRTF-A, and its translocation to nucleus. Induction of both Arhgef5 and MRTF-A by TGF-β2 is dependent on Smad4, and is required for TGF-β2-induced expression of α-SMA.

present study, for the first time, showed that Arhgef5 plays critical roles in TGF-β-induced EndMT. However, decreased expression of Arhgef5 did not completely abrogate the TGF-β-induced α-SMA expression while inhibition of Rho signals by Y27632 completely suppressed it, suggesting that Arhgef5 is not a sole component of TGF-β-induced activation of Rho GTPases.

MRTFs have been implicated in the TGF-β-induced α-SMA expression (*19*). This induction is mediated by stimulation of the nuclear transport of MRTF proteins by TGF-β-induced activation of Rho signals. In the present study, we, for the first time, found that TGF-β additionally enhanced the transcription of MRTF-A via Smad signals. Therefore, TGF-β-induced accumulation of MRTF-A proteins in the nuclei of MS-1 might be caused by both the elevated expression of MRTF-A and the induction of its nuclear transport by Rho signals.

We previously reported that TGF-β2 increased the expression of Snail, but not of other EMT-related transcription factors including Slug, δEF-1, SIP-1 and Twist in MESEC (*48*). Furthermore, Snail is necessary and sufficient for TGF-β2-induced EndMT of MESEC. Medici and colleagues recently reported that Snail is required for TGF-β2-induced α-SMA expression in human cutaneous microvascular endothelial cells (HCMEC) (*37*). However, over-expression of Snail was not sufficient to induce EndMT of HCMEC, suggesting that MESEC and HCMEC have differential responses to Snail expression. While we also found that Snail expression was induced by TGF-β2 in MS-1 cells, decreased expression of Snail in MS-1 cells did not suppress the TGF-β2-induced α-SMA expression, suggesting that MS-1 cells have less requirement of Snail for the induction of EndMT. Molecular mechanisms underlying these differential necessity and sufficiency of Snail for mesenchymal transition of various types of endothelial cells remain to be elucidated in the future.

TGF-β2 induces the expression of various mesenchymal markers during mesenchymal transition of epithelial and endothelial cells. In the present study, we found that TGF-β2 increased the expression of α-SMA, SM22α, fibronectin 1 and MMP2 in MS-1 cells. To our interest, while Smad4 is dispensable for the expression of all four mesenchymal markers above, inhibition of Rho signals by Y27632 and C3 exoenzyme suppressed the TGF-β2-induced expression of α-SMA and SM22α, but not of fibronectin 1 and MMP2. These results suggest that TGF-β2 triggers multiple signalling cascades that induce multiple groups of the target genes in different manners.

During EMT and EndMT, TGF-β2 not only induces the mesenchymal markers, but also decreases the expression of E-cadherin and VE cadherin, respectively. In the present study, TGF-β2 decreased the VE-cadherin expression in MS-1 cells. However, this decrease in VE-cadherin expression was not observed in all MS-1 cells treated with TGF-β2. In consistent with this finding, Hashimoto *et al.* (*36*) showed that the extent of TGF-β2-induced decrease in VE-cadherin expression was not as potent as that of TGF-β2-induced increase in α-SMA expression in MS-1 cells. TGF-β decreased the VE-cadherin expression in concert with Notch signals by inducing the expression of Slug, which directly binds to the VE-cadherin promoter in human microvascular endothelial cells (*55*).

EndMT has also been implicated in several pathological situations. During cardiac fibrosis, accumulated fibroblasts cause deposition of extracellular matrix, which can cause heart failure. Furthermore, activated fibroblasts promote the progression of cancers. Zeisberg and colleagues reported that the TGF-β-induced EndMT plays important roles in the formation of fibroblasts from endothelial cells during cardiac and renal fibrosis (*26*, *56–58*) and cancer progression (*59*). Since fibroblasts are culprits in various pathological situations, EndMT is expected to be a target for the therapies of cardiac dysfunction and cancer. Therefore, the present findings may lead to greater understanding of not only normal cardiovascular development but also such pathological situations, and eventually to development of the strategies to manipulate these signals for therapeutic benefit.

Supplementary Data

Supplementary Data are available at *JB* online.

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Conflict of interest

None declared.

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TGF-β-induced epithelial-mesenchymal transition of A549 lung adenocarcinoma cells is enhanced by pro-inflammatory cytokines derived from RAW 264.7 macrophage cells

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Cancer cells undergo epithelial-mesenchymal transition (EMT) during invasion and metastasis. Although transforming growth factor-β (TGF-β) and pro-inflammatory cytokines have been implicated in EMT, the underlying molecular mechanisms remain to be elucidated. Here, we studied the effects of proinflammatory cytokines derived from the mouse macrophage cell line RAW 264.7 on TGF-β-induced EMT in A549 lung cancer cells. Co-culture and treatment with conditioned medium of RAW 264.7 cells enhanced a subset of TGF-β-induced EMT phenotypes in A549 cells, including changes in cell morphology and induction of mesenchymal marker expression. These effects were increased by the treatment of RAW 264.7 cells with lipopolysaccharide, which also induced the expression of various proinflammatory cytokines, including TNF-α and IL-1β. The effects of conditioned medium of RAW 264.7 cells were partially inhibited by a TNF-α neutralizing antibody. Dehydroxy methyl epoxyquinomicin, a selective inhibitor of NFκB, partially inhibited the enhancement of fibronectin expression by TGF-β, TNF-α, and IL-1β, but not of N-cadherin expression. Effects of other pharmacological inhibitors also suggested complex regulatory mechanisms of the TGF-β-induced EMT phenotype by TNF-α stimulation. These findings provide direct evidence of the effects of RAW 264.7-derived TNF-α on TGF-β-induced EMT in A549 cells, which is transduced in part by NFκB signalling.

Keywords: EMT/lung adenocarcinoma/NFκB/TGF-β/TNF-α.

Abbreviations: δEF1, delta-crystallin/E2-box factor 1; DHMEQ, dehydroxy methyl epoxyquinomicin; EMT, epithelial-mesenchymal transition; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HMGA2, high mobility group AT-hook 2; ICAM-1, intercellular adhesion molecule-1; Id, inhibitor of differentiation; IL, interleukin; LPS, lipopolysaccharide; MMP, matrix metalloproteinase; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; RT, reverse transcription; SIP1, Smad interacting protein-1; siRNA, small interfering RNA; TβR, TGF-β receptor; TGF-β, transforming growth factor-β; TNF-α, tumour necrosis factor-α; TTF-1, thyroid transcription factor-1.

Cytokines of the transforming growth factor-β (TGF-β) family have multiple roles in development and diseases (*1–3*). TGF-β inhibits the proliferation of normal epithelial cells, but cancer cells often evade this control. Furthermore, TGF-β induces epithelial–mesenchymal transition (EMT) in cancer cells, enabling the cells to become motile and invasive (*2*, *4–7*). Since cancer cells are subjected to numerous extracellular stimulations *in vivo*, elucidating the roles of these factors on TGF-β-induced EMT is important for developing cancer treatments.

TGF-β binds to the TGF-β type II receptor (TβRII) on the cell membrane, forming a complex with the type I TGF-β receptor (TβRI) and activating it by phosphorylation (*8*, *9*). The intracellular signalling pathway of the TGF-β family is primarily induced by Smad family proteins. The receptor complex phosphorylates Smad2 and Smad3 on their C-terminal SSXS motifs, resulting in hetero-oligomer formation with Smad4, followed by translocation to the nucleus where they act as transcription factors.

The mechanism of TGF-β-induced EMT has been intensively examined, and each phenotype of EMT was found to be regulated by distinct regulatory factors. For example, expression of E-cadherin mRNA was suppressed by TGF-β via the induction of transcription factors Snail, Slug, high-mobility group AT-hook 2 (HMGA2), delta-crystallin/E2-box factor 1 (δEF1) and Smad interacting protein-1 (SIP1) (*10*, *11*). E12/E47 also represses E-cadherin when inhibitor of differentiation (Id) proteins are downregulated by TGF-β (*12*). Complex formation between Smads and Snail has been reported to be important for E-cadherin regulation by TGF-β (*13*). In contrast, the regulatory mechanisms involved in the expression of mesenchymal markers fibronectin and N-cadherin is poorly understood, and is generally not regulated by the

above factors (*11*, *14*). Induction of α-smooth muscle actin by TGF-β is reported to be induced by nuclear translocation of myocardin family proteins (*15*, *16*). We previously found that thyroid transcription factor-1 (TTF-1) is expressed in the lung epithelium and inhibits TGF-β-induced EMT in A549 lung adenocarcinoma cells, suggesting that TTF-1 is an intrinsic inhibitor of TGF-β-induced EMT (*17*).

Extracellular signals other than TGF-β have been shown to induce EMT in a variety of cells with or without the cooperation of TGF-β (*18*). FGF-2 cooperates with TGF-β to induce EMT and promotes invasion of cancer (*19*). Recent reports have suggested that inflammation plays an important role in tumour progression. Inflammatory cells in the tumour micro-environment produce various inflammatory cytokines, which are involved in the EMT of cancer cells. Previous reports have shown that various pro-inflammatory cytokines, including tumour necrosis factor-α (TNF-α), are produced from activated macrophages (*20*), and that these cytokines augment TGF-β-induced EMT in A549 cells (*21*), whereas TNF-α by itself does not induce EMT in A549 cells (*22*). However, the roles of macrophage-derived inflammatory cytokines in TGF-β-induced EMT of lung cancer cells and the molecular mechanisms underlying this process are not fully understood.

In the present study, we examined the effect of factors derived from a mouse macrophage cell line RAW 264.7 on TGF-β-induced EMT in A549 cells. We found that RAW 264.7-derived factors enhance some phenotypes of TGF-β-induced EMT in A549 cells, including upregulation of fibronectin and N-cadherin. We also showed that the effects of conditioned medium of RAW 264.7 cells on TGF-β-induced EMT is inhibited by a neutralizing antibody against TNF-α, suggesting that the secretion of TNF-α from RAW 264.7 cells is critical for TGF-β-induced EMT. We further found that interleukin (IL)-1β is produced by RAW 264.7 cells and augments TGF-β-induced EMT in A549 cells. Interestingly, the effect is partially mediated by nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) signalling which is suppressed by a specific inhibitor dehydroxy methyl epoxyquinomicin (DHMEQ). Effects of other pharmacological inhibitors on enhancement of TGF-β-induced EMT by TNF-α and IL-1β were also evaluated. Our findings suggest that TNF-α and IL-1β endogenously secreted from RAW 264.7 cells enhance TGF-β-induced EMT in A549 cells at least in part through NFκB signalling.

Materials and Methods

Cell culture and reagents

RAW 264.7, a macrophage-like cell line established from an ascites of tumour induced in mouse by intraperitoneal injection of Abelson leukaemia virus, was a kind gift from Dr Tadashi Muroi (NIH Sciences). A549 and RAW264.7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (GIBCO/Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin G and 100 μg/ml of streptomycin. NMuMG cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml of penicillin G, 100 μg/ml of streptomycin and 10 μg/ml of insulin. Cells were grown in a 5% CO_2 atmosphere at 37°C. Recombinant human TGF-β1, TNF-α and IL-1β were obtained from R&D Systems (Minneapolis, MN, USA). Recombinant human IL-6 was purchased from Peprotech (Rocky Hill, NJ, USA). Lipopolysaccharide (LPS) was from SIGMA (St Louis, MO, USA) and GM6001, a pan-matrix metalloproteinase (MMP) inhibitor, was from Calbiochem (San Diego, CA, USA). DHMEQ has been described previously (*23*, *24*). U0126 was from Promega (Fitchburg, WI, USA). SP600125 and SB203580 were from Calbiochem.

Preparation of conditioned medium of RAW 264.7 cells

RAW 264.7 cells (4.5×10^6) were cultured in 10 ml growth medium in a 100-mm dish with or without LPS for 24 h. A549 cells were pre-cultured in a 6-well plate for 24 h with or without TGF-β. Culture medium was replaced with 2 ml/well of the obtained conditioned medium containing TGF-β where indicated.

Quantitative reverse transcription polymerase chain reaction

Total RNAs were extracted using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) as described previously (*25*). First-strand cDNAs were synthesized using the SuperScript III First-Strand Synthesis System (Life Technologies). Quantitative real-time PCR (qRT–PCR) analysis was performed as described previously (*25*). Amplification data were quantified using the standard curve method. All samples were run in duplicate, and the results were averaged and normalized to expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primer sequences are available as Supplementary Table I.

Immunoblotting

A549 cells were washed with PBS and lysed with Radioimmunoprecipitation assay buffer [20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 10 mM EDTA, 1 mM EGTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS] or cell lysis buffer containing 0.5% Nonidet P-40, 20 mM Tris–HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulphonyl fluoride and 10 mg/ml aprotinin (*26*, *27*). SDS polyacrylamide gel-electrophoresis (SDS–PAGE) and immunodetection were performed as described previously (*28*). Antibodies used for immunoblotting included anti-fibronectin (SIGMA), anti-N-cadherin (BD Biosciences, San Jose, CA, USA), anti-E-cadherin (BD), anti-α-tubulin (SIGMA) and anti-phospho-Smad2 (Cell Signaling Technology, Danvers, MA, USA).

Determination of morphological changes

Morphological changes of the cells were quantitatively determined by calculating cell circularity as described (*29*) by Image J software. Circularity value decreases by morphological change from a pebble-like shape to an elongated shape.

ELISA

Amounts of TNF-α secreted by RAW 264.7 cells were determined in 24 h conditioned media using the Quantikine Mouse TNF-α Immunoassay (R&D Systems), according to the manufacturer's instructions.

Cell invasion assay

Cell invasion assay was performed as described previously with some modifications (*30*). Briefly, cells were pre-treated with or without 5 ng/ml TGF-β and 20 ng/ml TNF-α and seeded in the upper chambers of type-IV collagen-coated (Nitta Gelatin, Osaka, Japan) 12-well culture inserts. After 8 h, cells that had migrated through the collagen-coated-inserts were visualized using crystal violet staining.

Luciferase reporter assays

Luciferase reporter assays were performed as described previously (*31*), by using 9xCAGA-luc (*32*) and NFκB-luc (*33*) as a reporter and TK-Rluc as an internal control.

Neutralizing antibody

Goat anti-mouse TNF-α neutralizing antibody and goat control IgG were obtained from R&D systems. A549 cells were pre-treated with or without 5 ng/ml TGF-β for 24 h, and culture medium was replaced with conditioned medium of RAW 264.7 cells containing either neutralizing antibody or control IgG (10 μg/ml).

Statistical analysis

Student's *t*-test was used to calculate the significance of differences between the two samples. The Tukey–Kramer test of the R statistical analysis programme was used for multiple data comparisons (*34*). A $P<0.05$ was considered to indicate statistical significance.

Results

Secreted factors from RAW 264.7 cells enhance TGF-β-induced EMT of A549 cells

As previously reported (14), TGF-β induces EMT of human lung adenocarcinoma A549 cells that is characterized by morphological changes from a pebble-like shape to an elongated shape (Fig. 1A). These morphological changes were quantitatively determined by the circularity of the cells (*29*) (Fig. 1B). We examined whether secreted factors from mouse RAW 264.7 macrophage cells affect the TGF-β-induced EMT of A549 cells. As shown in Fig. 1A, morphological changes induced by TGF-β were further enhanced by co-culture with RAW 264.7 cells (Fig. 1A and B). To study these effects at a molecular level, qRT–PCR analyses to examine the expression of hallmark EMT genes were performed using human-specific primers. TGF-β-induced expression of the mesenchymal markers fibronectin and N-cadherin were enhanced by the co-culture of the cells (Fig. 1C). Conversely, expression of the epithelial marker E-cadherin was strongly down-regulated by TGF-β, but co-culture of the cells with RAW 264.7 did not elicit an additional effect.

We next determined whether the enhanced EMT was caused by secreted factors from RAW 264.7 cells. Since RAW 264.7 macrophage cells become activated upon treatment with *Escherichia coli*-derived LPS, we examined the effect of conditioned medium of RAW 264.7 cells treated with or without LPS on A549 cells. TGF-β-induced expression of fibronectin and N-cadherin mRNAs was enhanced by the addition of the conditioned medium, which was more significant when the conditioned medium of LPS-treated RAW 264.7 cells was used (Fig. 2A). The effect of the conditioned medium was not observed for repression of E-cadherin by TGF-β, which is in agreement with the

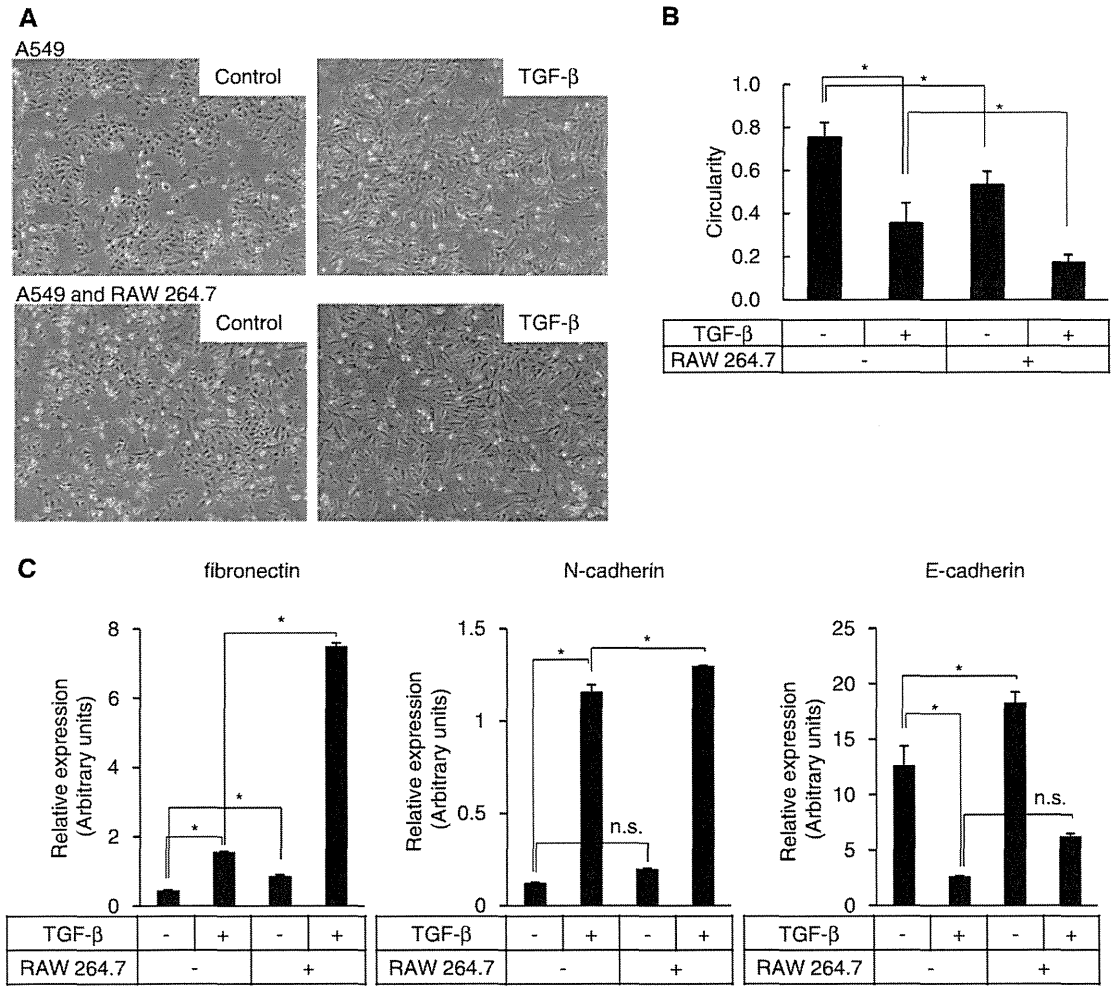

Fig. 1 Effect of co-culture with RAW 264.7 cells on TGF-β-induced EMT of A549 cells. (A) Human lung adenocarcinoma A549 cells were pre-cultured with or without 5 ng/ml TGF-β for 24 h and plated at a density of 5.0×10^4 cells/well in a 6-well plate. The same number of mouse macrophage RAW 264.7 cells were plated and incubated with or without TGF-β for 4 days followed by phase contrast microscopic imaging. (B) Cell circularity was calculated using ImageJ software. In total, 10 cells from each treatment in (A) were measured and the results were averaged. (C) qRT–PCR analysis of EMT marker expression by human-specific primers. A549 cells were co-cultured with RAW 264.7 cells for 3 days with or without TGF-β. *$P<0.05$; Error bars, SDs; n.s., not significant.

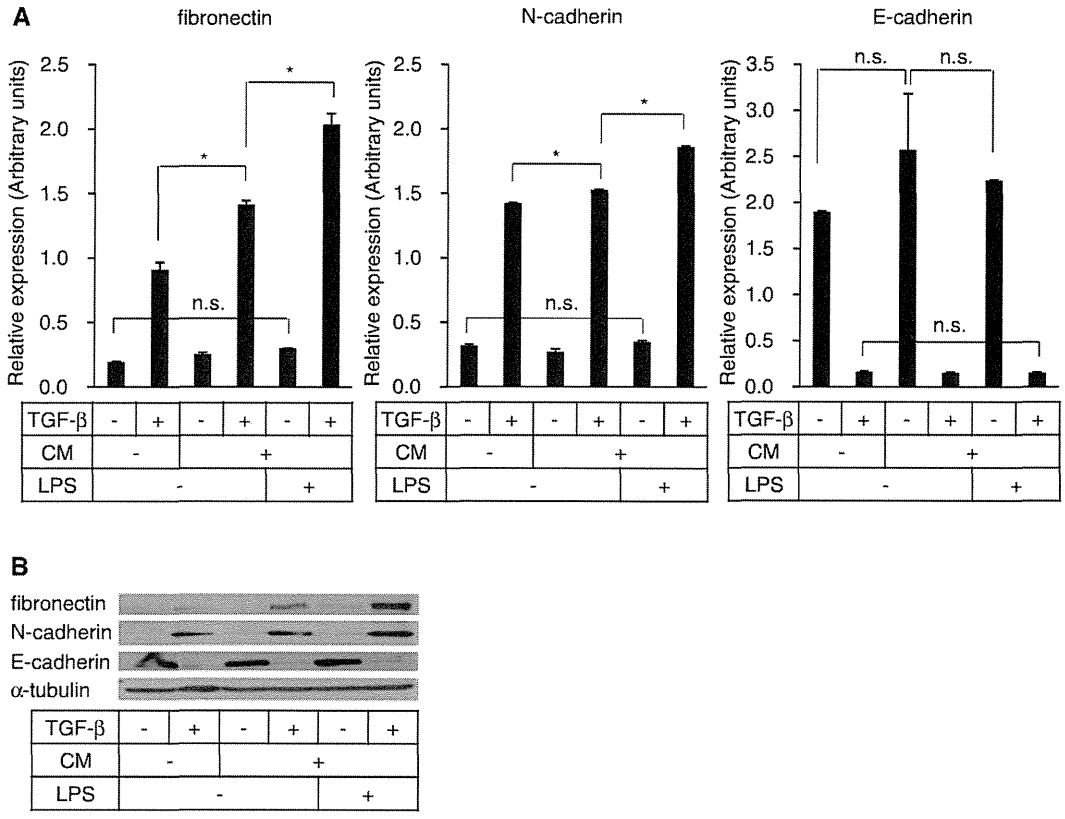

Fig. 2 Effect of conditioned medium of RAW 264.7 cells on TGF-β-induced EMT of A549 cells. (A) A549 cells were cultured with conditioned medium of LPS-activated (0.1 μg/ml) RAW 264.7 cells and stimulated with 5 ng/ml TGF-β. Expression of EMT markers was quantified using qRT–PCR. (B) Expression of fibronectin, N-cadherin and E-cadherin proteins in TGF-β-stimulated A549 cells cultured with conditioned medium of RAW 264.7 cells. A549 cells were cultured as in (A). α-tubulin expression is shown as the loading control. $^*P<0.05$; CM, conditioned medium; error bars, SDs; n.s., not significant.

results of co-culture assays (Fig. 1C). Of note, treatment of A549 cells with LPS alone did not affect TGF-β-induced EMT phenotypes (Supplementary Fig. 1). The effects of conditioned medium on the expression of fibronectin, N-cadherin and E-cadherin determined using qRT–PCR analysis were confirmed at the protein expression level by immunoblot analysis (Fig. 2B). Secreted factor(s) from activated RAW 264.7 cells therefore enhance EMT of A549 cells stimulated with TGF-β.

TNF-α is secreted from RAW 264.7 cells and enhances TGF-β-induced EMT of A549 cells

We next attempted to identify RAW 264.7-derived factors that enhance the TGF-β-induced EMT in A549 cells. We speculated that production of such factors is increased following treatment of RAW 264.7 cells with LPS. Since TNF-α is reported to be secreted from activated macrophages, we examined the effects of LPS on TNF-α expression in RAW 264.7 cells. We confirmed that TNF-α was expressed in RAW 264.7 cells, which were upregulated following LPS treatment (Fig. 3A). ELISA analysis showed that >600 pg/ml of TNF-α was present in the conditioned medium prepared from the RAW 264.7 cells and that TNF-α concentrations were increased following LPS treatment (Fig. 3B).

When recombinant TNF-α was added to A549 cells, cellular morphology changed both in the absence and presence of TGF-β (Fig. 3C and D). Induction of fibronectin and N-cadherin by TGF-β was also enhanced by TNF-α addition (Fig. 3E). In contrast, E-cadherin expression was strongly suppressed by TGF-β treatment alone and additional effects of TNF-α were not observed. The effects of TNF-α on cellular morphology and mesenchymal marker expression are similar to those observed in the co-culture experiments and those using conditioned medium of RAW 264.7 cells. Notably, E-cadherin expression was not repressed following both co-culture with RAW 264.7 cells and the use of conditioned medium without TGF-β (Fig. 1C and Fig. 2A and B). In contrast, addition of recombinant TNF-α partially inhibited E-cadherin expression without TGF-β stimulation, suggesting that secreted TNF-α was insufficient for effectively regulating E-cadherin expression.

TNF-α enhances TGF-β-induced motility of A549 cells

On the basis of the effect of TNF-α on TGF-β-induced EMT of A549 cells, we next examined its role in cell invasiveness that accompanies with EMT. Cell invasion assay showed that TNF-α enhanced the TGF-β-induced invasion of A549 cells (Fig. 4A). During EMT, MMPs play important roles in stimulating cell invasion. Expression of MMP-9 and MMP-2 was significantly enhanced by TNF-α and TGF-β, but not by TNF-α alone (Fig. 4B). The effect of TNF-α on

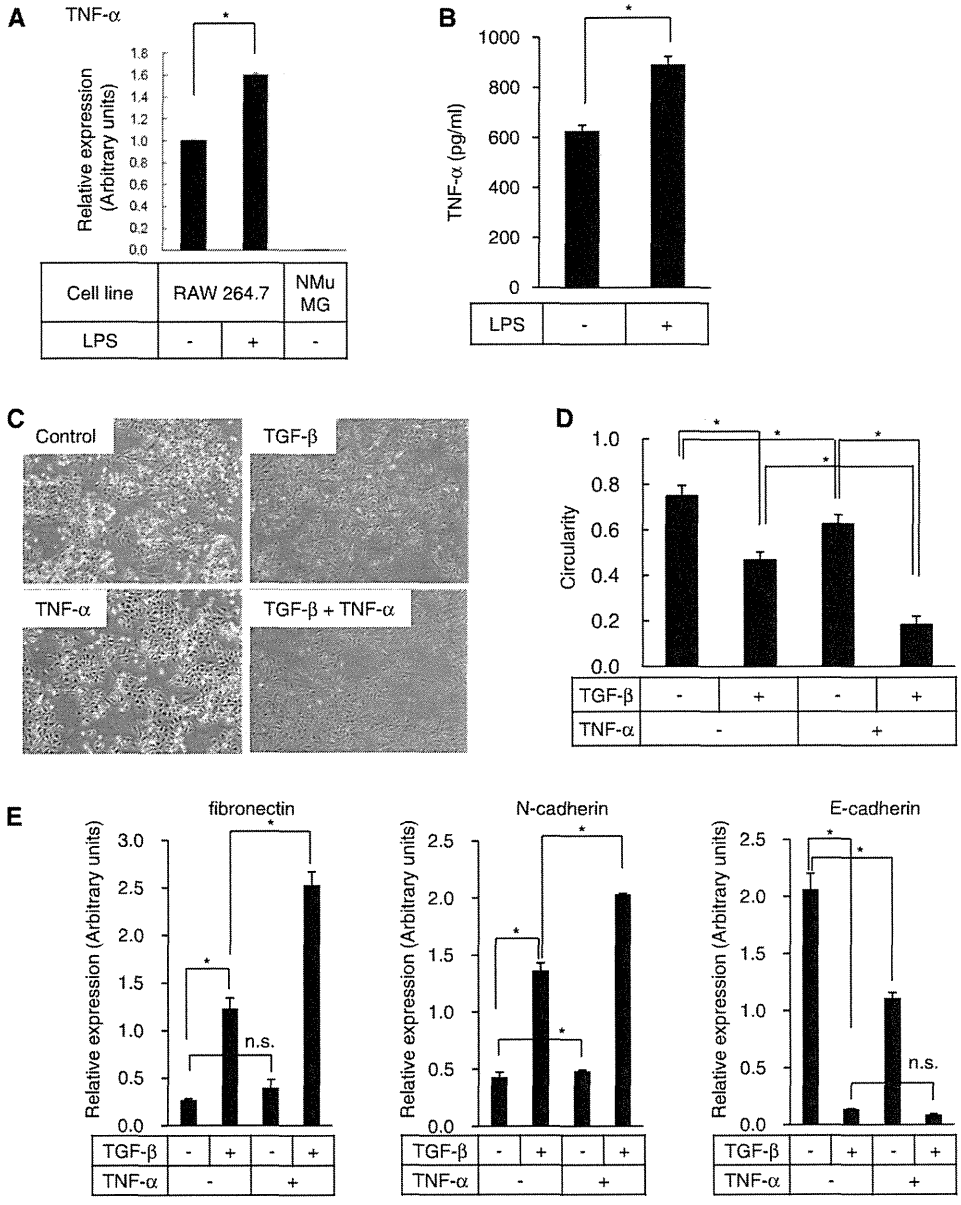

Fig. 3 Enhancement of TGF-β-induced EMT phenotype by TNF-α. (A) Expression of TNF-α mRNA in RAW 264.7 cells. Cells were treated with 0.1 μg/ml LPS, and TNF-α expression was quantified using qRT–PCR. Normal murine mammary gland NMuMG cells served as a negative control. (B) Quantification of secreted TNF-α protein from RAW 264.7 cells stimulated with LPS (0.1 μg/ml). TNF-α protein in the conditioned medium of RAW 264.7 cells was measured using ELISA. (C) Phase-contrast microscopic images of A549 cells treated with 5 ng/ml of TGF-β and 20 ng/ml of TNF-α. Cells were treated with the cytokines for 24 h. (D) Circularity of the cells in (C) was measured as in Fig. 1B. (E) qRT–PCR analysis of EMT marker expression in A549 cells. Cells were treated with 5 ng/ml of TGF-β and 20 ng/ml of TNF-α as indicated for 24 h. $^*P<0.05$; Error bars, SDs; n.s., not significant.

cell invasiveness was dependent in part on MMPs, as shown by the effect of the pan-MMP inhibitor GM6001 (Fig. 4C).

Blocking TNF-α partially abrogates the effects of RAW 264.7-derived factors on the TGF-β-induced EMT in A549 cells

We next evaluated to what extent the effect of conditioned medium of RAW 264.7 cells is attributable to TNF-α by adding anti-mouse TNF-α neutralizing antibody to the conditioned medium of RAW 264.7 cells. When A549 cells were incubated with conditioned medium treated with anti-TNF-α neutralizing antibody, TGF-β-induced expression of fibronectin and N-cadherin was partially suppressed, whereas their basal expression was not significantly affected (Fig. 5). On the basis of these findings, we concluded that TNF-α is secreted from RAW 264.7 cells and enhances the EMT phenotype of A549 cells induced by TGF-β.

IL-1β is produced by RAW 264.7 cells and enhances TGF-β-induced EMT of A549 cells

The observation that anti-TNF-α neutralizing antibody was capable of partially inhibiting the effects of RAW 264.7-derived conditioned medium prompted us

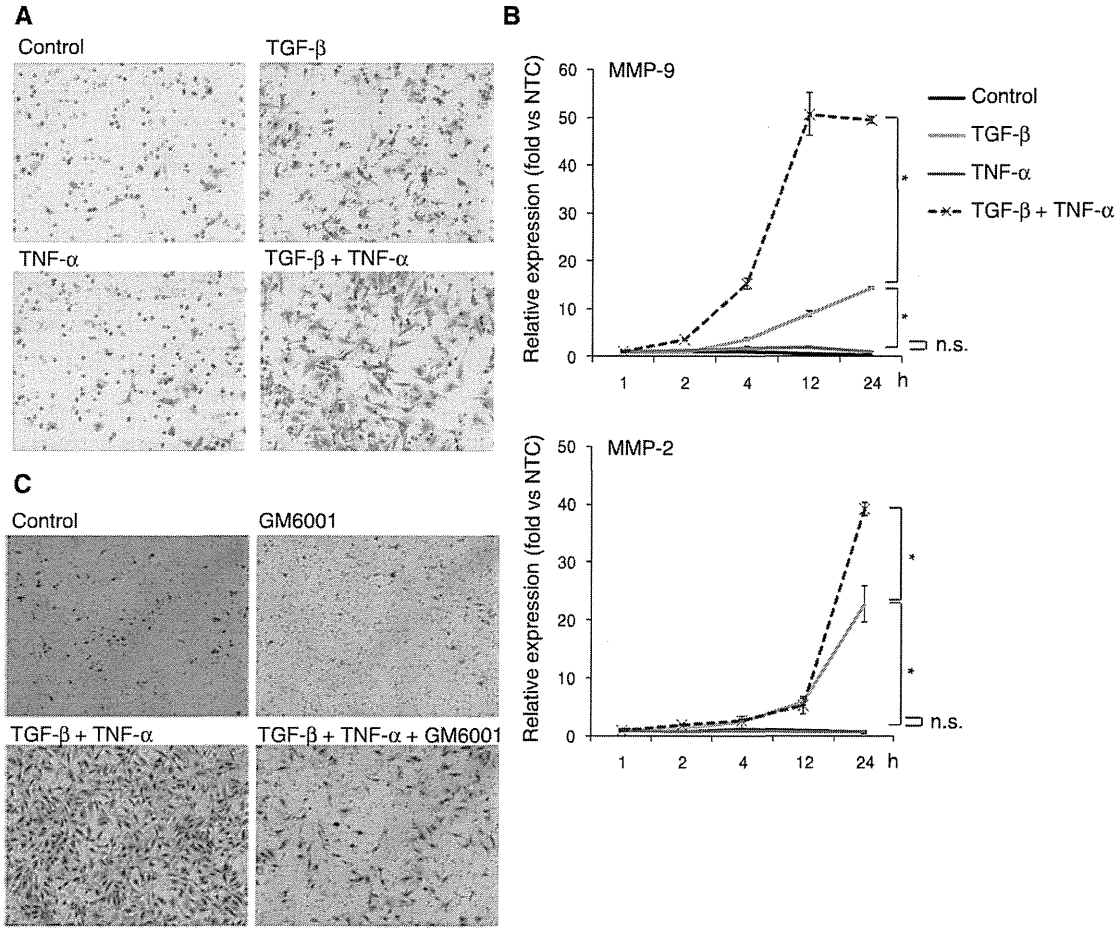

Fig. 4 Effect of TNF-α on the migration A549 cells. (A) Chamber cell invasion assay was performed using A549 cells stimulated with 5 ng/ml TGF-β and 20 ng/ml TNF-α. (B) Expression levels of MMP-2 and MMP-9 in A549 cells treated with TGF-β and TNF-α were analysed using qRT–PCR. (C) Effect of a pan-MMP inhibitor GM6001 on migration of A549 cells. Cells were seeded on Transwells as in (A), and cultured with 10 μM GM6001 in addition to TGF-β and TNF-α for 8 h. NTC, no treatment control at 1 h; $*P<0.05$; Error bars, SDs; n.s., not significant.

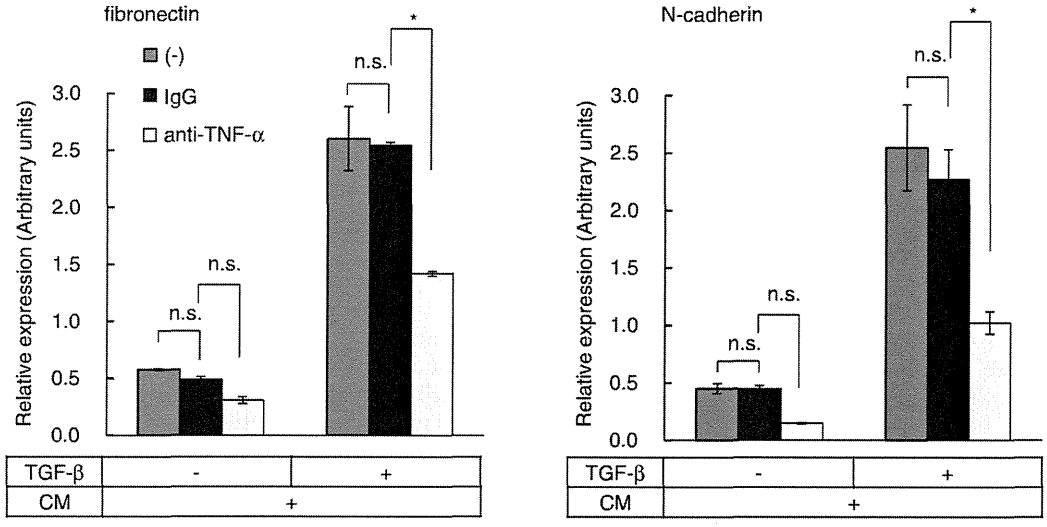

Fig. 5 Effect of TNF-α neutralizing antibody on the expression of mesenchymal markers. A549 cells were cultured in the conditioned medium of RAW 264.7 cells with anti-mouse TNF-α or control IgG. Total RNA was extracted and expression levels of fibronectin and N-cadherin were measured using qRT–PCR. $*P<0.05$; CM, conditioned medium; Error bars, SDs; n.s., not significant.

to search for other secreted factors in the conditioned medium of RAW 264.7 cells that are able to enhance EMT of A549 cells. As shown in Fig. 6, the expression of IL-1β and IL-6, inflammatory cytokines produced by activated macrophages, was also detected in RAW 264.7 cells and upregulated by LPS (Fig. 6A). Similar to TNF-α, IL-1β significantly enhanced the expression of TGF-β-induced fibronectin and N-cadherin (Fig. 6B). In contrast, we did not observe such an effect following IL-6 treatment. Additionally, no cooperative effect of TNF-α and IL-1β was observed for TGF-β-induced expression of EMT markers (Fig. 6C). These results suggest that RAW 264.7 cells secrete multiple proinflammatory cytokines, including TNF-α and IL-1β, to enhance TGF-β-induced EMT in A549 cells.

Effect of NFκB inhibitor DHMEQ on EMT of A549 cells

We further attempted to evaluate the molecular mechanisms underlying enhanced TGF-β-induced EMT by TNF-α. A mixture of inflammatory cytokines has been reported to increase the expression of TGFBR1 encoding TβRI in A549 cells, leading to enhanced Smad2 phosphorylation (*35*). We also observed the upregulation of TGFBR1 mRNA by TNF-α, IL-1β and TGF-β (Supplementary Fig. 2A). However, phosphorylation of Smad2 did not change under our experimental conditions (Supplementary Fig. 2B). Furthermore, we found that TNF-α did not enhance the transcriptional activity of the 9xCAGA-luc reporter, which consists of tandemly repeating Smad binding elements (Fig. 7A), suggesting that TNF-α failed to activate TGF-β signals in the present experimental conditions. Activation of TNF-α-induced NF-κB signals was confirmed by the NF-κB-luc reporter, which was not activated by TGF-β. We also quantified the expression levels of several EMT-related transcriptional regulators by qRT–PCR. We found that expression levels of δEF1 and SIP1 were highest when the cells were stimulated with TNF-α, IL-1β and TGF-β (Supplementary Fig. 2C). In contrast, co-stimulation with TNF-α and IL-1β did not enhance TGF-β-induced expression of other transcriptional regulators. Therefore, δEF1 and SIP1 might function as downstream components of the TGF-β-induced EMT enhanced by TNF-α and IL-1β.

Finally, we studied the involvement of signalling pathways downstream of TNF-α and IL-1β in the enhancement of TGF-β-induced EMT. We examined the effect of the NFκB inhibitor DHMEQ on the enhancement of EMT by TNF-α and IL-1β. As a positive control, induction of the intercellular adhesion molecule 1 (ICAM-1) by TNF-α and IL-1β was efficiently inhibited by DHMEQ addition (Fig. 7B). Fibronectin expression was partially inhibited by DHMEQ, whereas that of N-cadherin was not affected (Fig. 7C). We also performed qRT–PCR analysis of A549 cells transfected with RelA small interfering RNA (siRNA). We observed that the expression levels of fibronectin in A549 cells transfected with three different siRNAs for RelA were lower than those in the cells transfected with control siRNA, which was in agreement with the result using DHMEQ (Supplementary Fig. 3). We then used several kinase inhibitors to examine whether other signalling pathways downstream of TNF-α and IL-1β enhance TGF-β-induced EMT. We found that U0126, an mitogen-activated extracellular signal regulated kinase kinase (MEK) inhibitor, weakly inhibited the induction of fibronectin expression (Fig. 8). We also found that SB203580, a p38 mitogen-activated protein kinase inhibitor, inhibited the enhancement of TGF-β-induced N-cadherin expression by TNF-α and IL-1β, though it upregulated the expression of N-cadherin, as well as that of fibronectin induced by TGF-β alone. Thus, NFκB, ERK and p38 pathways appear to play different roles as downstream components for both TNF-α and IL-1β.

Discussion

Previous studies focused on the roles of TGF-β and inflammatory cytokines on EMT during lung fibrosis and used A549 cells as a cell line of alveolar epithelial origin (*22*). Kasai *et al.* reported that TGF-β, but not TNF-α or IL-1β, induces EMT of A549 cells. Subsequent studies, however, revealed that TGF-β-induced EMT is augmented by either TNF-α or IL-1β (*36*, *37*), or a mixture of inflammatory cytokines which include TNF-α and IL-1β (*35*). Enhanced TGF-β-induced EMT by TNF-α was observed not only in A549 cells, but also in normal bronchial epithelial cells, suggesting that enhanced EMT by TNF-α may be important in other pathological processes of lung diseases (*21*, *38*, *39*). Recently, Borthwick *et al.* (*21*) reported enhanced TGF-β-induced EMT by co-culture of A549 cells with THP-1 human macrophage cells. However, they did not provide direct evidence that THP-1 cell-derived TNF-α is involved in the enhancement of TGF-β-induced EMT and did not study the molecular mechanisms involved. We utilized a neutralizing antibody against TNF-α, and revealed that endogenous TNF-α derived from RAW 264.7 cells plays an important role in the enhancement of TGF-β-induced EMT of A549 cells. We observed enhancement of TGF-β-induced EMT of A549 cells by stimulation of RAW 264.7 cells with LPS. It has been reported that LPS directly affects epithelial cells via its receptor TLR4. However, LPS neither activated NFκB pathway nor enhanced TGF-β-induced EMT of A549 cells in our analysis (Supplementary Figs. 1 and 4). Absence or reduced CD14 and TLR4 possibly explains such an impairment of LPS response of A549 cells in our condition (*40*, *41*). Partial inhibition of the effects of RAW 264.7-derived conditioned medium by the TNF-α antibody also suggested the importance of other secreted factors. Multiple inflammatory cytokines, including IL-1β, IL-8 and IL-6, are produced from activated macrophages. Whether endogenous IL-1β secreted from RAW 264.7 enhances TGF-β-induced EMT, should be evaluated in future studies. It has been reported that IL-8 does not exhibit this enhancing effect (*21*), and we did not observe enhancement of EMT by IL-6. We have not ruled out the possibility that enhanced EMT resulted from crosstalk between A549 cells and RAW 264.7 cells. The effects of cancer cells on macrophages have been extensively studied. For example, cancer cells produce the chemoattractant MCP-1. Versican, an extracellular matrix

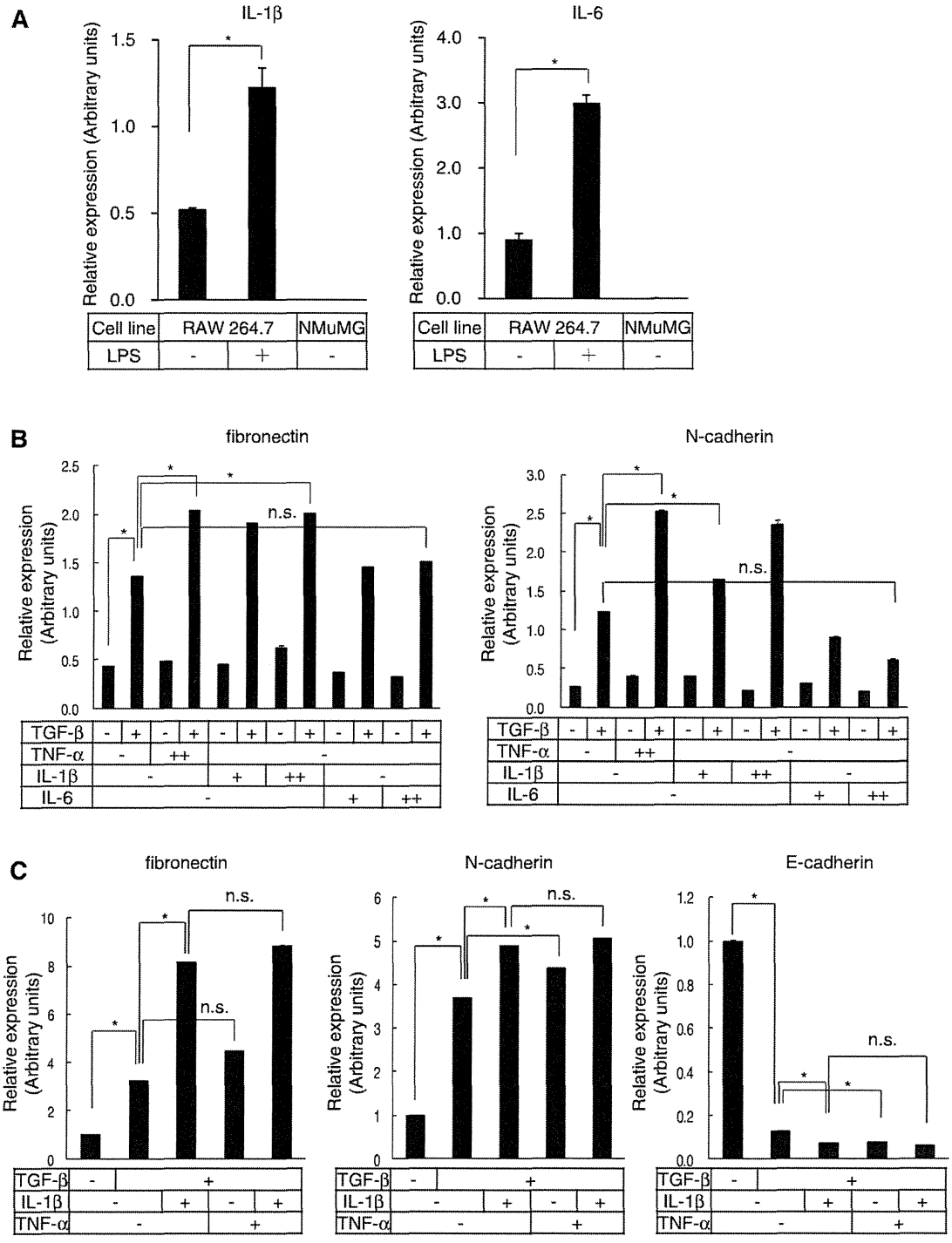

Fig. 6 Effect of IL-1β and IL-6 on TGF-β-induced expression of EMT markers. (A) qRT–PCR analysis of expression of IL-1β and IL-6 in RAW 264.7 cells treated with 0.1 μg/ml LPS was performed. NMuMG cells served as a negative control. The samples used in Fig. 3A were used for the analysis. (B) Effect of IL-1β and IL-6 on the expression of fibronectin and N-cadherin was determined using qRT–PCR in A549 cells. Cells were treated with 5 ng/ml TGF-β, 20 ng/ml TNF-α, 1 ng/ml (+) or 20 ng/ml (++) of IL-1β and IL-6 for 24 h. (C) A549 cells were treated with 1 ng/ml IL-1β, 20 ng/ml TNF-α, or both in the presence of TGF-β. $*P<0.05$; Error bars, SDs; n.s., not significant.

proteoglycan, is also secreted from cancer cells and is reported to activate macrophages and induce cancer cell invasion and metastasis (*42*). Whether A549 cells affect RAW 264.7 cells to secrete the factors that regulate EMT of A549 cells requires further investigation.

The mechanisms underlying the enhancement of TGF-β-induced EMT by TNF-α in A549 cells are poorly understood. Liu reported upregulation of TGFBR1 by inflammatory cytokines (*35*). However, upregulation of TGFBR1 mRNA was primarily induced by TGF-β in our analysis, and the effect of