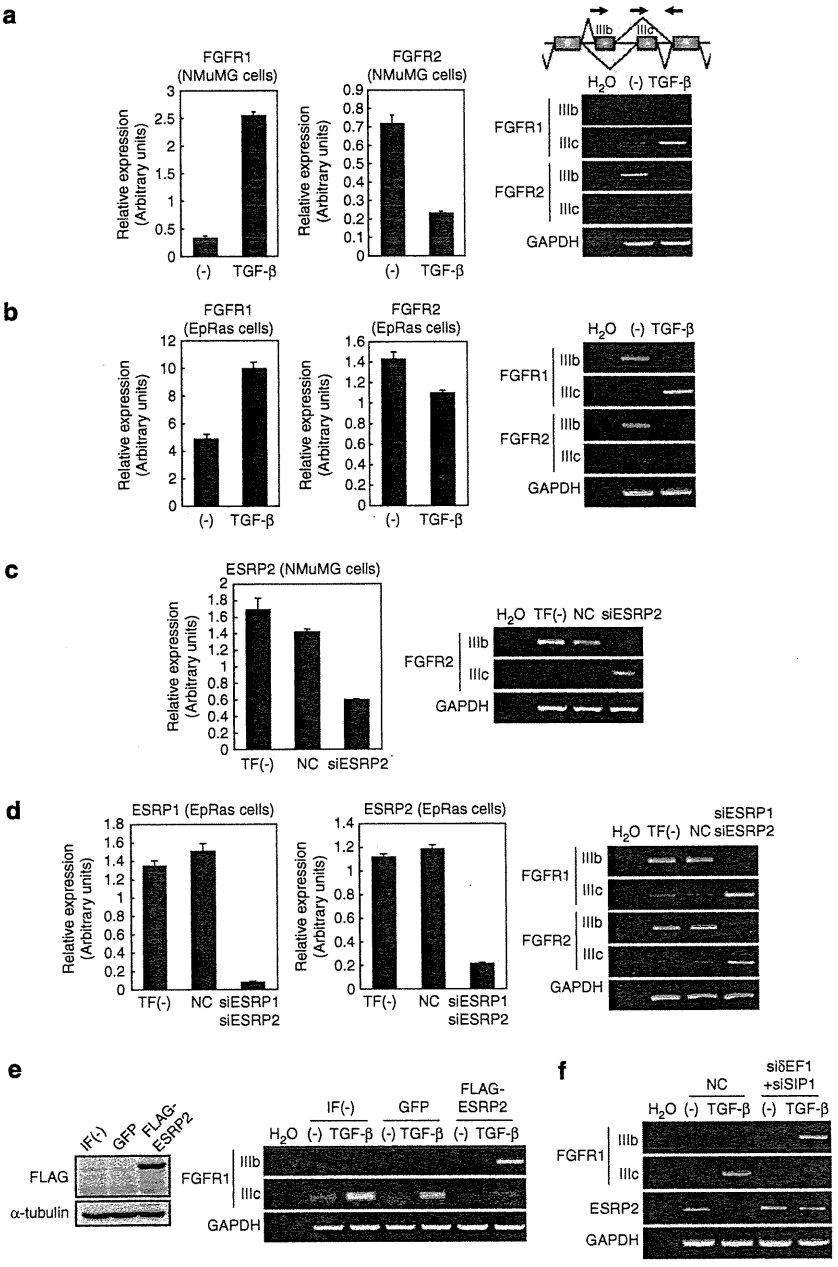


Figure 4 Isoform switching of FGFRs induced by TGF-β. (**a**) NMuMG cells were treated with 1 ng/ml TGF-β for 24 h and the expression of FGFR1 (left) and FGFR2 (center) was determined by quantitative RT–PCR. Expression of FGFR isoforms was analyzed by conventional RT–PCR using specific primers for IIIb or IIIc (right). (**b**) After EpRas cells were treated with 1 ng/ml TGF-β, the total levels of FGFR1 (left) and FGFR2 (center) and expression of FGFR isoforms (right) were examined. (**c**) NMuMG cells transfected with siRNA against mouse ESRP2 (siESRP2) were incubated for 48 h, and then analyzed by quantitative RT–PCR to determine the levels of endogenous ESRP2 (left). Expression of FGFR2 isoform was analyzed by conventional RT–PCR (right). TF(−), no transfection; NC, control siRNA. (**d**) EpRas cells transfected with siRNAs against both ESRP1 (siESRP1) and ESRP2 (siESRP2) were incubated for 48 h, and analyzed by quantitative RT–PCR to determine the levels of endogenous ESRP1 (left) and ESRP2 (center). Expression of FGFR isoforms was analyzed by conventional RT–PCR (right). TF(−), no transfection; NC, control siRNA. (**e**) NMuMG cells infected with GFP or FLAG-ESRP2 lentiviruses were treated with TGF-β for 24 h and analyzed by immunoblot analysis (left) and conventional RT–PCR to determine the levels of IIIb and IIIc isoforms of FGFR1 (right). IF(−), no infection. (**f**) After NMuMG cells were treated with 1 ng/ml TGF-β for 48 h or transfected with both δEF1 and SIP1 siRNAs, conventional RT–PCR were performed to detect expression of FGFR1 isoforms. NC, control siRNA

expressed in control or GFP-transfected cells, whereas it was replaced with the IIIb isoform in ESRP2-overexpressed cells (Figure 4e). Importantly, when δEF1 and SIP1 were silenced by their specific siRNAs in NMuMG cells, treatment with TGF-β did not result in the replacement of the IIIc isoform of FGFR1, due to

de-repression of the ESRP2 (Figure 4f). Switching of responses to FGF ligands was also confirmed by phosphorylation of Erk in NMuMG cells (Supplementary Figures S4a–f). These findings, thus, suggest that isoform switching of functional FGFRs through TGF-β-induced alternative splicing is mediated by δEF1/SIP1-repressed ESRPs.

Regulation of ESRP expression by δEF1 and SIP1 in human breast cancer cells

TGF-β-induced EMT appears to correlate with the progression of various cancers, especially breast cancer (Padua and Massague, 2009). We examined the expression of ESRPs and δEF1/SIP1 as well as that of other EMT regulators, including Snail, Twist and Slug, in 23 human breast cancer cell lines. As previously reported, the expression of ESRPs was correlated with E-cadherin expression (Supplementary Figure S5) (Warzecha *et al.*, 2009a,b). Interestingly, the expression levels of δEF1 and SIP1 mRNAs in these cell lines were inversely correlated to those of ESRPs (Figure 5a). However, the expression levels of neither Snail, Slug nor Twist were significantly correlated with those of ESRPs in human breast cancer cells used in our study (Supplementary Figure S5). Importantly, most of the cell lines with high levels of δEF1 and SIP1 expression and low levels of ESRPs expression appeared to be categorized into the 'basal-like' subtype of breast cancer (Charafe-Jauffret *et al.*, 2006; Neve *et al.*, 2006; Yamaguchi *et al.*, 2008). In contrast, most of the cell lines with low levels of δEF1 and SIP1 expression and high levels of ESRPs expression were categorized into the 'luminal' subtype of breast cancer. Among the 23 cell lines, we selected several cell lines and confirmed the expression of FGFR isoforms by RT–PCR. CRL1500 and UACC893 cells, which expressed low δEF1/SIP1 levels and high ESRP1/2 levels, exhibited constitutive expression of only IIIb isoforms of FGFR (Figure 5b). On the other hand, MDA-MB-231, MDA-MB-157, Hs578T, HCC1395 and BT549 cells, with low expression of ESRPs and high expression of δEF1/SIP1, expressed only IIIc isoforms of FGFRs (Figure 5b). Moreover, double knockdown of δEF1 and SIP1 increased the expression of ESRP1 and ESRP2 in MDA-MB231 and BT549 cells (Figure 5c), indicating that δEF1 and SIP1 downregulate ESRP expression in human breast cancer cells.

We next examined whether δEF1/SIP1 and ESRPs are reciprocally expressed in human breast tumors. Primary tumor tissues from cancer patients were subjected to immunohistochemical analyses with anti-δEF1 and anti-ESRP1 antibodies. The quality of anti-ESRP2 antibodies obtained in our study was not suitable for immunohistochemical analyses. The samples analyzed showed positive ESRP1 and cytokeratin 19 (K19) staining in cancer cells in tumor nest, whereas δEF1 was not detected in typical tumor cells, especially those in the tumor nest, but it was clearly detected in stromal cells and spindle-shaped cells at the degenerated tumor nests (Figure 5d). Therefore, these findings suggest that the expression levels of ESRP and δEF1 are reciprocally controlled in tumor tissues and/or stroma tissues, which was consistent with the expression profiles in breast cancer cell lines.

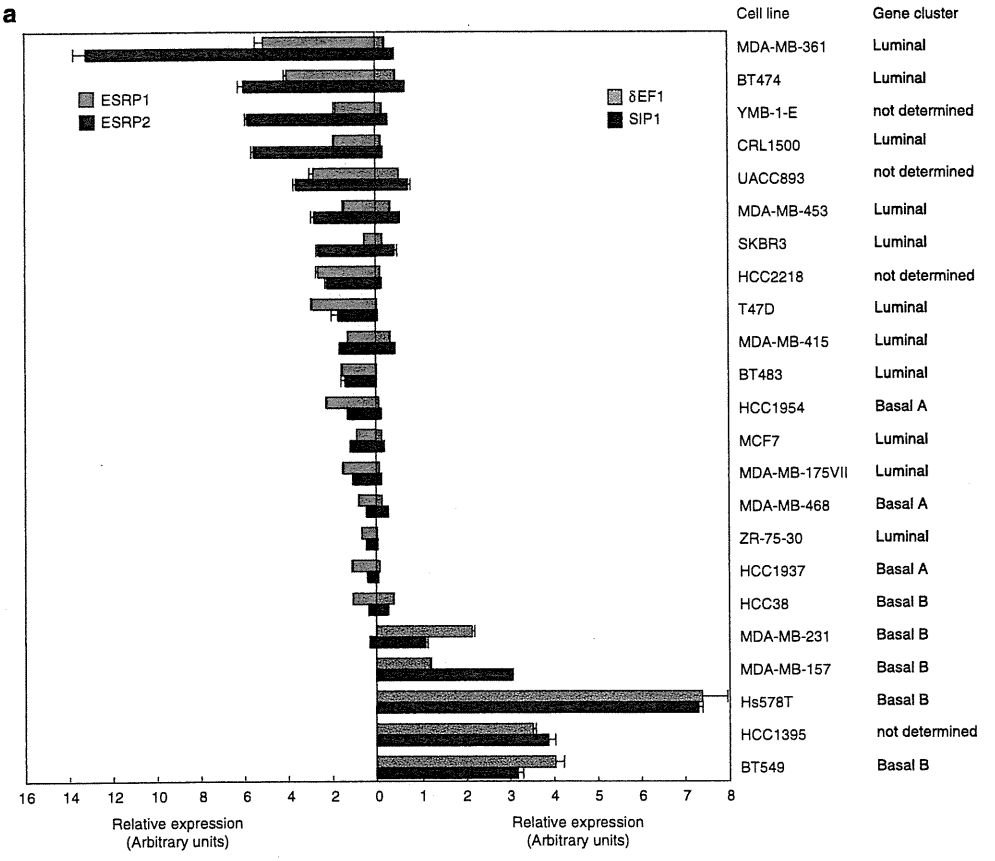
ESRPs attenuate malignant phenotypes of cancer cells as well as EMT

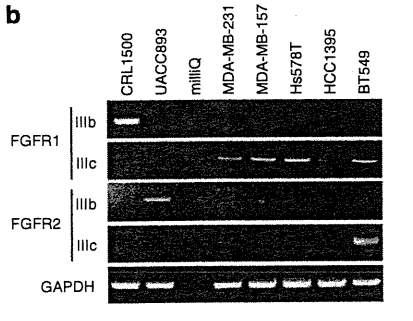
MDA-MB-231 cells are morphologically classified as poorly differentiated carcinoma cells (Neve *et al.*, 2006). We analyzed the anchorage-independent growth of MDA-MB-231 cells by cultivating the cells in soft agar. As shown in Figure 6a, these cells showed anchorage-independent growth, whereas the cells overexpressing ESRPs failed to efficiently proliferate in soft agar. Overexpression of ESRPs also switched the isoform expression of FGFR1 from IIIc to IIIb in MDA-MB-231 cells (Supplementary Figure S6a). The expression of E-cadherin was upregulated at the mRNA and protein levels in cells overexpressing ESRPs (Figures 6b, d and e), whereas reorganization of actin stress fiber and expressions of EMT regulators and mesenchymal marker proteins, including fibronectin and N-cadherin, were not significantly affected by ESRP overexpression (Figure 6e, Supplementary Figures S6b and c). In addition, morphology of the cells overexpressing ESRPs was altered to a cobblestone-like shape (Figure 6c), suggesting that ESRPs partially restored the well-differentiated phenotype in cells with a poorly differentiated phenotype. Moreover, these effects of ESRPs were also confirmed in NMuMG cells, in which the overexpression of ESRP2 restored TGF-β-mediated alteration of morphology and downregulation of E-cadherin (Figures 6f, g and h). Similar to MDA-MB-231 cells, ESRP overexpression failed to affect the expression of mesenchymal marker proteins and reorganization of actin stress fiber (Supplementary Figures S6d and e). These findings thus suggest that ESRPs attenuate the EMT phenotype mainly through upregulation of E-cadherin.

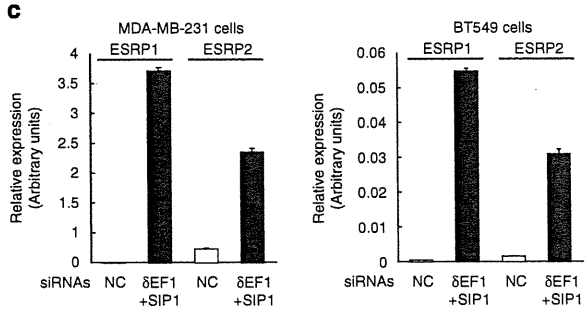
Discussion

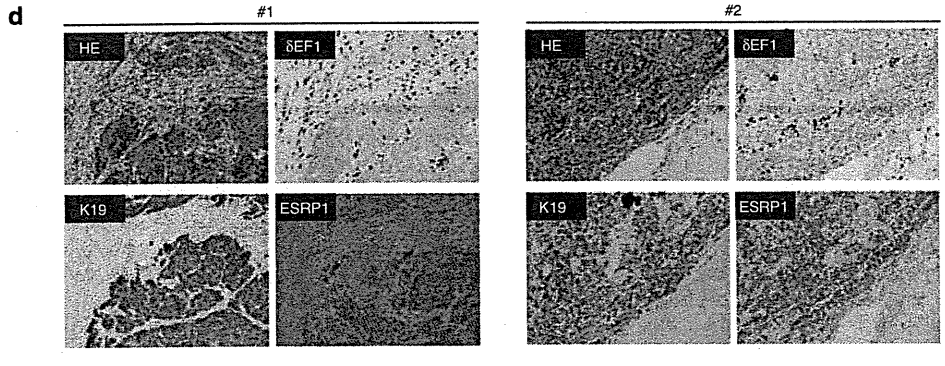
Roles of ESRPs in alteration in splicing during TGF-β-induced EMT

By comparing our data with the published database of the exon-array data of ESRP1/2-silenced PNT2 cells using high ARH scores ($P<0.01$), a subset of genes in NMuMG cells overlapped with those in PNT2 cells (Supplementary Table 2). Recently, profiling of ESRP-regulated splicing using a further sensitive analysis was reported (Warzecha *et al.*, 2010). In the report, Affymetrix human exon junction arrays were performed to profile splicing changes in response to ectopic expression of ESRP1 in MDA-MB-231 cells and knockdown of ESRP1 and ESRP2 in PNT2 cells. They identified 310 genes in MDA-MB-231 cells and 385 genes in PNT2 cells as ESRP-dependent targets of alternative splicing. When they were compared with our gene list of NMuMG cells ($P<0.01$), 55 genes in MDA-MB-231 cells and 92 genes in PNT2 cells matched our


a
ESRP1
ESRP2
δEF1
SIP1
Cell line
Gene cluster
MDA-MB-361 Luminal
BT474 Luminal
YMB-1-E not determined
CRL1500 Luminal
UACC893 not determined
MDA-MB-453 Luminal
SKBR3 Luminal
HCC2218 not determined
T47D Luminal
MDA-MB-415 Luminal
BT483 Luminal
HCC1954 Basal A
MCF7 Luminal
MDA-MB-175VII Luminal
MDA-MB-468 Basal A
ZR-75-30 Luminal
HCC1937 Basal A
HCC38 Basal B
MDA-MB-231 Basal B
MDA-MB-157 Basal B
Hs578T Basal B
HCC1395 not determined
BT549 Basal B
16 14 12 10 8 6 4 2 0
0 1 2 3 4 5 6 7 8
Relative expression (Arbitrary units)
Relative expression (Arbitrary units)


b
CRL1500
UACC893
milliQ
MDA-MB-231
MDA-MB-157
Hs578T
HCC1395
BT549
FGFR1
IIIb
IIIc
FGFR2
IIIb
IIIc
GAPDH


c
MDA-MB-231 cells
ESRP1
ESRP2
Relative expression (Arbitrary units)
siRNAs NC δEF1 +SIP1 NC δEF1 +SIP1
BT549 cells
ESRP1
ESRP2
Relative expression (Arbitrary units)
siRNAs NC δEF1 +SIP1 NC δEF1 +SIP1


d
#1
#2
HE
δEF1
K19
ESRP1
HE
δEF1
K19
ESRP1

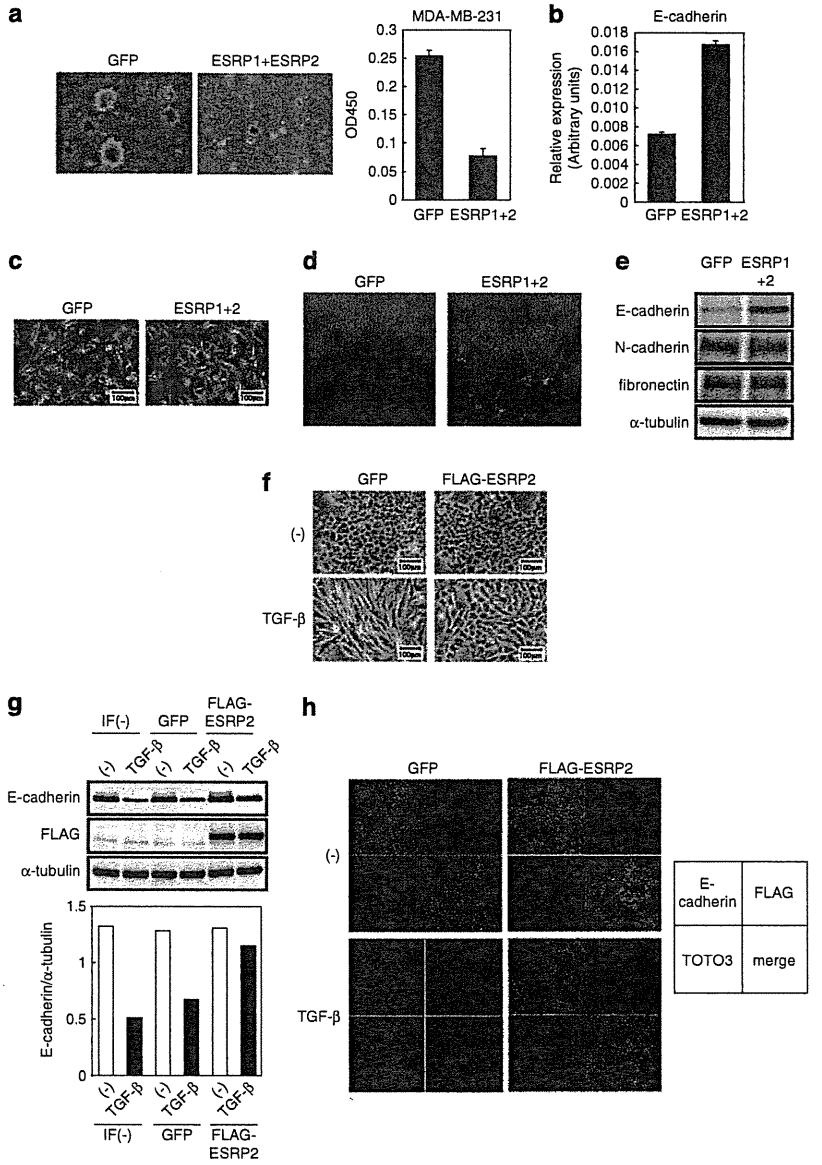


Figure 6 ESRPs attenuate malignant phenotypes of cancer cells. (**a–e**) MDA-MB-231 cells were infected with lentiviruses encoding ESRP1 and ESRP2. The cells were examined for anchorage-independent growth in soft agar (left in **a**) and quantified (right in **a**). Expression of E-cadherin in the cells was evaluated by quantitative RT–PCR (**b**), immunohistochemical (**d**) and immunoblot (**e**) analyses. (**c**) Morphology of the cells were analyzed by phase-contrast microscopy (**d**). α-tubulin levels were monitored as a loading control (**e**). (**f–h**) NMuMG cells infected with lentivirus encoding GFP or FLAG-ESRP2 were treated with TGF-β for 36 h. The cells were analyzed by phase-contrast microscopy (**f**), immunoblot analyses with the indicated antibodies (**g**), and immunohistochemical analyses with anti-E-cadherin (green in **h**) and anti-FLAG (red in **h**) antibodies, and by TOTO3 to detect nuclei (blue in **h**). α-tubulin levels were monitored as a loading control. Ratio of E-cadherin to α-tubulin is shown at bottom (**g**). IF(−), no infection.

Figure 5 Expression profiles of ESRP1/2 and δEF1/SIP1 in breast cancer cells. (**a**) mRNA levels of the expression of ESRP1, ESRP2, δEF1 and SIP1 were determined by quantitative RT–PCR and compared among 23 human breast cancer cell lines. Gene cluster shown is reported by Neve *et al.* (2006) and Charafe-Jauffret *et al.* (2006). Basal A subtype reveals basal-like signature with basal cytokeratin (K5/K14) positive, and basal B subtype exhibits a stem-cell like expression profile with vimentin positive and may reflect the clinical triple-negative tumor type (Neve *et al.*, 2006). (**b**) The expression of FGFRs isoforms in human breast cancer cell lines was determined by conventional RT–PCR. (**c**) MDA-MB-231 and BT549 cells were transfected with siRNAs against δEF1 and SIP1, and mRNA levels of ESRP1 and ESRP2 were examined by quantitative RT–PCR. NC, control siRNA. (**d**) Representative images of hematoxylin and eosin (HE) staining and immunohistochemical staining of cytokeratin 19 (K19), ESRP1, and δEF1 in primary tumor samples from breast cancer patients are shown (# 1 and 2).

gene list of NMuMG cells. Although it is difficult to further evaluate these data, due to the differences in species and tissues of the cells lines, these findings suggest that ESRPs play crucial roles in alteration in splicing variants during TGF-β-induced EMT.

Regulation of FGFRs by TGF-β at the levels of transcription and alternative splicing

Splicing of the second half of the third Ig-like domain of the FGFRs has been well documented (Eswarakumar *et al.*, 2005). ESRPs were identified through the screening of the proteins that regulate the splicing of FGFRs (Warzecha *et al.*, 2009a, b). We have recently reported that TGF-β induces isoform switching of FGFRs from the IIIb to IIIc type by alternative splicing during EMT in NMuMG cells, which results in enhanced EMT through the cooperative action of TGF-β and FGF-2 (Shirakihara *et al.*, 2011). NMuMG cells predominantly expressed FGFR2IIIb in the resting state. TGF-β repressed the expression of FGFR2IIIb isoform and induced the expression of the FGFR1IIIc isoform, but not that of the FGFR2IIIc (Figure 4a). Importantly, overexpression of ESRP2 in TGF-β-treated NMuMG cells led to an increase in FGFR1IIIb isoform (Figure 4e), and δEF1 and SIP1 siRNAs did not affect the upregulation of FGFR1 (Figure 4f). These findings suggest that δEF1 and SIP1 are dispensable in the TGF-β-mediated transcriptional regulation of FGFR1. Therefore, isoform switching of FGFRs during TGF-β-induced EMT requires ESRPs and other unidentified transcriptional factor(s) that are not regulated by δEF1/SIP1.

Splicing profiles of CD44 and Mena were also changed by ESRP2 siRNA (Supplementary Figures S7a and b). As described above, treatment by TGF-β alone induced partial EMT with about 50% reduction of ESRP2 mRNA (Figure 2a). Thus, addition of FGF-2 in TGF-β-treated cells further repressed the levels of ESRP2 mRNA and in turn changed the profile of alternative splicing of Mena (Supplementary Figure S7c). When ESRP2 was knocked down in TGF-β-treated cells, Mena was almost completely altered to its splicing variant (Mena 11a-) (Supplementary Figure S7d). These findings, therefore, suggest that TGF-β stimulation elicits partial EMT with repression of ESRP2 to about 50%, and that further reduction of ESRP2 expression induces enhanced EMT with aggressive phenotypes of mesenchyme.

Attenuation of EMT phenotype by ESRPs

Human breast cancer MDA-MB-231 cells are classified as poorly differentiated carcinoma cells, and express low levels of ESRP1/2 and high levels of δEF1/SIP1. Overexpression of ESRPs upregulated E-cadherin expression without affecting the levels of δEF1 and SIP1 (Figures 6b–e, and Supplementary Figure S6c). Among other EMT regulators, expression of Snail and Slug was not affected by ESRPs, whereas that of E47 and Twist was not detected in the cells (Supplementary Figure S6c and data not shown), suggesting that restoration of E-cadherin by ESRPs is not induced by de-repression of the EMT regulators. In addition, ESRP2 overexpression failed to downregulate mesenchymal-marker proteins and restore reorganization of actin stress fiber in MDA-MB-231 and TGF-β-treated NMuMG cells (Figures 6e, Supplementary Figures S6b, d and e). In the present study, some of polarity and adhesion proteins, including p120 catenin and scribbled, are regulated at splicing levels by ESRPs. Thus, alternative splicing variants of these proteins may regulate unidentified E-cadherin inducers or epithelial regulators, and alter the cells from mesenchymal to epithelial phenotype through increase in E-cadherin expression.

Regulation of ESRP expression in other types of cancer

δEF1 and SIP1 are necessary for TGF-β-induced EMT in NMuMG cells and in some breast cancer cells (Shirakihara *et al.*, 2007; Gregory *et al.*, 2008). Intriguingly, they were not upregulated by TGF-β and dispensable for TGF-β-induced EMT in pancreatic cancer Panc-1 cells, in which Snail was involved in TGF-β-induced EMT (Horiguchi *et al.*, 2009). Moreover, Twist induced EMT in human mammary epithelial HMLE cells (Yang *et al.*, 2004). Thus, expression of each EMT regulator appears to be variously regulated in the cells that have undergone EMT, depending on cell or tissue specificity. EMT regulators are not good markers to detect cells that have undergone EMT, because in certain cells it is difficult to determine which regulators specifically and preferentially contribute to EMT. However, ESRPs were repressed by Snail and Twist in certain cells that had undergone EMT, including Panc-1 cells (Supplementary Figure S8) and HMLE cells (Warzecha *et al.*, 2009a, b), respectively, and the expression of ESRPs was inversely correlated with progression of breast cancer (Figure 5a). Therefore, these findings suggest that ESRPs, rather than EMT regulators, may be useful negative markers for detecting cells that have undergone EMT or cancer cells with more aggressive phenotypes.

Expression of δEF1/SIP1 and ESRPs in the 'basal-like' and 'luminal' types of breast cancer cells

Our findings on a panel of 23 human breast cancer cell lines revealed an important phenomenon that the expression levels of ESRPs are reciprocally controlled by the expression levels of δEF1 family proteins. Importantly, most of the cell lines with high levels of δEF1 and SIP1 expression and low levels of ESRP expression were categorized into the 'basal-like' subtype of breast cancer (Charafe-Jauffret *et al.*, 2006; Neve *et al.*, 2006; Yamaguchi *et al.*, 2008). Thus, elevated expression of δEF1 and SIP1 appears to correlate with aggressive phenotypes and poor prognosis of cancer patients, which are most likely due to the reinforced invasive and metastatic properties of tumor cells via EMT. In contrast, most of the cell lines with low levels of δEF1 and SIP1 expression and high levels of ESRPs expression were categorized into the 'luminal' subtype of breast cancer. Thus, δEF1 and SIP1 are specifically expressed in 'basal-like' subtype and ESRPs are specifically expressed in 'luminal' subtype of breast cancer cells. Although some of the luminal-type breast cancer cells expressed high levels of Snail or Twist

mRNAs, it is still unknown why these EMT regulators failed to affect E-cadherin expression. In addition to mRNA profiling, determination of the protein levels of the EMT regulators will be required in the future.

Materials and methods

Cell culture, reagents and antibodies
All cells used in the present study were cultured as described previously (Shirakihara *et al.*, 2011). Recombinant human TGF-β1 was obtained from R&D Systems (Minneapolis, MN, USA). SB431542 was form Sigma-Aldrich (St Louis, MO, USA). Mouse monoclonal anti-FLAG M2, anti-α-tubulin and anti-ESRP1 antibodies were purchased from Sigma-Aldrich. Rabbit monoclonal anti-keratin 19 and polyclonal δEF1 antibodies were purchased from Epitomics (Burlingame, CA, USA) and Novus Biologicals (Littleton, CO, USA), respectively. Mouse anti-E-cadherin antibody was from BD Transduction Laboratories (Lexington, KY, USA).

RNA extraction, microarray and RT–PCR analyses
Total RNA was purified using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and used to perform microarray, conventional RT–PCR and quantitative RT–PCR analyses. Values were normalized to mouse TATA binding protein (TBP) or human hypoxanthine phosphoribosyltransferase 1. The primer sequences are shown in Supplementary Table 3. Oligonucleotide microarray analysis was performed using GeneChip Mouse Exon 1.0 ST Array (Affymetrix) according to the manufacturer's instructions. The ARH method was used to identify exons differentially expressed between non-treated and TGF-β-treated NMuMG cells (Rasche and Herwig, 2010). Exon-array data are available at Gene Expression Omnibus (GSE28184).

DNA construction and generation of lentiviruses
Mouse ESRP2 promoter, containing −1000 to +200 base pairs from transcription start site, was cloned by PCR using genomic DNA of NMuMG cells. The purified PCR fragment was cloned into pGL4 vector (Promega, Madison, WI, USA). Human ESRP1 and mouse ESRP2 were cloned by PCR using cDNA of A431 and NMuMG cells. All constructs were confirmed by sequencing. The mouse δEF1 and SIP1 cDNAs, and the adenoviral vector encoding δEF1 or SIP1 epitope-tagged with FLAG at their N-termini were described previously (Shirakihara *et al.*, 2007). We used a lentiviral expression system to establish stable expression of ESRP2 in NMuMG cells (NMuMG-ESRP2) and that of ESRP1/2 in MDA-MB-231 cells (Horiguchi *et al.*, 2009).

RNA interference
Transfection of siRNA was performed according to the protocol recommended for HiPerfect (Qiagen) or RNAiMAX (Invitrogen, Carlsbad, CA, USA). NMuMG cells were transiently transfected with siRNAs against mouse δEF1 (Stealth RNAi MSS210696; Invitrogen), mouse SIP1 (Stealth RNAi MSS216412; Invitrogen), mouse ESRP1 (Stealth RNAi MSS209488; Invitrogen), or mouse ESRP2 (Stealth RNAi MSS246490; Invitrogen). Human breast cancer cells were transiently transfected with siRNAs against human δEF1 (Stealth RNAi HSS110549; Invitrogen) and human SIP1 (Stealth RNAi HSS114854; Invitrogen). The final concentration of the siRNAs used was 20 nM. At 12 h after transfection, 1 ng/ml TGF-β was added and cultured for an additional 48 h.

Immunoblotting, luciferase assays, ChIP and immunohistochemistry analyses of tumor sample
The procedures used for immunoblotting, immunofluorescence, luciferase assays and ChIP were as previously described (Horiguchi *et al.*, 2009; Koinuma *et al.*, 2009). Formalin-fixed, paraffin-embedded primary breast tumor tissues were obtained as a part of routine clinical management of patients with breast cancer at the Hospital of University of Yamanashi. Hematoxylin and eosin-stained sections were examined for regions that contained tumor cells and stroma, which were then analyzed as serial sections for anti-δEF1, ESRP1 and K19 antibodies. All studies were conducted using the protocol approved by the Ethics Committee of the University of Yamanashi.

Colony-formation assay in soft agar
Agar (Nacalai Tesque, Kyoto, Japan) was dissolved with culture medium to a final concentration of 0.5% in six-well plates. Cells were seeded at a density of 3×10^4 cells per well in 0.3% agar. The cells were covered with culture media for 3 weeks. Cell viability was measured using Cell Count Reagent SF (Nacalai Tesque). The reagent was added in the media and incubated for 60 min. The aliquot was taken and colorimetrically measured at 450–650 nm wavelengths.

Conflict of interest

The authors declare no conflict of interest.

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References

Blencowe BJ. (2006). Alternative splicing: new insights from global analyses. *Cell* **126**: 37–47.

Chaffer CL, Dopheide B, Savagner P, Thompson EW, Williams ED. (2007). Aberrant fibroblast growth factor receptor signaling in bladder and other cancers. *Differentiation* **75**: 831–842.

Charafe-Jauffret E, Ginestier C, Monville F, Finetti P, Adelaide J, Cervera N *et al.* (2006). Gene expression profiling of breast cell lines identifies potential new basal markers. *Oncogene* **25**: 2273–2284.

Coumoul X, Deng CX. (2003). Roles of FGF receptors in mammalian development and congenital diseases. *Birth Defects Res C Embryo Today* **69**: 286–304.

Dutertre M, Lacroix-Triki M, Driouch K, de la Grange P, Gratadou L, Beck S *et al.* (2010). Exon-based clustering of murine breast

tumor transcriptomes reveals alternative exons whose expression is associated with metastasis. *Cancer Res* **70**: 896–905.

Ehata S, Hanyu A, Hayashi M, Aburatani H, Kato Y, Fujime M *et al.* (2007). Transforming growth factor-β promotes survival of mammary carcinoma cells through induction of antiapoptotic transcription factor DEC1. *Cancer Res* **67**: 9694–9703.

Eswarakumar VP, Lax I, Schlessinger J. (2005). Cellular signaling by fibroblast growth factor receptors. *Cytokine Growth Factor Rev* **16**: 139–149.

Gregory PA, Bert AG, Paterson EL, Barry SC, Tsykin A, Farshid G *et al.* (2008). The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. *Nat Cell Biol* **10**: 593–601.

Horiguchi K, Shirakihara T, Nakano A, Imamura T, Miyazono K, Saitoh M. (2009). Role of Ras signaling in the induction of Snail by transforming growth factor-β. *J Biol Chem* **284**: 245–253.

Hoshino Y, Katsuno Y, Ehata S, Miyazono K. (2011). Autocrine TGF-β protects breast cancer cells from apoptosis through reduction of BH3-only protein, Bim. *J Biochem* **149**: 55–65.

Kalluri R, Weinberg RA. (2009). The basics of epithelial-mesenchymal transition. *J Clin Invest* **119**: 1420–1428.

Koinuma D, Tsutsumi S, Kamimura N, Taniguchi H, Miyazawa K, Sunamura M *et al.* (2009). Chromatin immunoprecipitation on microarray analysis of Smad2/3 binding sites reveals roles of ETS1 and TFAP2A in transforming growth factor β signaling. *Mol Cell Biol* **29**: 172–186.

Lee B, Brown K, Hathout Y, Seo J. (2008). GOTreePlus: an interactive gene ontology browser. *Bioinformatics* **24**: 1026–1028.

Licatalosi DD, Darnell RB. (2010). RNA processing and its regulation: global insights into biological networks. *Nat Rev Genet* **11**: 75–87.

Matlin AJ, Clark F, Smith CW. (2005). Understanding alternative splicing: towards a cellular code. *Nat Rev Mol Cell Biol* **6**: 386–398.

Miyazono K. (2009). Transforming growth factor-β signaling in epithelial-mesenchymal transition and progression of cancer. *Proc Jpn Acad Ser B Phys Biol Sci* **85**: 314–323.

Moustakas A, Heldin CH. (2007). Signaling networks guiding epithelial-mesenchymal transitions during embryogenesis and cancer progression. *Cancer Sci* **98**: 1512–1520.

Neve RM, Chin K, Fridlyand J, Yeh J, Baehner FL, Fevr T *et al.* (2006). A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. *Cancer Cell* **10**: 515–527.

Padua D, Massague J. (2009). Roles of TGFβ in metastasis. *Cell Res* **19**: 89–102.

Pan Q, Shai O, Lee LJ, Frey BJ, Blencowe BJ. (2008). Deep surveying of alternative splicing complexity in the human transcriptome by high-throughput sequencing. *Nat Genet* **40**: 1413–1415.

Philippar U, Roussos ET, Oser M, Yamaguchi H, Kim HD, Giampieri S *et al.* (2008). A Mena invasion isoform potentiates EGF-induced carcinoma cell invasion and metastasis. *Dev Cell* **15**: 813–828.

Rasche A, Herwig R. (2010). ARH: predicting splice variants from genome-wide data with modified entropy. *Bioinformatics* **26**: 84–90.

Shirakihara T, Horiguchi T, Miyazawa M, Ehata S, Shibata T, Morita I *et al.* (2011). TGF-β regulates isoform switching of FGF receptors and epithelial-mesenchymal transition. *EMBO J* **30**: 783–795.

Shirakihara T, Saitoh M, Miyazono K. (2007). Differential regulation of epithelial and mesenchymal markers by δEF1 proteins in epithelial mesenchymal transition induced by TGF-β. *Mol Biol Cell* **18**: 3533–3544.

Thiery JP, Acloque H, Huang RY, Nieto MA. (2009). Epithelial-mesenchymal transitions in development and disease. *Cell* **139**: 871–890.

Thiery JP, Sleeman JP. (2006). Complex networks orchestrate epithelial-mesenchymal transitions. *Nat Rev Mol Cell Biol* **7**: 131–142.

Wang ET, Sandberg R, Luo S, Khrebtukova I, Zhang L, Mayr C *et al.* (2008). Alternative isoform regulation in human tissue transcriptomes. *Nature* **456**: 470–476.

Wang GS, Cooper TA. (2007). Splicing in disease: disruption of the splicing code and the decoding machinery. *Nat Rev Genet* **8**: 749–761.

Warzecha CC, Jiang P, Amirikian K, Dittmar KA, Lu H, Shen S *et al.* (2010). An ESRP-regulated splicing programme is abrogated during the epithelial-mesenchymal transition. *EMBO J* **29**: 3286–3300.

Warzecha CC, Sato TK, Nabet B, Hogenesch JB, Carstens RP. (2009a). ESRP1 and ESRP2 are epithelial cell-type-specific regulators of FGFR2 splicing. *Mol Cell* **33**: 591–601.

Warzecha CC, Shen S, Xing Y, Carstens RP. (2009b). The epithelial splicing factors ESRP1 and ESRP2 positively and negatively regulate diverse types of alternative splicing events. *RNA Biol* **6**: 546–562.

Yamaguchi N, Ito E, Azuma S, Honma R, Yanagisawa Y, Nishikawa A *et al.* (2008). FoxA1 as a lineage-specific oncogene in luminal type breast cancer. *Biochem Biophys Res Commun* **365**: 711–717.

Yang J, Mani SA, Donaher JL, Ramaswamy S, Itzykson RA, Come C *et al.* (2004). Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. *Cell* **117**: 927–939.

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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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Mikiko Kawata[1], Daizo Koinuma[1], Tomohiro Ogami[1], Kazuo Umezawa[2], Caname Iwata[1], Tetsuro Watabe[1] and Kohei Miyazono[1,*]

[1]Department of Molecular Pathology, Graduate School of Medicine, University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113-0033; and [2]Faculty of Science and Technology, Keio University, Yokohama 223-0061, Japan

*Kohei Miyazono, Department of Molecular Pathology, Graduate School of Medicine, University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113-0033, Japan. Tel: +81-3-5841-3356, Fax: +81-3-5841-3354, email: miyazono@m.u-tokyo.ac.jp

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

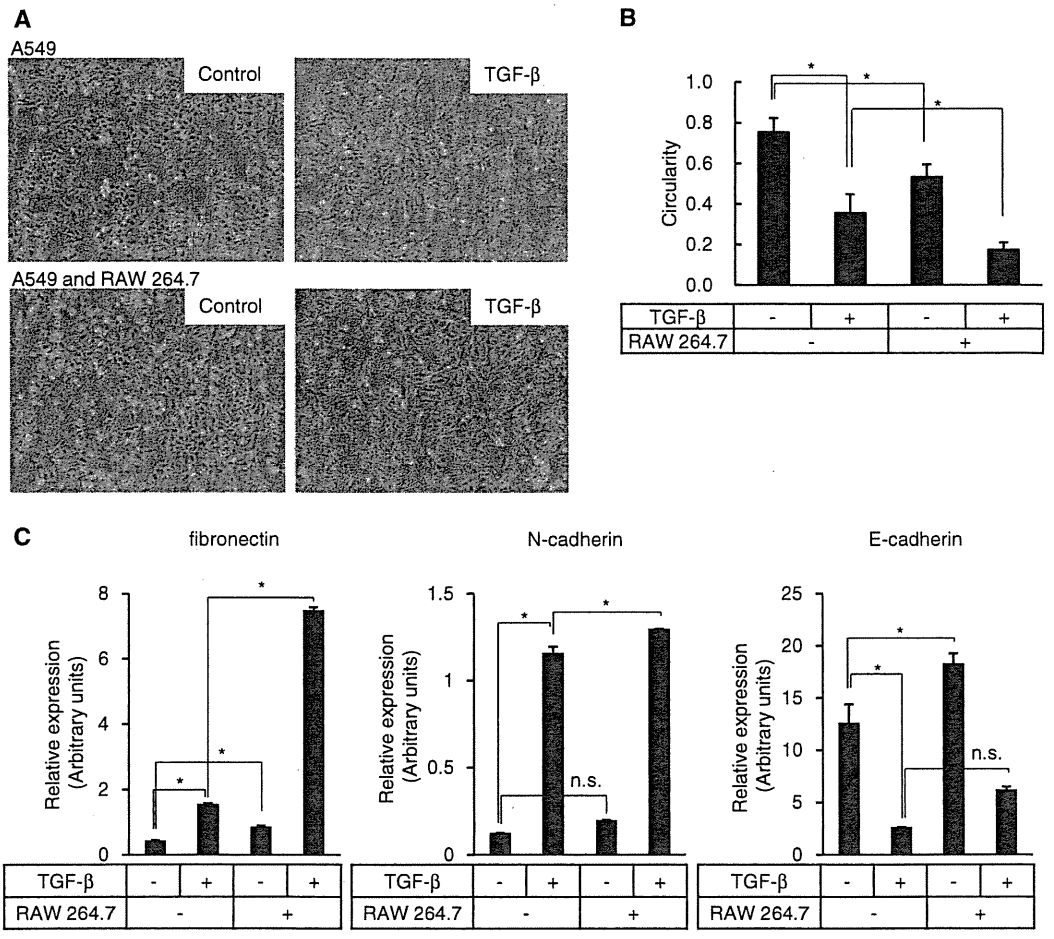

Fig. 1 Effect of co-culture with RAW 264.7 cells on TGF-β-induced EMT of A549 cells. (A) Human lung adenocarcinoma A549 cells were pre-cultured with or without 5 ng/ml TGF-β for 24 h and plated at a density of 5.0×10^4 cells/well in a 6-well plate. The same number of mouse macrophage RAW 264.7 cells 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.

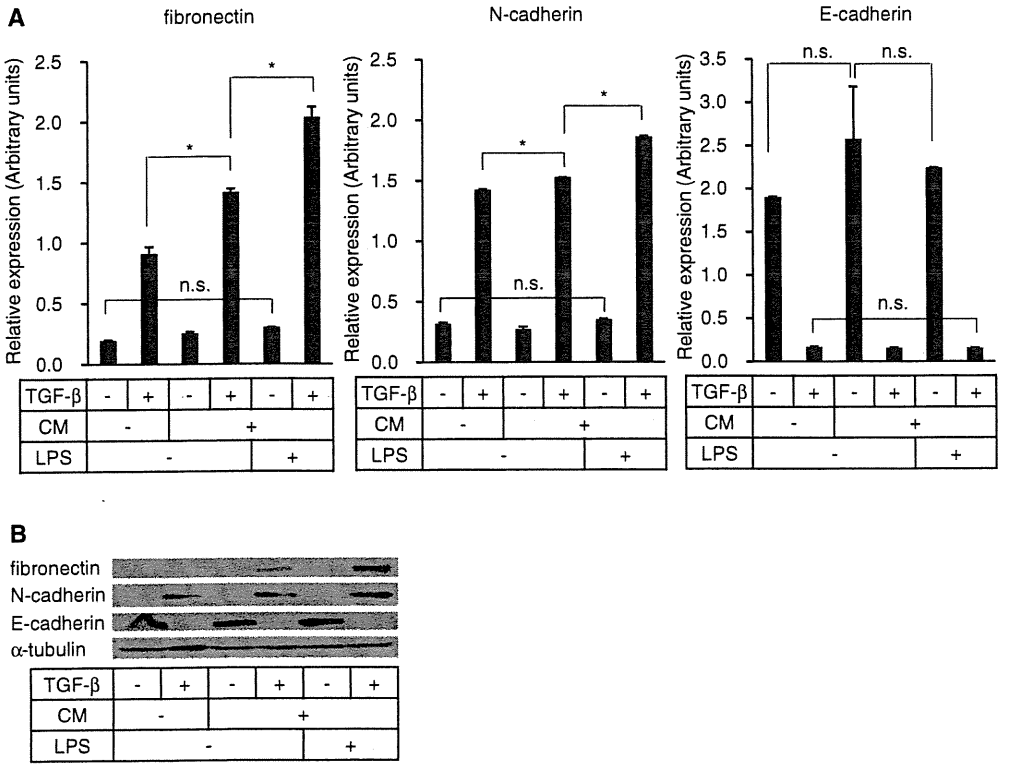

Fig. 2 Effect of conditioned medium of RAW 264.7 cells on TGF-β-induced EMT of A549 cells. (A) A549 cells were cultured with conditioned medium of LPS-activated (0.1 μg/ml) RAW 264.7 cells and stimulated with 5 ng/ml TGF-β. Expression of EMT markers was quantified using qRT–PCR. (B) Expression of fibronectin, 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

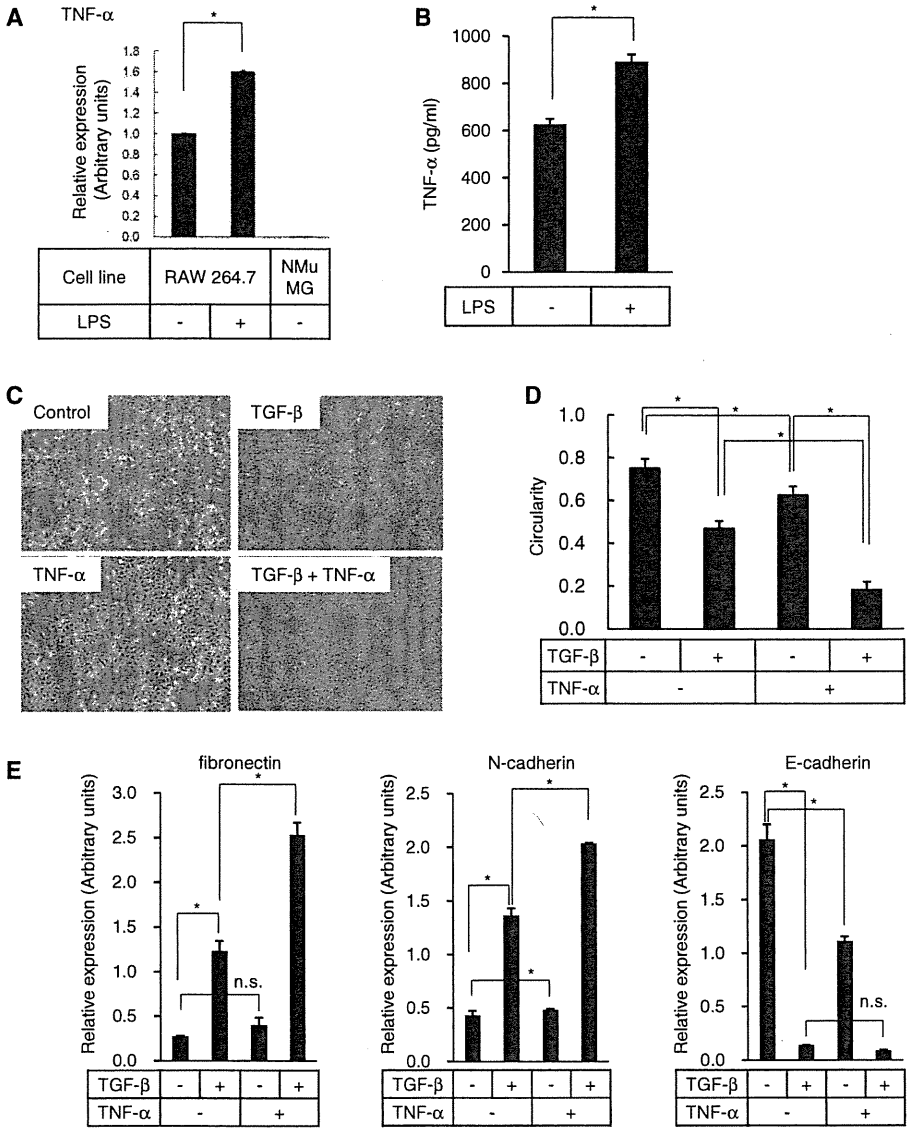

Fig. 3 Enhancement of TGF-β-induced EMT phenotype by TNF-α. (A) Expression of TNF-α mRNA in RAW 264.7 cells. Cells were treated with 0.1 μg/ml LPS, and TNF-α expression was quantified using qRT–PCR. Normal murine mammary gland NMuMG cells served as a negative control. (B) Quantification of secreted TNF-α 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

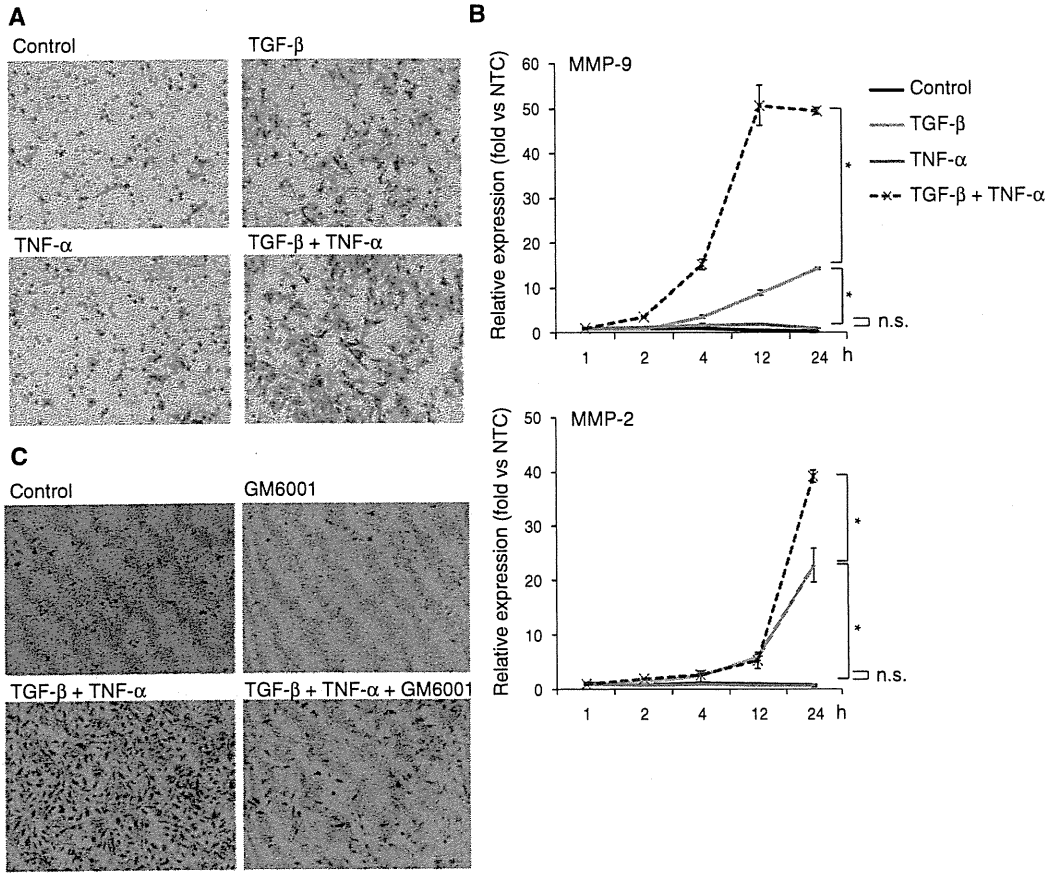

Fig. 4 Effect of TNF-α on the migration A549 cells. (A) Chamber cell invasion assay was performed using A549 cells stimulated with 5 ng/ml TGF-β and 20 ng/ml TNF-α. (B) Expression levels of MMP-2 and MMP-9 in A549 cells treated with TGF-β and TNF-α were analysed using qRT–PCR. (C) Effect of a pan-MMP 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.

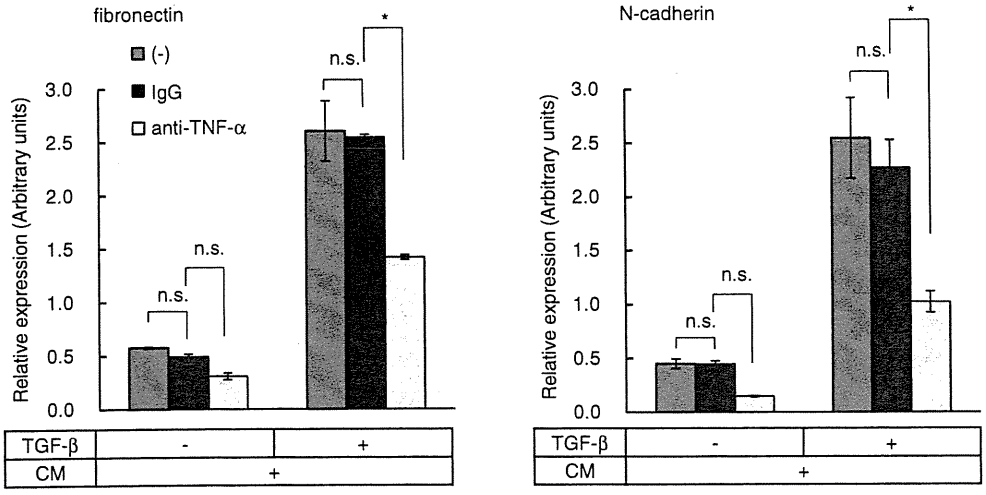

Fig. 5 Effect of TNF-α neutralizing antibody on the expression of mesenchymal markers. A549 cells were cultured in the conditioned medium of RAW 264.7 cells with anti-mouse TNF-α or control IgG. Total RNA was extracted and expression levels of fibronectin and N-cadherin were measured using qRT–PCR. $*P<0.05$; 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

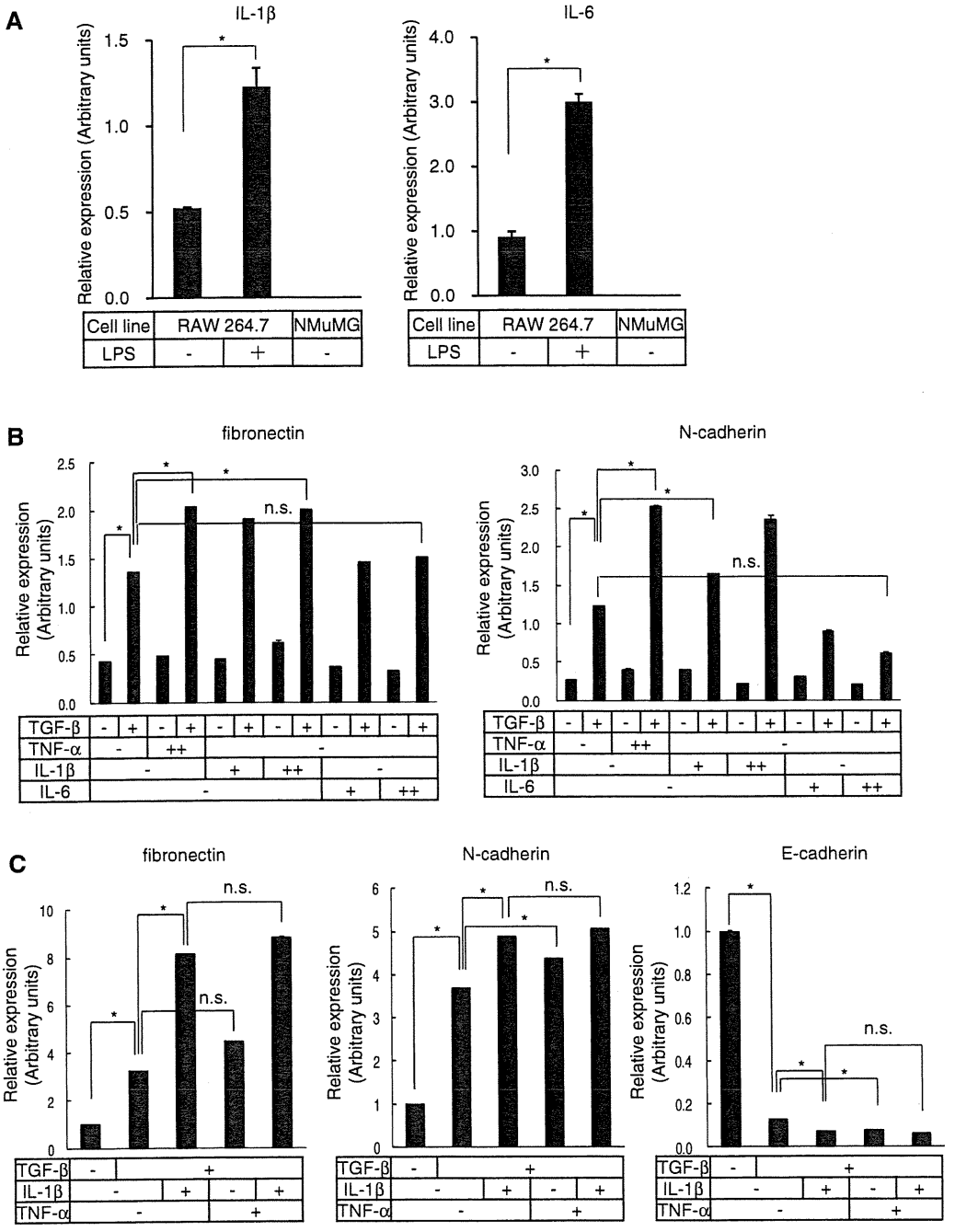

Fig. 6 Effect of IL-1β and IL-6 on TGF-β-induced expression of EMT markers. (A) qRT–PCR analysis of expression of IL-1β and IL-6 in RAW 264.7 cells treated with 0.1 μg/ml LPS was performed. NMuMG cells served as a negative control. The samples used in Fig. 3A were used for the analysis. (B) Effect of 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

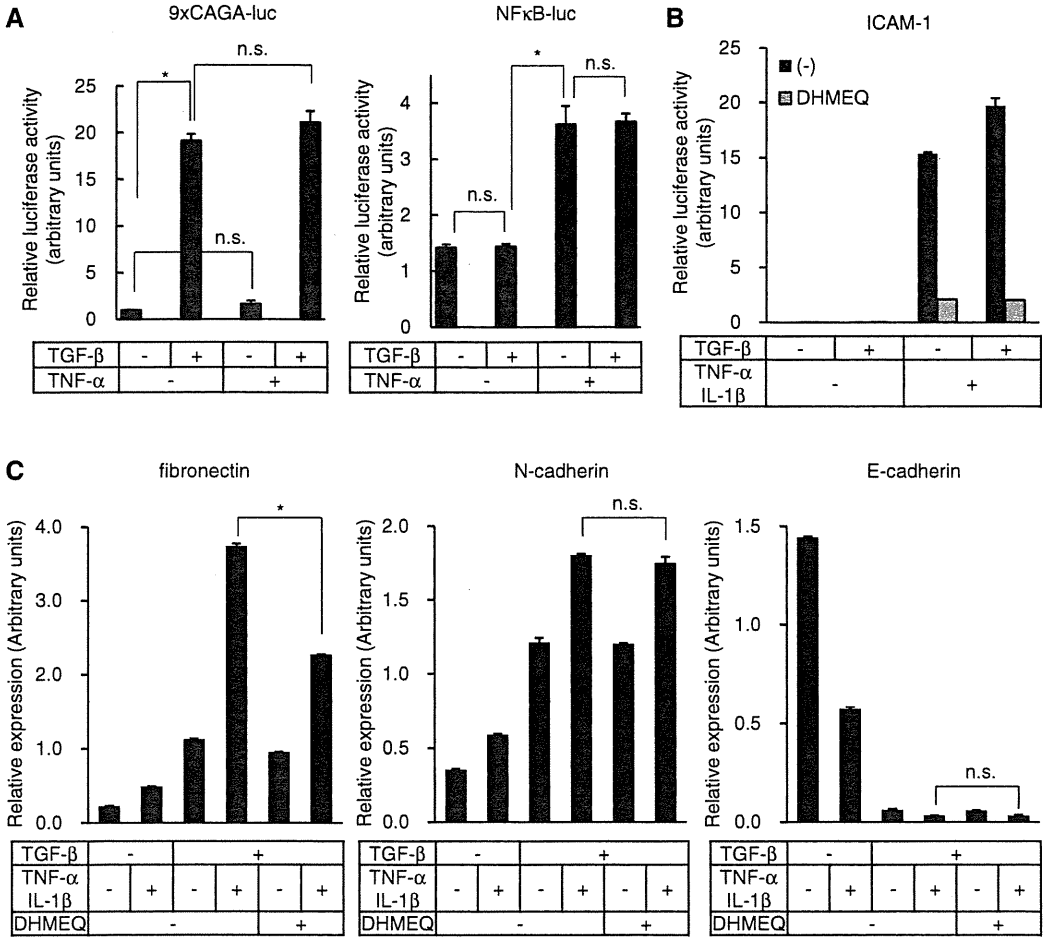

Fig. 7 Effect of DHMEQ on the enhancement of EMT marker expression by TNF-α and IL-1β. (A) A 9xCAGA-luc luciferase construct consisting of tandemly repeated Smad binding elements and NF-κB-luc luciferase reporter construct were transfected in A549 cells and stimulated with 5 ng/ml of TGF-β and 20 ng/ml of TNF-α for 24 h (9xCAGA-luc) or 6 h (NFκB-luc). (B) Inhibition of TNF-α and IL-1β-induced ICAM-1 expression by DHMEQ. A549 cells were pre-treated with 10 μg/ml of DHMEQ or DMSO as a control for 3.5 h and stimulated by TNF-α and IL-1β for 4 h. ICAM-1 expression was measured by qRT–PCR. (C) A549 cells were treated as in (B), and stimulated with TGF-β, TNF-α and IL-1β for 24 h. Expression of EMT markers was analysed by qRT–PCR. $*P<0.05$; Error bars, SDs; n.s., not significant.

TNF-α was observed only in the presence of TGF-β. Nevertheless, TGF-β-induced phosphorylation of Smad2, and Smad-induced 9xCAGA luciferase reporter activity did not change following the addition of TNF-α. We also observed that TGF-β does not enhance TNF-α-induced transcriptional activity of NFκB-luc, and that enhanced expression of target genes by TNF-α was selective to fibronectin and N-cadherin. Although many reports have revealed crosstalk between TNF-α and TGF-β signalling pathways in a variety of cells (*43*, *44*), the present analyses suggest that the cooperation of these cytokines appears to be exerted at the transcription level of each target gene depending on the context of their *cis*-regulatory elements, indicating differences in the transcriptional responses of target genes (*34*, *45*, *46*). Thus, neutralizing antibody against TNF-α inhibited the expression of both fibronectin and N-cadherin. In contrast, only the expression of fibronectin was inhibited by DHMEQ and U0126, whereas that of N-cadherin was inhibited by SB203580. Combinatorial effects of the several inhibitors on EMT phenotypes including expression of fibronectin and N-cadherin in the context of co-stimulation by TGF-β and inflammatory cytokines need to be evaluated in the future analyses.

The importance of EMT in cancer pathophysiology is not limited to cancer cell invasion and metastasis. Asiedu et al. reported generation of breast cancer stem cells by TNF-α and TGF-β (*47*). Based on our analyses suggesting the importance of TNF-α on TGF-β-induced EMT in A549 cells at a level of endogenous secretion from RAW 264.7 cells, complex mechanisms of enhancement by TNF-α require further examination to develop methods for controlling tumour cell invasion and cancer stem cells.

Supplementary Data

Supplementary Data are available at *JB* online.

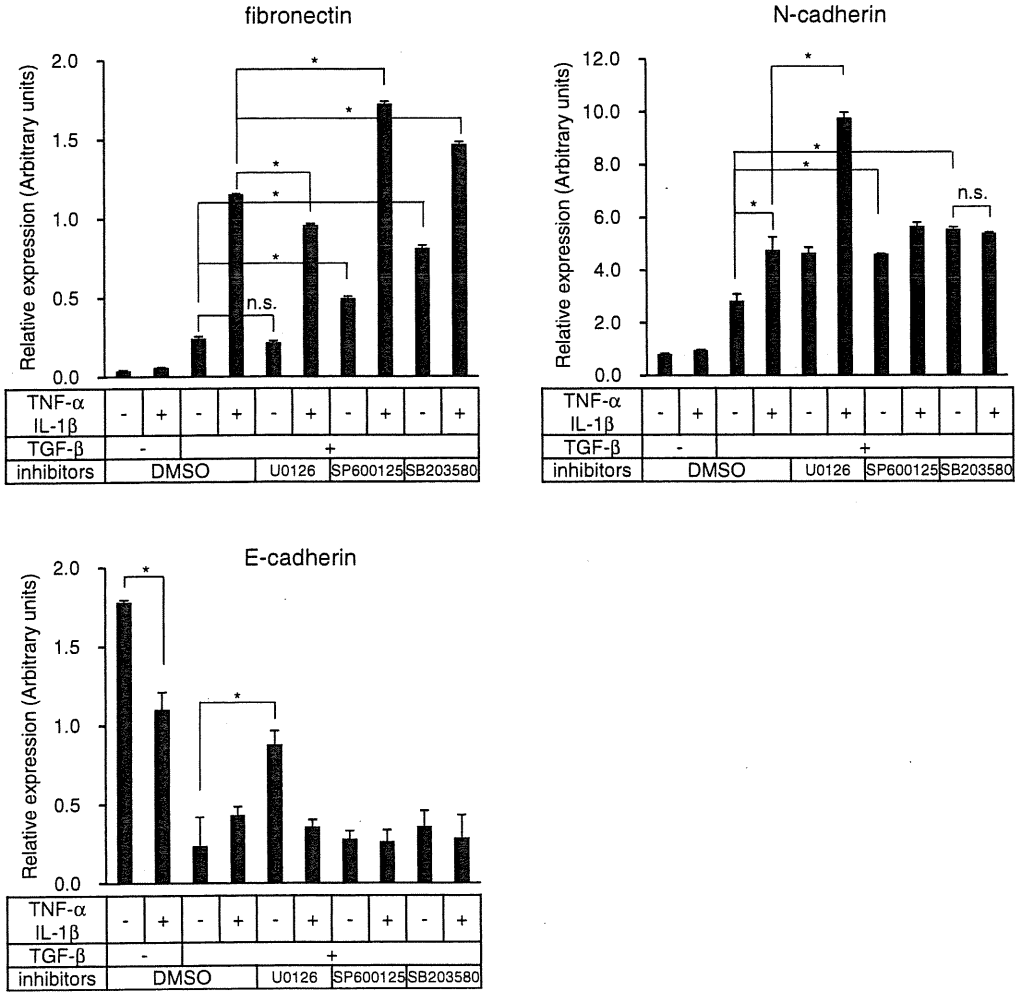

Fig. 8 Effect of kinase inhibitors on enhancement of TGF-β-induced EMT by TNF-α and IL-1β. A549 cells were pre-treated with either 10 μM U0126 (MEK inhibitor), 1 μM SP600125 (JNK inhibitor) or 1 μM SB203580 (p38 inhibitor) for 3.5 h. Cells were then stimulated with 5 ng/ml TGF-β, 20 ng/ml TNF-α and 1 ng/ml IL-1β for 24 h. Expression of EMT markers was determined by qRT–PCR after 24-h stimulation and normalized by GAPDH. $*P<0.05$; Error bars, SDs; n.s., not significant.

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Conflict of interest

None declared.

References

1. Bierie, B. and Moses, H.L. (2006) Tumour microenvironment: TGF-β: the molecular Jekyll and Hyde of cancer. *Nat. Rev. Cancer* **6**, 506–520
2. Ikushima, H. and Miyazono, K. (2010) TGF-β signalling: a complex web in cancer progression. *Nat. Rev. Cancer* **10**, 415–424
3. Yoshimura, A., Wakabayashi, Y., and Mori, T. (2010) Cellular and molecular basis for the regulation of inflammation by TGF-β. *J. Biochem.* **147**, 781–792
4. Derynck, R., Akhurst, R.J., and Balmain, A. (2001) TGF-β signaling in tumor suppression and cancer progression. *Nat. Genet.* **29**, 117–129
5. Sabe, H. (2011) Cancer early dissemination: cancerous epithelial-mesenchymal transdifferentiation and transforming growth factor-β signalling. *J. Biochem.* **149**, 633–639
6. Miyazono, K. (2009) Transforming growth factor-β signaling in epithelial-mesenchymal transition and progression of cancer. *Proc. Jpn. Acad. Ser. B Phys. Biol. Sci.* **85**, 314–323
7. Meulmeester, E. and ten Dijke, P. (2011) The dynamic roles of TGF-β in cancer. *J. Pathol.* **223**, 205–218
8. Heldin, C.H., Miyazono, K., and ten Dijke, P. (1997) TGF-β signalling from cell membrane to nucleus through SMAD proteins. *Nature* **390**, 465–471

9. Miyazono, K., Kamiya, Y., and Morikawa, M. (2010) Bone morphogenetic protein receptors and signal transduction. *J. Biochem.* **147**, 35–51
10. Thuault, S., Valcourt, U., Petersen, M., Manfioletti, G., Heldin, C.H., and Moustakas, A. (2006) Transforming growth factor-β employs HMGA2 to elicit epithelial-mesenchymal transition. *J. Cell Biol.* **174**, 175–183
11. Shirakihara, T., Saitoh, M., and Miyazono, K. (2007) Differential regulation of epithelial and mesenchymal markers by δEF1 proteins in epithelial mesenchymal transition induced by TGF-β. *Mol. Biol. Cell* **18**, 3533–3544
12. Kondo, M., Cubillo, E., Tobiume, K., Shirakihara, T., Fukuda, N., Suzuki, H., Shimizu, K., Takehara, K., Cano, A., Saitoh, M., and Miyazono, K. (2004) A role for Id in the regulation of TGF-β-induced epithelial-mesenchymal transdifferentiation. *Cell Death Differ.* **11**, 1092–1101
13. Vincent, T., Neve, E.P., Johnson, J.R., Kukalev, A., Rojo, F., Albanell, J., Pietras, K., Virtanen, I., Philipson, L., Leopold, P.L., Crystal, R.G., de Herreros, A.G., Moustakas, A., Pettersson, R.F., and Fuxe, J. (2009) A SNAIL1-SMAD3/4 transcriptional repressor complex promotes TGF-β mediated epithelial-mesenchymal transition. *Nat. Cell Biol.* **11**, 943–950
14. Thuault, S., Tan, E.J., Peinado, H., Cano, A., Heldin, C.H., and Moustakas, A. (2008) HMGA2 and Smads co-regulate SNAIL1 expression during induction of epithelial-to-mesenchymal transition. *J. Biol. Chem.* **283**, 33437–33446
15. Morita, T., Mayanagi, T., and Sobue, K. (2007) Dual roles of myocardin-related transcription factors in epithelial mesenchymal transition via slug induction and actin remodeling. *J. Cell Biol.* **179**, 1027–1042
16. Mihira, H., Suzuki, H., Akatsu, Y., Yoshimatsu, Y., Igarashi, T., Miyazono, K., and Watabe, T. (2011) TGF-β-induced mesenchymal transition of MS-1 endothelial cells requires Smad-dependent cooperative activation of Rho signals and MRTF-A. *J. Biochem.*, doi:10.1093/jb/mvr121
17. Saito, R.A., Watabe, T., Horiguchi, K., Kohyama, T., Saitoh, M., Nagase, T., and Miyazono, K. (2009) Thyroid transcription factor-1 inhibits transforming growth factor-β-mediated epithelial-to-mesenchymal transition in lung adenocarcinoma cells. *Cancer Res.* **69**, 2783–2791
18. Moustakas, A. and Heldin, C.H. (2007) Signaling networks guiding epithelial-mesenchymal transitions during embryogenesis and cancer progression. *Cancer Sci.* **98**, 1512–1520
19. Shirakihara, T., Horiguchi, K., Miyazawa, K., Ehata, S., Shibata, T., Morita, I., Miyazono, K., and Saitoh, M. (2011) TGF-β regulates isoform switching of FGF receptors and epithelial-mesenchymal transition. *EMBO J.* **30**, 783–795
20. Maier, H.J., Schmidt-Strassburger, U., Huber, M.A., Wiedemann, E.M., Beug, H., and Wirth, T. (2010) NF-κB promotes epithelial-mesenchymal transition, migration and invasion of pancreatic carcinoma cells. *Cancer Lett.* **295**, 214–228
21. Borthwick, L.A., McIlroy, E.I., Gorowiec, M.R., Brodlie, M., Johnson, G.E., Ward, C., Lordan, J.L., Corris, P.A., Kirby, J.A., and Fisher, A.J. (2010) Inflammation and epithelial to mesenchymal transition in lung transplant recipients: role in dysregulated epithelial wound repair. *Am. J. Transplant.* **10**, 498–509
22. Kasai, H., Allen, J.T., Mason, R.M., Kamimura, T., and Zhang, Z. (2005) TGF-β1 induces human alveolar epithelial to mesenchymal cell transition (EMT). *Respir. Res.* **6**, 56
23. Umezawa, K., Ariga, A., and Matsumoto, N. (2000) Naturally occurring and synthetic inhibitors of NF-κB functions. *Anticancer Drug Des.* **15**, 239–244
24. Matsumoto, N., Ariga, A., To-e, S., Nakamura, H., Agata, N., Hirano, S., Inoue, J., and Umezawa, K. (2000) Synthesis of NF-κB activation inhibitors derived from epoxyquinomicin C. *Bioorg. Med. Chem. Lett.* **10**, 865–869
25. Nagano, Y., Koinuma, D., Miyazawa, K., and Miyazono, K. (2010) Context-dependent regulation of the expression of c-Ski protein by Arkadia in human cancer cells. *J. Biochem.* **147**, 545–554
26. Horiguchi, K., Shirakihara, T., Nakano, A., Imamura, T., Miyazono, K., and Saitoh, M. (2009) Role of Ras signaling in the induction of snail by transforming growth factor-β. *J. Biol. Chem.* **284**, 245–253
27. Nagata, M., Nagata, S., Yuki, K., Isogaya, K., Saitoh, M., Miyazono, K., and Miyazawa, K. (2010) Identification of a phosphorylation site in c-Ski as serine 515. *J. Biochem.* **148**, 423–427
28. Mizutani, A., Saitoh, M., Imamura, T., Miyazawa, K., and Miyazono, K. (2010) Arkadia complexes with clathrin adaptor AP2 and regulates EGF signalling. *J. Biochem.* **148**, 733–741
29. Soll, D.R., Voss, E., Varnum-Finney, B., and Wessels, D. (1988) "Dynamic Morphology System": a method for quantitating changes in shape, pseudopod formation, and motion in normal and mutant amoebae of Dictyostelium discoideum. *J. Cell. Biochem.* **37**, 177–192
30. Katsuno, Y., Hanyu, A., Kanda, H., Ishikawa, Y., Akiyama, F., Iwase, T., Ogata, E., Ehata, S., Miyazono, K., and Imamura, T. (2008) Bone morphogenetic protein signaling enhances invasion and bone metastasis of breast cancer cells through Smad pathway. *Oncogene* **27**, 6322–6333
31. Nakano, A., Koinuma, D., Miyazawa, K., Uchida, T., Saitoh, M., Kawabata, M., Hanai, J., Akiyama, H., Abe, M., Miyazono, K., Matsumoto, T., and Imamura, T. (2009) Pin1 down-regulates transforming growth factor-β (TGF-β) signaling by inducing degradation of Smad proteins. *J. Biol. Chem.* **284**, 6109–6115
32. Dennler, S., Itoh, S., Vivien, D., ten Dijke, P., Huet, S., and Gauthier, J.M. (1998) Direct binding of Smad3 and Smad4 to critical TGF-β-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. *EMBO J.* **17**, 3091–3100
33. Lernbecher, T., Muller, U., and Wirth, T. (1993) Distinct NF-κ B/Rel transcription factors are responsible for tissue-specific and inducible gene activation. *Nature* **365**, 767–770
34. Mizutani, A., Koinuma, D., Tsutsumi, S., Kamimura, N., Morikawa, M., Suzuki, H.I., Imamura, T., Miyazono, K., and Aburatani, H. (2011) Cell-type specific target selection by combinatorial binding of Smad2/3 and hepatocyte nuclear factor 4-α in HepG2 cells. *J. Biol. Chem.* **286**, 29848–29860
35. Liu, X. (2008) Inflammatory cytokines augments TGF-β1-induced epithelial-mesenchymal transition in A549 cells by up-regulating TβR-I. *Cell Motil. Cytoskeleton* **65**, 935–944
36. Kim, J.H., Jang, Y.S., Eom, K.S., Hwang, Y.I., Kang, H.R., Jang, S.H., Kim, C.H., Park, Y.B., Lee, M.G., Hyun, I.G., Jung, K.S., and Kim, D.G. (2007)

Transforming growth factor β1 induces epithelial-to-mesenchymal transition of A549 cells. *J. Korean Med. Sci.* **22**, 898–904

37. Yamauchi, Y., Kohyama, T., Takizawa, H., Kamitani, S., Desaki, M., Takami, K., Kawasaki, S., Kato, J., and Nagase, T. (2010) Tumor necrosis factor-α enhances both epithelial-mesenchymal transition and cell contraction induced in A549 human alveolar epithelial cells by transforming growth factor-β1. *Exp. Lung Res.* **36**, 12–24
38. Camara, J. and Jarai, G. (2010) Epithelial-mesenchymal transition in primary human bronchial epithelial cells is Smad-dependent and enhanced by fibronectin and TNF-α. *Fibrogenesis Tissue Repair* **3**, 2
39. Kamitani, S., Yamauchi, Y., Kawasaki, S., Takami, K., Takizawa, H., Nagase, T., and Kohyama, T. (2011) Simultaneous stimulation with TGF-β1 and TNF-α induces epithelial mesenchymal transition in bronchial epithelial cells. *Int. Arch. Allergy Immunol.* **155**, 119–128
40. Schulz, C., Farkas, L., Wolf, K., Kratzel, K., Eissner, G., and Pfeifer, M. (2002) Differences in LPS-induced activation of bronchial epithelial cells (BEAS-2B) and type II-like pneumocytes (A-549). *Scand. J. Immunol.* **56**, 294–302
41. Tsutsumi-Ishii, Y. and Nagaoka, I. (2003) Modulation of human β-defensin-2 transcription in pulmonary epithelial cells by lipopolysaccharide-stimulated mononuclear phagocytes via proinflammatory cytokine production. *J. Immunol.* **170**, 4226–4236
42. Kim, S., Takahashi, H., Lin, W.W., Descargues, P., Grivennikov, S., Kim, Y., Luo, J.L., and Karin, M. (2009) Carcinoma-produced factors activate myeloid cells through TLR2 to stimulate metastasis. *Nature* **457**, 102–106
43. Takahashi, E., Nagano, O., Ishimoto, T., Yae, T., Suzuki, Y., Shinoda, T., Nakamura, S., Niwa, S., Ikeda, S., Koga, H., Tanihara, H., and Saya, H. (2010) Tumor necrosis factor-α regulates transforming growth factor-β-dependent epithelial-mesenchymal transition by promoting hyaluronan-CD44-moesin interaction. *J. Biol. Chem.* **285**, 4060–4073
44. Bitzer, M., von Gersdorff, G., Liang, D., Dominguez-Rosales, A., Beg, A.A., Rojkind, M., and Bottinger, E.P. (2000) A mechanism of suppression of TGF-β/SMAD signaling by NF-κ B/RelA. *Genes Dev.* **14**, 187–197
45. Gomis, R.R., Alarcon, C., He, W., Wang, Q., Seoane, J., Lash, A., and Massague, J. (2006) A FoxO-Smad synexpression group in human keratinocytes. *Proc. Natl. Acad. Sci. USA* **103**, 12747–12752
46. Koinuma, D., Tsutsumi, S., Kamimura, N., Taniguchi, H., Miyazawa, K., Sunamura, M., Imamura, T., Miyazono, K., and Aburatani, H. (2009) Chromatin immunoprecipitation on microarray analysis of Smad2/3 binding sites reveals roles of ETS1 and TFAP2A in transforming growth factor β signaling. *Mol. Cell. Biol.* **29**, 172–186
47. Asiedu, M.K., Ingle, J.N., Behrens, M.D., Radisky, D.C., and Knutson, K.L. (2011) TGF-β/TNF-α-mediated epithelial-mesenchymal transition generates breast cancer stem cells with a claudin-low phenotype. *Cancer Res.* **71**, 4707–4719

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REVIEW ARTICLE

Tumor-promoting functions of transforming growth factor-β in progression of cancer

KOHEI MIYAZONO[1,2], SHOGO EHATA[1] & DAIZO KOINUMA[1]

[1]*Department of Molecular Pathology, Graduate School of Medicine, University of Tokyo, Bunkyo-ku, Tokyo 113-0033, Japan, and* [2]*Ludwig Institute for Cancer Research, Box 595, SE 751-24 Uppsala, Sweden*

Abstract

Transforming growth factor-β (TGF-β) elicits both tumor-suppressive and tumor-promoting functions during cancer progression. Here, we describe the tumor-promoting functions of TGF-β and how these functions play a role in cancer progression. Normal epithelial cells undergo epithelial-mesenchymal transition (EMT) through the action of TGF-β, while treatment with TGF-β and fibroblast growth factor (FGF)-2 results in transdifferentiation into activated fibroblastic cells that are highly migratory, thereby facilitating cancer invasion and metastasis. TGF-β also induces EMT in tumor cells, which can be regulated by oncogenic and anti-oncogenic signals. In addition to EMT promotion, invasion and metastasis of cancer are facilitated by TGF-β through other mechanisms, such as regulation of cell survival, angiogenesis, and vascular integrity, and interaction with the tumor microenvironment. TGF-β also plays a critical role in regulating the cancer-initiating properties of certain types of cells, including glioma-initiating cells. These findings thus may be useful for establishing treatment strategies for advanced cancer by inhibiting TGF-β signaling.

Key words: *Angiogenesis, cancer-initiating cell, EMT, invasion, metastasis, TGF-β*

Introduction

Transforming growth factor-β (TGF-β) is a multifunctional regulator of cell growth, apoptosis, differentiation, and migration. TGF-β1 was originally discovered as a secreted protein that induces anchorage-independent growth in normal rat kidney NRK49F fibroblasts in the presence of epidermal growth factor (EGF) (1). TGF-β was shown to potently inhibit the proliferation of most cell types, including epithelial cells, endothelial cells, hematopoietic cells, and lymphocytes, and is widely known as a tumor suppressor. Studies investigating TGF-β signaling have revealed that perturbations of the TGF-β signaling pathway, such as mutations of TGF-β receptors or Smad proteins, lead to cancer progression and are related to poor prognosis of certain types of cancer. However, recent findings have shown that cancer cells become resistant to the growth inhibitory activity of TGF-β and that TGF-β facilitates invasion and metastasis of these cells both *in vitro* and *in vivo*.

Accumulating evidence has revealed that TGF-β plays a bidirectional role in cancer progression (2,3). TGF-β acts as a tumor suppressor by inhibiting cell growth through suppressing c-Myc expression and stimulating certain cyclin-dependent kinase inhibitors, including $p21^{WAF1}$ and $p15^{Ink4b}$, and by inducing cellular apoptosis through inducing DAP kinase, GADD45β, and Bim (4). Conversely, TGF-β functions as a tumor-promoting factor by stimulating extracellular matrix deposition and tissue fibrosis, perturbing immune and inflammatory function, stimulating angiogenesis, and promoting epithelial-mesenchymal transition (EMT).

In this review article, we discuss the tumor-promoting functions of TGF-β, particularly on EMT, on the basis of recent findings in our

Correspondence: Kohei Miyazono, Department of Molecular Pathology, Graduate School of Medicine, University of Tokyo, Bunkyo-ku, Tokyo 113-0033, Japan. Fax: +81-3-5841-3354. E-mail: miyazono@m.u-tokyo.ac.jp

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