

ORIGINAL ARTICLE

TGF- β drives epithelial-mesenchymal transition through δ EF1-mediated downregulation of ESRPK Horiguchi¹, K Sakamoto², D Koinuma¹, K Semba³, A Inoue⁴, S Inoue⁴, H Fujii⁴, A Yamaguchi², K Miyazawa⁵, K Miyazono¹ and M Saitoh^{1,5}

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Epithelial-mesenchymal transition (EMT) is a crucial event in wound healing, tissue repair and cancer progression in adult tissues. We have recently shown that transforming growth factor (TGF)- β -induced EMT involves isoform switching of fibroblast growth factor receptors by alternative splicing. We performed a microarray-based analysis at single exon level to elucidate changes in splicing variants generated during TGF- β -induced EMT, and found that TGF- β induces broad alteration of splicing patterns by downregulating epithelial splicing regulatory proteins (ESRPs). This was achieved by TGF- β -mediated upregulation of δ EF1 family proteins, δ EF1 and SIP1. δ EF1 and SIP1 each remarkably repressed ESRP2 transcription through binding to the ESRP2 promoter in NMuMG cells. Silencing of both δ EF1 and SIP1, but not either alone, abolished the TGF- β -induced ESRP repression. The expression profiles of ESRPs were inversely related to those of δ EF1 and SIP in human breast cancer cell lines and primary tumor specimens. Further, overexpression of ESRPs in TGF- β -treated cells resulted in restoration of the epithelial splicing profiles as well as attenuation of certain phenotypes of EMT. Therefore, δ EF1 family proteins repress the expression of ESRPs to regulate alternative splicing during TGF- β -induced EMT and the progression of breast cancers.

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Introduction

Splicing is a post-transcriptional process involved in the maturation of mRNAs and contributes to proteomic diversity by increasing the number of distinct mRNAs

generated from a single gene locus. Recent works suggest that more than 90% of human genes can produce different isoforms through alternative splicing (Pan *et al.*, 2008; Wang *et al.*, 2008). This process is tightly regulated in a tissue- and cell-type-dependent fashion (Matlin *et al.*, 2005; Blencowe, 2006), and alterations in this process are often linked to various types of diseases including cancer (Wang and Cooper, 2007; Dutertre *et al.*, 2010). Aberrations of splicing machinery result from mutations in splicing sites or dysfunction of splicing regulatory factors (Licatalosi and Darnell, 2010).

One of the well-known genes that are regulated by tissue-specific alternative splicing is the fibroblast growth factor receptors (FGFRs). Functional FGFRs are encoded by four genes (FGFR1–FGFR4), and the receptors consist of three extracellular immunoglobulin domains (Ig-I, Ig-II and Ig-III), a single transmembrane domain and a cytoplasmic tyrosine kinase domain (Eswarakumar *et al.*, 2005). FGFRs have several isoforms, as exon skipping removes the Ig-I domain. In addition, alternative splicing in the second half of the Ig-III domain in FGFR1–FGFR3 produces the IIIb (FGFR1IIIb–FGFR3IIIb) and IIIc (FGFR1IIIc–FGFR3IIIc) isoforms that have distinct fibroblast growth factor (FGF)-binding specificities and are predominantly expressed in epithelial and mesenchymal cells, respectively. FGF-2 (basic FGF) and FGF-4 bind preferentially to the IIIc isoforms, whereas FGF-7 (keratinocyte growth factor) and FGF-10 bind exclusively to the IIIb isoforms (Coulmoul and Deng, 2003; Chaffer *et al.*, 2007). Recently, epithelial splicing regulatory proteins (ESRPs) 1 and 2 were identified as coordinators of the epithelial cell-type-specific splicing program. ESRPs activate the splicing of exon IIIb and silence the splicing of exon IIIc of FGFR2, leading to the expression of proteins with the epithelial patterns of alternative splicing (Warzecha *et al.*, 2009a, b).

Epithelial-mesenchymal transition (EMT) is the differentiation switch directing polarized epithelial cells to trans-differentiate into mesenchymal cells (Thiery *et al.*, 2009). During the process of embryonic development, wound healing and reorganization in adult tissues, epithelial cells have been shown to lose their epithelial

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polarity and acquire mesenchymal phenotype. Further, EMT is involved in the process of invasion of tumor cells which also includes the loss of cell–cell interaction (Kalluri and Weinberg, 2009). Thus far, in nearly all cases, EMT appears to be regulated by extracellular matrix components and soluble growth factors or cytokines (Thiery and Sleeman, 2006). Among these factors, transforming growth factor- β (TGF- β) is considered as the key mediator of EMT during physiological processes. It is frequently and abundantly expressed in various tumors and also induces EMT in cancer cells during cancer progression. Recent studies revealed that TGF- β transcriptionally regulates expression of several transcription factors, including the zinc-finger factors Snail and Slug, the two-handed zinc-finger factors of δ EF1 family proteins δ EF1 and SIP1, the helix-loop-helix factors Twist and E12/E47, and the high motility group protein family HMGA2, which are involved in the induction of EMT particularly through the transcriptional repression of E-cadherin (Moustakas and Heldin, 2007; Miyazono, 2009).

We have recently reported that TGF- β induces isoform switching of FGFRs from IIIb to IIIc by alternative splicing during EMT in NMuMG cells, which results in enhanced EMT with aggressive phenotypes through cooperative action of TGF- β and FGF-2 (Shirakihara *et al.*, 2011). In the present study, we found that TGF- β regulates alternative splicing of numerous genes during EMT. The expression of δ EF1 family proteins, δ EF1 and SIP1, is increased after TGF- β treatment and subsequently represses the expression of the alternative splicing factor ESRP. Overexpression of ESRP in TGF- β -treated cells inhibits the conversion of alternative splicing pattern of epithelial types into those of mesenchymal types, as well as downregulation of the expression of E-cadherin. Repression of ESRP by δ EF1 family proteins is thus, a crucial process during EMT induced by TGF- β and in progression of breast cancers.

Results

Changes in splice variants during TGF- β -induced EMT

We have recently found that TGF- β primes isoform switching of FGFRs by alternative splicing during TGF- β -induced EMT, thereby changing the sensitivities of cells from FGF-7 to FGF-2 (Shirakihara *et al.*, 2011). Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of mouse mammary epithelial NMuMG cells revealed that, in addition to *FGFRs*, *CD44* splicing profile and the total level of *CD44* mRNA were changed after treatment with TGF- β (Figures 1a and b). There are multiple splice variants of the *Mena* gene (a member of Enabled (Ena)/vasodilator-stimulated phosphoprotein family of proteins) that are involved in cancer progression (Philippart *et al.*, 2008). We found that TGF- β also caused changes in splicing of the exon 11a of the *Mena* gene (Figure 1c). These findings suggest that alteration in splicing variants by TGF- β is not limited to FGFRs.

We next analyzed the expression of more than one million exons in NMuMG cells using mouse exon 1.0 ST

array and adapted ARH method to rank the splicing predictions across the different genes (Figure 1d) (Rasche and Herwig, 2010). We found that the expression of 3601 genes was altered at the exon level, which was classified by GO parameters (Lee *et al.*, 2008), suggesting that TGF- β induces a broad alteration in splicing patterns and generates a number of splicing variants during EMT in NMuMG cells (Supplementary Figure S1 and Supplementary Table 1). As *CD44*, *FGFRs*, *SLK* (ste 20-like kinase) and *CTNND1* (also known as δ -catenin or p120 catenin), of which splicing profiles have been reported to be regulated by ESRPs, were included in our exon-array data (Figure 1e and Supplementary Figure S2), we calculated ARH scores for the published exon-array data of ESRPs-silenced human prostate cancer PNT2 cells and compared the data with our exon-array data (Warzecha *et al.*, 2009b). We found that 227 genes and 75 genes in ESRP1/2-silenced cells overlapped with those of our data with $P < 0.05$ and $P < 0.01$, respectively (Supplementary Table 2). These findings suggest that TGF- β -induced changes in splice variants are partly mediated by ESRPs.

Repression of ESRPs by TGF- β

We next determined how TGF- β regulates the functions of splicing factors ESRP1 and ESRP2 during EMT. We found that TGF- β considerably downregulated the mRNA expression of ESRP2 in NMuMG cells, whereas the expression of ESRP1 mRNA could not be clearly detected (Figure 2a, left). We also examined the expression of ESRPs after TGF- β stimulation in other cells derived from mammary gland epithelial cells, including EpH4 cells expressing the viral H-Ras oncogene (EpRas cells) and breast cancer JygMC(A) cells (Ehata *et al.*, 2007). Treatment of EpRas cells with TGF- β repressed both ESRP1 and ESRP2 at the mRNA levels and ESRP1 at the protein level (Figures 2a, right and b). As JygMC(A) cells autonomously secrete TGF- β (Hoshino *et al.*, 2011), we treated the cells with TGF- β type I receptor (T β R-I) inhibitor, SB431542. The treatment increased the expression of ESRP1 and ESRP2 (Figure 2c). In addition, transfection of NMuMG cells with small interfering RNAs (siRNAs) against Smad2 and Smad3 attenuated the effects of TGF- β on the expression of ESRP2 (Figure 2d). Moreover, when *de novo* protein synthesis was inhibited by cycloheximide, which is an inhibitor of protein synthesis, downregulation of ESRP2 by TGF- β was attenuated (Figure 2e). PAI-1 and SIP1 have been reported as direct and indirect transcriptional targets of TGF- β /Smad pathway, respectively (Shirakihara *et al.*, 2007). Thus, these findings suggest that the suppression of ESRP2 by TGF- β involves *de novo* protein synthesis through the Smad pathway.

ESRP2 repression by δ EF1 and SIP1 in TGF- β -induced EMT

We examined the expression profiles of δ EF1, SIP1, E-cadherin and ESRP2 after TGF- β stimulation by quantitative RT-PCR. The levels of ESRP2 were

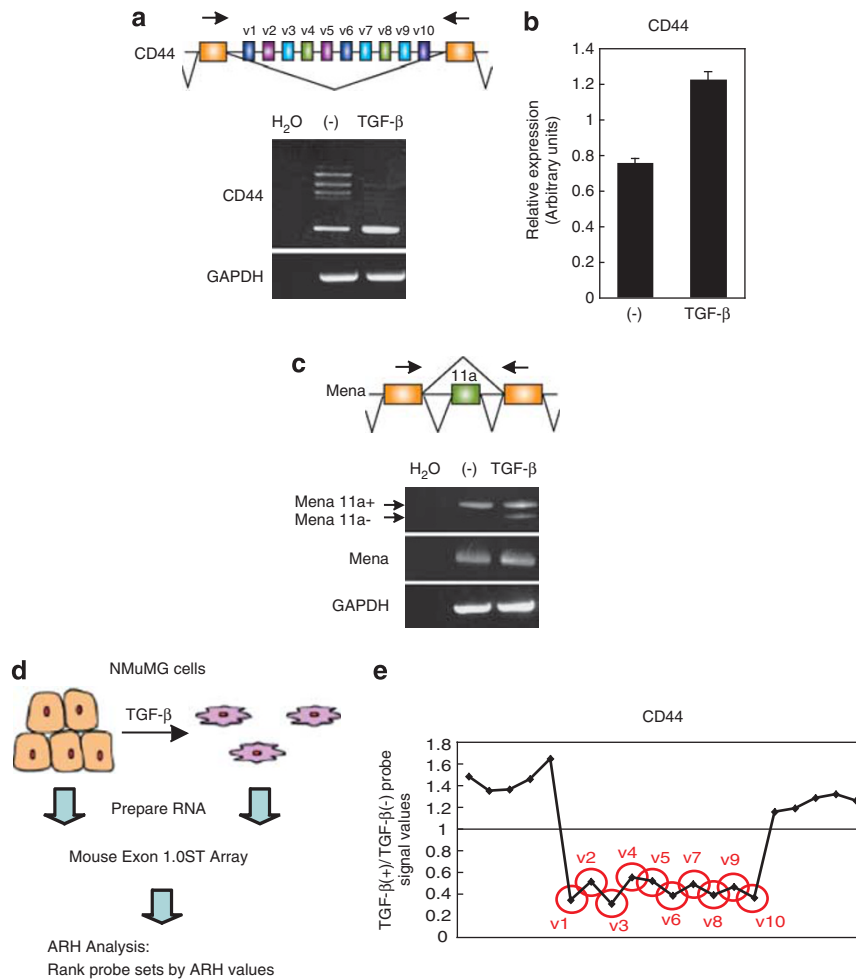


Figure 1 Changes in alternative splicing during TGF- β -induced EMT. (a) Changes in alternative splicing of CD44. Specific primers to detect v1-v10 variants of CD44 are shown as arrows (top panel). GAPDH was used as internal control. (b) The total level of CD44 mRNA was evaluated by quantitative RT-PCR analysis. (c) Specific primers to detect splicing variants of *Mena* are shown as arrows (top panel). GAPDH was used as internal control. (d) NMuMG cells treated with TGF- β for 24 h were prepared for Mouse Exon 1.0 ST Array. The ARH method was adapted to identify candidate genes at the exon level whose expressions changed during EMT. (e) The ratio of expression changes of each exon calculated by probe signal value in CD44 is shown. Red circles indicate the exons whose probe signals were altered by TGF- β treatment and reported to be spliced by ESRPs (Warzecha *et al.*, 2009a).

gradually decreased until 24 h upon TGF- β stimulation, with the expression profile similar to that of E-cadherin and reciprocal to that of δ EF1 and SIP1 (Figure 3a). To evaluate the mechanism of reciprocal regulation between δ EF1/SIP1 and ESRP2 expression, we prepared the ESRP2 promoter region from NMuMG cells by a PCR-based strategy. The activity of ESRP2 promoter in NMuMG cells was remarkably repressed by constitutively active mutant of T β R-I (caT β R-I), δ EF1 and SIP1. δ EF1 overexpression had a stronger effect than SIP1 overexpression, probably because the protein levels of transfected SIP1 were much lower than those of δ EF1 as determined by immunoblot analysis (Figure 3b and Supplementary Figure S3a). When we infected the cells with adenoviral vector encoding either δ EF1 or SIP1, δ EF1 or SIP1 each reduced the expression of endogenous ESRP2 mRNA with equivalent efficiencies (Figure 3c).

To determine whether δ EF1 and SIP1 interact with the promoter regions of ESRP2, we performed chromatin

immunoprecipitation (ChIP) assays in NMuMG cells after TGF- β treatment. The quality of commercially available anti- δ EF1 antibody was appropriate for ChIP assays, whereas that of anti-SIP1 antibodies was not suitable for this assay. Thus, we overexpressed FLAG-tagged SIP1 in NMuMG cells and immunoprecipitated it with anti-FLAG antibody. In the absence of TGF- β , the level of δ EF1 expression was very low and thus insufficient for ChIP (Figure 3d). After treatment with TGF- β , interactions of δ EF1 with DNA fragments of the ESRP2 promoter in NMuMG and EpRas cells (Figure 3d, left and data not shown) and the ESRP1 promoter in EpRas cells (Figure 3d, right) were observed. Moreover, SIP1 also interacted with the ESRP2 and ESRP1 promoters, whereas neither δ EF1 nor SIP1 associated with hemoglobin β gene (*HBB*) promoter that was used as a negative control (Figure 3d). In the competition assays of ChIP, overexpression of FLAG-SIP1 reduced the interaction of endogenous δ EF1

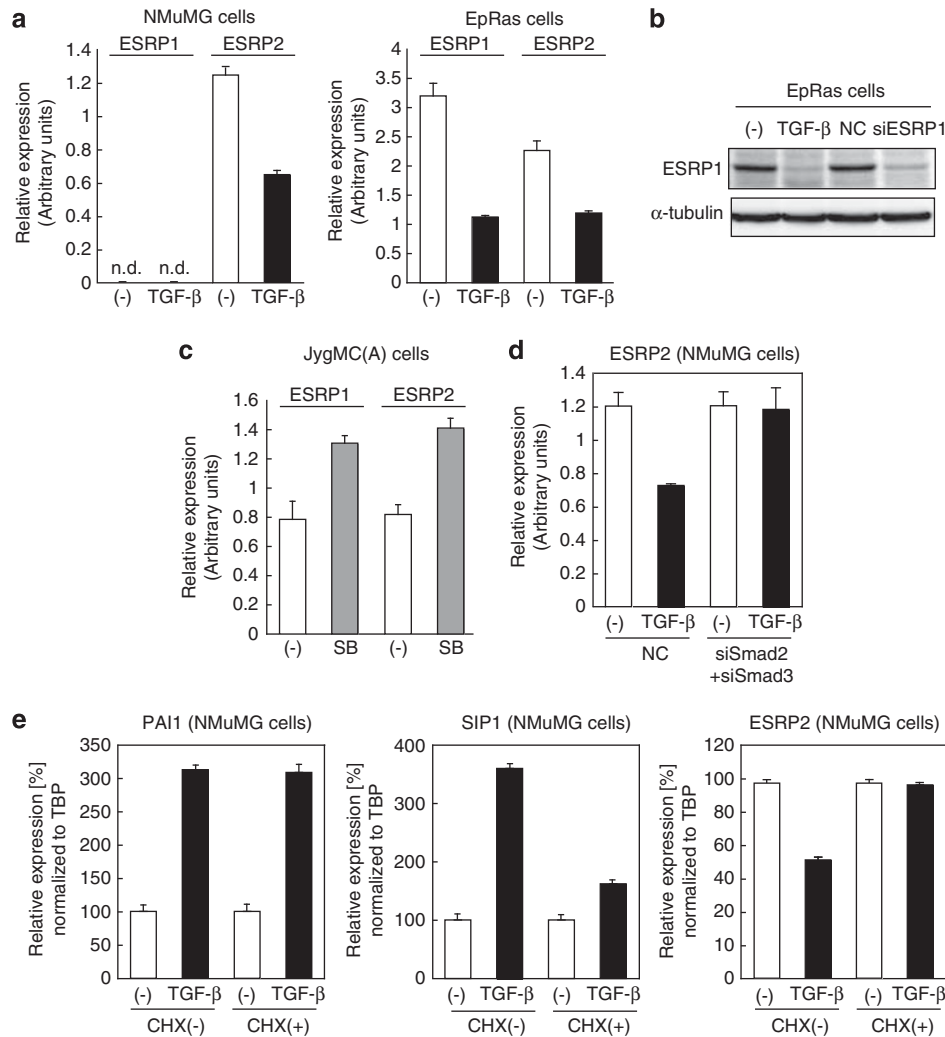


Figure 2 Requirement of *de novo* protein synthesis for downregulation of ESRP2 by TGF- β . (a) Effect of TGF- β on the expression of ESRPs in NMuMG cells (left) and EpRas cells (right) was examined by quantitative RT-PCR analysis. n.d., not detected. (b) After treatment of EpRas cells with TGF- β or transfection with ESRP1 siRNA, the levels of ESRP1 were evaluated by immunoblot analysis. α -tubulin was used as a loading control. (c) JygMC(A) cells were treated with 10 μ M of T β R-I inhibitor (SB431542) for 48 h. The levels of ESRP1 and ESRP2 were evaluated by quantitative RT-PCR analysis. SB, SB431542. (d) NMuMG cells transfected with both Smad2 and Smad3 siRNAs were stimulated with 1 ng/ml TGF- β for 24 h, and then examined by quantitative RT-PCR analysis for the expression levels of ESRP2. NC, control siRNA. (e) NMuMG cells pretreated with 3 μ M cycloheximide (CHX) for 1 h were stimulated with 1 ng/ml TGF- β for 24 h, and examined by quantitative RT-PCR analysis for PAI1 (left), SIP1 (center) and ESRP2 levels (right).

with the ESRP2 promoter (Supplementary Figure S3b), suggesting that δ EF1 family proteins recognize the same binding regions of ESRP2 promoter. Overall, these findings indicate that δ EF1 and SIP1 are preferentially recruited to the promoter region of ESRPs, and that they suppress the transcription of ESRPs in response to TGF- β treatment.

As double knockdown of δ EF1 and SIP1 is necessary to block the E-cadherin repression by TGF- β (Shirakihara *et al.*, 2007), we next analyzed the TGF- β -mediated ESRP2 repression in NMuMG cells in which both δ EF1 and SIP1 were silenced using their specific siRNAs (Figure 3e). TGF- β treatment induced the expression of δ EF1 and SIP1 mRNAs by about three-fold after 48 h and repressed the expression of ESRP2. In the cells transfected with either δ EF1 or SIP1 siRNA alone,

TGF- β -mediated ESRP2 repression was only partially blocked; however, transfection with both δ EF1 and SIP1 siRNAs completely abolished the TGF- β -mediated ESRP2 repression (Figure 3e). The δ EF1/SIP1-mediated ESRP repression was also detected in EpRas cells (Supplementary Figure S3c). Therefore, similar to the repression of E-cadherin, the transcription of ESRPs is accumulatively regulated by the δ EF1 family proteins during EMT by TGF- β .

Switching between FGFR isoforms by ESRPs during TGF- β -induced EMT

As we have previously reported (Shirakihara *et al.*, 2011), FGFR1 upregulated by TGF- β in NMuMG cells was the mesenchymal isoform, that is, FGFR1IIIc,

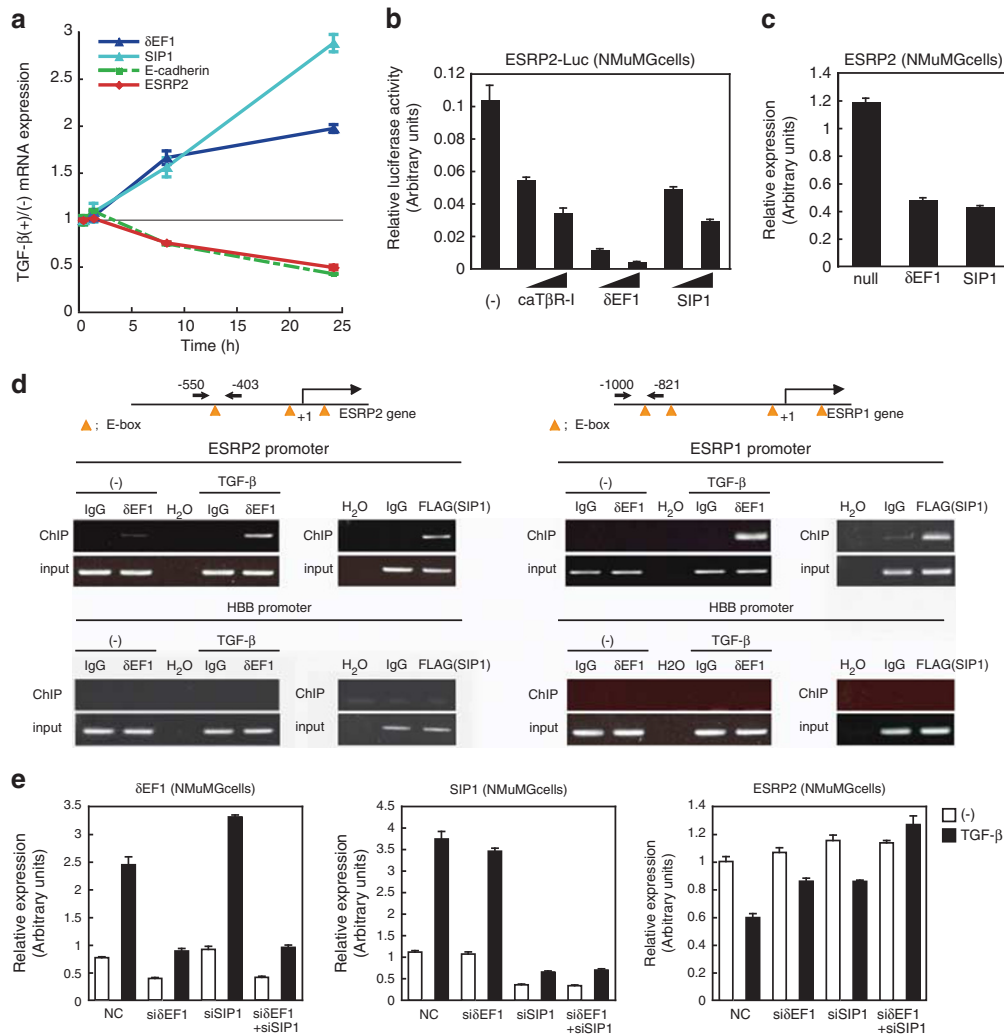


Figure 3 Regulation of ESRP2 expression by Δ EF1 and SIP1. **(a)** After treatment with 1 ng/ml of TGF- β , the kinetics of ESRP2, Δ EF1, SIP1 and E-cadherin expressions were examined in NMuMG cells by quantitative RT-PCR analysis. The ratio of the mRNA levels in TGF- β -treated cells as compared with that in non-treated cells is shown. **(b)** NMuMG cells were transfected with mouse ESRP2 promoter-reporter construct (ESRP2-Luc) in combination with various amounts of caT β R-I, Δ EF1 and SIP1 plasmids. At 48 h after transfection, cells were harvested and assayed for luciferase activities. **(c)** mRNA levels of ESRP2 in NMuMG cells infected with null, Δ EF1 or SIP1 adenoviruses were determined by quantitative RT-PCR. **(d)** ChIP analysis was performed using NMuMG and EpRas cells in the presence or absence of 1 ng/ml TGF- β . FLAG-SIP1 adenovirus was infected into NMuMG and EpRas cells 24 h before ChIP analysis. Endogenous Δ EF1 and FLAG-SIP1 were immunoprecipitated with anti- Δ EF1 antibody and with anti-FLAG antibody, respectively. Eluted DNAs from NMuMG cells and from EpRas cells were subjected to conventional PCR for ESRP2 promoter (left) and for ESRP1 promoter (right), respectively. HBB promoter was used as negative control. Primers used are shown as arrows. **(e)** NMuMG cells transfected with siRNA against Δ EF1, SIP1, or both (si Δ EF1 + siSIP1) were stimulated with 1 ng/ml TGF- β for 48 h and examined by quantitative RT-PCR analysis for Δ EF1 (left), SIP1 (center) and ESRP2 levels (right). NC, control siRNA.

whereas the FGFR2 downregulated by TGF- β was the epithelial isoform, that is, FGFR2IIIb (Figure 4a). Further, isoform switching of FGFR1 and FGFR2 was also observed in EpRas cells, in which both ESRP1 and ESRP2 were endogenously expressed (Figures 2a and 4b). Because TGF- β downregulated the total levels of FGFR2, the TGF- β -mediated induction of the IIIc isoform of FGFR2 was not clearly detected in both cells (Figures 4a and b). When ESRP2 was silenced by its specific siRNAs in NMuMG cells, ESRP2 siRNA changed the FGFR2IIIb isoform to FGFR2IIIc isoform without appearance of FGFR1IIIc in the absence of TGF- β (Figure 4c), suggesting that the TGF- β -mediated

conversion of FGFR2IIIb into FGFR1IIIc requires ESRPs as well as other unidentified transcriptional factor(s). In addition, transfection with both ESRP1 and ESRP2 siRNAs in EpRas cells resulted in the expression of IIIc isoform of FGFR2 as well as that of FGFR1 (Figure 4d). Taken together, these findings suggest that TGF- β increases FGFR1 expression and decreases FGFR2 expression, leading to the conversion of the IIIb isoform into the IIIc isoform of FGFRs through alternative splicing by ESRPs.

We next performed gain-of-function experiments after achieving ectopic expression of FLAG-tagged ESRP2. After TGF- β treatment, the FGFR1IIIc isoform was

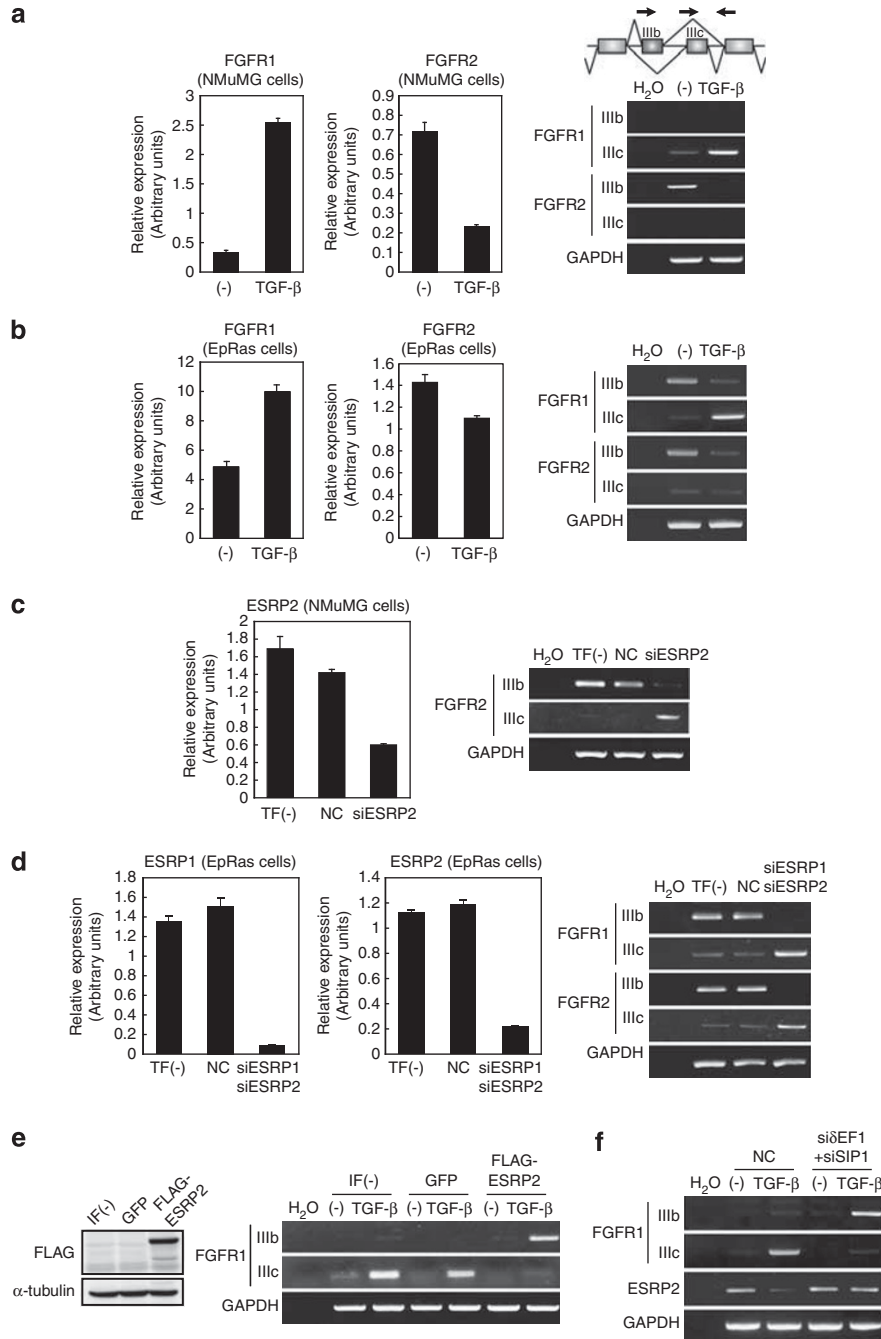


Figure 4 Isoform switching of FGFRs induced by TGF- β . **(a)** NMuMG cells were treated with 1 ng/ml TGF- β for 24 h and the expression of FGFR1 (left) and FGFR2 (center) was determined by quantitative RT-PCR. Expression of FGFR isoforms was analyzed by conventional RT-PCR using specific primers for 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 δ EF1 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

δ EF1 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 δ EF1/SIP1-repressed ESRPs.

Regulation of ESRP expression by δ EF1 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 δ EF1/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 δ EF1 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 δ EF1 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 δ EF1 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 δ EF1/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 δ EF1/SIP1, expressed only IIIc isoforms of FGFRs (Figure 5b). Moreover, double knockdown of δ EF1 and SIP1 increased the expression of ESRP1 and ESRP2 in MDA-MB231 and BT549 cells (Figure 5c), indicating that δ EF1 and SIP1 downregulate ESRP expression in human breast cancer cells.

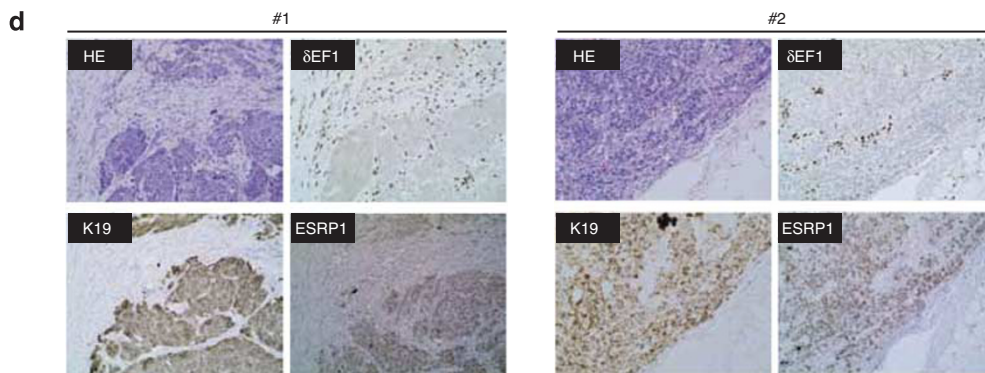
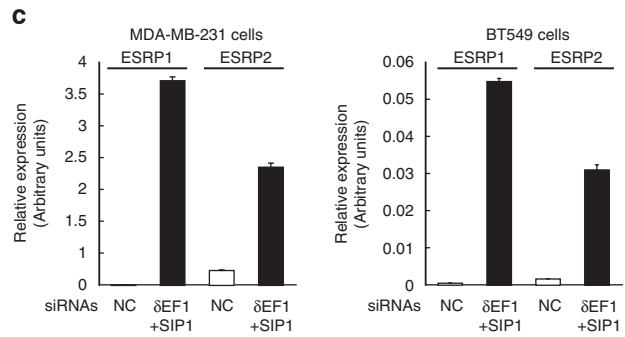
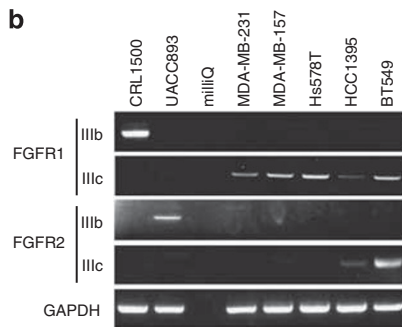
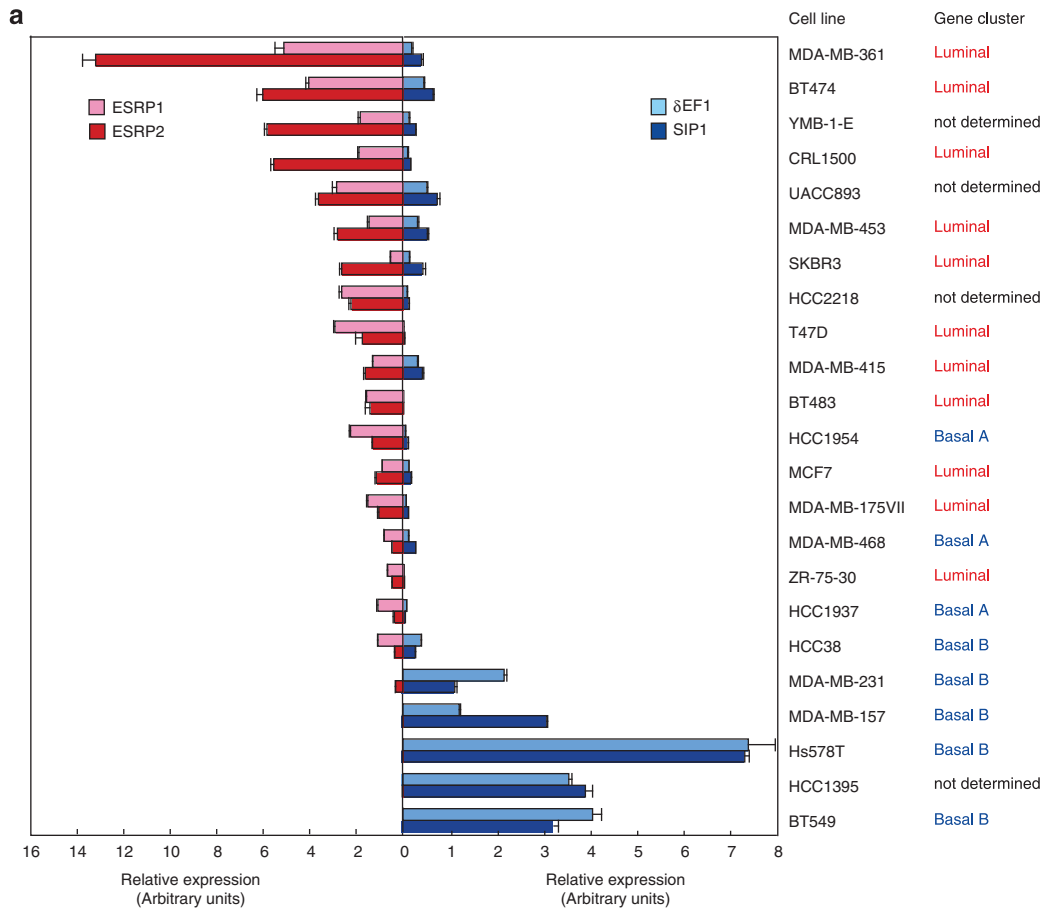
We next examined whether δ EF1/SIP1 and ESRPs are reciprocally expressed in human breast tumors. Primary tumor tissues from cancer patients were

subjected to immunohistochemical analyses with anti- δ EF1 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 δ EF1 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 δ EF1 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.

Figure 5 Expression profiles of ESRP1/2 and δ EF1/SIP1 in breast cancer cells. (a) mRNA levels of the expression of ESRP1, ESRP2, δ EF1 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 δ EF1 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 δ EF1 in primary tumor samples from breast cancer patients are shown (# 1 and 2).



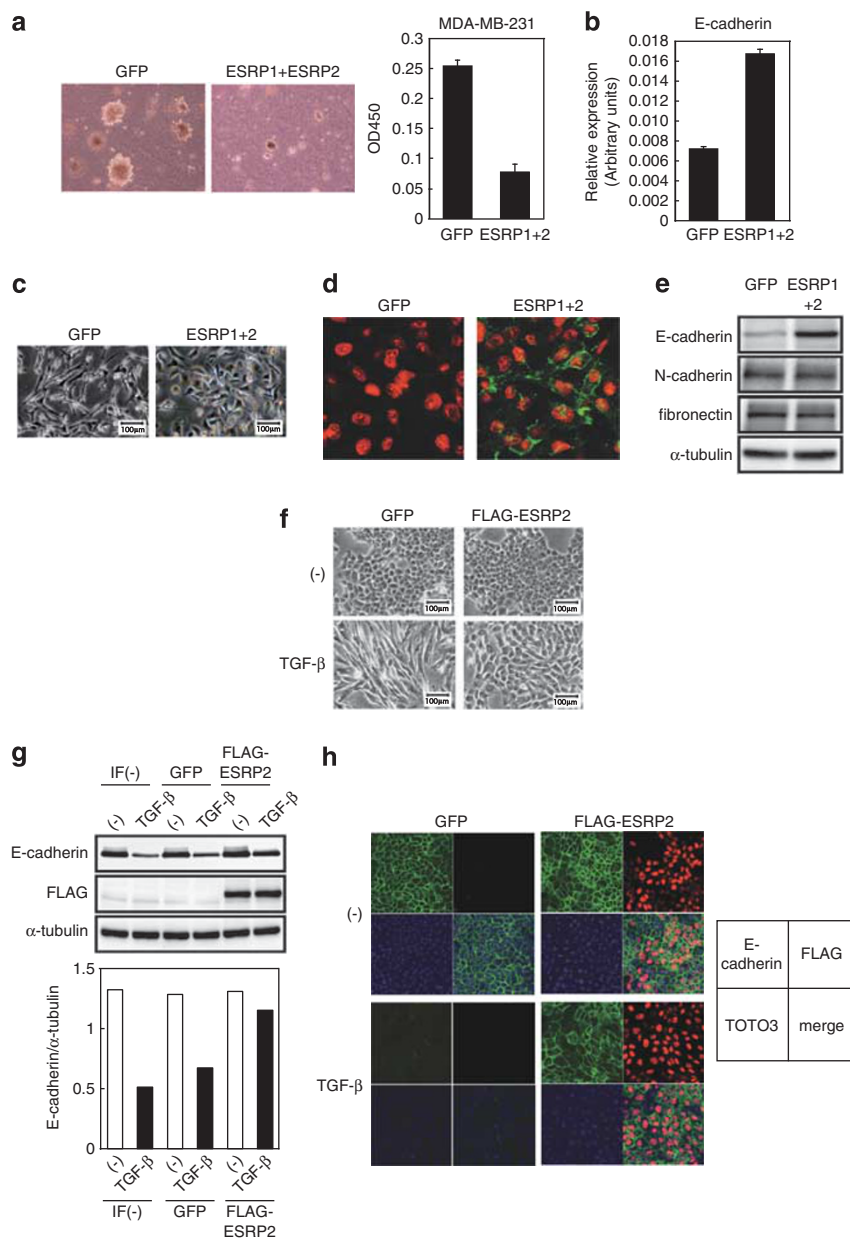


Figure 6 ESRPs attenuate malignant phenotypes of cancer cells. (a–e) MDA-MB-231 cells were infected with lentiviruses encoding ESRP1 and ESRP2. The cells were examined for anchorage-independent growth in soft agar (left in a) and quantified (right in a). Expression of E-cadherin in the cells was evaluated 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.

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

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 δ EF1 and SIP1 siRNAs did not affect the upregulation of FGFR1 (Figure 4f). These findings suggest that δ EF1 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 δ EF1/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 δ EF1/SIP1. Overexpression of ESRPs upregulated E-cadherin expression without affecting the levels of δ EF1 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
 δ EF1 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 δ EF1/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 δ EF1 family proteins. Importantly, most of the cell lines with high levels of δ EF1 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 δ EF1 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 δ EF1 and SIP1 expression and high levels of ESRPs expression were categorized into the 'luminal' subtype of breast cancer. Thus, δ EF1 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- β 1 was obtained from R&D Systems (Minneapolis, MN, USA). SB431542 was from 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 δ EF1 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 δ EF1 and SIP1 cDNAs, and the adenoviral vector encoding δ EF1 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 δ EF1 (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 δ EF1 (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- δ EF1, 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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