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Transcriptional and Post-transcriptional Regulation in TGF-β-mediated epithelial-mesenchymal transition

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Epithelial-mesenchymal transition (EMT) is a crucial event in appropriate embryonic development as well as in wound healing, tissue repair and cancer progression in adult tissues. EMT endows cells with migratory and invasive properties, inhibits apoptosis and senescence, contributes to immunosuppression and induces stress resistance and stem cell properties. Many secreted polypeptide factors act in a sequential or cooperative manner to elicit EMT. Transforming growth factor (TGF)-β can initiate and maintain EMT by activating intracellular signalling pathways. Recent studies have provided new insights into molecular mechanisms by which TGF-β mediates changes in transcription of EMT regulators and EMT marker proteins, as well as changes in alternative splicing controlled by epithelial splicing regulatory proteins 1 and 2. Here, we present some of the emerging molecular mechanisms that mediate EMT upon exposure to TGF-β.

Keywords: TGF-β/EMT/ESRP alternative splicing.

Abbreviations: CBP, cAMP-response element-binding protein (CREB)-binding protein; δEF1, δ-crystallin/E2-box factor1; EMT, epithelial-mesenchymal transition; FGF, fibroblast growth factor; HLH, helix-loop-helix; R-Smad, receptor-regulated Smad; SIP1, smad interacting protein1; Id, inhibitors of differentiation or inhibitors of DNA binding; SBD, Smad-binding domain; αSMA, smooth muscle α-actin; TGF-β, transforming growth factor-β; TNF, tumour necrosis factor.

Bidirectional roles of TGF-β in cancer progression

Transforming growth factor (TGF)-β, the prototypical member of the TGF-β family, regulates a broad range of cellular responses, including cell proliferation, differentiation, adhesion, migration and apoptosis. TGF-β exhibits its pleiotropic effects through binding to transmembrane serine-threonine kinase receptors type I (TβR-I) and type II (TβR-II). Upon ligand-induced heteromeric complex formation between TβR-I and TβR-II, TβR-I is phosphorylated and activated by TβR-II. Smads are the major transducers of TGF-β signalling; Smad2 and Smad3 are phosphorylated by TβR-I, and form complexes with Smad4. These complexes translocate to and accumulate in the nucleus, where they control transcription of target genes in conjunction with various transcription factors, co-activators and co-repressors (*1*, *2*). TGF-β suppresses proliferation of normal epithelial cells but promotes that of normal fibroblasts. In the early stages of tumourigenesis, TGF-β inhibits the proliferation of epithelial cells; insensitivity to this inhibitory effect on cell proliferation is associated with tumourigenesis. These aberrations in TGF-β signalling are due to mutations in signalling components; such mutations are observed in various types of tumours including head and neck, breast, gastric, colorectal and pancreatic cancers (*3*). Increases in expression of inhibitory molecules of TGF-β signalling, e.g. Smad7 and c-Ski, have also been reported in certain cancers (*4*, *5*). Ectopic expression of a dominant-negative TβR-II in epidermis promotes malignant conversion of epithelial cells and tumour formation (*6*). Moreover, the ubiquitin ligase Arkadia enhances TGF-β and bone morphogenetic protein (BMP) signalling by promoting degradation of repressors of these pathways (*7*); Arkadia ± heterozygous mice exhibit increased susceptibility to carcinogen-induced tumour formation (*8*). Based on these observations, TGF-β is considered as a tumour suppressor. In contrast, in the advanced stages of tumourigenesis, TGF-β is often overexpressed in tumour tissues, where it induces migration and invasion of cancer cells. Moreover, TGF-β facilitates immunosuppression, lymphangiogenesis and deposition of extracellular matrix proteins in various types of cells in the cancer microenvironment (*9*, *10*). Blockade of TGF-β signalling suppresses cell survival, intravasation and metastasis of tumour cells (*11*, *12*). In tumour cells, chronic exposure to TGF-β results in loss of TGF-β-mediated growth inhibition and apoptosis and marked changes in cell morphology (*13*). One mechanism by which TGF-β promotes tumour cell motility and invasion involves the epithelial-mesenchymal transition (EMT) (*14*–*16*).

Transcriptional regulators of EMT

EMT is a phenotypic conversion that facilitates embryonic development and wound healing in physiological processes, and that is also associated with fibrotic diseases and cancer progression (*17*). EMT involves dramatic cellular changes, including decreased intercellular adhesion and cell polarity as well as an increase in matrix remodelling and migratory and invasive

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properties. Recently, it has been proposed that EMT can be classified into three subtypes based on the biological and biomarker context (*18*). Type 1 EMT involves primitive epithelial cells that differentiate into motile mesenchymal cells during the process of gastrulation, and primitive neuroepithelial cells that generate migrating neural crest cells. Type 2 EMT involves secondary epithelial or endothelial cells that differentiate into resident tissue fibroblasts in association with wound healing, tissue regeneration or organ fibrosis. Type 3 EMT occurs in epithelial carcinoma cells in primary nodules, which differentiate into metastatic tumour cells. The process of tumour cell invasion, which is also often associated with EMT, involves the loss of cell–cell interactions together with acquisition of migratory properties. Besides acquisition of mesenchymal behaviour, cancer cells undergoing EMT show more aggressive phenotypes including resistance to drugs, stresses and anoikis, inhibition of senescence, immunosuppression and acquisition of stem cell-like features. Together, these changes promote cancer progression (Fig. 1) (*17*, *19*). Thus, outcomes produced by Type 3 EMT are far different from those of the other types of EMT.

Formation of tight cell–cell adhesions is mainly dependent on the E-cadherin system; loss of E-cadherin-mediated cell–cell interaction is thus essential for EMT. EMT is characterized by down-regulation of epithelial markers, including cytokeratins and tight junction components and by up-regulation of mesenchymal markers such as fibronectin, N-cadherin and vimentin (*17*). Recent studies of the molecular mechanism by which expression of E-cadherin is repressed in epithelial cells have revealed that several transcription factors are involved in this process; these include the Snail family of zinc-finger transcription factors (Snail, Slug and Smuc), the two-handed zinc-finger factors of δEF1 family proteins [δ-crystallin/E2box factor (δEF1)]/zinc-finger E-box-binding homeobox (ZEB) 1 and Smad-interacting protein (SIP)1/ZEB2 and the basic helix-loop-helix (bHLH) factors Twist and E12/E47 (*17*, *20*). The consensus binding site for Snail family proteins contains a hexamer core (CAGGTG) that is identical to the E-box sequence, the binding site for bHLH proteins and δEF1 family proteins. These transcription factors repress expression of E-cadherin by direct binding to the E-box sites in its promoter, and thereby elicit EMT when over-expressed in normal epithelial cells. In addition, if over-expressed in cancer cells, these factors induce EMT and consequently promote development of metastatic properties such as migration and invasion. Consistent with this, the metastatic phenotypes of aggressive cancer cells are considerably rendered by knockdown of Snail and δEF1; therefore, these transcriptional factors are now considered as key regulators of EMT.

Transcriptional regulators in TGF-β-mediated EMT

So far it has been demonstrated that nearly all cases of EMT in adult tissues are regulated by extracellular matrix components and soluble growth factors or cytokines, including Wnt, fibroblast growth factors (FGFs), hepatocyte growth factor, epidermal growth factor (EGF) and TGF-βs (*17*). Among them, TGF-β was first described as an inducer of EMT during development (*21*), and is now thought to promote invasion and metastasis of cancer cells through induction of EMT. TGF-β up-regulates expression of key regulators of EMT, including Snail and δEF1/SIP1, in several epithelial and cancer cells. It is currently unclear which key regulators play the most critical roles in the EMT programme; these regulators may independently act in a cell context-dependent fashion, or may act cooperatively and/or hierarchically in the induction of EMT.

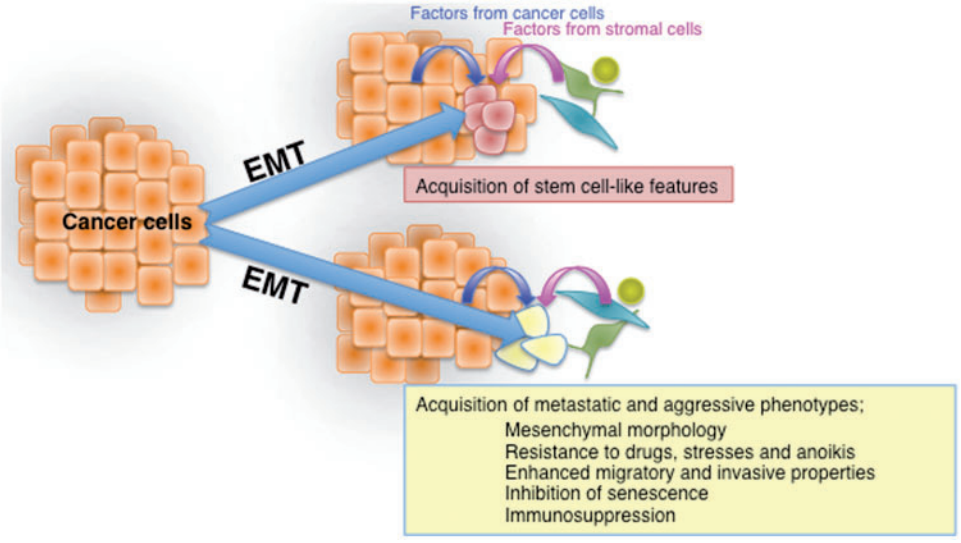

Fig. 1 EMT in cancer cells. Many secreted polypeptide factors derived from cancer or stromal cells act in a sequential or cooperative manner to elicit EMT. Cancer cells undergoing EMT exhibit stem cell-like features and more aggressive phenotypes including mesenchymal morphology, resistance to drugs, stress and anoikis, enhanced migration and invasion, inhibition of senescence and immunosuppression.

By microarray expression profiling, Id (inhibitors of differentiation or inhibitors of DNA binding) was identified as a negative regulator of TGF-β-mediated EMT. The expression of Id proteins is down-regulated by TGF-β, and ectopic expression of Id2 and Id3 maintains epithelial phenotypes by inhibiting TGF-β-mediated suppression of E-cadherin (*22*, *23*). Id proteins have a bHLH domain lacking the basic DNA-binding region; in humans, the family consists of four homologues, Id1-4 (*24*). Id proteins interact with and inhibit the transcriptional activity of other bHLH proteins. E47 and E12, two Id target proteins that are derived from a single gene (*E2A*) by alternative splicing, suppress the expression of E-cadherin through direct binding to its promoter. Repression of Ids by TGF-β is partially mediated through interaction of JunB with activating transcription factor 3 (*25*). This down-regulation relieves this inhibition by liberating E2A, and permits conversion of epithelial cells to cells with mesenchymal phenotypes, leading to EMT.

δEF1 and SIP1 are members of the δEF1 family, well-characterized factors involved in TGF-β-induced EMT in mouse mammary epithelial NMuMG cells. SIP1 was originally identified as a SIP1 and interacts with Smads through the Smad-binding domain (SBD) at its N-terminus (*26*). Compared with SIP1, δEF1 interacts only weakly with Smads, possibly due to the low degree of sequence similarity in the SBD (our unpublished data). Expression levels of δEF1 and SIP1 are increased by TGF-β, with profiles of expression reciprocal to that of E-cadherin. Both δEF1 and SIP1 redundantly repress the transcription of E-cadherin through direct binding to two E-box sites in the E-cadherin promoter, which is essential for TGF-β-induced EMT in NMuMG cells. A SIP1 deletion mutant lacking the SBD fails to regulate representative TGF-β target genes, whereas it is still able to repress the transcription of E-cadherin (*27*). Thus, binding to Smads is dispensable for E-cadherin repression. Ectopic expression of Id reduces TGF-β-mediated up-regulation of δEF1/SIP1 and other bHLH transcription factors, Ets proteins, enhance δEF1/SIP1 induction. Since E2A collaborates with Ets to regulate gene expression, Id blocks this cooperation by capturing E2A and reduces δEF1/SIP1 induction by TGF-β. In addition to TGF-β-induced EMT, δEF1, but not SIP1, positively regulates the transcription of calponin and smooth muscle α-actin (αSMA), representative markers for myofibroblasts, in epithelial-myofibroblastic transition and in vascular smooth muscle cell differentiation (*28*, *29*). Even though δEF1 and SIP1 possess high sequence similarity each other, they appear to have distinct functions. A functional distinction between them has not been well elucidated (*30*). Recently, several microRNAs involved in specifying epithelial phenotypes have been identified. The microRNAs of the miR-200 family target and repress δEF1 and SIP1, whereas δEF1 and SIP1 inhibit the transcription of the two loci (miR-200b/200a/429 and miR-200c/141) that encode the five members of the miR-200 family, resulting in forming a negative feedback loop (*31*). Thus, expression levels of miR-200 and δEF1/SIP1 are independently regulated by EMT stimuli including TGF-β, and they hierarchically and cooperatively control EMT in many different cell types.

Snail (Snai1), Slug (Snai2) and Smuc (Snai3) are members of the Snail family; all three genes function to repress E-cadherin expression (*20*). In NMuMG cells, TGF-β up-regulates Snail mRNA >10-fold after 1 h; moderate induction of Snail is observed until 24 h of exposure. The rapid induction is insensitive to cycloheximide treatment, suggesting direct target of TGF-β/Smad pathway (*27*), whereas the sustained induction appears to be regulated by *de novo* synthesis of proteins such as high-mobility group transcription factor 2 (HMGA2) (*32*). Slug induction by TGF-β has been observed in some oesophageal cancer cells (our unpublished data), whereas the induction of Smuc has not yet been determined. The Snail family proteins bind to E-box sites in the E-cadherin promoter, and locally modify chromatin structure by recruitment of homologue of yeast suppressor SWI-independent 3 A (SIN3A), histone deacetylases HDAC1 and 2 and components of the Polycomb 2 complex (*33*, *34*). In addition, Snail forms a complex with Smad3 and Smad4, and interacts with the gene promoters of a tight-junction protein Car and E-cadherin during TGF-β-mediated EMT (*35*). Snail undergoes post-transcriptional modifications that control its localization and degradation. LIV1, a breast cancer-associated zinc transporter protein, is required for the nuclear localization of Snail (*36*). Phosphorylation of Snail mediated by p21-activated kinase (PAK1), casein kinase 1 (CK1) and glycogen synthase kinase 3β (GSK-3β) promotes proteasomal degradation by β-TrCP-dependent ubiquitylation, whereas dephosphorylation is regulated by the small C-terminal domain phosphatase (SCP) (*37*–*39*). Snail is stabilized by the inflammatory cytokine TNF-α through the activation of the NF-κB pathway, which is required for the induction of the COP9 signalosome 2 (CSN2) and, in turn, blocks the ubiquitylation and degradation of Snail (*40*). Lysine oxidation of Snail is regulated by lysyl oxidase-like 2 (LOXL2), and is essential for its stability and Snail-dependent EMT (*41*). Translation of Snail mRNA is controlled in a cap-independent manner, and is activated by the transcription/translation regulator Y-box-binding protein-1 (*42*). p53 suppresses the expression of Snail by inducing miR-34, and reduces expression of Slug by promoting Slug degradation by the ubiquitin ligase murine double minute 2 (MDM2) in a ternary complex of p53, Slug and MDM2 (*43*, *44*). When p53 is mutated in cancer cells, Snail and Slug are up-regulated. However, it is still unknown whether TGF-β regulates post-translational modifications of Snail family proteins during EMT.

Another regulator of EMT is Twist, a gene in which mutations can cause Saethre Chotzen Syndrome, an autosomal dominant human craniosynostosis syndrome (*45*). Six orthologues of Twist have been identified in mammals: Twist1, Twist2, Hand1, Hand2, Paraxis and Scleraxis. Twist1 null mice die around E11.5 with a number of phenotypes including dysmorphic facial features (*45*). In addition to its essential role in development, Twist1 is associated with a number of aggressive tumours including gastric, liver and breast cancers. In a mouse mammary tumour model, Twist1

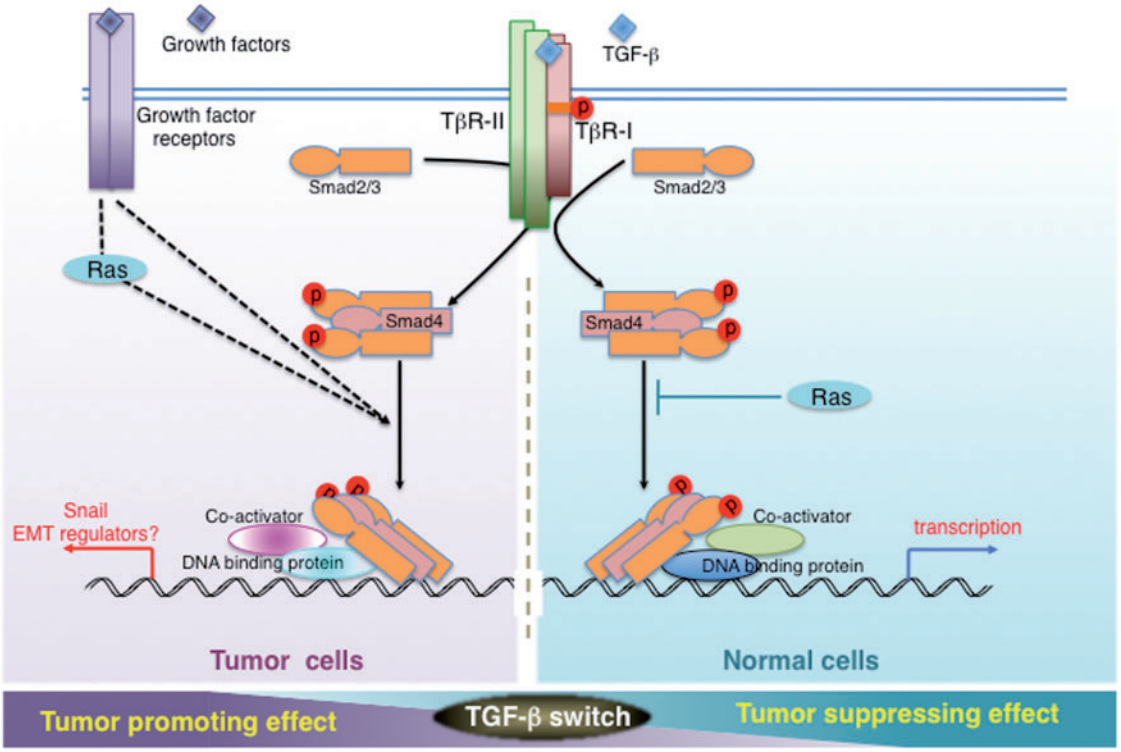

Fig. 2 TGF-β signalling is transduced through the Smad pathways. Smad pathway (right): TGF-β binds to TβR-II and TβR-I. TβR-I is phosphorylated by TβR-II, and activates Smad2 and Smad3. Activated Smad2/3 form a complex with Smad4 and translocate into the nucleus. The Smad complexes interact with various transcriptional factors and transcriptional co-activators. Phosphorylation at the linker regions of Smads by specific kinases downstream of Ras prevents nuclear translocation of and stimulates degradation of the R-Smads. Snail induction by the Smad pathway (left). Smad complexes are activated by unknown mechanisms downstream of Ras and growth factor receptors, and subsequently activate the transcription of Snail in tumour cells. These discrepancies may be associated with the so-called 'TGF-β switch' that has not been identified yet.

expression was identified as a predictor of metastatic behaviour. Most aggressive metastatic cells could be rendered by Twist1 siRNA, whereas ectopic expression of Twist1 in epithelial cells drives EMT (*46*). Twist is phosphorylated by protein kinase A (PKA), which modulates its biological activity by altering its interaction with other bHLH proteins, E12 and Hand2 (*47*). It is likely that, in addition to Twist1, other members of the Twist family play critical roles in EMT. Recently, it has been reported that induction of αSMA by TGF-β is mediated through CBP-dependent interaction between Smad3 and β-catenin (*48*). Transcription factors involved in TGF-β-induced EMT appear to be highly dependent on types or origins of epithelial cells. More and more novel players in EMT may be found from now.

Cooperation between TGF-β and other growth factors in EMT

Some intracellular signals, including Wnt, EGF and TNF-α, cooperate with TGF-β signalling, to promote tumour invasion/metastasis and EMT (*49*). As described below, in the case of FGF-2, TGF-β induces isoform switching of FGF receptors (FGFRs) and sensitizes cells to FGF-2, resulting in induction of enhanced EMT with more aggressive characteristics by this cooperation (*28*). In addition, the synergism between TGF-β and Ras signalling has been extensively investigated. When epithelial cells are transformed by constitutively active mutant Ras, they not only become resistant to growth inhibition by TGF-β but also undergo EMT with invasive and metastatic phenotypes (*50*). In Madin-Darby canine kidney (MDCK) cells and human pancreatic cancer Panc-1 cells, induction of Snail by TGF-β occurs in cooperation with active Ras signalling (*51*, *52*). Constitutively active Ras dramatically enhances TGF-β-induced expression of Snail, whereas representative direct targets of TGF-β, including Smad7 and plasminogen activator inhibitor 1, are either unaffected or slightly inhibited by Ras signalling. Consistent with these observations, cancer cells harbouring activated alleles of Ras reveal remarkable induction of Snail upon exposure to TGF-β alone. This finding is intriguing, because phosphorylation at the linker regions of Smad2 and Smad3 by kinases downstream of Ras prevents nuclear translocation of the R-Smads and stimulates their degradation (*53*). Ablation of these putative phosphorylation sites in R-Smads enhances the responsiveness of representative TGF-β target genes, but does not affect the induction of Snail by a combination of TGF-β with Ras (our unpublished data) (*51*). Thus, this synergism between TGF-β and Ras signalling selectively leads to the induction of Snail, which is dependent on Smads but independent of phosphorylation at the linker region of Smads. Although the underlying mechanism is not well understood, it may be associated with the so-called 'TGF-β switch' that converts the effects of TGF-β from tumour suppressor to tumour promoter (Fig. 2) (*54*).

Post-transcriptional regulators in TGF-β-mediated EMT

Splicing is a post-transcriptional process involved in maturation of mRNAs and it contributes to proteomic diversity by increasing the number of distinct mRNAs generated from a single gene. Recent studies have shown that >90% of human genes undergo at least one alternative splicing event that produces distinct isoforms (*55*, *56*). This process is tightly regulated in a tissue- and cell type-dependent fashion; alterations in this process have been linked to various types of diseases including cancer (*57*). Aberrations in the splicing machinery can result from mutations in splicing sites or dysfunction of splicing regulatory factors.

One of the well-known examples regulated by tissue-specific alternative splicing involves the mutually exclusive alternative exons IIIb and IIIc of FGFR, which are expressed in epithelial and mesenchymal tissues, respectively. FGF-2 (also known as basic FGF) and FGF-4 bind preferentially to the mesenchymal IIIc isoforms, whereas FGF-7 (also known as KGF, keratinocyte growth factor) and FGF-10 bind exclusively to the epithelial IIIb isoforms (*58*). Although several studies identified many ubiquitously expressed splicing factors that influence the choice of exon inclusion in the FGFRs, none of these factors explains how the alternative splicing of FGFRs is tightly regulated in a tissue-specific manner. ESRP 1 and 2 have been recently identified as epithelial-specific splicing factors by Warzecha *et al.* (*59*); those authors performed a high-throughput cDNA expression screen and identified 18 clones that converted the specificity of exon inclusion from IIIc to IIIb. Among them, ESRP1 and 2, which were previously known as RNA-recognition motif-containing proteins Rbm35a and Rbm35b, were subjected to further analysis. ESRP mRNA is epithelial-specific in mouse embryo, and is expressed in skin and gastrointestinal tissue in the adult mouse. ESRPs bind directly to hexamers containing repeats of UGG or GGU motifs, which are enriched in alternatively spliced regions. In the case of the *FGFR2* gene, ESRPs are capable of binding to the UGG-rich motif located in the intron between exons IIIb and IIIc. When ESRPs bind to the motif, they direct splicing to exon IIIb, upstream of the motif, resulting in skipping of exon IIIc downstream of the motif, thereby generating IIIb isoform of FGFR2 (Fig. 3). The function of similar UGG-rich motifs, UGGUG, GGUGG and GUGGU, which are present within ESRP-silenced exons, has not yet been fully elucidated, but ESRPs may promote exon skipping by binding to the motif within the exon. Recently, it was reported that ESRPs regulate hundreds of alternative splicing events in transcripts of numerous genes with function in cell–cell adhesion, cell polarity and cell migration/invasion (*60*, *61*). Loss of ESRPs causes a switch from the epithelial splicing programme to the mesenchymal splicing programme, and induces changes in cell morphology that are observed during the EMT. RBFOX2, another splicing factor, is also involved in EMT-associated alternative splicing (*60*). Taken together, these observations demonstrate that the splicing machinery is required for changes in cellular morphology during EMT. More recently, it has been reported that activation of mTOR pathway by TGF-β is implicated in regulation of the EMT phenotype including cytoskeletal changes, cell size control and increased migration/invasion. Therefore, translational pathway mediated by mTOR may complement the transcription pathway by Smads during TGF-β-induced EMT (*62*, *63*).

Down-regulation of ESRPs during EMT by TGF-β

Since the expression of more than 3500 genes is altered at the exon level in mouse epithelial NMuMG cells

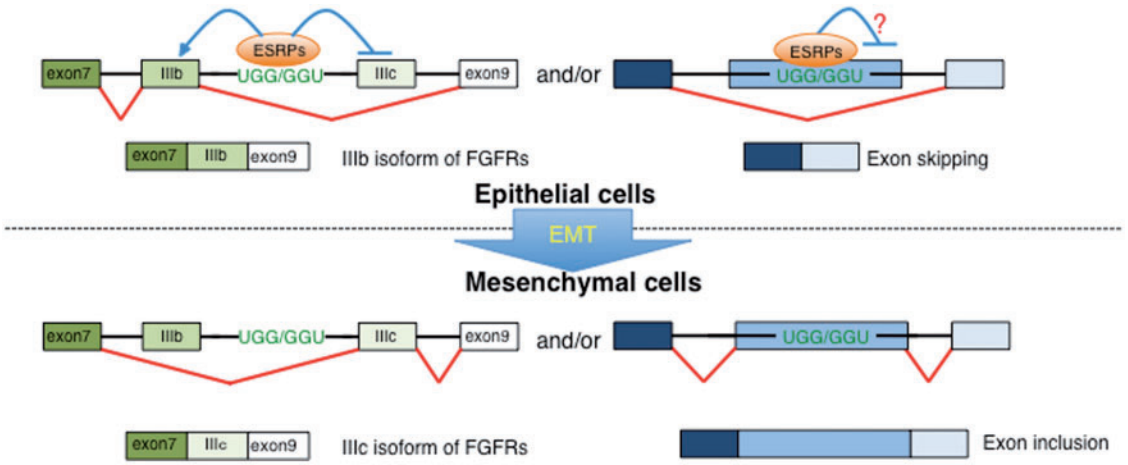

Fig. 3 ESRPs promote the epithelial-specific splicing programme. In epithelial cells, ESRPs bind to hexamer sequences enriched in UGG/GGU motifs, which are located in the intron between IIIb and IIIc of FGFRs. This binding enhances exon inclusion of the upstream exon, IIIb, while silencing the downstream exon, IIIc (left in top). Since similar motifs are enriched within ESRPs-silenced exons, ESRPs may promote exon skipping by binding to the motif within the exon (right in top). In mesenchymal cells, the repression of ESRP proteins promotes an alternative pattern of splicing that contributes to an EMT (bottom).

during TGF-β-mediated EMT, TGF-β induces broad alteration in splicing patterns and generates a number of splicing variants during EMT (*64*). TGF-β down-regulates the expression of ESRPs, and in turn changes the splicing profiles of FGFRs, CD44, Ste 20-like kinase, CTNND1 (also known as δ-catenin or p120 catenin) and Mena [a member of the Enabled (Ena)/vasodilator-stimulated phosphoprotein (VASP) family of proteins] (Fig. 4A). Intriguingly, over-expression of ESRP inhibits TGF-β-induced appearance of EMT phenotypes (*64*). Thus, down-regulation of ESRP appears to be one of the key events during EMT. In addition, TGF-β increases FGFR1 expression and decreases FGFR2 expression, and causes cells to switch from the IIIb isoform to the IIIc isoform of FGFR1, through alternative splicing as described above (Fig. 4B). Therefore, TGF-β primes change in sensitivities of cells to FGF ligands during EMT, resulting in more aggressive phenotypes in cells undergoing EMT (*28*).

The expression of ESRP was suppressed by TGF-β, which was highly sensitive to treatment with cycloheximide. In addition, the expression profile of ESRP by TGF-β was very similar to that of E-cadherin and reciprocal to that of δEF1/SIP1. Similar to δEF1/SIP1-repressed E-cadherin, δEF1/SIP1 interact with the promoter regions of ESRPs and suppress ESRPs. siRNAs against ESRP cause cells to switch from the IIIb isoform of FGFR2 to the IIIc isoform, whereas over-expression of ESRP hampers the effect of TGF-β on FGFRs (Fig. 4B). Moreover, siRNAs against δEF1/SIP1 abolish the TGF-β-mediated ESRP repression and isoform switching of FGFRIIIc (Fig. 4B). Thus, up-regulation of δEF1/SIP1 suppresses ESRPs via binding to its promoter regions during EMT. Breast cancers are classified into two subtypes, 'luminal' and 'basal-like', corresponding to two distinct types of epithelial cells found in the normal mammary gland. The 'basal-like' subtype is associated with aggressive behaviour and poor prognosis, and typically does not express oestrogen and progesterone receptors, and ERBB2 ('triple-negative' subtype). In 23 human breast cancer cell lines and some human clinical samples, the expression of ESRP was inversely correlated with both expression of δEF1/SIP1 and progression of breast cancer. Additionally, most of the cell lines with low ESRP levels and high δEF1/SIP1 levels were categorized into the 'basal-like' subtype of breast cancer. In contrast, most of the cell lines with high ESRPs levels and low δEF1 and SIP1 were categorized into the 'luminal' subtype of breast cancer. Thus, δEF1/SIP1 and ESRPs are specifically expressed in the 'basal-like' and 'luminal' subtypes of breast cancer cells, respectively. Although expression levels of Snail, Slug, and Twist are not relevant in breast cancer cells, Snail and Twist repress the expression of ESRPs in other types of cells undergoing EMT including Panc-1 cells and HMLE cells, respectively (*59*, *64*). Since each of the EMT regulators are tightly controlled in a cell context-dependent manner, ESRPs represent useful negative markers for detecting cells that have undergone EMT, or cancer cells with more aggressive phenotypes.

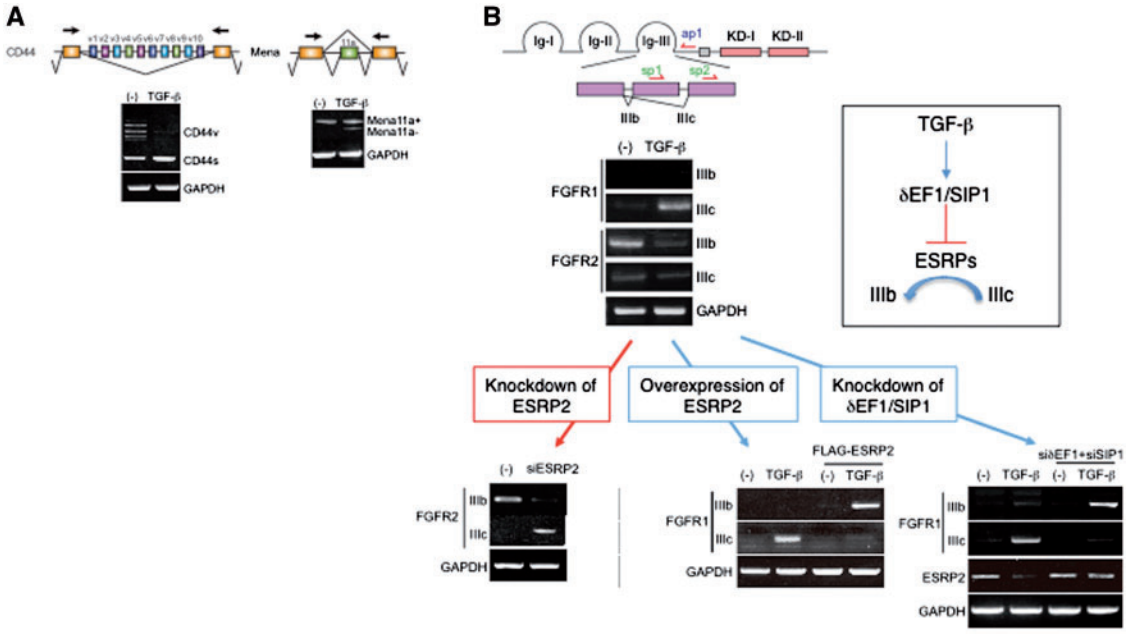

Fig. 4 Changes in alternative splicing during TGF-β-induced EMT. (originally published in *Oncogene*) (*64*) (**A**) Changes in alternative splicing of CD44 and Mena. Specific primers are shown as arrows (top panel). GAPDH was used as an internal control. CD44s, standard form of CD44; CD44v, splicing variants of CD44. (**B**) Isoform switching of FGFRs induced by TGF-β. Expression of the alternatively spliced forms of FGFR1 and FGFR2 was examined by RT–PCR. A schematic illustration of the primers is shown at the top. The primers for the IIIb and IIIc isoforms were the sp1–ap1 pair and sp2–ap1 pair, respectively. When ESRP2 is knocked down in mouse epithelial NMuMG cells (which do not express ESRP1), conversion from FGFR2IIIb to FGFR2IIIc can be observed. When ESRP2 is over-expressed or δEF1 and SIP are knocked down in TGF-β-treated NMuMG cells, FGFR1 is expressed as the IIIb rather than the IIIc isoform. Ig, extracellular immunoglobulin-like domain; KD, kinase domain.

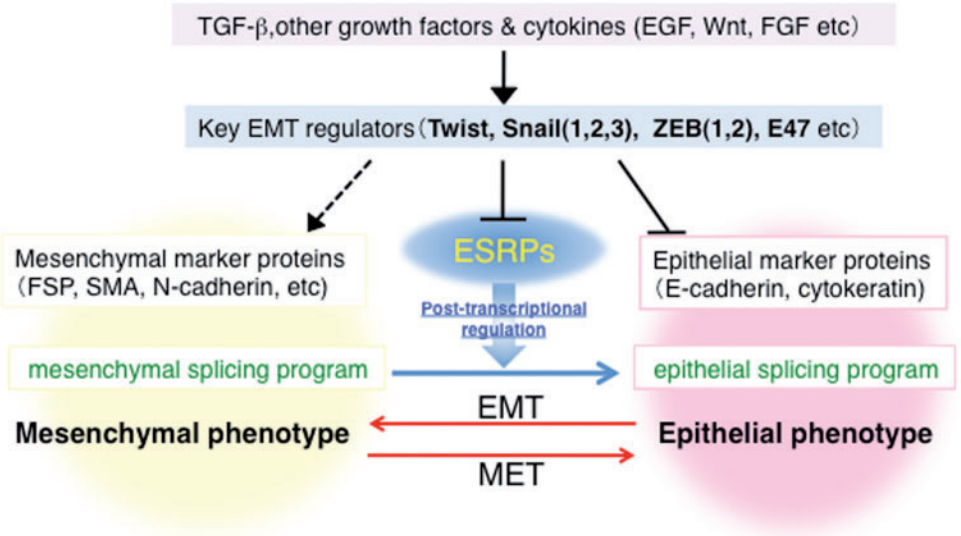

Fig. 5 Schematic illustration of TGF-β-mediated EMT. TGF-β, in collaboration with other growth factors or cytokines, modulates the expression of the key EMT regulators, and in turn represses the expression of ESRPs, leading to the EMT. FSP, fibroblast-specific protein; SMA, smooth muscle α-actin.

Conclusion

Understanding the mechanisms of EMT induction is clinically important with regard to both the diagnosis and future treatment of cancer. TGF-β, probably in collaboration with other growth factors or oncogenic signals, dramatically elicits EMT not only through transcriptional regulation of key EMT regulators such as Snail, Twist and δEF1/SIP1, but also through changes mediated by down-regulation of ESRPs in the alternative splicing programme of a range of genes (Fig. 5).

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Conflict of interest

None declared.

References

1. Miyazawa, K., Shinozaki, M., Hara, T., Furuya, T., and Miyazono, K. (2002) Two major Smad pathways in TGF-β superfamily signalling. *Genes Cells* **7**, 1191–1204
2. Feng, X.H. and Derynck, R. (2005) Specificity and versatility in TGF-β signaling through Smads. *Annu. Rev. Cell Dev. Biol.* **21**, 659–693
3. Derynck, R., Akhurst, R.J., and Balmain, A. (2001) TGF-β signaling in tumor suppression and cancer progression. *Nat. Genet.* **29**, 117–129
4. Yan, X. and Chen, Y.G. (2011) Smad7: not only a regulator, but also a cross-talk mediator of TGF-β signalling. *Biochem. J.* **434**, 1–10
5. Inoue, Y. and Imamura, T. (2008) Regulation of TGF-β family signaling by E3 ubiquitin ligases. *Cancer Sci.* **99**, 2107–2112
6. Gorska, A.E., Jensen, R.A., Shyr, Y., Aakre, M.E., Bhowmick, N.A., and Moses, H.L. (2003) Transgenic mice expressing a dominant-negative mutant type II transforming growth factor-β receptor exhibit impaired mammary development and enhanced mammary tumor formation. *Am. J. Pathol.* **163**, 1539–1549
7. Nagano, Y., Mavrakis, K.J., Lee, K.L., Fujii, T., Koinuma, D., Sase, H., Yuki, K., Isogaya, K., Saitoh, M., Imamura, T., Episkopou, V., Miyazono, K., and Miyazawa, K. (2007) Arkadia induces degradation of SnoN and c-Ski to enhance transforming growth factor-β signaling. *J. Biol. Chem.* **282**, 20492–20501
8. Sharma, V., Antonacopoulou, A.G., Tanaka, S., Panoutsopoulos, A.A., Bravou, V., Kalofonos, H.P., and Episkopou, V. (2011) Enhancement of TGF-β signaling responses by the E3 ubiquitin ligase Arkadia provides tumor suppression in colorectal cancer. *Cancer Res.* **71**, 6438–6449
9. 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
10. Massague, J. (2008) TGFβ in cancer. *Cell* **134**, 215–230
11. Muraoka, R.S., Dumont, N., Ritter, C.A., Dugger, T.C., Brantley, D.M., Chen, J., Easterly, E., Roebuck, L.R., Ryan, S., Gotwals, P.J., Koteliansky, V., and Arteaga, C.L. (2002) Blockade of TGF-β inhibits mammary tumor cell viability, migration, and metastases. *J. Clin. Invest.* **109**, 1551–1559
12. Hoshino, Y., Katsuno, Y., Ehata, S., and Miyazono, K. (2011) Autocrine TGF-β protects breast cancer cells from apoptosis through reduction of BH3-only protein, Bim. *J. Biochem.* **149**, 55–65
13. Caulin, C., Scholl, F.G., Frontelo, P., Gamallo, C., and Quintanilla, M. (1995) Chronic exposure of cultured transformed mouse epidermal cells to transforming growth factor-β 1 induces an epithelial-mesenchymal transdifferentiation and a spindle tumoral phenotype. *Cell Growth Differ.* **6**, 1027–1035

14. 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
15. Moustakas, A. and Heldin, C.H. (2007) Signaling networks guiding epithelial-mesenchymal transitions during embryogenesis and cancer progression. *Cancer Sci.* **98**, 1512–1520
16. Sabe, H. (2011) Cancer early dissemination: cancerous epithelial-mesenchymal transdifferentiation and transforming growth factor β signalling. *J. Biochem.* **149**, 633–639
17. Thiery, J.P., Acloque, H., Huang, R.Y., and Nieto, M.A. (2009) Epithelial-mesenchymal transitions in development and disease. *Cell* **139**, 871–890
18. Kalluri, R. and Weinberg, R.A. (2009) The basics of epithelial-mesenchymal transition. *J. Clin. Invest.* **119**, 1420–1428
19. Kudo-Saito, C., Shirako, H., Takeuchi, T., and Kawakami, Y. (2009) Cancer metastasis is accelerated through immunosuppression during Snail-induced EMT of cancer cells. *Cancer Cell* **15**, 195–206
20. Nieto, M.A. (2002) The Snail superfamily of zinc-finger transcription factors. *Nat. Rev. Mol. Cell Biol.* **3**, 155–166
21. Miettinen, P.J., Ebner, R., Lopez, A.R., and Derynck, R. (1994) TGF-β induced transdifferentiation of mammary epithelial cells to mesenchymal cells: involvement of type I receptors. *J. Cell Biol.* **127**, 2021–2036
22. Kowanetz, M., Valcourt, U., Bergstrom, R., Heldin, C.H., and Moustakas, A. (2004) Id2 and Id3 define the potency of cell proliferation and differentiation responses to transforming growth factor β and bone morphogenetic protein. *Mol. Cell. Biol.* **24**, 4241–4254
23. 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
24. Miyazono, K. and Miyazawa, K. (2002) Id: a target of BMP signaling. *Sci. STKE* **2002**, PE40
25. Gervasi, M., Bianchi-Smiraglia, A., Cummings, M., Zheng, Q., Wang, D., Liu, S., and Bakin, A.V. (2012) JunB contributes to Id2 repression and the epithelial-mesenchymal transition in response to transforming growth factor-β. *J. Cell Biol.* **196**, 589–603
26. Verschueren, K., Remacle, J.E., Collart, C., Kraft, H., Baker, B.S., Tylzanowski, P., Nelles, L., Wuytens, G., Su, M.T., Bodmer, R., Smith, J.C., and Huylebroeck, D. (1999) SIP1, a novel zinc finger/homeodomain repressor, interacts with Smad proteins and binds to 5′-CACCT sequences in candidate target genes. *J. Biol. Chem.* **274**, 20489–20498
27. 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
28. Shirakihara, T., Horiguchi, T., Miyazawa, M., 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
29. Nishimura, G., Manabe, I., Tsushima, K., Fujiu, K., Oishi, Y., Imai, Y., Maemura, K., Miyagishi, M., Higashi, Y., Kondoh, H., and Nagai, R. (2006) δEF1 mediates TGF-β signaling in vascular smooth muscle cell differentiation. *Dev. Cell* **11**, 93–104
30. Postigo, A.A. (2003) Opposing functions of ZEB proteins in the regulation of the TGF-β/BMP signaling pathway. *EMBO J.* **22**, 2443–2452
31. Bracken, C.P., Gregory, P.A., Kolesnikoff, N., Bert, A.G., Wang, J., Shannon, M.F., and Goodall, G.J. (2008) A double-negative feedback loop between ZEB1-SIP1 and the microRNA-200 family regulates epithelial-mesenchymal transition. *Cancer Res.* **68**, 7846–7854
32. 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
33. Cano, A., Perez-Moreno, M.A., Rodrigo, I., Locascio, A., Blanco, M.J., del Barrio, M.G., Portillo, F., and Nieto, M.A. (2000) The transcription factor Snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nat. Cell Biol.* **2**, 76–83
34. Herranz, N., Pasini, D., Diaz, V.M., Franci, C., Gutierrez, A., Dave, N., Escriva, M., Hernandez-Munoz, I., Di Croce, L., Helin, K., Garcia de Herreros, A., and Peiro, S. (2008) Polycomb complex 2 is required for E-cadherin repression by the Snail1 transcription factor. *Mol. Cell. Biol.* **28**, 4772–4781
35. 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
36. Yamashita, S., Miyagi, C., Fukada, T., Kagara, N., Che, Y.S., and Hirano, T. (2004) Zinc transporter LIVI controls epithelial-mesenchymal transition in zebrafish gastrula organizer. *Nature* **429**, 298–302
37. Zhou, B.P., Deng, J., Xia, W., Xu, J., Li, Y.M., Gunduz, M., and Hung, M.C. (2004) Dual regulation of Snail by GSK-3β-mediated phosphorylation in control of epithelial-mesenchymal transition. *Nat. Cell Biol.* **6**, 931–940
38. Wu, Y., Evers, B.M., and Zhou, B.P. (2009) Small C-terminal domain phosphatase enhances Snail activity through dephosphorylation. *J. Biol. Chem.* **284**, 640–648
39. Xu, Y., Lee, S.H., Kim, H.S., Kim, N.H., Piao, S., Park, S.H., Jung, Y.S., Yook, J.I., Park, B.J., and Ha, N.C. (2010) Role of CK1 in GSK3β-mediated phosphorylation and degradation of Snail. *Oncogene* **29**, 3124–3133
40. Wu, Y., Deng, J., Rychahou, P.G., Qiu, S., Evers, B.M., and Zhou, B.P. (2009) Stabilization of Snail by NF-κB is required for inflammation-induced cell migration and invasion. *Cancer Cell* **15**, 416–428
41. Peinado, H., Del Carmen Iglesias-de la Cruz, M., Olmeda, D., Csiszar, K., Fong, K.S., Vega, S., Nieto, M.A., Cano, A., and Portillo, F. (2005) A molecular role for lysyl oxidase-like 2 enzyme in Snail regulation and tumor progression. *EMBO J.* **24**, 3446–3458
42. Evdokimova, V., Tognon, C., Ng, T., Ruzanov, P., Melnyk, N., Fink, D., Sorokin, A., Ovchinnikov, L.P., Davicioni, E., Triche, T.J., and Sorensen, P.H. (2009) Translational activation of Snail1 and other developmentally regulated transcription factors by YB-1 promotes an epithelial-mesenchymal transition. *Cancer Cell* **15**, 402–415

43. Kim, N.H., Kim, H.S., Kim, N.G., Lee, I., Choi, H.S., Li, X.Y., Kang, S.E., Cha, S.Y., Ryu, J.K., Na, J.M., Park, C., Kim, K., Lee, S., Gumbiner, B.M., Yook, J.I., and Weiss, S.J. (2011) p53 and microRNA-34 are suppressors of canonical Wnt signaling. *Sci. Signal.* **4**, ra71
44. Wang, S.P., Wang, W.L., Chang, Y.L., Wu, C.T., Chao, Y.C., Kao, S.H., Yuan, A., Lin, C.W., Yang, S.C., Chan, W.K., Li, K.C., Hong, T.M., and Yang, P.C. (2009) p53 controls cancer cell invasion by inducing the MDM2-mediated degradation of Slug. *Nat. Cell Biol.* **11**, 694–704
45. Miraoui, H. and Marie, P.J. (2010) Pivotal role of Twist in skeletal biology and pathology. *Gene* **468**, 1–7
46. Yang, J., Mani, S.A., Donaher, J.L., Ramaswamy, S., Itzykson, R.A., Come, C., Savagner, P., Gitelman, I., Richardson, A., and Weinberg, R.A. (2004) Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. *Cell* **117**, 927–939
47. Firulli, A.B. and Conway, S.J. (2008) Phosphoregulation of Twist1 provides a mechanism of cell fate control. *Curr. Med. Chem.* **15**, 2641–2647
48. Zhou, B., Liu, Y., Kahn, M., Ann, D.K., Han, A., Wang, H., Nguyen, C., Flodby, P., Zhong, Q., Krishnaveni, M.S., Liebler, J.M., Minoo, P., Crandall, E.D., and Borok, Z. (2012) Interactions between beta-catenin and transforming growth factor-β signaling pathways mediate epithelial-mesenchymal transition and are dependent on the transcriptional co-activator cAMP-response element-binding protein (CREB)-binding Protein (CBP). *J. Biol. Chem.* **287**, 7026–7038
49. Kawata, M., Koinuma, D., Ogami, T., Umezawa, K., Iwata, C., Watabe, T., and Miyazono, K. (2011) TGF-β-induced epithelial-mesenchymal transition of A549 lung adenocarcinoma cells is enhanced by pro-inflammatory cytokines derived from RAW 264.7 macrophage cells. *J. Biochem.* **151**, 205–216
50. Oft, M., Akhurst, R.J., and Balmain, A. (2002) Metastasis is driven by sequential elevation of H-ras and Smad2 levels. *Nat. Cell Biol.* **4**, 487–494
51. 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
52. Peinado, H., Quintanilla, M., and Cano, A. (2003) Transforming growth factor-β 1 induces Snail transcription factor in epithelial cell lines: mechanisms for epithelial mesenchymal transitions. *J. Biol. Chem.* **278**, 21113–21123
53. Mulder, K.M. (2000) Role of Ras and Mapks in TGFβ signaling. *Cytokine Growth Factor Rev.* **11**, 23–35
54. Inman, G.J. (2011) Switching TGFβ from a tumor suppressor to a tumor promoter. *Curr. Opin. Genet. Dev.* **21**, 93–99
55. Pan, Q., Shai, O., Lee, L.J., Frey, B.J., and Blencowe, B.J. (2008) Deep surveying of alternative splicing complexity in the human transcriptome by high-throughput sequencing. *Nat. Genet.* **40**, 1413–1415
56. Wang, E.T., Sandberg, R., Luo, S., Khrebtukova, I., Zhang, L., Mayr, C., Kingsmore, S.F., Schroth, G.P., and Burge, C.B. (2008) Alternative isoform regulation in human tissue transcriptomes. *Nature* **456**, 470–476
57. Dutertre, M., Lacroix-Triki, M., Driouch, K., de la Grange, P., Gratadou, L., Beck, S., Millevoi, S., Tazi, J., Lidereau, R., Vagner, S., and Auboeuf, D. (2010) Exon-based clustering of murine breast tumor transcriptomes reveals alternative exons whose expression is associated with metastasis. *Cancer Res.* **70**, 896–905
58. Eswarakumar, V.P., Lax, I., and Schlessinger, J. (2005) Cellular signaling by fibroblast growth factor receptors. *Cytokine Growth Factor Rev.* **16**, 139–149
59. Warzecha, C.C., Sato, T.K., Nabet, B., Hogenesch, J.B., and Carstens, R.P. (2009) ESRP1 and ESRP2 are epithelial cell-type-specific regulators of FGFR2 splicing. *Mol. Cell* **33**, 591–601
60. Warzecha, C.C., Jiang, P., Amirikian, K., Dittmar, K.A., Lu, H., Shen, S., Guo, W., Xing, Y., and Carstens, R.P. (2010) An ESRP-regulated splicing programme is abrogated during the epithelial-mesenchymal transition. *EMBO J.* **29**, 3286–3300
61. Warzecha, C.C., Shen, S., Xing, Y., and Carstens, R.P. (2009) The epithelial splicing factors ESRP1 and ESRP2 positively and negatively regulate diverse types of alternative splicing events. *RNA Biol.* **6**, 546–562
62. Lamouille, S., Connolly, E., Smyth, J.W., Akhurst, R.J., and Derynck, R. (2012) TGF-β-induced activation of mTOR complex 2 drives epithelial-mesenchymal transition and cell invasion. *J. Cell Sci.* **125**, 1–15
63. Lamouille, S. and Derynck, R. (2007) Cell size and invasion in TGF-β-induced epithelial to mesenchymal transition is regulated by activation of the mTOR pathway. *J. Cell Biol.* **178**, 437–451
64. Horiguchi, K., Sakamoto, K., Koinuma, D., Semba, K., Inoue, A., Inoue, S., Fujii, H., Yamaguchi, A., Miyazawa, K., Miyazono, K., and Saitoh, M. (2012) TGF-β drives epithelial-mesenchymal transition through δEF1-mediated downregulation of ESRP. *Oncogene*, in press