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TGF-β in developmental and fibrogenic EMTs

Jun Ho Lee,

Joan Massagué*

Cancer Biology and Genetics Program, Sloan Kettering Institute, Memorial Sloan Kettering Cancer Center, New York, NY 10065, USA

Abstract

TGF-β plays a prominent role as an inducer of epithelial-mesenchymal transitions (EMTs) during development and wound healing and in disease conditions such as fibrosis and cancer. During these processes EMT occurs together with changes in cell proliferation, differentiation, communication, and extracellular matrix remodeling that are orchestrated by multiple signaling inputs besides TGF-β. Chief among these inputs is RAS-MAPK signaling, which is frequently required for EMT induction by TGF-β. Recent work elucidated the molecular basis for the cooperation between the TGF-β-SMAD and RAS-MAPK pathways in the induction of EMT in embryonic, adult and carcinoma epithelial cells. These studies also provided direct mechanistic links between EMT and progenitor cell differentiation during gastrulation or intra-tumoral fibrosis during cancer metastasis. These insights illuminate the nature of TGF-β driven EMTs as part of broader processes during development, fibrogenesis and metastasis.

Keywords

TGF-β; EMT; Development; Cancer; Fibrosis

1. Introduction

EMT is a key cellular process during embryogenesis, wound healing, fibrosis, and tumor progression. Epithelial cells undergoing EMT lose apicobasal polarity and adherent junctions while gaining mesenchymal traits including anteroposterior polarity, migration, and invasion of adjacent tissue. These changes additionally remodel cell contacts with neighboring cells and with basement membrane and other extracellular matrix (ECM) structures.

The ability to undergo EMT reflects the intrinsic phenotypic plasticity of epithelial stem and progenitor cells, which is essential for remodeling epithelial structures during developmental and regenerative processes. Cells that undergo an EMT frequently return to an epithelial

*Correspondence to: Memorial Sloan Kettering Cancer Center, Box 116, 1275 York Avenue, New York, NY 10065, USA. j-massague@ski.mskcc.org (J. Massagué).

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state, regaining apicobasal polarity and adherent contact with neighboring cells through a mesenchymal-to-epithelial transition (MET). Notably, EMT is no longer thought to be a binary alternation between extreme epithelial and mesenchymal phenotypic states. Rather, EMTs represent departures from the polarized epithelial phenotype the extent of which depends on the type of cell and the signals it receives [1,2]. Distinct epithelial progenitors undergoing an EMT may manifest different degrees of departure from the epithelial state, expressing different types of mesenchymal markers and phenotypic features.

EMT is orchestrated by transcription factors (TFs) called EMT-TFs, which repress epithelial genes and stimulate expression of mesenchymal components. Combinatorial expression of the core EMT-TFs Snail (encoded by *SNAI1*), Slug (*SNAI2*), ZEB1, ZEB2, TWIST1, and TWIST2 drives the mesenchymal state and represses the epithelial state [1,2]. Other transcription factors also contribute to EMT such as GATA3 and GATA6 [3,4], SOX9 [5], PRRX1 [6], Goosecoid (GSC) [7], Brachyury [8] and LBX1 [9], EMT is additionally balanced by a network of countervailing transcription factors and microRNAs.

Expression of EMT-TFs is regulated by extracellular signals and frequently involves cooperation between various signaling pathways that shape epithelial plasticity in a context-dependent manner. Although multiple signals can modulate EMTs, transforming growth factor-β (TGF-β) frequently plays a dominant role [1,10,11]. TGF-β activates the expression of EMT-TFs and triggers EMTs is diverse developmental and regenerative processes and in the diseases that coopt these mechanisms.

EMTs do not occur as isolated cellular events. The complex biological processes that involve EMTs also involve profound changes is epithelial tissue structure. For example, EMT and migration accompany mesoderm and endoderm differentiation of pluripotent epiblast cells during embryo gastrulation, re-epithelialization and tissue remodeling during wound healing, and collective cell invasion, proliferation, and intra-tumoral fibrosis during metastasis. In these diverse contexts, epithelial cells undergo changes in proliferation, differentiation, extracellular matrix remodeling, and secretome-mediated crosstalk with fibroblast, immune, and other stromal cell types. Mechanisms likely exist that coordinate EMT with these accompanying changes for tissue formation during development and regeneration.

The diversity of EMT-TFs, modulatory signals, and associated cellular responses poses a challenge in delineating the mechanistic basis for EMT and its coupling with distinct events. Recent work on the coordinated induction of EMTs during gastrulation and tumorigenesis provided insights into the molecular basis for EMT induction by collaborative TGF-β and RAS signals and how EMT is coupled with cell differentiation, apoptosis or fibrogenesis depending on the context [11–13]. Here we review the contextual nature of EMTs and their associated programs in the light of these insights and discuss the implications for a better understanding of TGF-β regulated EMTs as fundamental events in development and disease.

2. TGF-β as a regulator of EMTs in different contexts

The existence of epithelial-mesenchymal interface regulation in developmental processes controlled by TGF-β family members was observed in Müllerian duct regression in rat [14]. Direct evidence of the ability of TGF-β to induce epithelial phenotypic transitions was first reported in embryonic heart formation [15,16], in mouse mammary epithelial cells in culture [17], and later in many other epithelial cell types in culture and during development, fibrosis, and cancer [1,18]. During development, the TGF-β related Nodal and bone morphogenetic protein (BMP) pathways establish the body axes and direct the subsequent patterning of tissues. Nodal induces expression of *Snai1* and *Twist1* and drives an EMT program including loss of E-cadherin expression and gain of N-cadherin, key events during gastrulation [19]. Mouse embryos deficient for Snail fail to downregulate E-cadherin and exhibit defects in cell migration and the formation of mesoderm [20]. Additionally, Nodal induces the expression of Eomesodermin (Eomes) and Mesp, TFs that contribute to EMT during gastrulation. Different levels of Nodal signaling are required to specify and pattern the mesendoderm through effects beyond EMT [21,22]. For example, formation of the endoderm and the anterior mesoderm in the mouse requires more Nodal stimulation than posterior mesoderm, in line with the observation of Nodal gradient-dependent induction of endodermal TF *Foxa2* and mesodermal TFs *Gsc* and *brachyury*.

TGF-β induces Snail and Slug to drive EMT during cardiac valve formation [23]. TGF-β is associated with cells undergoing EMT at the interfaces of injured tissues and invasive tumors with the surrounding stroma [24,25]. TGF-β, and EMT, play a dual role in cancer, as clearly observed during pancreatic tumorigenesis. TGF-β acts as a tumor suppressor that eliminates pre-malignant cells harboring oncogenic KRAS mutations by triggering apoptosis, which results from a conflict between TGF-β dependent EMT and an epithelial progenitor enforcing program in these cells [12]. Cancer cell clones can bypass this pressure by decoupling TGF-β-induced EMT from apoptosis, which consequently allows EMT to support cancer cell migration, invasiveness, and metastasis, by turning TGF-β from a tumor suppressor into a tumor promoter [11,13, 26]. Moreover, cancer cells undergoing a TGF-β-induced EMT show resistance to chemotherapy and iradiation [27,28], and overexpression of Snail impairs cell cycle progression and apoptosis [29,30].

Thus, TGF-β (or Nodal signaling though the same pathway) is a potent inducer of different EMTs with different outcomes in different contexts (Fig. 1): morphogenesis and differentiation in gastrulation, organogenesis later in development, balanced epithelial regeneration in wound healing, apoptosis in pre-malignant stages, and invasive growth in tumor progression and metastasis.

3. The SMAD pathway as a transcriptional activator of EMTs

Gene regulation by TGF-β is executed through SMAD transcription factors. The context-dependent determinants and operating logic of the TGF-β-SMAD signaling pathway have been reviewed [10,11,31], and are summarized here only briefly (Fig. 2A). SMAD2 and SMAD3 are directly phosphorylated by the TGF-β receptor complex (or the Nodal/Activin receptor complex). Thus activated, SMAD2 and SMAD3 form trimeric complexes with

SMAD4 to transcriptionally regulate nuclear target genes increasing gene expression in most cases or repressing it in others. SMADs bind with low affinity to GC-rich DNA sequences but are directed to specific loci by interaction with context-dependent DNA-binding cofactors that recruit activated SMADs to loci genome-wide. Some of these cofactors are lineage-determining transcription factors (LDTFs) expressed in a developmentally programmed manner, while others are signal-driven transcription factors (SDTFs) activated by specific extracellular stimuli. The repertoire of specific DNA-binding SMAD partners in a cell, together with signal crosstalk inputs modulating SMAD activity and chromatin accessibility controlling the access to SMAD target loci, collectively shape the TGF-β response and determine the highly contextual and pleiotropic nature of TGF-β effects [10].

The forkhead box protein H1 (FOXH1) in mesendoderm precursors, zinc-finger protein 423 (ZFP423, also known as OAZ) in ventral mesoderm, MyoD1 in myoblast precursors, CCAAT/enhancer-binding protein-α (C/EBPα) in myeloid precursors, and GATA in erythroid precursors are classical examples of LDTFs dictating the genome binding pattern of SMADs to determine lineage commitment and cell differentiation within the lineage [32–35]. These LDTFs occupy cis-regulatory elements targeted by TGF-β-activated SMADs. FOXH1 acts as a pioneer transcription factor that binds to repressive chromatin to remodel it for subsequent recruitment and binding of activated SMADs [32,36,37]. In mesendoderm differentiation genes, FOXH1 can recruit SMAD3 in the absence of Nodal signaling, but Nodal is still required for the activation and recruitment of SMAD2 and SMAD4 to the FOXH1-SMAD3 pre-bound complex [32]. Other LDTFs may similarly act as pioneer factors that locally remodel target loci for the eventual recruitment of ligand-activated SMADs.

Alongside LDTFs, SDTFs activated by certain signals also participate in directing SMADs to specific loci. Examples include AP-1 transcription factors in the regulation of the plasminogen activator protease inhibitor SerpinE1 (also known as PAI-1) [38] and matrix metalloproteinases [39, 40], and FOXO transcription factors in the regulation of cyclin-dependent kinase (CDK) inhibitors p15INK4b (encoded by *CDKN2B*) and p21CIP1 (*CDKN1A*) [41]. Activated SMAD complexes cooperate with different co-activators, co-repressors, chromatin remodelers, and chromatin readers to shape transcriptional regulation of target genes. Early examples include SMAD interactions with the CBP and p300 histone acetyltransferases [42,43], the TGIF histone deacetylase adaptor [44], and the BRG subunit of the SWI-SNF chromatin remodeler complex [45,46].

TGF-β triggers EMTs by inducing the expression of the EMT-TFs Snail and/or Slug, which repress the expression of the epithelial adherens junction molecule E-cadherin (encoded by *CDH1*) [47–49] and the epithelial progenitor transcription factor KLF5 [12]. SMAD transcription factors are central mediators of TGF-β-induced EMTs in many contexts. For example, SMADs are required for TGF-β induction of *Snai1* and/or *Snai2* in kidney epithelial cells [50,51], mammary epithelial and breast carcinoma cells [51–53], hepatocytes [54], lens epithelial cells [55], and human pancreatic ductal adenocarcinoma (PDAC) cells [56]. TGF-β-activated SMADs may cooperate with EMT-TFs in subsequent waves of transcriptional regulation. For example, TGF-β activated SMADs cooperate with Snail to downregulate the expression of epithelial markers such as E-cadherin, occludin, and

claudin-3 in mouse mammary epithelial cells [57], and *Klf5* in mouse pancreatic ductal adenocarcinoma cells [12]. In turn ZEB1 represses epithelial markers by recruiting co-repressors together with SMAD3 [58].

Various additional cofactors have been identified as important participants in SMAD-mediated induction of EMT-TF expression, including the enhancer organizing factor HMGA2 [59–61], the histone demethylases KDM1A (LSD1) [62] and KDM6B (JMJD3) [63], the COMPASS complex component RBBP5 [64], the histone methyltransferase EZH2 [65], and the SWI/SNF chromatin remodeling complex core component BRG1 [45]. ZEB expression by TGF-β additionally involves the transcription factor ETS1 [66,67].

4. TGF-β and RAS cooperate in the induction of EMTs

Notably, TGF-β induces EMT in epithelial cells under certain conditions and not in others depending on inputs from other pathways. RAS-MAPK signaling is a particularly important determinant of a cell's competence to undergo EMT in response to TGF-β, as first observed in mouse mammary epithelial cells [68–70]. Mammary ductal morphogenesis and gland formation involves EMT [71] and mammary gland development requires EMT-TFs [72]. TGF-β induction of EMT in mammary epithelial cells requires ERK MAPK activity [73]. TGF-β cooperates with RAS-MAPK signaling to induce EMTs in normal and malignant mammary, kidney, liver, pancreatic and lung epithelial cells [12,70, 74–81]. Oncogenic RAS mutations enable TGF-β-SMAD signaling to induce EMT in cancer cells [12,13,77,82]. The cooperation of TGF-β and RAS pathways allows carcinoma cells at the tumor invasive front to undergo EMT, migration and hematogenous dissemination [83]. During gastrulation, Nodal requires FGF and WNT signals to activate mesendoderm differentiation and drive an EMT [18,84–87]. Epiblast cells in Fgfr1-null mice fail to downregulate E-cadherin and do not undergo EMT [84]. In sum, although various pathways cooperate with the TGF-β pathway during processes that involve EMTs, the RAS-MAPK pathway stands out as a potent partner of TGF-β in triggering EMTs.

In principle, the involvement of RAS signaling in TGF-β induction of EMT could be due to a role of RAS-MAPK directly downstream of TGF-β receptors. TGF-β addition to cells in culture can activate the ERK MAPK, p38 MAPK and PI3K pathways [88,89], and several reports suggested that TGF-β receptors trigger EMT by activating ERK MAPK [73], p38 MAPK [88] or PI3K pathways [90]. While the biochemical and structural basis for the coupling of TGF-β receptors to these pathways is not clear, each of these pathways is potently and directly activated by well-established receptor tyrosine kinase ligands, cell metabolism effectors, and cellular stresses. In developing tissues and tumors TGF-β operates in the presence of these potent MAPK and PI3K agonists, therefore raising questions about the significance of TGF-β as an activator of MAPKs or PI3K for the induction of EMTs in vivo.

An alternative hypothesis is that the synergy between TGF-β-SMAD and RAS-MAPK as inducers of EMTs is based on a convergence of these pathways, each activated by its corresponding agonists, to bring about strong activation of EMT-TF expression. Support for this model comes from experiments using inducible expression of an oncogenic RAS allele

(KRAS^{G12D}) in mouse pancreatic organoids. In these experiments, TGF-β strongly induced Snail expression and EMT when KRAS^{G12D} was turned on, but not when it was turned off [12,13,26]. Intriguingly, other work showed that knockdown of KRAS in human PDAC cells prevented the downregulation of E-cadherin, while sparing other TGF-β gene responses intact [77], suggesting a selective participation of RAS in some but not all TGF-β gene responses.

5. TGF-β and RAS pathways converge on RREB1 for EMT induction

The preceding observations raised the possibility that a RAS-dependent transcription factor is required for SMAD-driven expression of EMT-TFs, but not for other SMAD-dependent responses (Fig. 2B). This hypothesis was confirmed with the identification of RAS-responsive element binding protein 1 (RREB1) as a RAS-MAPK-activated partner of SMADs in the transcriptional activation of *Snai1* together with a specific subset of TGF-β target genes [13] (Fig. 2A). Motif analysis of SMAD binding sites in RAS-dependent and RAS-independent TGF-β target genes revealed an enrichment for RREB1 binding elements in genes requiring RAS input for activation. In an orthogonal approach, a genetic screen for transcription factors required in PDAC cells for TGF-β-dependent apoptosis identified SOX4 and RREB1 as hits. Biochemical characterization showed that N-terminal phosphorylation of RREB1 by ERK induces its binding to DNA. In cells with high levels of RAS-MPK activity, RREB1 is pre-bound to these target genes prior to stimulation of cells by TGF-β [13]. RREB1 is essential for SMAD activation of *Snai1* in PDAC cells, lung adenocarcinoma (LUAD) cells, mammary epithelial cells (which harbor high ERK activity levels), and mouse embryonic stem cells. Smad4-restored PDAC cells harboring oncogenic KRAS mutant, where TGF-β-induced EMT triggers apoptosis [12], grew poorly as subcutaneous tumors in mice compared to *Rreb1*-knockout counterparts. In contrast, TGF-β- and RREB1-dependent EMT in KRAS-mutant LUAD is decoupled from apoptosis and instead promotes the growth of pulmonary metastases [13].

RREB1 contains 15 C2H2 zinc-fingers (ZFs) distributed in three clusters (Fig. 2C) and exists in various splice variants. The RREB1 orthologue in *Drosophila*, known as *hindsight* or *pebbled*, mediates collective migration of border cells [91]. Previous work on this relatively obscure RAS effector showed that RREB1 activates or represses transcription depending on context. In mammalian adipose tissues, RREB1 selectively recruits JMJD3 to brown fat-specific genes and removes H3K27me3 to activate their gene expression, which leads to development of beige adipocytes [92]. In pancreatic endocrine β cells, RREB1 occupies the promoter of insulin genes and recruits CtBP-LSD1 to stimulate gene transcription by removal of repressive histone marks [93]. In colorectal adenocarcinoma, RREB1 mediates KRAS repression of miR-143/145 expression [94].

RREB1 shows alterations in cancer, and particularly in PDAC tumors, 95 % of which harbor *KRAS* mutations. Heightened RAS signaling in premalignant pancreatic progenitor cells leads to TGF-β-induced EMT coupled with apoptosis. Nearly half of human PDAC show a genetic loss of *SMAD4* or other components of the TGF-β pathway, thus disabling TGF-β-mediated tumor suppression. Expression of *RREB1* is markedly downregulated in

early stage of PDAC [95], and truncating RREB1 mutations occur in a small but significant proportion of human PDAC [96].

6. TGF-β EMTs are part of broad developmental and regenerative programs

6.1. TGF-β EMTs in gastrulation and other developmental stages

EMT is essential for gastrulation, which generates the three germ layers –endoderm, mesoderm, and ectoderm–from pluripotent epiblast cells. Epithelial epiblast cells exhibit apical-basal polarity and express E-cadherin, as these cells lose pluripotency they undergo EMT to migrate to different locations. The first stage of gastrulation is the formation of a primitive streak within the epiblast epithelial layer where EMT is induced by Nodal-activated SMAD signaling [97].

Nodal-activated SMADs integrate multiple inputs by cooperating with other transcription factors, resulting in the parallel induction of EMT-TFs and differentiation genes, which orchestrate epiblast cell migration and differentiation. Genome-wide prebound FoxH1 directs Nodal-activated SMAD2/3 to target loci and jointly binds to the regulatory elements of mesendoderm differentiation genes, including *Eomes*, *Brachyury*, *Gsc*, *Mixl1*, and *Foxa2* [32]. FoxH1-occupied *cis*-elements are poised along with repressive histone mark H3K9me3. To resolve chromatin accessibility, Nodal-activated SMAD3 cooperates with E3 ubiquitin-protein ligase TRIM33. TRIM33 interacts with histone marks at regulatory loci and displaces the interaction of histone reader heterochromatin protein 1 (HP1) with the histone marks, resulting in the activation of mesendodermal gene expressions [98].

The neural crest is a transient group of progenitor cells characterized by multipotency and migratory ability. After specification, pre-migratory neural crest precursors undergo EMT and delaminate from the neuroepithelium to migrate within the embryo and in a context-specific manner, where they contribute to the development of a variety of organs. BMP secreted from the dorsal neural tube induces segregation of pre-migratory neural crest cells and initiates the EMT program [99]. WNT induces BMP expression and provides ground for the cooperation of the BMP-Wnt pathways to induce *Snai1*, *Snai2*, and *Sox9* [100]. TGF-β also endows endothelial plasticity in heart development via endothelial-mesenchymal transition (EndMT) which leads to heart valve formation [101].

6.2. TGF-β EMTs in wound healing and tissue fibrosis

Wound repair is a fundamental property of multicellular organisms to restore tissue integrity and homeostasis. Postnatal wound repair results in a non-functioning mass of fibrotic scar conceivably due to inflammatory responses. Fibrosis, defined by the excessive accumulation of ECM components, is an outcome of dysregulated regenerative responses, such as pathological activation of repair with sustained inflammation and impaired termination. TGF-β-induced EMT plays a prominent role in the regenerative processes, as a high level of TGF-β is linked to EMT-like changes in tissue fibrosis [50,102,103]. Both in wound healing and tissue fibrosis, EMT is associated with a fibrogenic program to restore tissue architecture, and TGF-β signaling drives pro-fibrotic events in wound healing and tissue

fibrosis, such as inflammation, epithelial cell migration, fibroblast recruitment, and ECM deposition.

During wound healing, re-epithelialization, characterized by the migration and proliferation of epithelial cells at the edge of damaged tissue serves to replenish tissue integrity and structure, and involves EMTs [1,104]. Single-cell transcriptome analysis shows induction of EMT gene signatures at wound sites in mice [105]. A loss of epithelial features and increased cell motility provided by a reversible EMT supports tissue homeostasis [106–108]. Injured tissue is exposed to multiple growth factors and inflammatory cytokines. Platelets, neutrophils, and monocyte-derived macrophages are key initial sources of TGF-β, which induces EMT [109]. EGF, FGF, and HGF stimulate corresponding receptors and activate downstream signaling cascades, such as MAPK pathways [110]. Activated MAPK upregulates EMT-TFs such as Snail, Slug, and ZEB [1,111], and accelerates cutaneous wound healing [112], likely in combination with TGF-β signaling.

In cutaneous wounds, TGF-β is not only an inducer of keratinocyte migration but also a potent chemoattractant for endothelial cells and fibroblasts. The dermis is restored by invading and proliferating fibroblasts that generate ECM components, mainly collagen, which ultimately forms the bulk of the mature scar. Myofibroblasts, the differentiated form of fibroblasts and the primary cell type supporting matrix-preservation, aid in the synthesis of ECM proteins and wound closure. A dominant pro-fibrotic role of TGF-β is the differentiation of fibroblasts into myofibroblasts [113], and pro-fibrotic factors such as tenascin-C, which is induced by TGF-β, support the recruitment and differentiation of fibroblasts at the wound site [114].

The pro-fibrotic actions of TGF-β are linked, in part, to their effects on epithelial cells during wound healing in which pro-fibrotic factors are secreted in response to TGF-β. TGF-β activates a matrix-preserving program including production of interstitial collagens [115] and fibronectin [116]. Scar formation after a stab wound to the cerebral cortex is reduced in *Smad3*-null mice [117]. Recent studies on the contribution of EMT to renal fibrosis suggest that epithelial cells undergoing EMT activated other gene programs that resulted in tissue fibrosis [118,119]. Snail-induced partial EMT in kidney epithelial cells gave rise to inflammation during fibrosis. Partial EMT in tubular epithelial cells is not coupled to their differentiation into myofibroblasts, rather, it is coupled to inflammatory gene activation. A parallel regulation of EMT and fibrogenesis by may be beneficial in acute tissue injury, while chronically it may lead to reduced organ function in tissue fibrosis or tumor progression in malignancy

6.3. TGF-β EMTs in tumor suppression

The tumor-suppressive function of TGF-β is manifest through genetic loss-of-function in TGF-β pathway components. Prominent examples include *SMAD4* mutations in PDAC [96,120] and *TGFBR2* mutations in colorectal cancers [121]. Genetic inactivation of TGF-β signaling components allows pre-malignant cells to avoid apoptosis induced by TGF-β. TGF-β induces expression of cyclin-dependent kinase inhibitors, which slow down the cell cycle, in normal epithelial cells [122–124], whereas carcinoma cells bypass the effectiveness of these CDK inhibitors by harboring CDK activating signals. However, TGF-β signaling

induces apoptosis in pancreatic premalignant progenitor cells that harbor KRAS oncogenic mutations [125]. Recent work delineated how TGF-β-induced EMT is coupled to apoptosis (Fig. 3A). TGF-β induces Snail, which then suppresses the expression of transcription factor KLF5. SOX4 is a genome-wide binding partner of KLF5 in pancreatic epithelial progenitors. Downregulation of KLF5 by Snail deprives SOX4 from this cofactor and leads SOX4 to activate expression of proapoptotic factors including BIM and BMF, a process termed lethal EMT [12].

6.4. TGF-β EMTs in tumor progression and metastasis

TGF-β-induced EMT is implicated in tumor progression and metastasis [1,126]. Carcinoma cells that progress by decoupling TGF-β from apoptosis can undergo TGF-β dependent EMT and adopt a highly plastic phenotype that facilitates tumor growth and metastasis (Fig. 3B), in addition to immune suppressive protection and other supportive effects of TGF-β in the tumor microenvironment. Carcinoma cells undergoing EMT at the primary site gain invasive and migratory activity for intravasation into blood and lymphatic vessels becoming circulating tumor cells (CTCs) [127], and may further benefit from this EMT during extravasation after lodging in distant organ capillaries minutes later. However, carcinoma cells can also depart from tumors as small epithelial clusters which are highly metastatic [128,129]. CTC clusters become coated with platelets in the blood circulation [130]. It has been shown that platelet-derived TGF-β induces EMT in associated CTCs to stimulate cancer cell extravasation [131]. After CTCs infiltrate distant organs, TGF-β controls dormancy of the disseminated cells [132,133]. TGF-β induces the entry of disseminated carcinoma cells into a non-proliferative dormant state [134]. Dormancy is an immune evasive state that protects cancer cells from elimination by the immune system [135]. It is not known whether TGF-β induced dormancy is accompanied by EMT. However, the outgrowth of metastasis initiating cells requires downregulation of *Twist*, *Prrx1* and other EMT-TFs and return to an epithelial phenotype through an MET [6,136,137].

Cancer cell populations are heterogeneous in their EMT response to TGF-β. Although EMT is a common mechanism for metastatic dissemination, the cells that are most competent at forming metastases are not necessarily those which adopt the most mesenchymal-like phenotype as they undergo EMT [138]. The basis for this heterogeneity of EMT responses to TGF-β, and which forms of EMT are most effective at mediating specific steps in the metastatic cascade [139,140] are currently unknown.

TGF-β-induced EMTs are accompanied by fibrogenic activation of stromal fibroblasts not only during wound healing and fibrotic diseases, but also in primary and metastatic tumors. Cancer cells undergoing an EMT in response to TGF-β secrete pro-fibrotic factors [13], suggesting that EMT and fibrogenesis are parts of an orchestrated program (Fig. 3B). Intra-tumoral fibrosis promotes tumor growth by supporting collective cancer cell migration and cancer cell proliferation on stiff extracellular matrices. An association between fibrosis and cancer has been noted [141]. Studies have shown a strong association between fibrotic microenvironment and tumor progression [142–144]. Cancer cells in primary tumors orchestrate the recruitment and activation of stromal cells that build up desmoplasia. A fibrotic environment with increased deposition of fibrillar collagen,

hyaluronic acid, fibronectin, and tenascin-C is conducive to multiple steps of metastasis. A TGFβ-driven, LRRC15^{+} cancer-associated fibroblasts (CAFs) lineage portends poor outcome in immunotherapy trials in different types of carcinoma [145].

7. RREB1 coordinates EMTs with developmental and fibrogenic gene expression programs

The preceding sections highlight the fact that TGF-β pathway drives cells into EMTs as part of broader developmental or regenerative programs which are closely associated with other changes that TGF-β is also known to induce, notably the differentiation of germ layers, and fibrogenesis in the context of wound healing, fibrosis, and cancer. If TGF-β signaling drives EMT together with fibrogenesis or differentiation in a context-dependent fashion, might the activation of EMT be directly and specifically coupled with the activation of these accompanying programs and an essential feature of the developmental or regenerative processes?

The identification of RREB1 as a common nexus for the TGF-β-SMAD and RAS-MARK pathways in the induction of Snail expression and EMT unexpectedly shed light on this question [13]. The subset of TGF-β responsive genes that require RAS activity and RREB1 in PDAC, LUAD and mammary epithelial cells includes not only *Snai1,* but also a set of genes encoding secreted fibrogenic factors and matrix remodeling molecules. These factors include interleukin-11, PDGFB, WISP1, SerpinE1 and HAS2. RREB1 binds to the regulatory regions of these genes in a RAS-MAPK-dependent manner, as it does in the regulatory region of *Snai1* (Fig. 4A). Moreover, inhibiting EMT by knockout or knockdown of *Snai1* and *Zeb1* in LUAD cells did not interfere with the induction of these fibrogenic factors by TGF-β [13]. Thus, RREB1 connects EMT and fibrogenic effects of TGF-β as important yet distinct facets of a common program in adult epithelial cells, providing a mechanism for the coordination of EMT and fibrogenesis during normal tissue remodeling and in cancer. In similar but contrasting fashion, the set of Nodal responsive genes that RREB1 activates in mouse embryo mesendoderm precursor cells include not only *Snai1* and *Snai2,* but also the key mesendoderm differentiation genes including *Eomes, Gsc, Mixl1 and Brachyury* [13]. The coupled induction of EMT-TFs and mesendoderm differentiation genes provides a mechanism for the coordination of EMT and differentiation during gastrulation (Fig. 4B).

Insight into why different sets of genes are co-regulated by SMAD and RREB1 together with EMT-TFs in different contexts was provided by chromatin accessibility analysis in mouse mesendoderm embryoid bodies and in PDAC cells [13]. The chromatin at SMAD2/3 and RREB1 binding sites in the *Snai1* promoter was accessible both in mesendoderm progenitors and PDAC cells. In contrast, the chromatin at SMAD2/3 and RREB1 binding sites in *Gsc* and *Mixl1* was accessible in mesendoderm progenitors, but not in PDAC cells, and the contrary was true of chromatin accessibility in *Wisp1* and *Serpine1* (Fig. 4C). Thus, different chromatin patterns enable signal-activated SMAD and RREB1 to access either fibrogenic or mesendoderm programs in these two distinct cellular contexts, and access *Snai1* in both contexts.

8. Perspectives

In this review, we have highlighted how cells utilize TGF-β signals to trigger EMTs in different contexts and for different purposes. The regulation of EMT and its associated programs provides a clear example of convergence of three proposed classes of determinants shaping the contextual nature of TGF-β effects [10], namely, SMAD regulatory signals (RAS-MAPK in this case), SMAD transcriptional partners (RREB1), and the chromatin status of target loci. Combined, these three classes of determinants shape TGF-β induced EMTs as part of broader programs in different physiological and pathological contexts [13].

Despite the recent progress in understanding the basic principles of TGF-β signaling and biology, we still lack a complete understanding of transcriptional regulation by TGF-β and its diverse roles under different conditions. For example, we do not know why certain SMAD target genes require RREB1 for activation and others do not. Although RREB1 is required for transcriptional activation of all RAS-dependent TGF-β gene responses, it is required for SMAD binding to the regulatory regions of only some of these, and more so in PDAC cells than in mammary epithelial cells [13]. If the main function of RREB1 is not recruiting SMADs to target genes (FOXH1 serves this function in mesendoderm progenitors [32,98]), what biochemical functions then does RREB1 bring to its collaboration with SMADs in the transcriptional activation of these genes?

In the context of fibrosis, if TGF-β is a direct and potent activator of stromal fibroblasts, what is the role of the TGF-β-induced production of fibrogenic factors by epithelial cells undergoing EMT? Do the epithelial derived fibrogenic factors serve to complement the direct effects of TGF-β on stromal fibroblasts? How important is this contribution, and when in the course of a wound healing or a fibrotic process?

The simplicity of the scheme in Fig. 2A belies hidden complexities that our current knowledge cannot explain. For example, in mouse embryonic stem cells which form embryoid bodies recapitulating signaling and lineage specification events of gastrulation, absence of RREB1 causes a complete loss of EMT and mesendoderm differentiation responses to Nodal [13]. However, when these *Rreb1*$^{-/-}$ ESCs were injected into wild-type mouse blastocysts and allowed to develop in utero, the resulting chimeric embryos showed gastrulation defects, but the *Rreb1*$^{-/-}$ cells in these embryos did not exhibit an absolute EMT block [13]. Moreover, germline *Rreb1*-null mice showed altered organization of actin filaments and adherens junctions within the epiblast, perturbed epithelial architecture, and ectopic migration through the underlying basement membrane, but many of the cells still underwent EMTs [146]. These observations suggest that Nodal- and RREB1-dependent EMTs are amalgamated with other EMT programs in embryos, where the absence of RREB1 activity may be partly buffered by other transcriptional cofactors.

To address some of these questions, it will be important to identify other critical components of SMAD-RREB1 complexes. This would provide a more complete understanding of the biochemical function of RREB1 in transcriptional regulation and reveal factors that modulate the function of this complex and partly buffer the loss of RREB1 in the precise execution of TGF-β dependent EMT programs. Defining the distinct chromatin features

of TGF-β target genes in different EMT-associated contexts will also further clarify the requirement for a collaboration between TGF-β-SMAD and RAS-MAPK pathways through RREB1. Addressing these and other related questions will be important towards advancing our understanding of TGF-β EMTs in development, regeneration, fibrosis, and cancer.

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Data availability

No data was used for the research described in the article.

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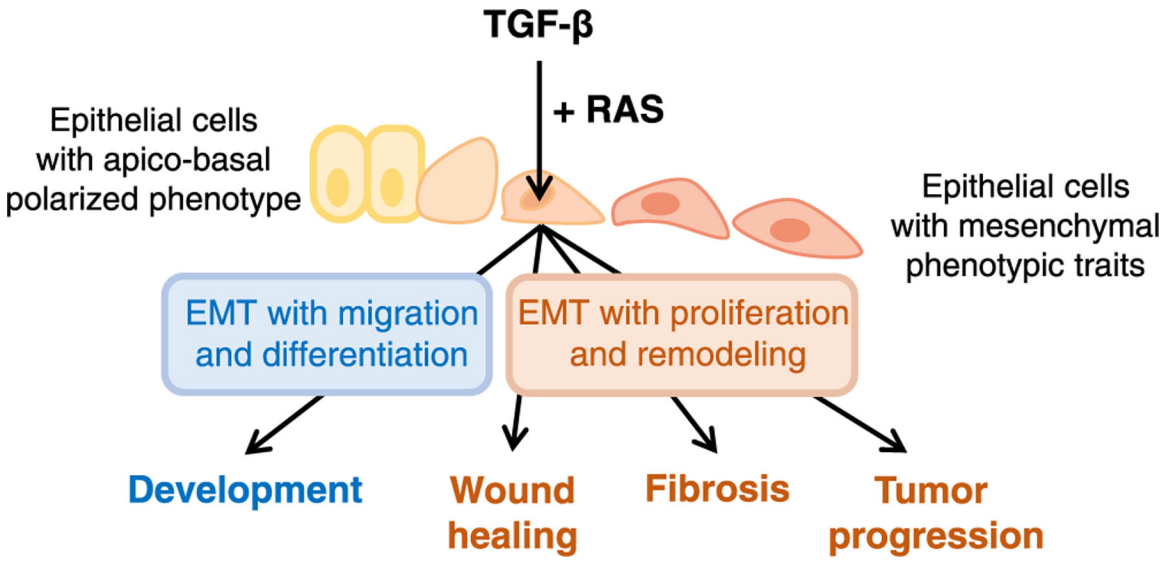

Fig. 1.

TGF-β as regulator of EMTs in different contexts. TGF-β triggers EMTs in the context of complex developmental, regenerative, and pathological processes that also involve profound changes in cell proliferation, differentiation, position, and extracellular matrix remodeling. In developmental contexts such as gastrulation, EMT driven by Nodal TGF-β signaling occurs together with mesendodermal cell differentiation. In regenerative contexts such as wound healing and their derivative pathologies –fibrosis and cancer–TGF-β driven EMT occurs alongside fibrogenic effects that remodels the extracellular matrix. Other signals converge with TGF-β on the regulation of EMT. RAS signaling is a particularly powerful collaborating signal in TGF-β induction of EMTs in diverse contexts.

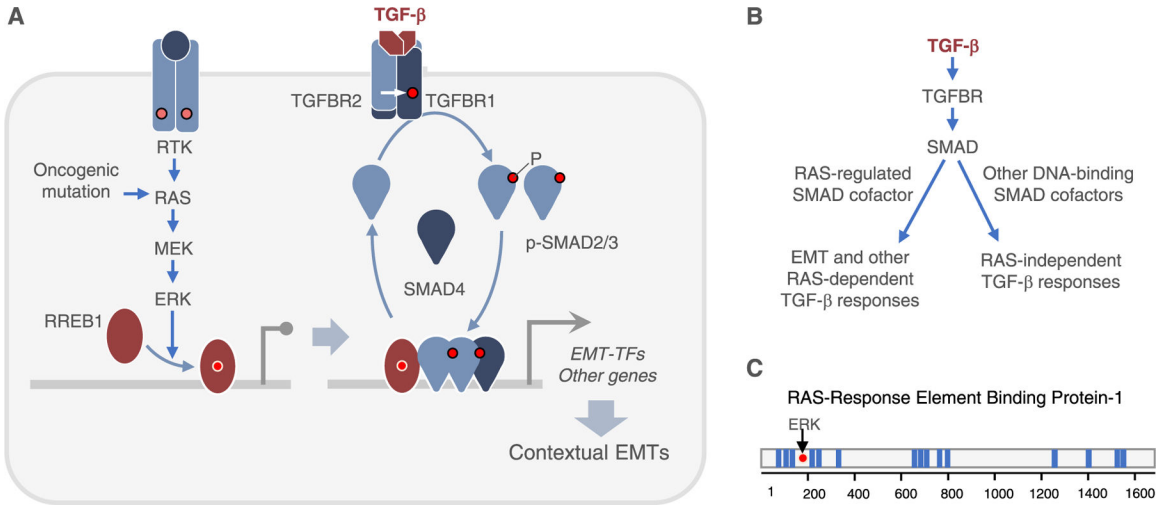

Fig. 2.

TGF-β and RAS collaboration in the induction of EMTs. A. RAS-MAPK signaling is an determines a cell's competence to undergo EMT in response to TGF-β. RAS activation by receptor tyrosine kinases (RTK) or oncogenic mutation leads the ERK phosphorylation of RREB1, enabling its binding to cognate sites genome-wide. RREB1 binds near SMAD binding sites. TGF-β receptors activate SMAD transcription factors for activation of EMT-TFs and other genes in collaboration with RREB1, triggering different types of EMTs in different contexts. B. Only a subset of TGF-β-SMAD target genes depend on RAS-MAPK input for activation. RREB1 collaborated with SMADs in the activation of these RAS-dependent genes. C. RREB1 contains 15 zinc-finger domains (*blue bars*) distributed in three clusters. ERK phosphorylation of a canonical MAPK site in the N-terminal domain of RREB1 stimulates binding to DNA.

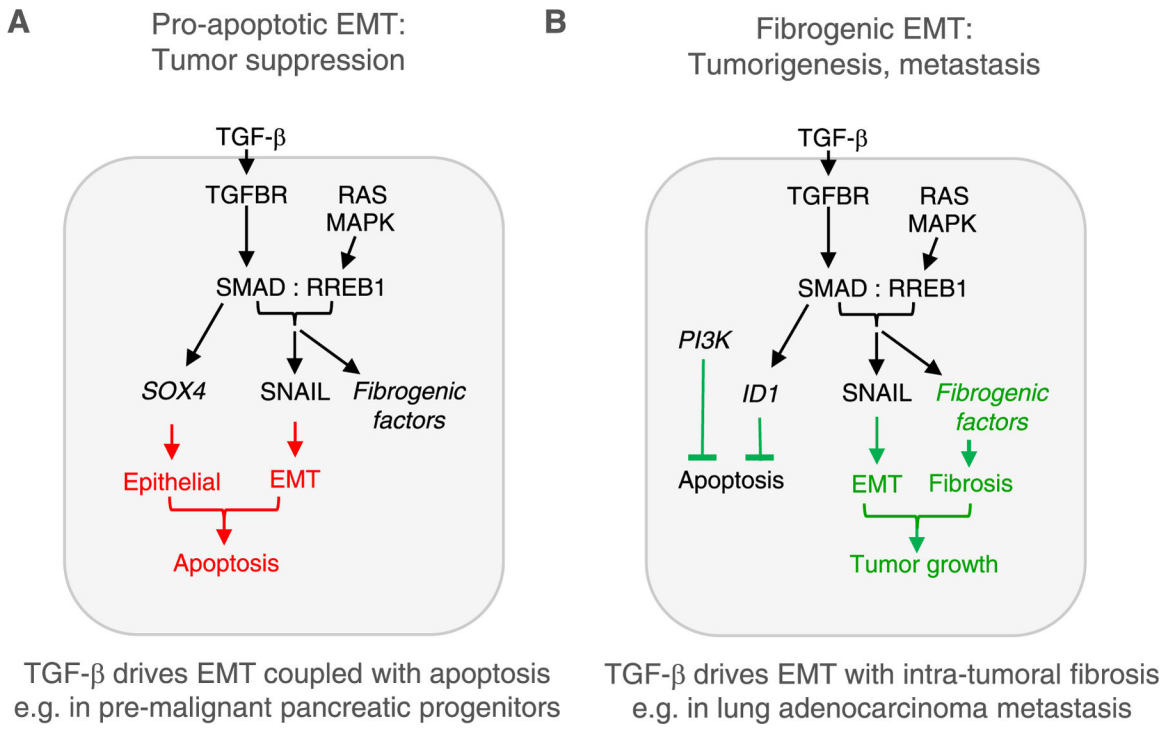

Fig. 3.

TGF-β EMTs in tumor suppression and tumor progression. TGF-β induces EMTs in malignant cells in collaboration with RAS signaling, a frequently hyperactivated pathway in carcinoma cells. A. In pre-malignant pancreatic progenitors, strong activation of EMT by collaboration of TGF-β with oncogenic, hyperactive RAS signaling enter into a conflict with a SOX4-dependent epithelial program and this conflict triggers apoptosis. A pro-apoptotic or lethal EMT thus explains the tumor suppressive action of TGF-β in pancreatic cancer. B. Carcinoma cells decouple EMT from apoptosis by various mechanisms and use TGF-β induced EMT and associated fibrogenic gene response for invasion and metastatic outgrowth, as observed in lung adenocarcinoma models.

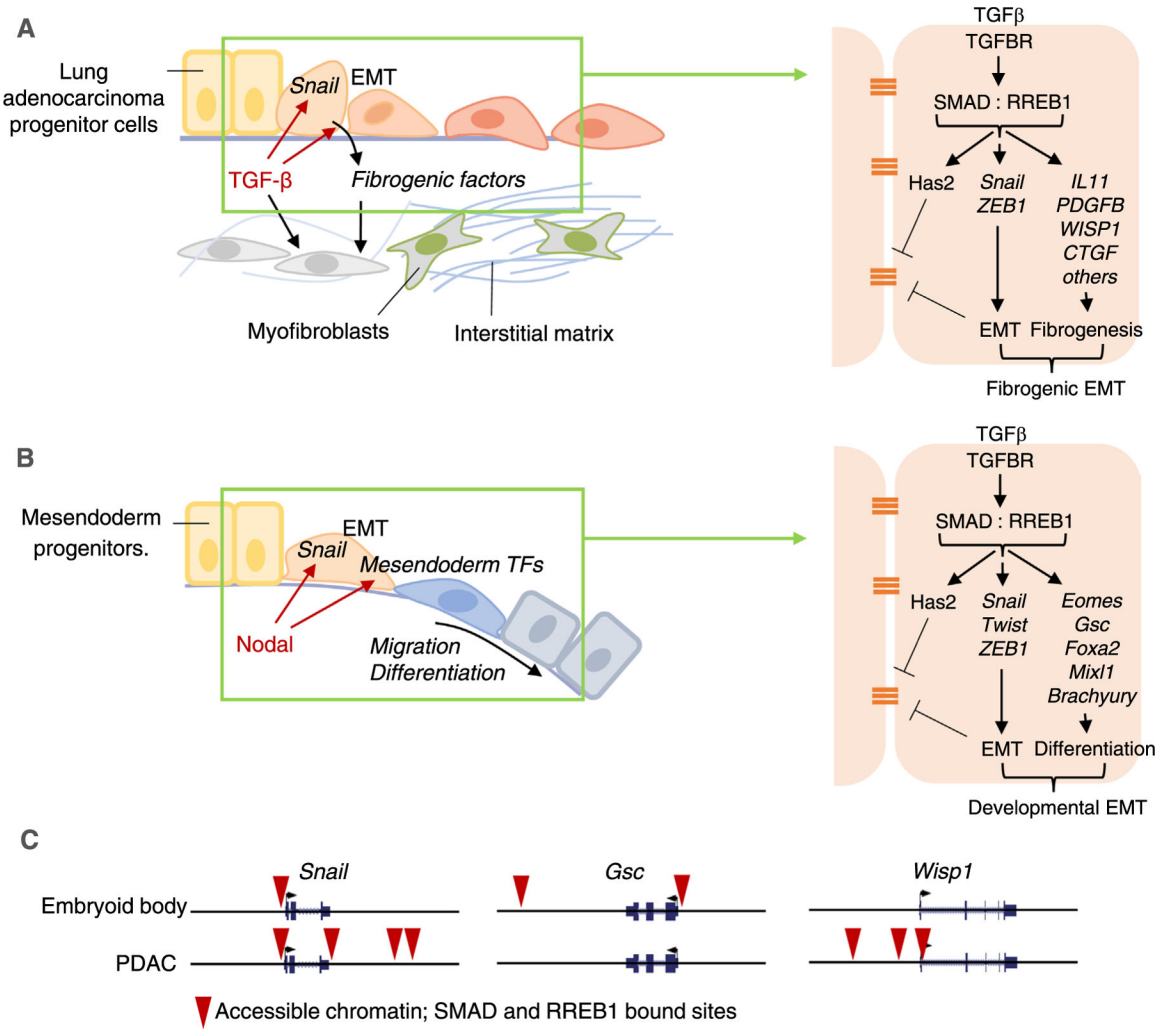

Fig. 4.

RREB1 coordinates EMTs with developmental and fibrogenic gene expression programs. A. In various types of adult epithelial cells and carcinoma cells, TGF-β induces a fibrogenic EMT coordinated through SMAD and RREB1, which activate the expression of EMT-TFs and the cell disjunction enzyme hyaluronan synthase 2 (Has2) as well as the expression of various fibrogenic factors. Together, these two arms coordinated by SMAD and RREB1 drive a fribogenic EMT. B. Similarly, in embryonic mesendoderm progenitors Nodal receptor signaling acting through SMADs and RREB1 activates the expression of EMT-TFs together with the expression of mesendoderm differentiation TFs, two key events in gastrulation. C. The chromatin status at relevant loci determines the accessibility of SMAD and RREB1 to the EMT-TF *Snai1,* the lineage differentiation TF *Gsc* and the fibrogenic factor *Wisp1* in lung adenocarcinoma cells and mesendoderm progenitors.