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The contribution of TGF-β in Epithelial–Mesenchymal Transition (EMT): Down-regulation of E-cadherin via snail

Minireview

H. YU, Y. SHEN, J. HONG, Q. XIA, F. ZHOU, X. LIU*

Institute of Biomedical Engineering, School of Preclinical and Forensic Medicine, Sichuan University, Chengdu 610041, P.R. China

**Correspondence: liuxiaohg@scu.edu.cn*

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TGF-β plays a central role in epithelial–mesenchymal transition (EMT), which is a highly conserved and reversible process that governs tumor development, invasion and metastasis. Through this transition, the epithelial cell acquires a migratory behavior which allows it to move away from the cell community and to integrate into the surrounding tissue. The cells lost epithelial phenotypes including the change of cell polarity and the loss of specialized cell-cell contacts, which related with the shortage of E-cadherin directly. The increasing reports indicated that TGF-β down-regulated the expression of E-cadherin through snail signaling pathway, which played an important role in the development of EMT. In this review, we summarized the contribution of TGF-β in EMT and discussed the molecular mechanism of snail signaling participating in the regulation of E-cadherin triggered by TGF-β.

Key words: TGF-β, Epithelial-mesenchymal-transition (EMT), snail family, E-cadherin

Overview of the epithelial-mesenchymal transition (EMT)

It had been found that the interconversion of the epithelial cells and mesenchymal cells in their morphology and phenotype during the embryonic development [1], which was defined as epithelial-mesenchymal transition (EMT) by Greenburg and Elisabeth Hay in the 1980s [2-4]. The occurrence of EMT is integral in development, wound healing and stem cell behavior, and is related to fibrosis and cancer progression [5]. Based on the biological context in which they occur, EMT were classified into three types at the EMT meeting in Poland (2007) and Cold Spring Harbor Laboratories (2008). The type I EMT that is associated with implantation, embryo formation, and organ development is organized to generate diverse cell types that share common mesenchymal phenotypes. The type II EMT is associated with wound healing, tissue regeneration and organ fibrosis. The Type III EMT that occurs in neoplastic cells has previously undergone genetic and epigenetic changes, especially in the genes that favor clonal outgrowth and the development of localized tumors. Three types of EMT showed a series of hallmarks, including down-regulation of E-cadherin resulting in the loss of cell-cell adhesion, up-regulation of matrix-degrading proteases and mesenchymal-related proteins such as Vimentin and N-cadherin, the reorganization of actin cytoskeleton mediated by Rho small GTPases to acquire motility ability. Furthermore, the up-regulation and/or nuclear translocation of transcription factors underlying the specific gene program of EMT, such as β-catenin and members of the Snail, ZEB and basic helix-loop-helix (bHLH) families also can be found [6].

Type I EMT. The EMT plays a crucial role in the differentiation and development of multiple tissues and organs. The development of the most primitive species, such as the sponges, is based on the reconstructing of a simple epithelium to form a two-layered epithelium. This process contains the delamination, invagination or cavitation of an aggregate of cells [7]. However, in most metazoans, EMT occurs in gastrulation at first and allows the formation of a third germ layer, which is called the mesoderm between the ectoderm and the endoderm [1]. In addition, EMT is fundamental to many other histogenic processes including bones and smooth muscle formation [8].

The evolution of EMT allows an increase in embryonic complexity by morphogenesis from diploblastic to triploblastic grades of organization. There are three sequential rounds (re-

ferred as primary, secondary and tertiary EMT) during critical phases of the development of embryo [9, 10]. Primary developmental EMT drives germ layer reorganization of the initial primary embryonic epithelium during gastrulation, neurulation and neural crest formation. For example, the mesoderm formation and neural crest development which are two typical progresses representing the primary EMT programs, occured in the development of early embryo [11].

Secondary developmental EMT involves cells that secondarily adopted an epithelial organization and then experienced a mesenchymal transition during organogenesis. The secondary EMT is also essential to the development of somite liver [12], secondary palate and reproductive tracts [10]. For example, somite undergoes various secondary EMT processes to acquire the morphogenesis of the axial skeleton [13], which is controlled by the high expression of some transcription factors [14, 15].

An example of the tertiary EMT arises during the invasion of endothelial cells from the atrioventricular canal into the cardiac jelly and the formation of the endocardial cushion [16]. Endocardial epithelium transforms into cushion mesenchyme, and remodels the simple tubular heart into a four-chambered organ [17]. In addition, the tertiary EMT also has been found in the border cells of ovarian follicles in the fruit fly [18-20]. In tissue culture models *in vitro*, the capillary endothelial cells drive a process of EMT and lose the endothelial markers (CD31 and integrin αVβ3), as well as the acquisition of fibroblast- and myofibroblast-specific markers (FSP1, α-SMA, DDR2, Collagen I and Vimentin) [21].

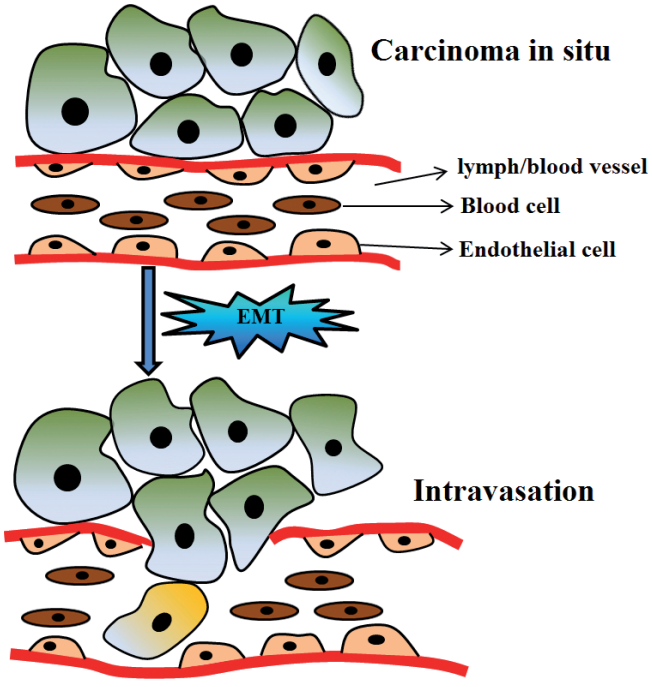

Figure1. EMT plays a critical role in cancer progression. Through the EMT, the cancer cells acquire the ability to overcome the physical constraint from cell–cell adhesion and basement membrane barrier and to invade into lymph or circulation, allowing their passive transport and metastasis to distant organs (single dissociated cell indicated by yellow in Figure).

Type II EMT. Furthermore, the EMT also can be activated in association with pathological stresses, organ fibrosis and tissue repair through a variety of mechanisms, which called Type II EMT [21]. During wound healing, keratinocytes at the border of the wound acquire an intermediate phenotype called the "metastable" state, which allows them to move while maintaining loose contacts rather than migrating as individual cells. It depends on the Snail at the migratory front in keratinocytes. The inactivation or overexpression of Snail can inhibit or accelerate the process of wound repair, respectively [22].

Not only in response to injury repair, is EMT an essential event in other pathologic developments involving organ degeneration, such as fibrosis. EMT is also found to be associated with fibrosis in kidney, liver, lung and intestine. The lineage-tagging experiments demonstrated that during the course of kidney fibrosis in mice, about 30% are derived from the tubular epithelial cells of the kidney via EMT [23]. Indeed, EMT in chemotactic factors (CCL4)-induced liver fibrosis has taken place in hepatocytes, which has been revealed in transgenic mice [24], so do the alveolar epithelial cells during the pulmonary fibrosis induced by transforming growth factor β (TGF-β) [25]. The occuring of EMT in humans' fibrosis tissue has also been demonstrated [26]. In patients with Crohn disease, the EMT was confirmed in the areas of fibrosis in the colon [27]. Furthermore, the EMT has been observed in lens epithelial cells contributing to capsular opacification after cataract surgery [10].

Interestingly, recent accumulating evidences suggested that EMT had a critical role in cancer progression, through which tissue epithelial cancers acquired the abilities of invasiveness and metastasis.

Type III EMT. Although EMT processes are documented in many cancer cells *in vitro*, the significance of EMT during cancer progression and its relevance in human cancer tissues raised debates. Increasing evidences indicated that tumor cells would decrease E-cadherin-dependent intercellular adhesion and enhance their motility under some certain stimuli, then invade into circulation and metastasize to surrounding tissues (Figure 1), which defined as Type III EMT.

Most cancers are comprised of cohesive malignant cells derived from epithelial cells. Under certain conditions, the malignant cells lose epithelial characters, and gain mesenchymal phenotype to enhance their migratory and invasive properties. Carcinoma cells undergoing EMT in primary tumor lose the expression of E-cadherin and other components of epithelial cell-cell junctions, and break through the basement membrane with increased invasive properties, and enter the circulation through intravasation. Cancer cell invasion is the first event in the development of metastasis. At the invasive edge of tumors, cancer cells secrete cytokines and proteases that promote angiogenesis, remodel the peri-tumoral extra-

cellular matrix (ECM), and activate non-neoplastic stromal cells [27, 28].

An important hallmark of EMT is down-regulation of E-cadherin, which has been considered as an epithelial marker in most EMT studies. Other epithelial specific genes in cancer cells, like components of tight and gap junctions, desmosomes, cytokeratins, also showed a decreased expression. Meanwhile, up-regulation of mesenchymal markers (e.g., N-cadherin) were induced in carcinoma cells, associated with reorganization of the cytoskeleton (e.g., switch from cytokeratins to Vimentin), and the synthesis of ECM components and metalloproteases [10, 21].

The characterization of EMT

Epithelial cells generally form sheets like line surface and interface between the interior and exterior of the organism. To maintain structural integrity as a physical barrier, cell junctions consist of multi-protein complexes that provide contact between neighboring cells (cell-cell contacts) or between cell and ECM (cell-substrate interactions), which is essential for the development of multicellular organisms [29, 30]. It is known that intercellular junction as an important structural basis of cell functions is mediated by adhesion structures, which can be divided into tight junction, desmosomes and adherens junction. The loss and disassembly of intercellular junctions and polarity in epithelial cell is the crucial characterization of EMT.

The loss of junctions in EMT

Tight junctions and EMT. Tight junctions build a physical border between apical and lateral domains, which prevent the diffusion of solutes through the intercellular space and create a boundary between the apical and the basolateral plasma membrane domains. For recruiting various signaling molecules to their cytoplasmic surface [31], tight junctions are involved in transcription, cell proliferation, cell polarity and cell differentiation [32]. The decomposition of tight junctions is an early event in EMT, in which the components of tight junctions including ZO proteins, Claudins and Occludins are redistribution. Some reports illustrated the relationship between Snail and the promoter activity of Claudins and Occludins. Elamin et al [33] demonstrated that Snail mediated acetaldehyde-induced tight junction disruption and increased paracellular permeability. It has been shown that accompanied with Snail over-expression, EMT was induced and the expression of tight junction proteins, including Claudin-1, Occludin and ZO-1, were down-regulated in Madin-Daby canine kidney cells (MDCKs) that exogenously expressing Snail protein [34, 35]. Therefore, the disassembly of tight junctions is a key step in EMT.

E-cadherin is essential for regulating tight junctions. The absence of E-cadherin results in permeable tight junctions and thus changed the epidermal resistance. Tunggal et al [36] showed that the loss of E-cadherin in the epidermis *in vivo* given rise to perinatal death of mice that is due to the inability to retain a functional epidermal water barrier.

Adherens junction and EMT. Adherens junctions are specialized forms of cadherin-based adhesive contacts, which are important for tissue organization in developed aduit organisms [37]. Cadherin superfamily has a lot of molecular members, including E-cadherin, P-cadherin, and H-cadherin and so on [38-40]. The loss of E-cadherin is regarded as an important marker event in EMT, which is associated with disassembly of adherens junctions. During the development, the segregation of cells into distinct tissues is accompanied with the changes in the complement of cadherins expressed by the cells. Moreover, pathological examinations suggested that the down-regulation of cadherin expression is associated with the invasiveness of tumor cells [41]. Researches have already shown that E-cadherin-mediated cell-cell contacts inhibited invasive cellular migration [42]. There is an inverse relationship of the cell lines between E-cadherin protein and tumorigenicity, together with a complete absence of E-cadherin protein and mRNA expression in three carcinoma cell lines (the epithelioid HaCa4, the fibroblastoid CarB and CarC cells) [43]. Actually, the loss of adherens junction induced by E-cadherin promotes metastasis via multiple follow stream transcriptional pathways [44]. In conclusion, the down-regulation of E-cadherin is a potential main hallmark event in EMT.

Desmosomes and EMT. As a type of junctional complex, desmosomes belong to anchoring junctions, which connect cytoskeletal elements to the plasma membrane at cell-cell or cell-substrate interactions [45]. All desmosomes contain desmoplakin, plakoglobin and at least one isoform each of plakophilin and the desmosomal cadherins desmocollin and desmoglein [46]. They provide mechanical integrity to tissues to resist mechanical stress. Furthermore, they may act as signaling platform, regulating the availability of signaling molecules and thereby participating in fundamental processes such as cell proliferation, differentiation and morphogenesis. During EMT, the desmosomes structure is partially dissociated [47]. Savagner et al [48] found that transient or stable transfection of Slug cDNA in Nara Bladder Tumor No. 2 (NBT-II) cells resulted in a prominent disappearance of the desmosomal markers desmoplakin and desmoglein from cell-cell contact areas, which like the initial steps of fibroblast growth factor-1 (FGF-1) or hepatic growth factor (HGF)/scatter factor (SF)-induced EMT. It indicated that the zinc-finger protein Slug causes desmosome dissociation, which is an essential step for growth-factor induced EMT. Proteasome inhibition was found to inhibit SF/HGF-induced EMT in MDCK cells [49], suggesting that desmosomal component recycling is necessary for this EMT process.

The polarity of epithelial cells. Another distinct characterization of cells undergoing EMT is the loss and deregulation of epithelial polarity. The epithelial cells establish an apical-basal polarity through the intimate association with lamina layer at their basal surface facing cells and ECM, which calls

the basement membrane. Cell polarity is mediated by three interacting protein complexes including protease-activated receptor/atypical protein kinase C (Par/PKC) complex, crumbs complex, and scribble complex [50]. It is important for many aspects of cell function and developmental biology in both unicellular and multicellular organisms [51].

With the loss of epithelial cell-cell contact structures and actin reorganization, epithelial cells undergoing EMT acquire a mesenchymal identity and lost apical-basal polarity. Cells acquire a mesenchymal front-back polarity which allows them to migrate in a directional fashion, and the apical actin-binding transmembrane protein mucin-1 (MUC1), as epithelial polarity markers, are either redistributed or down-regulated [52]. In addition, the receptors in cellular membrane, integrins have been indicated to participate in regulating changes of cell polarity. Maschler et al [53] found that EpRas cells (Ha-Ras-transformed EpH4 cells (nontumorigenic mammary epithelial cells) established α5β1 complexes and deposited their ligand fibronectin into the ECM, and FibRas cells (fibroblastic, migratory cells displaying a mesenchymal gene expression program) failed to deposit the α6β4 ligand laminin 5, which suggested that α6β4 had no function after EMT and replaced by mesenchymal integrins such as α5β1. In conclusion, epithelial cells undergoing EMT alter their phenotype and polarity, and then acquire the ability of invasion and metastasis (Figure 2).

Snail family down-regulates E-cadherin in EMT

It has been described that Snail family of transcription factors involved in EMT, is a strong repressor of transcription of the E-cadherin gene [40]. As we known, E-cadherin is essential for regulating cell–cell adhesion including tight junction and adherens junction, and cell polarity. The down-regulation of E-cadherin expression occurs concomitantly with the acquisition of migratory properties in EMT.

Snail family in biological behaviors. The transcription factors include Zinc finger proteins Snail family, the basic helix–loop–helix factors including Twist1, Twist2, E47 and E2-2, the negative regulators including Id1, Id2 and Id3, and the Zinc finger homeobox proteins including ZEB1 and ZEB2, which could orchestrate the nuclear reprogramming in EMT [10]. The Zinc-finger factor Snail, as a transcriptional repressor of Cadherin-1 (E-cadherin), was an important inducer of EMT [40, 54]. Understanding the roles of Snail provides new insights into the molecular mechanisms of tumor invasion and the development of embryo in the process of EMT.

There are three proteins in Snail family (Snail1, Snail2 also known as Slug, and Snail3) have been identified. Snail1 and Slug showed the similar function and played critical roles in various processes of physiology and pathology. It has been found that slug and snail 1 genes participated in regulating carcinoma progression in several cancers [55-57]{Come, 2006 #2031}. In addition, Slug is essential for epithelial cell motility in wound healing, and Slug-deficient mice do not re-epithelialize in an *ex vivo* assay [48]. Slug and Snail also involved in developmental processes leading to the formation of mesoderm and of neural crest in Xenopus and chick [58-62], which is required in large-scale cells movements. Furthermore, Snail homologues highlighted their roles in promoting the cell

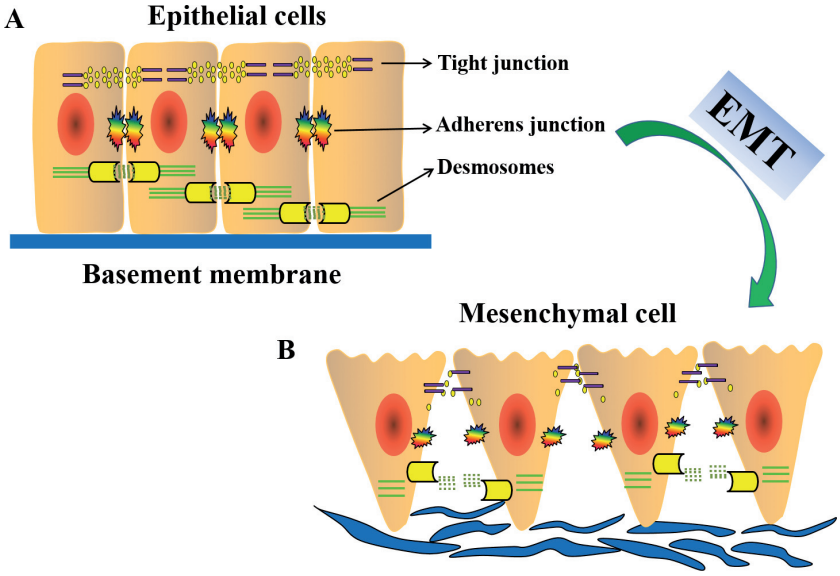

Figure 2. The alterations of cells' phenotype and polarity and increase their ability of metastasis and invasion in EMT. Cells connect each other in the epithelial phenotype (A), and lose their epithelial phenotype through the EMT, in which they dissociate from the basement membrane, and are divided into single cell losing the cell-cell adhesion (B) including tight junction (Up), adherens junction (Middle), desmosomes (Down). In addition, the formations of mesenchymal cells lead to the change of cell polarity, which acquire front-back polarity instead of the apical-basolateral polarity.

movement in EMT [40, 54], which was demonstrated by using either antisense or dominant-negative mutants.

The loss of E-cadherin in EMT. E-cadherin is important for tissue morphogenesis and polarity for establishing cell-cell adhesion, cadherins cluster in specialized cell junctions, which called adherens junctions associated with mediating actin cytoskeleton by α-catenin and β-catenin [63]. The β-catenin signaling is activated by the loss of E-cadherin-induced EMT. The activation of β-catenin downstream depends on the association of the Wnt co-receptor and the E-cadherin/catenin complex [64]. The translocation and accumulation of β-catenin into nucleus followed the progressive loss of E-cadherin and acquisition of mesenchymal markers such as Fibronectin [65]. On the other hand, the knockdown of β-catenin in shEcad cells showed significantly decreased expression of the mesenchymal proteins including N-cadherin, Vimentin, and Fibronectin [44]. As a result, E-cadherin is considered a tumor suppressor protein because its loss or inactivation by mutations is frequently seen in invasive epithelial cell cancers. Sang et al found that 61.9% pancreatic cancer tissue show a loss of E-cadherin. The loss of E-cadherin was observed in EMT of biliary epithelial cells suggesting its major role in cholangiopathies [66].

With injection of three tumor cell lines into the nude mice, Onder et al [44] confirmed that E-cadherin was suppressed and the expression of the dominant-negative construct was maintained during the course of tumor growth. Derksen et al [67] confirmed that loss of E-cadherin contributed to both mammary tumor initiation and metastasis. In addition, diffuse gastric carcinomas showed high E-cadherin gene mutations [68, 69]. It can be concluded that the loss of E-cadherin expression is essential for the EMT in cancer cells (Figure 3).

Snail family controls the expression of E-cadherin in EMT. In normal oral squamous cell carcinoma cell line HSC-4, the expression of E-cadherin is stable, but in the Snail/HSC-4 cells (stable Snail transfectants) which have completed the conversion of EMT, the rapid degradation was observed [70]. Galvan et al [71] found that the loss of E-cadherin/β-catenin andesion complex integrity at the cell membrane associated with high Snail1 protein levels emerge in gastroenteropancreatic neuroendocrine tumors. It can be concluded that the loss of E-cadherin implicated the transcription factor Snail in EMT. Interestingly, recent studies indicated that Slug down-regulated E-cadherin in chick gastrula as Snail does in mouse gastrula. Overexpression of Slug can directly inhibit the E-cadherin promoter in various transformed cell lines. It has been found that activation of Slug induced by down-regulation of integrin α3β1 resulted in a significant reduction in cell proliferation [72], and the ligands (urokinase receptor, uPAR) binding to integrin α3β1 up-regulated expression of Slug, resulted in down-regulation of E-cadherin and γ-catenin, regulated matrix adhesion and cell–cell contact [73]. In addition, there are several intracellular signaling pathways involved in regulation of Slug, which can be activated by EGF, FGFs, TGF, and oncogenic Ras [74, 75]. It is indicated that the ectopic expression of Slug blocked E-cadherin and promoted Vimentin and Fibronectin expression, resulting in a full EMT phenotype [52, 54, 76, 77], while silencing of Slug expression increased expression of E-cadherin and reversed EMT process [78, 79]. Similar to Slug, Snail was found to convert normal epithelial

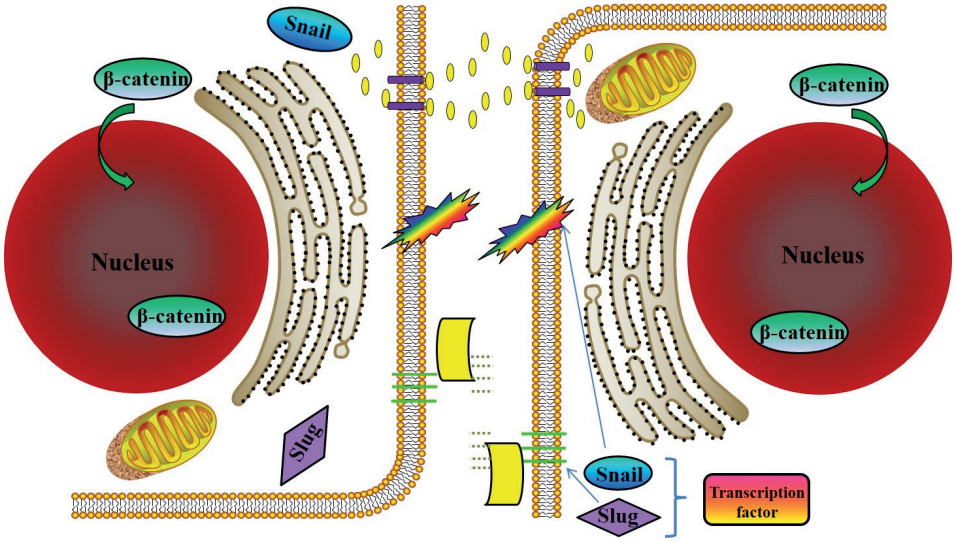

Figure 3. The loss of cell junctions induced by Snail and Slug through transcriptional regulation. The Zinc-finger Snail and slug share a similar DNA binding structure of zinc finger motifs, and trigger EMT process as transcription factors. Overexpression of Snail and Slug directly inhibits the E-cadherin promoter in various cell lines and results in translocation and accumulation of β-catenin in nucleus. The snail and slug have a negative effect on the cellular junction, which can be controlled by the regulation of E-cadherin. The down-regulation of E-cadherin leads to the loss of adherens junctions (Middle), and then the tumor cell increase mobility, which can facilitate their invasion and metastasis.

cells into mesenchymal cells through the direct repression of E-cadherin expression [40, 54]. The histone deacetylase activity recruited to Snail, and the interaction between Snail and transcriptional co-repressor Sin3A are essential for inhibition of E-cadherin mRNA expression [80]. Snail plays a critical role in the development of embryo and cancer progresses in the EMT. For example, Snail knockout mice showed defects in the EMT process of development, and die at gastrulation stages. The E-cadherin remained expression in the Snail mutant mice [81], and no down-regulation of E-cadherin during gastrulatio was found in Snail mutants in Drosophilan [82]. Moreover, Snail expression exhibited a tight correlation with the invasive phenotype in tumor cells. The decreased expressions of E-cadherin with the up-regulation of Snail levels were found in breast carcinoma and oral squamous-cell carcinoma, respectively [83, 84].

Not only Snail family members show a central role in down-regulating the expression of E-cadherin, but other cadherin repressors act simultaneously with Snail.

bHLH-type transcription factors such as SIP1 [85] and E47 [86] are the candidates for repressing E-cadherin expression, and are also expressed in the embryonic mesoderm. As a potent suppressor of E-cadherin, the activation of ZEB1 and ZEB2 led by the nuclear accumulation of β-catenin can increase cell migratory and invasiveness in thyroid cancer cells [87]. In the EMT, E-cadherin is not the only target for Snail. It has been demonstrated that Snail transfection down-regulate epithelial markers including desmoplakin, the epithelial mucin Muc-1, cytokeratin-18, while up-regulate and redistribute mesenchymal markers such as Vimentin and Fibronectin [40]. It suggests that the loss of E-cadherin is not enough to induce a conversion to a mesenchymal phenotype, and other targets were triggered by Snail, which can induce EMT.

TGF-β regulates EMT in development and cancer progression

The TGF-β signaling in EMT. TGF-β induced EMT through the transcription factors Snail and Slug which function in this progression through phosphorylation of the cell-cell contact regulator Par6. TGF-β also stimulates the differentiation of mesenchymal progenitor cells toward adipocyte and musculo-skeletal lineages [88].

There are 33 members of TGF-β family in humans, including TGF-β isoforms, activins, bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs) and Nodal, which play an important role in regulating the expression of specific genes. The members of TGF-β family can be activated by many factors. For example, Zhang et al illustrated that Wnt/β-catenin signaling pathway was an upstream activator of BMP2 expression in osteoblasts [89].

TGF-β family members form heterotetrameric complexes of type I and type II serine/threonine kinase receptors, and two TGF-β monomers are assembled in an antiparallel manner with a disulfide bond for stabilizing their structure. The ligand receptor complex was constructed with one dimeric ligand and two units of TβR-I and TβR-II. The evidences indicated that TGF-β bound to its type II receptor tightly, and then assembled type I receptor to establish complex [90]. However, it should be noted that TGF-β could also bind to other type I receptors, such as ALK1 in endothelial cells [91] and ALK2 and ALK3 in certain epithelial cells [92], respectively. TGF-β family members showed important roles in the embryonic development, and the adult organism to control the immune system and stimulate angiogenesis. In addition, the TGF-β signaling was also emphasized on the effects of growth arrest, cell differentiation and apoptosis, and EMT [90].

There are two pathways in TGF-β signaling containing Smad-dependent pathway and Smad-independent pathway (Figure 4). In the early endosomes, cytoplasmic Smad2/3 can be transported to the TGF-β receptors with the help of a protein called SARA to form TGF-βIR/SARA/Smad2/3 complexes. Smad2/3 is phosphorylated and enters the nucleus with the help of Smad4, which is a key factor in the signaling pathway of the TGF-β inducing EMT and can regulate the E-cadherin expression, and then bound with Smad binding elements (SBE) to activate EMT genes. Studies of invasive colon carcinoma cell lines that are null for Smad4 have demonstrated that they express no E-cadherin suggesting that Smad4 is essential for maintaining the epithelial phenotype and Smad4 has been shown to be necessary for inducing expression of the LEF-1, which is an EMT master gene [93]. Additionally, Kawakita et al proved that the emergence of the irreversible EMT was a result of Smad-mediated TGF-β signaling in the murine corneal/limbal epithelial progenitors cells [94].

TGF-β can induce mesenchyme-like cells containing actin stress fibers independent of Smads, using the Ras-Raf-MEK- phosphoralation of extracellular signal-regulated kinases (ERK) [95, 96] and RhoA-MAP kinase pathways [97]. Moreover, the activation of the TRAF6–TAK1–MKK3/6–p38 (P38 mitogen-activated protein kinases) pathway has been demonstrated in the apoptosis of prostate cancer cells induced by TGF-β [98, 99], which indicates that TβR-I activates two different signaling pathways mediated by R-Smads and non-R-Smads, respectively. This finding provides a novel way for future explorations of the mechanisms of signal transduction downstream of TGF-β receptors. TGF-β isoforms have been shown to induce signaling through G protein/MAPK cascades, as well as phosphatidylinositol-3-kinase (PI3K) [100-102]. Both PI3K and the G protein signaling molecule Grb2 might bind the TGF-β receptor leading a activation of Ras-GTP, which can signal other G proteins such as Rac or Rho, or phosphorylate an MAP kinase kinase kinase (MAPKKK) like Raf. After a sequential signaling progress, members of the *jun* and *fos* families of transcription factors can be induced to form heterodimers creating various forms of activator protein-1 (AP-1) [96, 103]. AP-1 has been demonstrated to up-regulate expression of Snail, a key activator of EMT, in the process of EMT induced by Smad-independent way [104].

Above all, TGF-β plays an important role in the induction of EMT. On the contrary, inhibition of TGF-β signaling molecules such as TGF-β receptors has been shown to prevent EMT and the metastatic phenotype in several cancer cell lines [105].

Furthermore, TGF-β is activated the protein tyrosine phosphatase Pezs and thus promotes EMT [106]. Except for TGF-β, EMT can be regulated by various growth and differentiation factors, including FGF, HGF, platelet derived growth factor (PDGF), and Notch proteins [107]. Accordingly, TGF-β signaling induced the program of EMT in development and cancer progression will be discussed below.

TGF-β and EMT in development and diseases. The TGF-β superfamily signaling pathways are required for the development of multicellular animals, which contribute to diversity and complexity of organisms. As we known, BMPs as an important member of TGF-β family play a central role in embryogenesis, osteogenesis and cell differentiation. The specification and patterning of Dorsal-ventral (D/V) axis require the BMP orthologs Decapentaplegic (Dpp) and Screw (Scw) in Drosophila embryos [108]. Interestingly, different levels of Dpp lead to different differentiation of embryo. Higher levels of Dpp specify the amnioserosa, whereas lower levels specify dorsal ectoderm, and the shortage of Dpp signaling results in the formation of neural ectoderm [109]. In the vertebrate ectoderm, high BMP activity induces epidermis and low activity specifies neural tissue, respectively. Cells exhibiting intermediate BMP signaling have been specified as the neural crest cells at the border among these tissues [110]. In addition, another TGF-β member Nodal is required in vertebrates for the induction of three germ layers: endoderm, mesoderm, and ectoderm. Similar to the mechanism of different levels of Dpp contributing to the D/V axis formation, Nodal first induces mesendoderm, and then high Nodal level mediates endoderm while low level of Nodal regulates mesoderm [111].

TGF-β family members are also known for their ability to induce EMT in cardiogenesis [112]. The key roles of TGF-β have been indicated in the EMT that occurs in the atrioventricular canal and the outflow tract region. In chicken heart, TGF-β2 is considered as a candidate inducer of EMT in the atrioventricular canal of the embryonic heart, which has been demonstrated by using neutralizing antibodies, and TGF-β2 can activate Slug, which is a key transcription factor in EMT [75]. TGF-β3 is responsible for EMT following palate fusion [113]. TGF-β1, 2, and 3 are expressed in different phases of the developing heart in mice. The results of knockout for each of the three TGF-β ligands showed that only the TGF-β2 null

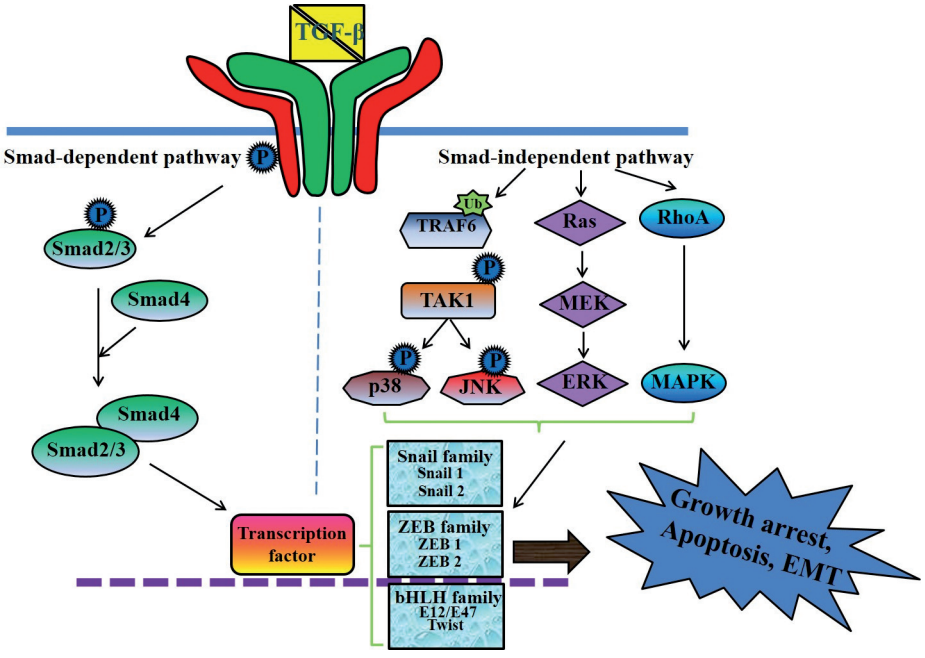

Figure 4. Two branches (Smad-dependent pathway and Smad-independent pathway) of signaling pathways participate in TGF-β-induced cell functions. Smad-dependent pathway: Two TGF-β monomers assembled together and induced the combination of TβR-II (marked by green in figure), which activate TβR-I (red in figure) to form a complex. The complex then phosphorylates Smad2/3 and binds them to Smad4. Smad-independent pathway: In addition, the oligomerization of ligand-induced TGF-β receptor brings together TRAF6 molecules that are constitutively bound to TβR-I, leading to their activation by autoubiquitination. TRAF6 then phosphorylates the TAK1 kinase which causes activation of the c-Jun N-terminal kinases (JNK) and p38 MAP kinase pathways. Both of the Smad-dependent and Smad-independent pathways contribute to cell growth arrest, apoptosis, and EMT. The mechanism of TGF-β promotes EMT by a combination of Smad-dependent and Smad-independent transcriptional event. In the transcriptional regulation, TGF-β activates Smad2/3 and then forming complexes with Smad4 to regulate the transcription of target genes through interactions with other DNA binding transcription factors. In result, the epithelial marker gene including Id has been repressed; on the contrary, the mesenchymal marker gene such as the dEF1, SIP1, and Snail have been activated.

mouse presented EMT-specific phenotypes. While TGF-β3 affects invasion/migration in a sequential manner, compliance with the order of expression of these ligands. Besides, TGF-β2 and 3 are required for inducing programmed cell death during interdigital webbing regression [114].

TGF-β superfamily signaling can also function in pathological contexts in the adult organism. The normal unstressed tissue can release sustained basal level of TGF-β by local sources to suffice for the maintenance of homeostasis. Under the conditions of injury, TGF-β is abundantly released by blood platelets and various stromal components to prevent proliferation and inflammation of runaway regenerative cells. Some studies indicated that mutations in a TGF-β receptor (such as Activin receptor-like kinase 1, ALK1) and its co-receptor Endoglin were implicated in the Hereditary Hemorrhagic Telangiectasia disease or Rendu-Oslr-Weber syndrome [115].

Myofibroblasts can contract by using smooth muscle type actin-myosin complex, rich in a form of actin called alpha-smooth muscle actin. These cells are then capable of speeding wound repair by contracting the edges of the wound. Myofibroblasts are capable of accelerating wound-healing and tissue repair by clustering α-smooth muscle actin-myosin complex, which is required for the induction of TGF-β. The disturbances of TGF-β signaling in overactive myofibroblasts result in increased deposition of ECM and then lead to excessive scarring. In renal fibrosis, TGF-β can promote the production of ECM components and convert renal epithelial cells to myofibroblasts by inducing EMT, leading to tubular atrophy [116]. In addition, BMP type II receptor, BMPR2 induced increased susceptibility of endothelial cells and proliferation of myofibroblast and smooth muscle cell, which can result in damage and abnormal repair of vessels eventually [117].

TGF-β-induced EMT in cancer. TGF-β is also strongly implicated in cancer, which shows a bidirectional role in the tumor micro-environment. TGF-β can be tumor suppressive, but it can also enhance tumor progression by stimulating the complex process of EMT. It is a tumor suppressor since it inhibits the growth of cells and induces apoptosis. On the other side, TGF-β that acts as a tumor promoter, can suppress the immune system and stimulate the angiogenesis to promote the tumor progression. At later stages of tumor progression, with the loss of TGF-β suppression, tumor cells sustain their potentiality to undergo EMT, which correlates to the acquirement of the ability of invasiveness and metastasis [88, 107]. It has been found that TGF-β could inhibit the formation of benign tumors during skin carcinogenesis, but enhance the progression to invasive spindle tumors [118]. Kojima et al [119] demonstrated that tumor cells could produce TGF-β to induce the conversion of fibroblasts into myofibroblasts known as carcinoma associated fibroblasts, and autocrine TGF-β signaling in myofibroblasts makes them maintain this conversion and greatly promote tumor development. To investigate the mechanism of TGF-β-induced EMT, Bhowmick et al [120] established a model using mouse mammary epithelial cells, which could produce autocrine TGF-β signaling to enhance cell invasion and metastasis. In addition, as the important components in TGF-β signaling pathways, both alteration of TGF-β receptors and downstream SMAD signaling contribute to the tumor progression [121]. Colon cancers with shortage of TGF-βRII showed a better prognosis, suggesting that TGF-β signaling might be involved in colon cancer progression as well [122]. Furthermore, loss of TGF-β receptor expression increases motility and invasiveness associated with EMT in pancreatic cancer [123].

Loss of the expression of TβRII is another highly frequent alteration observed in tumors, which could be found in 44% of NSCLCs (Non-small-cell lung carcinoma), in 44% of bladder cancers, in about 30% of head and neck squamous cell carcinomas of the esophageus, in 23% of ovarian carcinomas, 12.5% of prostate cancers and also in breast cancers, respectively [124-126]. Despite the occurrence of TGF-β receptor mutations in cancer, tissue-specific inactivation of TβRII alone in mouse models seldom leads to spontaneous tumor formation. In addition, missense mutations in the TβRI coding region are present in subsets of ovarian, esophageal, and head and neck cancers. Interestingly, not all types of tumors show the alteration of mutation. For example, endometrial tumors with microsatellite instability do not accumulate TβRII mutations [88]. Actually, MADH4/DPC4 (hereafter SMAD4) was identified as a tumor suppressor gene encoding Smad4 in pancreatic cancer [127], and germline mutations in SMAD4 in some individuals can lead to predisposition to hamartomatous polyps and gastrointestinal cancer [128]. Therefore, TGF-β is a major inducer of EMT during embryogenesis, even in fibrosis and cancer progression.

The switch of the EMT is controlled at the transcriptional, post-transcriptional, translational and post-translational levels. TGF-β shows a predominant role in inducing EMT involving in development, diseases and tumor. Both Smad-dependent and Smad-independent signals pathway are activated by TGF-β to build crosstalks in various signal transduction events.

TGF-β regulates the Snail family to down-regulate E-cadherin in EMT

The increasing evidences indicated that growth factors, such as TGF-β, PDGF, EGF, FGF-2 [129] and ECM molecules could bind to membrane receptors of tumor cells to generate cascades of intracellular signals that could finally trigger the down-regulation of E-cadherin and the activation of cytoskeleton. Other several factors including hypoxia [130], reactive oxygen species [131], and insulin-like growth factor-2 [132] also can induce EMT *in vitro* and *in vivo*. In particular, the mechanism of TGF-β inducing EMT in various cell types has been well-studied.

Epistasis analyses in Drosophila have established the central mechanisms of TGF family proteins regulate gene expres-

sion [133]. As described above, TGF-β promotes EMT by a combination of Smad-dependent and/or Smad-independent transcriptional event affecting on cell junction complexes, which induces the expression of several transcription factors involved in EMT, including dEF1, SIP1, and Snail, and represses Id proteins in NMuMG cells [134, 135]. However, the effect of TGF-β on the expression of E12/E47 and Twist is unclear in these cells [136].

The Snail family of transcription factors has been considered as the main controller in the EMT by repressing the E-cadherin expression, which is directly or indirectly mediated by TGF-β (Figure 4). All of the TGF-β family members including TGF-β1, 2, 3 could regulate the development of EMT. TGF-β1 induces EMT state in mature hepatocytes *in vitro* by inducing the Snail-1 transcription factor and activates the Smad2/3 pathway [137]. With the combination of FGF-2, TGF-β1 induces Snail expression in MDCKs and triggers EMT depending on the MAPK signaling pathway [138]. It has been shown that the expression of transcription factor Slug in the atrioventricular canal was required for initial steps of EMT, while the implanatation of cultured atrioventricular canal showed no Slug expression in the presence of anti-TGF-β2 antibody [75]. Therefore, it can be concluded that Slug was an essential target of TGF-β2 signaling during EMT in the developing chicken heart. In addition, TGF-β3 null mutant mice (TGF-β3 -/-) showed a cleft palate phenotype, but TGF-β1 could induce the expression of Snail in the medial edge epithelial (MEE) cells in TGF-β3-/-mouse embryo palates [139].

It has been found that TGF-β has a tight association with Snail family factors. The mechanisms of TGF-β regulating Snail family factors expression have been explored in previous reports. Emerging evidences suggested that TGF-β1 initiated the transition of renal tubular epithelial cells to myofibroblasts. To name a few, the re-expression of Snail mRNA could be found in TGF-β1 treated wild-type epithelial cells, but not in Smad3-null cells. These results indicated that the Smad3 pathway played an essential role in TGF-β1-induced EMT in the primary culture of renal tubular epithelial cells [140]. Myocardin-related transcription factors (MRTFs; also known as MAL and MKL) are critical mediators of TGF-β1-induced EMT. The complexes of MRTFs and Smad3 bound to the promoter region of the human slug gene, which could activate slug transcription and thereby dissociate cell–cell contacts. The dominant-negative MRTF-A or knockdown of MRTF-A and -B prevents the TGF-β 1-induced EMT [141]. The interaction of Smad3 and Smad4 formed a complex with Snail1, which was targeted to the gene promoters of tight-junction protein Coxsackievirus-adenovirus receptor (CAR) and E-cadherin, resulted in downregulation of CAR, E-cadherin, occluding and claudin-3 during TGF-β-driven EMT in breast epithelial cells. Conversely, co-silencing of Snail1 and Smad4 by siRNA inhibited repression of CAR and occludin [142].

Interestingly, induction of Snail occurs in cooperation with activated Ras signaling in MDCK cells and pancreatic carcinoma Panc-1 cells, indicating that activation of Snail is not only induced by TGF-β signaling [140]. Except for TGF-β, Snail is induced by various signaling events, including the EGFR and Notch pathways [80]. Furthermore, Cheng et al demonstrated that the cells treated with Hypoxia-inducible factor 1a (HIF-1a) siRNA attenuated their invasion ability by decreasing the expression of Snail and Slug as well as the down-regulation of E-cadherin by EGF [143].

Perspective

In the recent years, EMT has been noted prominently because of its significant role in the development of multi-cell organisms and cancer progression. Although the complicate networks orchestrating EMT *in vivo* has been studied well, the molecular mechanisms of EMT *in vitro* have not yet been described in depth. A limitation is that only few immortalized epithelial cell lines can undergo EMT and are used as experimental model *in vitro.* Another problem consists of the kinetics of EMT conversion, which varies considerably from hours to a week [144]. To address these concerns, a preclinical mouse model of human invasive lobular carcinoma was developed for novel intervention strategies to treat invasive breast cancer [67]. The epithelial cells cultured in three-dimensional condition can polarize and form functional glandular structures, which provide better models for *in vitro* study EMT [144].

TGF-β is a main inducer of EMT in development and cancer progression, which controls the transcriptional factors such as Snail, ZEB, and bHLH family through Smad-dependent and Smad-independent signaling pathway. The transcription factor Snail is the repressor of E-cadherin gene expression. The hypothesis has been established that TGF-β modulate the expression of E-cadherin by regulating the transcription factor Snail family members. Understanding EMT associated with signaling pathway, the new therapeutic strategy targeted EMT is expected to develop to inhibit cancer metastasis.

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