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Mechanisms of Epithelial-Mesenchymal Transition by TGF-β

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Summary

The formation of epithelial cell barriers results from the defined spatiotemporal differentiation of stem cells into a specialized and polarized epithelium, a process termed mesenchymal-epithelial transition. The reverse process, epithelial-mesenchymal transition (EMT), is a metastable process that enables polarized epithelial cells acquire a motile fibroblastoid phenotype. Physiological EMT also plays an essential role in promoting tissue healing, remodeling, or repair in response to a variety of pathological insults. On the other hand, pathophysiological EMT is a critical step in mediating the acquisition of metastatic phenotypes by localized carcinomas. Although metastasis clearly is the most lethal aspect of cancer, our knowledge of the molecular events that govern its development, including those underlying EMT, remain relatively undefined. Transforming growth factor-β (TGF-β) is a multifunctional cytokine that oversees and directs all aspects of cell development, differentiation, and homeostasis, as well as suppresses their uncontrolled proliferation and transformation. Quite dichotomously, tumorigenesis subverts the tumor suppressing function of TGF-β, and in doing so, converts TGF-β to a tumor promoter that stimulates pathophysiological EMT and metastasis. It therefore stands to reason that determining how TGF-β induces EMT in developing neoplasms will enable science and medicine to produce novel pharmacological agents capable of preventing its ability to do so, thereby improving the clinical course of cancer patients. Here we review the cellular, molecular, and microenvironmental mechanisms used by TGF-β to mediate its stimulation of EMT in normal and malignant cells.

Keywords

Epithelial-mesenchymal Transition; Metastasis; Signal Transduction; Transforming growth factor-β; Tumor Microenvironment

1. INTRODUCTION

The epithelium is comprised of highly specialized and diverse cells that play critical roles in nearly all biological processes [1,2]. Indeed, epithelial cells serve as protective barriers that line both the outer (*i.e.*, skin) and inner (*i.e.*, airways, gastrointestinal tract, etc.) body cavities, as well as behave as secretory and glandular tissues. In addition, epithelial cell function varies widely between tissues and ranges from nutrient absorption in the intestines to gaseous exchange in the lungs to lactogenesis in the mammary gland. Equally important is the role of the epithelium in providing the first line of defense against exterior insults and infections, while simultaneously enabling the exchange of vital nutrients needed to maintain tissue homeostasis. The fidelity and function of the epithelium is maintained through its

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continual renewal and repair, and as such, it perhaps is not surprising to learn that the majority (*i.e.*, ~90%; [3]) of cancers arise in cells derived from epithelial origins. Thus, it is imperative that science and medicine uncover the sequence of events that enable specialized and polarized epithelial cells to dedifferentiate along a tumorigenic pathway that terminates in their acquisition of metastatic phenotypes.

Recent evidence has linked the development of tissue fibrosis and cancer metastasis to the inappropriate reactivation of epithelial-mesenchymal transition (EMT), which is the process whereby immotile, polarized epithelial cells transition into highly motile, apolar fibroblastoid-like cells (Figure 1; [1,2,4-6]). Indeed, EMT is a normal physiological process essential for proper embryogenesis and tissue morphogenesis, particularly for the formation of the mesoderm, neural crest, cardiac valve, and secondary palate [1,2,7]. With respect to adult tissues, EMT also is engaged in wounded epithelia to facilitate their healing, remodeling, and repair in response to tissue damage. Thus, fully differentiated epithelial cells harbor a dormant embryonic transcriptional EMT program that can be reinitiated in response to a variety of specific environmental cues and signals, one of which is the pleiotropic cytokine, transforming growth factor-β (TGF-β). Interestingly, these same cellular and morphological features are observed in cells undergoing pathophysiological EMT, which underlies the development of several human pathologies, such as chronic inflammation, rheumatoid arthritis, and chronic fibrotic degenerative disorders of the lung, liver, and kidney [1,2,4-6,8,9]. Along these lines, aberrant reinitiation of EMT also engenders the acquisition of invasive and metastatic phenotypes in developing and progressing carcinomas, leading to their dissemination and colonization of distant organ sites suitable to support their metastatic growth. A commonality of physiological and pathophysiological EMT is their ability to be induced by TGF-β, which now is recognized as a master regulator of this transdifferentiation process.

TGF-β is a ubiquitously expressed and multifunctional cytokine that not only regulates EMT, but also oversees the development, differentiation, and survival of essentially all cell types and tissues [10-13]. TGF-β also is a powerful suppressor of cell growth and proliferation, particularly in cells of epithelial, endothelial, and hematopoietic origins [10-13]. Quite dichotomously, aberrations in the TGF-β signaling system regularly take place during tumorigenesis and elicit resistance to its anti-proliferative activities, contributing to the formation of human neoplasms. Upon being liberated from the cytostatic activities of TGF-β, cancer cells proliferate, invade, and metastasize beyond their tissue of origin when stimulated by TGF-β. How TGF-β suppresses these processes in normal epithelial cells is unclear, as is how TGF-β promotes these processes in their malignant counterparts. Despite the continued uncertainty of the molecular events associated with the diametric activities of TGF-β, it is absolutely clear that this cytokine stimulates the two deadliest aspects of cancer, namely cell invasion and metastasis. Moreover, recent studies indicate that acquisition of metastatic phenotypes by carcinoma cells is critically dependent upon their ability to undergo EMT [4-6,8,14]. Indeed, TGF-β stimulation of EMT was demonstrated originally by Miettinen *et al* [15] who observed normal mammary epithelial cells (MECs) to acquire fibroblastoid phenotypes in response to TGF-β. In addition, TGF-β3-deficient mice develop cleft palate due to defective palatogenesis associated with aberrant EMT [16]. Similar inactivation of TGF-β2 function impairs endocardial cushion development in chick hearts due to their absence of Slug expression and its ability to activate EMT [17]. Finally, Smad3-deficiency affords protection against EMT-driven retinal [18,19] and renal [20] fibrosis in mice. Thus, these and other seminal studies have clearly established TGF-β as a master regulator of EMT. This review focuses on the myriad of evidence supporting this designation for TGF-β, particularly the cellular, molecular, and microenvironmental mechanisms that underlie the ability of TGF-β to induce EMT in normal and malignant cells.

2. TGF-β SIGNALING & EMT

The general mechanisms whereby TGF-β activates responsive cells and regulates their behavior is depicted in Figures 2 and 3. As shown, transmembrane signaling by TGF-β commences *via* its binding to three high-affinity receptors, namely the TGF-β type I (TβR-I), type II (TβR-II), and type III (TβR-III or betaglycan). When and where it is expressed, TβR-III clearly is the most abundant TGF-β receptor on the cell surface where it functions as an accessory receptor that binds and presents TGF-β to its signaling receptors, TβR-I and TβR-II, both of which possess intrinsic Ser/Thr protein kinase activity in their cytoplasmic domains [11,12,21-23]. The binding of TGF-β to TβR-II enables the recruitment and activation of TβR-I, leading to its induction of canonical Smad2/3-dependent signaling. Once activated, Smad2/3 form heterocomplexes with Smad4 and translocate into the nucleus where they regulate the cell type-specific expression of TGF-β-responsive genes [11,12,21-23]. It is interesting to note that the variety of cell responses exhibited in response to TGF-β are governed primarily by the cell type-specific expression of various Smad2/3-interacting transcription factors (*e.g.*, AP-1 and Forkhead family members, Stats, etc. [11,22]), as well as their association with additional transcriptional activators or repressors [11,12,21-23]. Moreover, the amplitude and duration of Smad2/3 signaling is modulated by several mechanisms, including the expression of *(i)* adapter and/or anchoring proteins SARA [24], Hgs [25], and Dab2 [26] that enable Smad2/3 phosphorylation by TβR-I, and *(ii)* the inhibitory Smad, Smad7, which prevents the phosphorylation of Smad2/3 [27-29] and induces the degradation of TGF-β receptors [30,31]. In addition, the inhibitory functions of Smad7 are regulated by its interaction with STRAP [32], which potentiates the anti-TGF-β activity of Smad7, and by its association either with AMSH2 [33] or Arkadia [34-36], both of which negate the anti-TGF-β activity of Smad7. As alluded to above, the activation of Smad2/3 by TGF-β represents the canonical TGF-β signaling system, which is shown diagrammatically in Figure 3.

Also depicted in Figure 3 is the coupling of TGF-β to a variety of noncanonical signaling systems, including *(i)* the MAP kinases ERK1/ERK2, p38 MAPK, and JNK; *(ii)* the growth and survival kinases PI3K, AKT/PKB, and mTOR; and *(iii)* the small GTP-binding proteins Ras, RhoA, Rac1, and Cdc42 [37-45]. In addition, TGF-β typically represses NF-κB activity in normal epithelial cells [46,47], but readily activates this transcription factor in their malignant counterparts [47-51]. More recently, TGF-β has been shown to activate a number of protein tyrosine kinases (PTKs), including FAK [52,53], Src [43-45,54], and Abl [55,56], which results in the inappropriate amplification of noncanonical TGF-β signaling in mesenchymal or dedifferentiated epithelial cells. Moreover, imbalances in the activation status of canonical and noncanonical TGF-β signaling systems may very well underlie the ability of TGF-β to induce EMT in normal and malignant cells. The importance of canonical and noncanonical TGF-β signaling systems to promote physiological and pathophysiological EMT is presented in greater detail below.

3. DEFINING EMT

The phenomenon of EMT is defined by the morphologic and genetic transition of epithelial cells to fibroblastoid- or mesenchymal-like cells. An inherent characteristic or hallmark of EMT, including that stimulated by TGF-β, is the dramatic phenotypic change in epithelial cell morphology [4-6,8,14]. Typically, fully differentiated epithelium manifests as a single layer of polarized epithelial cells comprised of well-defined apical and basolateral surfaces, as well as a clearly demarcated actin cytoskeleton arranged into discrete “cobblestones” that reflect regions of concentrated actin fibers at cell-cell junctions. In response to the initiation of EMT, cell-cell junctions disassemble and filamentous actin undergoes a dramatic redistribution to form prominent stress fibers, which is tracked experimentally *via* the use of

a fluorescently-labeled mushroom toxin, phalloidin. The combined effect of these various cell biological activities is a loss of epithelial cell polarity (Figure 1).

Examining the biochemical and molecular alterations in cell-cell junction formation and dissolution has enabled science and medicine to garner a more complete assessment of the events underlying EMT. Indeed, a number of recent examinations have elucidated a variety of molecular complexes and scaffolds that govern the development of cell-cell junctions, including tight junctions, adherens junctions, and desmosomes [5]. Not surprisingly, a series of coordinated and dynamic processes underlie formation of these macromolecular complexes during the development and maintenance of the epithelium, while changes in the expression and localization of junctional proteins constitute useful measures to track the progression of EMT. For instance, tight junctions are formed by the actions of the transmembrane proteins, claudins, occludins, and JAMs (**J**unctional **A**dhesion **M**olecules), which are linked to the actin cytoskeleton *via* the scaffold proteins ZO-1, −2, −3 [57,58]. Moreover, following their formation, tight junctions and their constituents play essential roles in regulating the biology, homeostasis, and architecture of epithelial cells, and in preventing the initiation of EMT and tumorigenesis [59]. In contrast, the initiation of EMT induces a drastic modulation of tight junction localization in epithelial cells [15,38]. For instance, the function of Par6 (partitioning-defective 6), which governs the formation of tight junctions, the establishment of apical-basolateral polarity, and the initiation of polarized cell migration [60], is compromised by its physical interaction with TβR-I and subsequent phosphorylation by TβR-II in epithelial cells stimulated with TGF-β [61]. Once phosphorylated, Par6 recruits and interacts with E3 ubiquitin ligase, Smurf1, which ubiquitinates the small GTPase, RhoA, leading to its degradation and subsequent dissolution of tight junctions during EMT stimulated by TGF-β [62]. The importance of Par6 to EMT induced by TGF-β is highlighted by the ability of TβR-II-resistant Par6 mutants (*i.e.*, S345A-Par6) to prevent MECs from undergoing EMT in response to TGF-β [61].

Unlike tight junctions, adherens junctions consist of transmembrane E-cadherin (**E**pithelial-cadherin) proteins that are linked to the actin cytoskeleton by α- and β-catenins [63]. TGF-β stimulation of EMT represses E-cadherin transcription (*see below*), as well as disrupts its localization at the plasma membrane in part *via* diminished activation of the small GTPase, Rac1 [62]. The net effect of altered E-cadherin function during EMT is the dissolution of adherens junctions. In addition, the loss of cell-cell contacts parallels the development of prominent actin filaments and the appearance of fibroblastoid-like phenotypes in transitioning epithelial cells, processes requiring the activation of RhoA by TGF-β [64,65]. The mechanisms underlying TGF-β regulation of adherens junction expression and function are discussed below.

4. EMT, TGF-β, & CELL MICROENVIRONMENTS

Maintaining homeostasis within cell microenvironments is essential to alleviating disease development in humans, particularly cancer. Tumor development has been likened to that of dysfunctional miniature organs that house a mixture of malignant and normal cells, including fibroblasts, endothelial, and immune cells [66]. It also is important to remember that the growth and progression of tumors are not inherent properties of the cancer cells themselves, but instead are dictated in large part by a delicate balance between positive and negative proliferative signals produced by diverse cell types within tumor microenvironments. Indeed, alterations within tumor microenvironments can either suppress or promote cancer progression in a manner that mirrors the acquisition of oncogenic signaling by TGF-β in developing neoplasms. Biologically, TGF-β is a master inhibitor of cell cycle progression; however, this cytokine also functions as a master regulator of ECM production, deposition, and remodeling, all of which are essential processes during EMT.

Along these lines, recent evidence has shown that TGF-β stimulation of cancer progression proceeds in part *via* its reprogramming of cell microenvironments, particularly by its ability to target the behaviors of neighboring endothelial cells (ECs) and fibroblasts. Moreover, ECs and fibroblasts typically respond to TGF-β by synthesizing and secreting numerous cytokines, growth factors, and ECM components capable of driving the progression of tumors from indolent to aggressive states [67,68]. A vital component of normal and malignant cell microenvironments is the ECM, which functions as *(i)* a gel-like structural scaffold for cells comprised of polysaccharides and fibrous proteins, including collagen, fibronectin, and elastin; and *(ii)* a molecular sensor that monitors, detects, and responds rapidly to physiological and pathophysiological changes within cell microenvironments. Indeed, under physiological conditions, the ECM serves as a storage reservoir that sequesters numerous growth factors and cytokines that can be rapidly released in response to ECM perturbations or insults, thereby circumventing the need for *de novo* protein synthesis to elicit biological behaviors [69]. Thus, the microenvironment of epithelial cells plays a critical role in maintaining their polarization and differentiation, processes that are disrupted temporarily during physiological EMT and its modification of epithelial cell microenvironments. In contrast, chronic disruptions within carcinoma cell microenvironments elicits pathologic EMT and its ability to support cancer cell invasion and metastasis. Table 1 identifies numerous EMT-associated genes whose expression is regulated by TGF-β, and readers desiring more in depth discussions of the activities and functions of these genes in governing EMT and epithelial cell biology are directed to several recent reviews [1,2,4-6]. In the following sections, we highlight many of the mechanisms that underlie the ability of TGF-β to induce EMT and its associated alterations within the microenvironments of transdifferentiating cells.

4.1. Matrix Metalloproteinases

Matrix **m**etallo**p**roteinases (MMPs) comprise a large family of proteases that regulate essential steps of embryogenesis and tissue morphogenesis, and of wound healing and cell growth. MMPs also possess the ability to degrade nearly all ECM and basement membrane components, as well as the ability to promote the development and progression human malignancies [70,71]. Along these lines, TGF-β enhances the tumorigenicity and invasiveness of breast cancer cells by inducing their expression of MMPs 2 and 9 [72,73], which is consistent with the general importance of upregulated MMP expression in mediating the acquisition invasive phenotypes in several cancers [74]. Indeed, aberrant MMP expression (*e.g.*, MMP-7 or matrilysin) facilitates the development of mammary fibrosis and desmoplasia, which increase tumor rigidity and the selection, expansion, and dissemination of metastatic cells [75,76]. Similarly, upregulated MMP-3 expression is sufficient to induce lung and mammary fibrosis [77,78], and to stimulate EMT in carcinomas [79]. Thus, elucidating the connections between aberrant MMP expression and the development of fibrosis and/or EMT will offer important clues as to how EMT promotes cancer progression. For instance, does pathophysiologic EMT solely mediate the acquisition of invasive phenotypes by developing carcinomas, or does this event simply reflect the transdifferentiation of a subset of carcinoma cells into tumor supporting stroma cells (*e.g.*, myofibroblasts) [80]? Indeed, tumor-associated myofibroblasts upregulate their production and secretion of TGF-β, which may serve in establishing a positive feedback loop that drives the selection and expansion of metastatic carcinoma cells [81-83]. Collectively, these findings point to the need for additional studies to fully address these questions, particularly since the expression and activity of MMPs alters the expression of E-cadherin, Snail, vimentin, and TGF-β in a manner consistent with the induction of EMT [79].

4.2. Neuronal Cell Adhesion Molecule

Neuronal **c**ell **a**dhesion **m**olecule (NCAM) is a member of the immunoglobulin superfamily and has been implicated as a mediator of tumor progression and metastasis [84]. Recently, TGF-β stimulation of EMT was observed to induce NCAM expression in a manner correlated with downregulated expression E-cadherin [85]. Functionally, upregulated expression of NCAM during EMT facilitates the formation of β1 integrin-containing focal adhesion complexes [85]. Interestingly, the extracellular domain of NCAM is cleaved proteolytically by MMP-28 (epilysin), which also induces EMT through its ability activate latent TGF-β complexes from inactive ECM depots [86]. In addition, MMP-28 expression also is upregulated in a EMT-dependent manner in wounded epithelial cells, and in metastatic breast cancer cells [87]. Thus, future studies need to determine the physiological and pathophysiological connections between NCAM, MMP-28, and TGF-β during the initiation of EMT in normal and malignant epithelial cells.

4.3. Urokinase Plasminogen Activator

Urokinase **p**lasminogen **a**ctivator (uPA) is a serine protease whose elevated expression in human cancer correlates with advanced disease sates and poor clinical outcomes, presumably through its ability to promote cancer cell invasion and metastasis [88,89]. Accordingly, uPA expression is essential for breast and ovarian cancer metastasis in mice [90,91], and for hypoxia-induced EMT in breast cancer cells *via* uPA receptor-mediated activation of AKT and Rac1 [92]. TGF-β is a potent inducer of uPA expression, yet the role of this event in mediating EMT and metastasis stimulated by TGF-β remains to be elucidated fully. Recently, the activation of JNK1/2 was shown to be essential for TGF-β stimulation of uPA expression and EMT [93], which is consistent with the notion that noncanonical TGF-β signaling promotes its oncogenic activities in epithelial cells.

4.4. Plasminogen Activator Inhibitor-1

Plasminogen **a**ctivator **i**nhibitor-1 (PAI-1) is an antagonist of tissue-type plasminogen activator (tPA) and uPA, as well as a physical interactor of the ECM ligand, vitronectin [94,95]. tPA and uPA both activate the serine protease activity of plasminogens (or plasmins), resulting in the degradation of blood plasma proteins, such as fibrin and von Willebrand factor, and of ECM proteins, such as fibronectin, thrombospondin, and laminin [95]. Through its ability to inhibit tPA and uPA, PAI-1 prevents the activation of intravascular and cell-associated plasminogen, and as such, impedes the breakdown of blood clots and ECM proteins necessary to enable carcinoma cells to undergo invasion and extravasation reactions during metastasis [95].

TGF-β is principal player involved in stimulating PAI-1 transcription in part *via* activation of p53, which binds and stabilizes PAI-1 transcripts [96,97]. Quite dichotomously, overexpression of PAI-1 has been observed to reduce the migration and invasion of breast and ovarian cancers [94,98]; however, PAI-1 polymorphisms or its aberrantly elevated expression also have been associated with a poor prognosis and the increased risk of metastasis in breast cancer patients [99]. Thus, the precise mechanisms underlying the dynamic relationship between PAI-1, plasminogen, and TGF-β regulatory loops, as well as their impact on cancer cell motility, remain an active and important topic of investigation.

4.5. Collagen

Collagen is an abundant ECM molecule that assembles into tensilely strong fibers that provide mechanical support to tissues. The major types of collagen, types I-IV, are distributed differentially in specific tissues of the body. For instance, collagen IV is a major component of the basal lamina, a specialized component of the basement membrane in the

mammary gland. Invading breast cancer cells must degrade collagen IV to migrate into surrounding tissue. Interestingly, Endo180 is a cell surface receptor that promotes the uptake of collagen for its degradation intracellularly. Moreover, Endo180 expression is *(i)* elevated significantly in highly invasive breast cancer cells; *(ii)* induced transcriptionally by TGF-β stimulation in breast cancer cells; and *(iii)* reduced the collagen content and enhanced the growth of mammary tumors produced in mice [100]. In addition, TGF-β also governs collagen function by upregulating the expression of MMP-2 and other collagenases in normal and malignant MECs, leading to their enhanced migration and invasion [72,73,101].

4.6. Fibronectin

Fibronectin is a large and critical ECM glycoprotein whose elevated production by cancer cells classically is associated with the acquisition of EMT, and more recently, with the development of the metastatic niche [67]. TGF-β is a potent inducer of fibronectin production and deposition into the ECM [102], where it binds integrins and regulates cell adhesion and motility. The synthesis and secretion of fibronectin into the ECM is primarily mediated by fibroblasts, and by epithelial cells induced to undergo EMT (Table 1; [103]). With respect to the latter, nontumorigenic EpH4 MECs engineered to express oncogenic Ras (*i.e.*, EpRas cells) significantly upregulate their expression of fibronectin and its receptor, α5β1 integrin when stimulated with TGF-β [104]. More importantly, administration of neutralizing α5 integrin antibodies to TGF-β treated EpRas cells inhibited their migration and induced a significant apoptotic response [104]. Thus, the synthesis and deposition of fibronectin, coupled with changes in expression and activation of integrins (*see below*), clearly represent an important mechanism that enables TGF-β to stimulate invasive migration during EMT.

4.7. Cadherin Switching

A phenotypic hallmark of EMT is its ability to downregulate the expression and function of E-cadherin, which is critical in mediating epithelial cell integrity and cell-cell adhesion [105]. Reduced E-cadherin expression in developing and progressing carcinomas takes places through several mechanisms that function en masse to promote cancer cell invasion [5]. For instance, E-cadherin can be inactivated by genetic mutations, and humans harboring these E-cadherin mutations have significantly increased risk of developing cancer [106]. In addition, epigenetic silencing of the E-cadherin *(CDH1)* promoter *via* hypermethylation of its 5′ CpG island also enhances the development of carcinomas [107]. Along these lines, TGF-β stimulation of EMT also represses the synthesis of E-cadherin transcripts in large part *via* its ability to induce the expression of the Snail/ZEB family of basic helix-loop-helix transcription factors, including that of Snail1, ZEB1, Snail2/Slug, Twist, and ZEB2/SIP1 [105,108-110]. Although the relative contribution of canonical and noncanonical TGF-β signaling in mediating transcriptional activation of these E-cadherin repressors remains to be determined definitely, recent evidence suggests that these events do take place in a cell type-specific manner in response to TGF-β. For example, activation of Smad2/3 by TGF-β in MECs induces their expression of the nuclear high mobility group A2 (HMGA2), which promotes EMT by stimulating the expression of Snail1, Snail2/Slug, and Twist, and by inhibiting the expression of ID2 (inhibitor of differentiation 2) [111]. In addition, while the functional consequences of diminished E-cadherin expression on the behavior of transitioning epithelial cells is well established, recent studies have determined that these same cells also exhibit upregulated expression of N-cadherin (*i.e.*, **N**euronal-cadherin) [65], an event linked to elevated cell motility and poor clinical outcomes in cancer patients [112-114]. At present, the necessity of increased N-cadherin expression in mediating EMT, particularly that stimulated by TGF-β, remains to be clarified. Indeed, we [45] and others [115,116] recently established murine 4T1 breast cancer cells as a model of advanced stage breast cancer whose increased malignancy is governed by TGF-β. Interestingly, while 4T1

cells undergo EMT and downregulate E-cadherin when stimulated by TGF-β [44,45], these cells fail to express and/or elevate their expression of N-cadherin during EMT initiated by TGF-β (M.K. Wendt and W.P. Schiemann, *unpublished observation*). Thus, future studies aimed at determining the exact nature of N-cadherin in promoting the acquisition of EMT and metastatic phenotypes clearly are warranted.

4.8. Vimentin

The intermediate filament protein vimentin is expressed in all primitive cell types, but not in their differentiated counterparts. In light of its role as a master regulator of EMT, it perhaps is not surprising to learn that TGF-β stimulation of EMT reactivates vimentin expression in dedifferentiating epithelial cells, an event that serves as a canonical marker for detecting fully transitioned epithelial cells and their acquisition of fibroblastoid-like phenotype [117].

4.9. α-Smooth Muscle Actin

A major component of contractile microfilaments is α-smooth muscle actin (α-SMA), which also serves as a canonical marker for detecting fibroblasts/mesenchymal cells, particularly myofibroblasts. Indeed, during its induction of EMT, TGF-β stimulates α-SMA expression in transitioning epithelial cells [118], an event associated with increased tumor invasion and decreased patient survival rates [119].

5. TRANSMEMBERANE & MEMBRANE PROXIMAL PROTEIN COMPLEXES THAT IMPACT TGF-β SIGNALING & EMT

Recent evidence suggests that cell surface signaling receptors, such as receptor tyrosine kinases (RTKs) and G-protein-coupled receptors, do not function in isolation and instead require accessory signaling inputs that arise from interacting receptors and signaling modules. As shown in Figure 3, the function and behavior of TGF-β receptors also are modulated *via* their association with an ever expanding array of receptor-interacting molecules and scaffolding proteins. Included in this growing list of TGF-β receptor regulators are members of the integrin superfamily of heterodimeric transmembrane adhesion receptors, which function as direct physical conduits that link the ECM to the cytoskeleton of the cell [120,121]. Integrin signaling commences upon their clustering and subsequent stimulation of the Ser/Thr protein kinase ILK (**i**ntegrin-**l**inked **k**inase), as well as members of the Src family of PTKs and FAK (**f**ocal **a**dhesion **k**inase), leading to the activation of a vast array of downstream effectors, including members of the MAP kinase family of protein kinases, members of the Ras/Rho family of small GTPases, and members of the PI3K and AKT signaling axes [120-124]. Integrins also regulate cell behavior through their ability to form complexes with RTKs [125,126]. For instance, β1 integrins form FAK-dependent complexes with the receptors for EGF, PDGF, and HGF [126,127], and in doing so, enables growth factor-mediated induction of cell migration and invasion [126]. Interestingly, the scaffolding function of FAK is independent of its PTK activity, but does require its N-terminal FERM (**F**AK **E**zrin **R**adixin **M**oesin) domain and C-terminal FAT (**F**ocal **A**dhesion **T**argeting) domain to bind RTKs and β1 integrins, respectively [126]. Lastly, the establishment of EMT phenotypes in cultured cells, as well as the development of late stage cancers and their acquisition of invasive and metastatic phenotypes both have been linked to dramatic changes in the expression and localization of integrins in epithelial cells [104,128].

In addition to its regulation of cell cycle progression, TGF-β also figures prominently in mediating ECM remodeling and repair *via* its ability to regulate integrin expression [129-131]. Moreover, αvβ6 and αvβ8 integrin ligation promotes the activation of TGF-β1 and TGF-β3 from inactive ECM depots [132-137], which regulates alveolar development,

wound closure, fibrosis, and EMT [130,132,138-140]. In addition, epidermal transgenic expression of α6β4 integrin also elicits elevated development of metastatic papillomas and carcinomas in a chemical carcinogenesis model of skin cancer. Importantly, the tumorigenicity associated with α6β4 integrin expression was linked to its ability to uncouple TGF-β from activating Smad2/3 and preventing cell cycle progression [141]. Similar reciprocity between integrins and TGF-β is observed in cancers of the prostate, whose metastasis to bone is stimulated by TGF-β and its induction of α2β1 integrin, which binds to bone-derived type I collagen [142]. Thus, the ability of TGF-β to stimulate cancer progression and metastasis requires an intricate interplay between signals arising from TGF-β receptors and those initiated by integrins. Accordingly, integrins have been found to associate with TGF-β receptors and play a critical function in coupling TGF-β to activation of its noncanonical effectors, and to its induction of EMT. For instance, neutralizing β1 integrin antibodies abrogated the ability of TGF-β to activate p38 MAPK and induce EMT in MECs [39]. Similarly, hepatocellular carcinoma cells elevate their expression of α3β1 integrin in response to TGF-β, an event that enhances their motility and invasiveness [143]. Moreover, administering laminin-5 together with TGF-β stimulated hepatocellular carcinoma cells to undergo EMT in an α3 integrin-dependent manner [144], further demonstrating the necessity of integrins to cooperate with TGF-β to induce EMT and invasion in transitioning cells.

We also described the functional cooperation between integrins and TGF-β in promoting EMT, as well as in stimulating the development and progression of mammary tumors. For instance, we found the expression and activity of αvβ3 integrin and its downstream effector Src to be essential for TGF-β stimulation of MEC proliferation, invasion, and EMT [43-45]. In addition, transgenic expression of αvβ3 integrin not only negated the cytostatic response of normal MECs to TGF-β, but also enhanced its stimulation of MEC invasion and p38 MAPK activation. Importantly, inactivation of either αvβ3 integrin or Src function abolished the ability of TGF-β to stimulate EMT and invasion in normal and malignant MECs [43,44]. Mechanistically, β3 integrin interacts physically with TβR-II, leading to its *(i)* phosphorylation on Y284 by Src; *(ii)* interaction with Grb2 and Shc at phosphorylated Y284; *(iii)* activation of p38 MAPK; and *(iv)* stimulation of EMT and invasive migration in normal and malignant MECs [44]. Along these lines, the growth and metastasis of breast cancer cells in mice absolutely required TβR-II to be phosphorylated on Y284, a phosphotransferase reaction that disrupts the delicate balance between canonical and noncanonical TGF-β signaling inputs activated during mammary tumorigenesis [45]. In addition to its ability to promote pulmonary metastasis stimulated by TGF-β [45], αvβ3 integrin expression also directs breast cancer cell metastasis to bone [145,146] and lung [146] in part through a TGF-β-dependent pathway. Collectively, these findings suggest that pharmacological targeting of noncanonical TGF-β effectors, particularly αvβ3 integrin, Src, and p38 MAPK, may prove efficacious in treating metastatic breast cancers.

Besides integrins, a growing number of intracellular proteins also have been shown to interact with and regulate the activity of TGF-β receptors. For instance, two members of the focal adhesion complex, namely FAK and its downstream effector p130Cas (p130**C**rk-**a**ssociated **s**ubstrate), both influence the cellular response to TGF-β through dramatically different mechanisms. Indeed, TGF-β stimulates FAK and its relative PYK2 during EMT [147], leading to the activation of JNK and the subsequent upregulation α-SMA in fibroblasts [148]. In addition, FAK activation in hepatocytes is necessary for the transcription of mesenchymal and invasive gene expression profiles, as well as for the delocalization of E-cadherin from the plasma membrane [149]. Finally, we recently established FAK as a molecular scaffold that facilitates the formation of oncogenic β3 integrin:TβR-II complexes and their activation of Src and interaction with Grb2 (M.K. Wendt and W.P. Schiemann, *unpublished observation*). Moreover, the ability of FAK to

form these signaling complexes is essential for TGF-β stimulation of p38 MAPK in breast cancer cells, as well as for their induction of EMT and metastasis stimulated by TGF-β (M.K. Wendt and W.P. Schiemann, *unpublished observation*; [45]). Thus, the aberrant recruitment of FAK to TGF-β receptors readily influences the oncogenic conversion of TGF-β from a tumor suppressor to a tumor promoter, including its stimulation of pathophysiological EMT in carcinoma cells.

In stark contrast to FAK, the incorporation of p130Cas into active TGF-β receptor complexes alters the coupling of TGF-β to the canonical Smad2/3 pathway. Indeed, the activation and phosphorylation of p130Cas following cellular adhesion to ECM matrices led to its association and inactivation of Smad3, and to diminished cytostatic activity of TGF-β [150]. Similarly, we find that rendering malignant, metastatic MECs deficient in p130Cas enhances Smad2/3 activation by TGF-β, but fails to alter its coupling to p38 MAPK; however, this same cellular condition selectively inhibited breast cancer metastasis only in cells that possessed heightened TGF-β signaling (M.K. Wendt and W.P. Schiemann, *unpublished observation*), suggesting that p130Cas acts as a molecular integrator of canonical Smad2/3 signaling when confronted with elevated oncogenic behavior mediated by the receptors for TGF-β or EGF [151].

Recently, the regulation of TGF-β signaling has been shown to be modulated by two additional adapter proteins that localize to focal adhesions, namely Hic5 and Disabled-2 (Dab2). Indeed, Hic5 is a member of the paxillin superfamily and, like paxillin, functions as an adapter protein at focal adhesions [152], as well as resides in the nucleus where functions as a transcriptional coactivator in regulating gene expression induced by the androgen [153] and glucocorticoid [154,155]. Moreover, Hic5 expression is low in quiescent MECs, but is induced rapidly *via* a RhoA/ROCK-dependent pathway following administration of TGF-β [152]. In addition, uncoupling Hic5 from TGF-β regulation prevents its induction of EMT in normal MECs [156]. Thus, Hic5 plays an essential role in coupling TGF-β receptors to activation of RhoA/ROCK and, consequently, to the induction of EMT. Along these lines, Dab2 was identified originally as an ovarian tumor suppressor gene [157,158] that regulates the actin cytoskeletal architecture during cell migration and adhesion [159]. More recently, Prunier *et al* [160] established Dab2 as a novel gene target of TGF-β in MECs undergoing EMT in part *via* its ability to *(i)* associate with TGF-β receptor complexes [26]; *(ii)* promote Smad2/3 activation by TGF-β receptors [26]; and *(iii)* stimulate the activation of TAK1 and JNK, which induced fibronectin expression and enhanced cell motility [161]. Along these lines, TGF-β stimulates Dab2 expression in MECs undergoing EMT, which promotes the formation of Dab2:β1 integrin complexes and their activation of FAK [160]. Importantly, measures capable of disrupting Dab2 function prevents EMT stimulated by TGF-β, as well as promotes its ability to induce apoptosis in MECs. Although the molecular mechanisms underlying the ability of TGF-β to stimulate Dab2 expression remains to be defined, these studies do provide interesting insights into the connections that govern alterations in cell survival and morphology regulated by TGF-β.

Finally, two laboratories recently identified a novel collaboration between signaling molecules activated by TNF-α and those activated by TGF-β. Indeed, both studies demonstrated the ability of TGF-β to induce the physical association of its receptors with that of TRAF6 (**T**NF **r**eceptor-**a**ssociated **f**actor 6) [162,163], leading to K63-linked polyubiquitination and activation of TAK1 and its subsequent stimulation of p38 MAPK and JNK. Moreover, whereas TRAF6-deficiency had no effect on the coupling of TGF-β to Smad2/3, this same cellular condition uncoupled TGF-β from activation of MAP kinases and prevented this cytokine from inducing EMT in normal MECs [163]. Taken together, these studies reinforce the notion that imbalances in the TGF-β signaling system that favor its activation of noncanonical effectors over that of its canonical Smads are crucial to its

induction of EMT in normal and malignant epithelial cells. These findings also point to the need for additional studies to define precisely how these aberrant protein complexes and modules impact the epithelial cell response to TGF-β, and how science and medicine can better target these effector molecules that promote oncogenic signaling and EMT initiation by TGF-β.

6. SIGNALING SYSTEMS INVOLVED IN EMT STIMULATED BY TGF-β

Transmembrane signaling by TGF-β traditionally is associated with its activation of Smad2/3 and their ability to alter the transcription of TGF-β-responsive genes, which clearly play an important role in mediating the ability of TGF-β to induce EMT, tumor formation, and cancer cell metastasis [164]. The necessity of Smads 2 and 3 for TGF-β stimulation of EMT has been reviewed extensively in the scientific literature, and readers desiring a more in depth description of Smad2/3 function in regulating EMT in normal and malignant cells are directed to several recent reviews [4,5,11]. As alluded to above, the enhanced coupling of TGF-β to its noncanonical effectors figures prominently in mediating its biological and pathological behaviors, particularly its ability to induce EMT and cancer cell metastasis. Table 2 lists a variety of noncanonical effectors targeted by TGF-β during its activation of EMT, while the role of these signaling molecules during epithelial cell EMT induced by TGF-β is discussed below.

6.1. Rho Family of Small GTPases

The Rho family of small GTPases is comprised of RhoA, Rac1, and Cdc42, which regulate the formation of stress fibers, lamellipodia, or filopodia, respectively [165,166]. Indeed, Rac1 is an established inducer of cell-cell adhesions in epithelial cells [167], which contrasts sharply with the ability of RhoA to dissolve these adhesive complexes to facilitate times of cell migration [62]. Given the importance of these small GTPases in overseeing cell adhesion, morphology, and migration, it is fitting to find that these effectors are intimately involved in EMT stimulated by TGF-β. For instance, the activation of RhoA by TGF-β enables MECs to undergo EMT, while measures capable of inhibiting RhoA function or that of its downstream effector, $p160^{ROCK}$, uncouple TGF-β from EMT in MECs [65]. Moreover, RhoA activation also is essential for TGF-β stimulation of α-SMA expression in renal epithelial cells undergoing EMT [118]; however, completion of this same cellular event in lens epithelial cells requires signaling inputs from both RhoA/ROCK and Smad2/3 [168]. Taken together, these studies point to the overall importance of noncanonical TGF-β signaling, particularly that induced by RhoA/ROCK, to induce EMT in epithelial cells.

6.2. PI3K/AKT

The tumor suppressing activity of TGF-β not only reflects its ability to induce cytostasis, but also its propensity to activate apoptosis in epithelial cells [10,11,13,169]. Interestingly, the ability of TGF-β to stimulate apoptosis frequently is subverted during tumorigenesis, leading to enhanced cancer cell survival *via* activation of the PI3K and AKT signaling systems by TGF-β. Indeed, administration of PI3K inhibitors to MECs inhibits their activation of AKT and ability to undergo EMT in response to TGF-β [38]. The activation of AKT by TGF-β can transpire directly *via* TGF-β receptors or indirectly *via* the transactivation of EGF [170] and PDGF [171] receptors, which induces the expression of genes operant in mediating cancer cell EMT, metastasis, and survival. In addition to altering gene expression profiles, AKT also regulates mRNA translation when impacting the response of epithelial cells to TGF-β. For instance, TGF-β stimulation of EMT in MECs is accompanied by an increase in cell size and protein content, both of which correlate with the rapid activation of mTOR (**m**ammalian **t**arget **o**f **r**apamycin) in transitioning MECs [40]. Somewhat unexpectedly, administering the mTOR inhibitor, rapamycin, to MECs failed to affect their acquisition of

an EMT morphology in response to TGF-β; however, this same cellular condition completely prevented the ability of TGF-β to increase MEC size and protein production, as well as inhibited their migration and invasion [40]. Taken together, these findings highlight an important bifurcation in the TGF-β signaling system that dissociates the ability of TGF-β to alter cell morphology from its ability to elevate cell motility. Future studies need to identify the transcriptional and translational objectives targeted by TGF-β, as well as determine their relative contribution to oncogenic signaling stimulated by TGF-β in normal and malignant cells.

6.3. Integrin-linked Kinase (ILK)

In addition to their stimulation of PTKs, the ECM engagement of β1 and β3 integrins also activates the Ser/Thr protein kinase ILK and its ability to mediate the *(i)* stimulation of MAP kinases, PI3K/AKT, and small GTPases; and *(ii)* the inhibition of GSK3β [172-174]. Accordingly, targeting ILK expression to mouse mammary glands elicited a hyperplastic reaction that progressed to full-blown breast cancer in part *via* constitutive activation of ERK1/2 and AKT, which inactivated GSK3β [175]. Elevated ILK expression is associated with the acquisition of EMT phenotypes by MECs, including reductions in their expression of E-cadherin and adhesion, as well as increases in their formation of actin stress fibers and invasion [176]. ILK also participates in EMT stimulated by TGF-β by coupling this cytokine to its activation of AKT [177], and to its elevated expression of MMP-2 and uPA [91]. Collectively, these findings suggest that ILK may function analogously to FAK in mediating oncogenic signaling by TGF-β, and as such, suggest that ILK interfaces integrin signaling with that stimulated by TGF-β in epithelial cells undergoing EMT.

6.4. NF-κB

NF-κB is a principal player involved in regulating the production of proinflammatory cytokines [178], and in stimulating tumor growth, vascularization, survival, and invasion [178]. In addition, NF-κB activity was observed to be essential in mediating the ability of Ras-transformed breast cancer cells to undergo EMT and colonize the lung when stimulated by TGF-β [48,179]. Along these lines, NF-κB activity also associates with several hallmarks of EMT, including downregulated E-cadherin expression and upregulated expression of vimentin [180]. It is interesting to note that TGF-β typically represses NF-κB activity in normal epithelial cells [47,181,182], but readily induces the activation of this transcription factor in their malignant counterparts [47,182]. Recently, we demonstrated that the activation of NF-κB by TGF-β transpires *via* the aberrant formation of a TAB1:xIAP:TAK1:IKKβ signaling module that only materializes in malignant MECs, or in normal MECs following their induction of EMT by TGF-β [47]. Functionally, the formation of TAB1:xIAP:TAK1:IKKβ complexes is essential for TGF-β stimulation of *(i)* Cox-2 expression and its induction of EMT and invasion in normal and malignant MECs [47,182]; and *(ii)* mammary tumor growth in immunocompetent and immunocompromised mice [47], suggesting a potentially important role of NF-κB in regulating innate immunity by TGF-β. Collectively, these findings demonstrate the role of NF-κB in supporting the development of oncogenic signaling by TGF-β in normal and malignant cells, particularly its ability to drive the growth, metastasis, and EMT of tumors in response to TGF-β.

6.5. MAP Kinases

Members of the MAP kinase family of protein kinases, which includes ERK1/2, JNKs, and p38 MAPKs, all have been implicated in mediating EMT and metastasis stimulated by TGF-β [39,183,184]. For instance, pharmacological inhibition of ERK1/2 in MECs uncouples TGF-β from inducing EMT and its associated formation of stress fibers and delocalization of ZO-1 and E-cadherin [183]. Similarly, inactivation of JNK also prevents the ability of TGF-β to stimulate the morphological and transcriptional changes that drive EMT in epithelial

cells [93,161]. Indeed, the activation of JNK by TGF-β induces fibronectin expression during EMT, and during fibroproliferative disorders that may progress to carcinoma [185]. Along these lines, collagen I and other ECM proteins can promote EMT *via* their activation of PI3K, Rac1, and JNK [186]; however, while it remains to be determined whether TGF-β is intimately involved in this ECM-dependent induction of EMT, it seems likely that the ability of TGF-β to stimulate the synthesis and secretion of ECM components is reciprocated by the ability of the ECM to establish paracrine and autocrine TGF-β signaling loops that perpetuate EMT in normal and malignant epithelial cells.

Besides its ability to activate ERK1/2 and JNK, TGF-β also stimulates p38 MAPK during its induction of EMT in normal and malignant cells [39]. Interestingly, the activation of p38 MAPK by TGF-β requires the expression and activity of either β1 or β3 integrins [39,43,44]. Indeed, we established the necessity of β3 integrin to form oncogenic signaling complexes with TβR-II, resulting in its phosphorylation on Y284 by Src [43,44]. Once phosphorylated, Y284 functions as a SH2-binding site that coordinates the recruitment of either ShcA or Grb2, as well as their subsequent activation of p38 MAPK [44]. Most importantly, pharmacologic or genetic inactivation of this oncogenic signaling axis prevented TGF-β from stimulating the growth and pulmonary metastasis of breast cancers produced in mice [45]. Finally, the activation of p38 MAPK not only induces EMT, but it also stimulates the expression of prometastatic genes, particularly TβR-II and MMPs 2 and 9 [187,188], which collectively points to the importance of inappropriate p38 MAPK activation in mediating the conversion of TGF-β from a tumor suppressor to a tumor promoter.

7. MECHANISMS OF GENE REGULATION BY TGF-β

The importance of aberrant genetic and epigenetic events in promoting tumorigenesis is highlighted by the consistent and repeated finding that cancer cells that have lost their ability to regulate various rate-limiting steps that normally suppress malignant development. These untoward events typically are associated with the *(i)* mutational activation of oncogenes, *(ii)* mutational inactivation of tumor suppressor genes, or *(iii)* amplified or silenced expression of genes coupled to the development of cancer hallmarks [189]. Although many of the signaling systems and genes targeted by TGF-β during its activation of EMT have been discussed above, the succeeding sections focus on the transcriptional mechanisms that orchestrate its transitioning of epithelial cells into their mesenchymal counterparts (Figure 3).

7.1. Nuclear Factors

The Snail family of transcription factors are master regulators of EMT and include *(i)* SNAI1 (Snail) and SNAI2 (Slug); *(ii)* two ZEB factors, ZEB1 and ZEB2 (SIP1); and *(iii)* FOXC2 [110,190]. Indeed, the binding of Snail to conserved E-box sequences present in E-cadherin promoter is classically associated with EMT and the repression of E-cadherin expression, as well as that of the aforementioned cell polarity genes, occludin and claudin [191]. The essential function of various Snail family members in mediating EMT and cancer metastasis have been extensively reviewed, and as such, readers desiring a more in-depth description of their functions and behaviors in governing EMT are directed several recent reviews [109,190]. Besides Snail family members, emerging evidence also implicates dysregulated Myc expression in promoting the ability of epithelial cells to undergo EMT in response to TGF-β. Indeed, the tumor suppressing activity of TGF-β is intimately linked to its ability to rapidly repress Myc expression in epithelial cells [11,13]. Accordingly, uncoupling TGF-β from regulation of Myc expression is a common occurrence in developing carcinomas, resulting in their insensitivity to cytostasis mediated by TGF-β [192,193]. Somewhat unexpectedly, Myc recently was observed to function cooperatively with Smad4 to induce Snail expression during TGF-β stimulation of EMT in MECs [194].

Taken together, these findings suggest the Myc functions as a molecular detector that enables epithelial cells to sense TGF-β as a mediator of cytostasis or EMT.

7.2. STAT3

Signal **t**ransducer and **a**ctivator of **t**ranscription 3 (STAT3) is a critical component of cell survival and proliferative responses, and its inappropriate activation can endow this transcription factor with oncogene-like properties in developing and progressing neoplasms [195]. A recent study has suggested that TGF-β couples to STAT3 phosphorylation and activation *via* a PKA-dependent mechanism [196]. Moreover, STAT3 activation by TGF-β is necessary for its ability to induce apoptosis and EMT [196], and to stimulate the invasion and metastasis of Smad4-deficient pancreatic cancer cells [197]. In addition, carcinoma cells that overexpressed EGFR readily acquired EMT phenotypes when stimulated with EGF, a cellular reaction that required EGF/EGFR to activate STAT3 and its subsequent upregulation of TWIST [198]. Thus, while several studies have shown EGF to cooperate with TGF-β in mediating tumorigenesis, the extent to which this tumor-and EMT-promoting alliance requires STAT3 remains to be determined definitively.

7.3. Estrogen Receptor-α

Aberrant repression of the nuclear hormone receptor, estrogen receptor-α ER-α) has long been recognized as a major event that promotes the development and progression of mammary tumors, as well as significantly worsens the clinical prognosis of patients with metastatic breast cancer [199,200]. In addition to its prominent role in regulating mammary gland development and homeostasis, ER-α also prevents the ability of malignant MECs to acquire EMT and metastatic phenotypes, doing so *via* its stimulation of MTA3 (metastasis tumor antigen 3) expression, which in turn represses the expression of Snail [201]. Thus, inactivation or loss of ER-α in MECs promotes their EMT and invasion by allowing for their expression of Snail. Somewhat surprisingly, constitutive Snail expression in breast cancer cells was observed to inhibit ER-α expression [202], leading to enhanced invasion of these ER-α-deficient breast cancer cells. It is interesting to note that physiological actions of estrogen in mammary tissues typically oppose those of TGF-β. Accordingly, inactivation of ER-α signaling led to elevated expression of components of the TGF-β signaling system and, presumably, to enhanced EMT in breast cancer cells [202]. Thus, Snail appears to function as a novel molecular sensor that integrates the opposing cellular functions of ER-α and TGF-β, particularly their ability to inhibit and stimulate EMT, respectively.

7.4. TGF-β, microRNAs, & EMT

A number of recent studies have established microRNAs as important players that participate in cell and tissue development, as well as control cell proliferation and motility through their ability to repress mRNA translation, or to induce mRNA degradation [203-206]. These studies also have shown that a single microRNA can repress the translation of multiple transcripts, and as such, dysregulated expression of a single microRNA, either positively or negatively, could initiate a cascade of gene silencing events capable of eliciting disease development in humans, including cancer. Accordingly, aberrant regulation of several microRNAs (or miRs) is observed in human cancers (see [207]), especially in those of the breast, which can in fact be subtyped based on their differential expression of various microRNAs [205,208]. Along these lines, microRNAs also play a prominent role in regulating the expression of EMT-related genes. For instance, members of the miR-200 family suppress EMT by downregulating the expression of ZEB1 and ZEB2 (SIP1), which as mentioned above function in repressing the expression of E-cadherin [209-211]. Indeed, miR-200 family member expression marks epithelial cells that express E-cadherin and not vimentin, as well as identifies cancer cells that are poorly motile [212]. With respect to EMT and its regulation by TGF-β, a recent study established that this

cytokine downregulates the expression of microRNA-200 family members and miR-205, which promotes ZEB1 and ZEB2 expression and their initiation of EMT [211]. In addition, these same microRNAs are frequently downregulated in invasive human breast cancer cells that exhibit a mesenchymal-like morphology [211]. Somewhat surprisingly, elevated ZEB1 expression also was found to repress that of miR-41 and miR-200c, both of which belong to the miR-200 family and whose absence establishes a negative feedback loop that stabilizes the acquisition of EMT phenotypes in epithelial cells [213].

In contrast to the miR-200 family of microRNAs, metastatic breast cancers were found to preferentially upregulate their expression of miR-10b, which promotes the invasion and metastasis of malignant MECs both *in vitro* and *in vivo* [214]. Mechanistically, Twist was observed to induce miR-10b expression that results in the *(i)* diminished translation of HoxD10 transcripts, and *(ii)* induction of the prometastatic gene, RhoC [214]. More recently, administration of TGF-β to normal MECs induced miR-155 expression *via* a Smad4-dependent mechanism, an event that elicited EMT in cytokine-stimulated MECs [215]. Once expressed, miR-155 abrogated MEC expression of RhoA and prevented their ability to undergo EMT in response to TGF-β [215]. Similar overexpression of miR-21 also is observed in human cancers and results in the repression of the tumor suppressor, tropomyosin-1 [216,217]. The net effect of these events is the enhanced ability of breast cancer cells to grow in an anchorage-independent fashion [218], and to resist apoptotic stimuli in part *via* upregulated expression of the survival factor, Bcl-2 [216-218]. As above, the ability of TGF-β to induce EMT has been linked to its induction of miR-21 [219], which enhances cancer cell motility and invasive migration by downregulating tropomyosin expression [220-222].

Taken together, these findings suggest that the ability of TGF-β to govern microRNA expression plays an important role in dictating whether this cytokine propagates tumor suppressing or promoting signals to responsive cells; they also suggest that the development of chemotherapeutic agents capable of targeting microRNAs may function in “normalizing” carcinoma cells and, consequently, rendering them insensitive to the oncogenic activities of TGF-β.

7.5. DNA Hypermethylation

DNA hypermethylation is well established in its ability to aberrantly silence the expression of tumor suppressor genes in developing and progressing carcinomas [107]. Importantly, epigenetic silencing of the E-cadherin promoter *via* hypermethylation leads to morphological and differential gene expression profiles indicative of EMT phenotypes [107,223]. Besides silencing of the E-cadherin promoter, EMT and mammary tumorigenesis usurp the inactivation of p16INK4a as a means to promote expanded DNA hypermethylation. Indeed, Roberts *et al* [224] observed the loss of p16INH4a expression to depress that of the polycomb genes, EZH2 and SUZ12, which collectively enhance DNA hypermethylation and the generation of MECs locked into a perpetual plastic state. Interestingly, the repression of E-cadherin expression during EMT appears to function as a prerequisite for directed gene hypermethylation during the development and progression of mammary tumorigenesis [225]. Moreover, hypermethylation of the E-cadherin promoter served to mark stable EMT in Ras-transformed MECs that was induced by serum *versus* a transient EMT induced in these same MECs by TGF-β [225]. Clearly, additional investigations are warranted to further our understanding of the linkages between TGF-β and DNA hypermethylation in mediating EMT in normal and malignant cells. Indeed, upregulated ZEB1 expression and its ability to induce EMT is tightly correlated with the loss of E-cadherin expression in cultured epithelial cells, and in metastatic carcinoma cells *in vivo* [226]. Based on these findings, it is tempting to speculate that initiation of EMT results in *(i)* the expression of Snail family members that collectively function in repressing

that of E-cadherin, and *(ii)* the subsequent recruitment of DNA methyltransferases that potentiate and stabilize the EMT phenotype.

8. FUTURE PERSPECTIVE

Embryogenesis and its associated EMT creates progenitor cells that ultimately give rise to every cell- and tissue-type within mature organisms. For instance, EMT underlying gastrulation results in the generation of the mesoderm, which subsequently develops along distinct differentiation pathways that elicit the production of muscle, bone, and connective tissues [7]. Similarly, a single mammary stem cell can give rise to both the outer myoepithelial and inner luminal layers that comprise the branched structure of these glands[227-229]. These and other studies suggest an important link between physiologic EMT and the generation and maintenance of stem cells, of which both phenomena require signaling inputs elicited by the TGF-β signaling system [230]. Given the parallels between physiologic and pathophysiologic EMT, it is fitting to find that the inappropriate reactivation of EMT in malignant tissues also promotes the selection and expansion of cancer stem cells. For instance, aggressive and poorly differentiated breast cancer and glioma cells exhibit gene signatures characteristic of stem cells [231]. In addition, TGF-β stimulation of EMT in human and mouse MECs established a population of transitioning cells that possessed stem cell-like properties [232,233], suggesting that EMT induced by TGF-β promotes “stemness.” Along these lines, inactivation of TGF-β signaling in cancer stem cells induced a mesenchymal-epithelial transition that reestablished a more epithelial-like morphology in aggressive cancer cells [234]. Thus, these intriguing findings suggest that the ability of TGF-β to stimulate the selection and expansion of stem cell-like progenitors in post-EMT epithelial cells may represent the molecular crux that endows TGF-β with oncogenic activity. Clinically, these findings also suggest that the development of chemoresistance may reflect the induction of EMT and its expansion of cancer stem cells by TGF-β. If correct, then the studies reviewed herein offer important insights into how science and medicine may one day target the TGF-β signaling system and its coupling to EMT in order to *(i)* regulate the behaviors and activities of normal and cancer stem cells, and *(ii)* alleviate the devastating effects of TGF-β in promoting the acquisition of invasive and metastatic phenotypes in human cancers.

Executive Summary

Defining EMT

- EMT is defined by the morphologic and genetic transition of epithelial cells to fibroblastoid- or mesenchymal-like cells.
- The major cell-cell junctions include tight junctions, adherens junctions, and desmosomes.
- Tight junctions are composed of claudins, occludins, and JAMs, which are linked to the actin cytoskeleton *via* ZO-1, −2, and −3.
- During EMT, Par6 recruits the E3 ubiquitin ligase, Smurf1, which ubiquitinates RhoA, leading to its degradation and subsequent dissolution of tight junctions.
- Adherens junctions consist E-cadherin that is linked to the actin cytoskeleton by α- and β-catenins.
- EMT represses E-cadherin transcription and disrupts its localization at the plasma membrane.

EMT, TGF-β, and Cell Microenvironments

- Tumors house a mixture of malignant and normal cells, including fibroblasts, endothelial, and infiltrating immune cells, which collectively comprise the tumor microenvironment.
- Alterations within tumor microenvironments can either suppress or promote cancer progression in a manner that mirrors the acquisition of oncogenic signaling by TGF-β.
- Transient disruption of the ECM and epithelial cell microenvironments are characteristic of physiological EMT. In contrast, chronic disruptions within carcinoma cell microenvironments elicits pathologic EMT and its ability to support cancer cell invasion and metastasis.
- MMPs comprise a large family of proteases that regulate essential steps of embryogenesis and tissue morphogenesis, wound healing, and cell growth. MMPs also degrade nearly all ECM and basement membrane components, leading to the development and progression human malignancies.
- NCAM is a member of the immunoglobulin superfamily whose expression is increased during EMT.
- The extracellular portion of NCAM is cleaved by MMP-28.
- uPA is a serine protease whose expression is elevated during EMT and associates with advanced disease states and poor clinical outcomes.
- PAI-1 antagonizes tPA and uPA; its expression also is increased during EMT and associates with advanced disease states and poor clinical outcomes.
- EMT leads to the upregulation of collagen and fibronectin, whose expression drastically alters cell microenvironments.
- Vimentin is an intermediate filament protein, while α-smooth muscle actin is a component of contractile microfilaments. Upregulated expression of both proteins are considered to be markers of fully transitioned cells.

Transmembrane and Membrane Proximal Protein Complexes the Impact TGF-β Signaling and EMT

- α and β integrin heterodimers function in linking the ECM to intracellular signaling pathways, and to the cellular cytoskeletal system.
- Integrins interact with several intracellular kinases, as well as several transmembrane RTKs.
- Integrin β1 and β3 interact with TβR-II and profoundly affect downstream signaling events stimulated by TGF-β.
- β3 integrin is upregulated dramatically during EMT induced by TGF-β.
- Interaction between αvβ3 integrin and TβR-II leads to Src-mediated phosphorylation of TβR-II at Tyr284, which binds Grb2 and promotes the activation of downstream MAP kinases.
- The ability of TGF-β to stimulate cancer progression and metastasis requires an intricate interplay between signals arising from TGF-β receptors and those initiated by integrins.
- FAK is required for EMT stimulated by TGF-β.

- p130Cas inhibits Smad3 activity and alters cytostasis induced by TGF-β.
- Hic5 is a member of the paxillin superfamily that is induced by and required for EMT stimulated by TGF-β.
- TRAF6 interacts physically with both TβR-I and TβR-II, leading to TAK1 activation and the stimulation of p38 MAPK and JNK.

Signaling Systems Involved in EMT Stimulated by TGF-β

- Transmembrane signaling by TGF-β activates Smad2/3 and their ability to alter the transcription of TGF-β-responsive genes, which play important roles during TGF-β stimulation of cancer cell EMT and metastasis.
- Small GTPases RhoA, Rac1, and Cdc42 regulate the formation of stress fibers, lamellipodia, or filopodia, respectively, and are intimately involved in EMT stimulated by TGF-β.
- The ability of TGF-β to stimulate apoptosis frequently is subverted during tumorigenesis, leading to enhanced cancer cell survival *via* activation of the PI3K and AKT signaling systems.
- ECM engagement of β1 and β3 integrins activates the Ser/Thr protein kinase ILK and its ability to mediate the *(i)* stimulation of MAP kinases, and *(ii)* the inhibition of GSK3β, PI3K/AKT, and small GTPases.
- ILK participates in EMT stimulated by TGF-β by coupling this cytokine the activation of AKT, and to the elevated expression of MMP-2 and uPA.
- NF-κB activity enables Ras-transformed breast cancer cells to undergo EMT and colonize the lung when stimulated by TGF-β.
- Activation of NF-κB also associates with several hallmarks of EMT, including the downregulated expression of E-cadherin and upregulated expression of vimentin.
- MAP kinase family members, including ERK1/2, JNK, and p38 MAPK, have all been implicated in mediating EMT and metastasis stimulated by TGF-β.

Mechanisms of Gene Regulation by TGF-β

- Aberrant genetic and epigenetic events promote tumorigenesis by circumventing various rate-limiting cellular steps that normally suppress neoplastic development. These key steps are known as the “Hallmarks of Cancer” and include *(i)* oncogene activation; *(ii)* tumor suppressor inactivation; *(iii)* apoptosis resistance; *(iv)* angiogenesis activation; *(v)* invasion and metastasis initiation; and *(vi)* immortality acquisition.
- Snail transcription factor family members are master regulators of EMT and include *(i)* SNAI1 (Snail) and SNAI2 (Slug); *(ii)* ZEB1 and ZEB2 (SIP1); and *(iii)* FOXC2.
- Evidence implicates dysregulated Myc expression in promoting the ability of epithelial cells to undergo EMT in response to TGF-β. The tumor suppressing activity of TGF-β is intimately linked to its repression of Myc expression in epithelial cells.
- STAT3 mediates cell survival and proliferative signals, and serves as an oncogene in several human cancers.

- TGF-β activates STAT3 *via* a PKA-dependent mechanism, leading to the induction of EMT.
- ER-α promotes mammary gland develop and homeostasis, and suppresses EMT by inducing the expression of MTA3 (metastasis tumor antigen 3), which represses the expression of Snail.
- microRNAs are essential mediators of all facets of cell and tissue development, and of cell proliferation, motility, and survival.
- Members of the miR-200 family suppress EMT by downregulating the expression of ZEB1 and ZEB2.
- Epigenetic silencing of the E-cadherin promoter *via* hypermethylation promotes the acquisition of EMT phenotypes and gene expression profiles.
- EMT and mammary tumorigenesis usurp the inactivation of p16INK4a as a means to expand aberrant DNA hypermethylation.

Redefining EMT Induced by TGF-β

- Inappropriate reactivation of EMT by TGF-β in malignant tissues promotes the selection and expansion of cancer stem and progenitor cells.
- Targeting the molecular links between TGF-β, EMT, and stemness reduces breast cancer tumorigenicity.
- The development of pharmacological agents that inhibit EMT stimulated by TGF-β may provide new avenues to manipulate the behaviors of normal and cancer stem cells, and to alleviate the acquisition of cancer metastasis.

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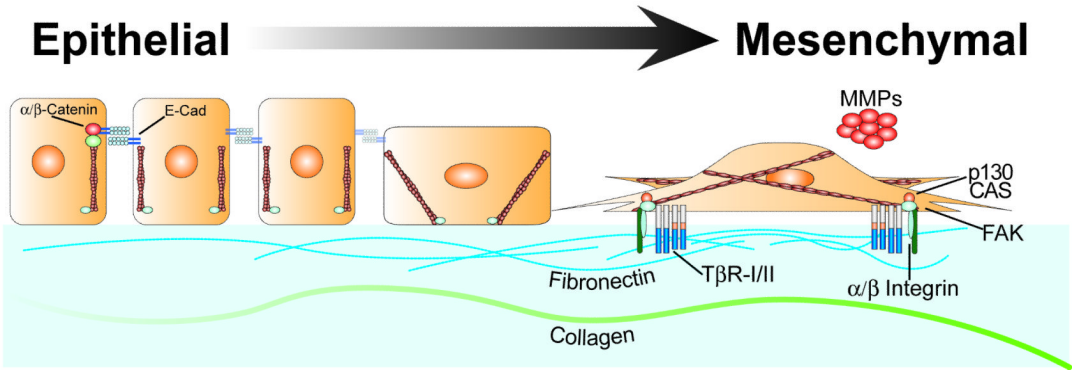

Figure 1. Epithelial Cells Transition to Mesenchymal-like Cells in Response to TGF-β

This schematic depicts polarized epithelial cells and their cuboidal structure that is maintained *via* cell-cell junctions comprised of homotypic E-cadherin molecules that are linked to the cortical actin cytoskeleton by α- and β-catenins. TGF-β stimulation of EMT during wound healing or tumor invasive migration results in the delocalization, degradation, and/or downregulation of cell-cell junctions and, consequently, a loss of epithelial integrity. In addition, the morphologic transition of epithelial cells also is supported by the simultaneous formation of actin stress fibers, the upregulation of integrins, and the activation of focal adhesion complexes. Moreover, the increased production and section of ECM proteins, such as fibronectin and collagen, coupled with the elevated expression and activation of MMPs enables transitioned fibroblastoid-like cells to exhibit invasive and motile phenotypes.

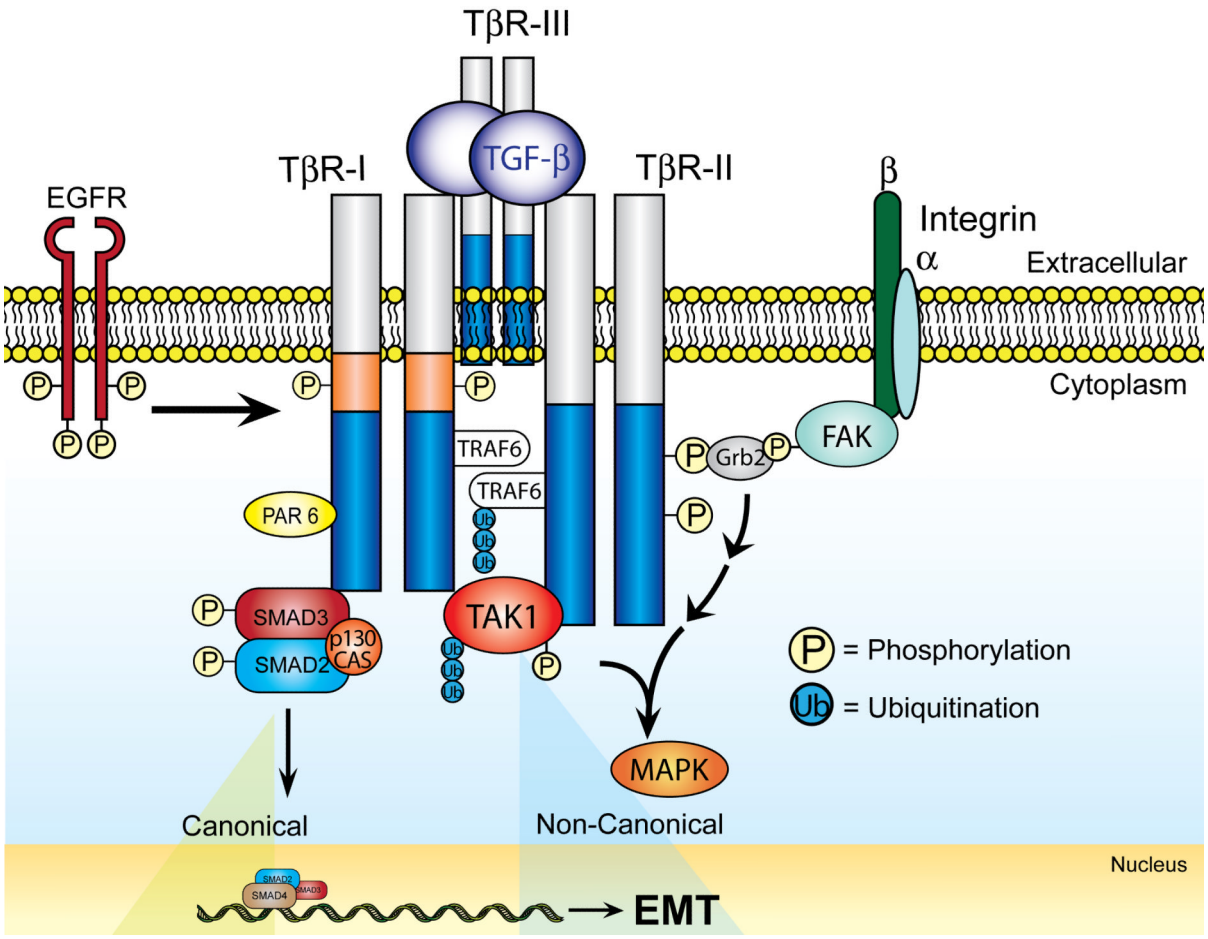

Figure 2. Differential Interactions of TGF-β Receptors with Transmembrane and Membrane Proximal Proteins Complexes Facilitate the Diversity TGF-β Signaling

β1 and β3 integrins interact physically with TβR-II [43-45]. The association of TβR-II with β3 integrin is mediated by FAK, which facilitates the binding of TβR-II to the SH2-binding protein, Grb2. In addition, TβR-II also interacts physically with EGFR (M.K. Wendt and W.P. Schiemann, *unpublished observation*), which also is activated indirectly by TGF-β through its increased synthesis and secretion of EGFR ligands. The cytoplasmic tails of both TβR-I and TβR-II interact with TRAF6, which ubiquitinates itself and the MAPKKK, TAK1. Additional interactions include the binding of p130Cas to Smad3, as well as that of PAR6 with TβR-I. Importantly, the differential composition of TGF-β receptor and scaffolding complexes directs the coupling of TGF-β to canonical and noncanonical effector activation, as well as underlies the pathophysiological conversion of TGF-β signaling and EMT in malignant epithelial cells. The biological outcomes of these various protein-protein interactions are discussed in the text.

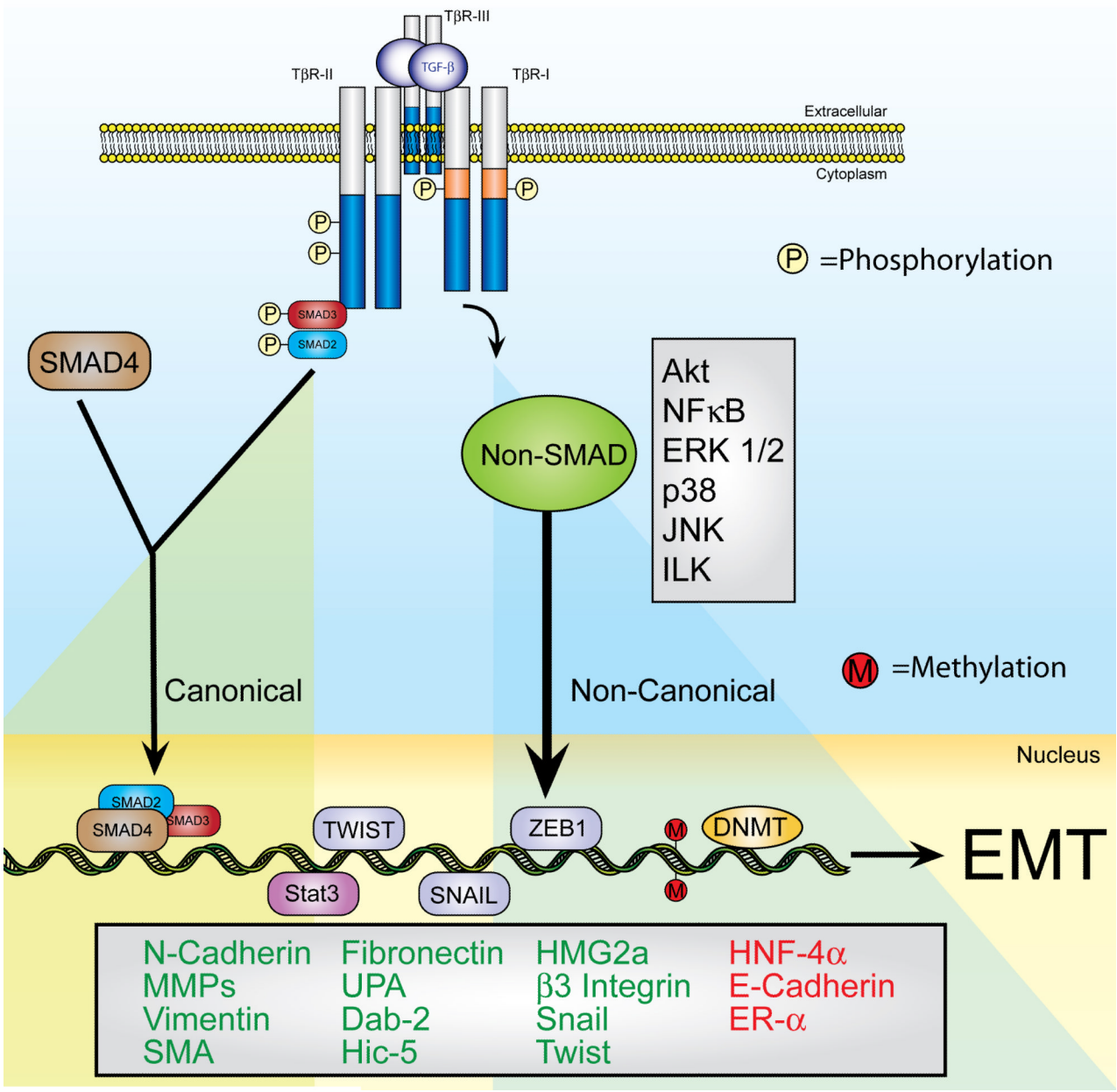

Figure 3. Diverse TGF-β Signaling Pathways Support a Complex Transcriptional Response During EMT

TGF-β stimulates epithelial cells by binding and activating two transmembrane Ser/Thr protein kinase receptors, namely TGF-β type I (TβR-I) and type II (TβR-II). Activation of these ligand:receptor ternary complexes requires TβR-II to transphosphorylate TβR-I, which phosphorylates and activates Smad2/3. Once activated, Smad2/3 form heterocomplexes with Smad4, which collectively translocate to the nucleus to mediate canonical signaling events by TGF-β (left panel). Noncanonical (right panel) TGF-β signaling takes place through its ability to stimulate various alternate signaling pathways discussed in detail herein. Activation of canonical Smad2/3 signaling results in their nuclear translocation with Smad4 and subsequent regulation of gene expression through their numerous interactions with additional transcriptional activators and repressors. Alternatively, activation of noncanonical TGF-β signaling, such as MAP kinases, small GTPases, PI3K/AKT, and NF-κB, also

couples TGF-β to its regulation of gene expression profiles operant in mediating EMT. Finally, activation of the transcription factors belonging to the Snail family (*e.g.*, Snail, Twist, or ZEB1), or of Stat3 elicit EMT-gene expression, which ultimately promotes the prolonged induction of EMT and fibroblastoid-like phenotypes of carcinoma cells *via* DNA methylation-mediated silencing of E-cadherin expression. Altered coupling of TGF-β to its canonical and noncanonical effector pathways leads to differential gene expression patterns that ultimately contribute to the development of oncogenic signaling by TGF-β. Indeed, the initiation of oncogenic signaling by TGF-β converts its regulation of physiological EMT in normal epithelial cells to one of pathophysiologic EMT in their malignant counterparts.

Table 1

Expression of EMT-associated Genes Targeted by TGF-β

Gene Name	Expression Change	Reference
E-cadherin	Decrease	Miettinen *et al* [15]
β3 integrin	Increase	Galliher *et al* [43]
N-cadherin	Increase	Hazan *et al* [112]
NCAM	Increase	Lehembre *et al* [85]
MMP-2	Increase	Duivenvoorden *et al* [72]
MMP-3	Increase	Farina *et al* [235]; Radisky et al [79]
MMP-9	Increase	Farina *et al* [235]; Kim *et al* [73]
Vimentin	Increase	Grunert *et al* [117]
α-Smooth Muscle Actin	Increase	Masszi *et al* [118]
Fibronectin	Increase	Ignotz *et al* [102]
Estrogen Receptor-α	Decrease	Dhasarathy *et al* [202]
Urokinase Plasminogen Activator	Increase	Farina *et al* [235]
Dab2	Increase	Hocevar *et al* [26]
Hic5	Increase	Tumbarello *et al* [156]
HMG2A	Increase	Thuault *et al* [111]

Table 2

Signaling Pathways Activated During EMT Stimulated by TGF-β

Pathway	Reference
Smad2/3	Piek *et al* [236]
Rho family of small GTPases	Bhowmick *et al* [65]
PI3K and AKT	Bakin *et al* [38]
NF-κB	Huber *et al* [179]
ERK1/2	Xie *et al* [183]
p38 MAPK	Bhowmick *et al* [39]; Galliher and Schiemann [43-45]
JNK	Hocevar *et al* [185]
Integrin-linked kinase	Lee *et al* [177]; Lin *et al* [91]