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The Pathophysiology of Epithelial-Mesenchymal Transition Induced by Transforming Growth Factor- β in Normal and Malignant Mammary Epithelial Cells

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Abstract

Epithelial-mesenchymal transition (EMT) is an essential process that drives polarized, immotile mammary epithelial cells (MECs) to acquire apolar, highly migratory fibroblastoid-like features. EMT is an indispensable process that is associated with normal tissue development and organogenesis, as well as with tissue remodeling and wound healing. In stark contrast, inappropriate reactivation of EMT readily contributes to the development of a variety of human pathologies, particularly those associated with tissue fibrosis and cancer cell invasion and metastasis, including that by breast cancer cells. Although metastasis is unequivocally the most lethal aspect of breast cancer and the most prominent feature associated with disease recurrence, the molecular mechanisms whereby EMT mediates the initiation and resolution of breast cancer metastasis remains poorly understood. Transforming growth factor- β (TGF- β) is a multifunctional cytokine that is intimately involved in regulating numerous physiological processes, including cellular differentiation, homeostasis, and EMT. In addition, TGF- β also functions as a powerful tumor suppressor in MECs, whose neoplastic development ultimately converts TGF- β into an oncogenic cytokine in aggressive late-stage mammary tumors. Recent findings have implicated the process of EMT in mediating the functional conversion of TGF- β during breast cancer progression, suggesting that the chemotherapeutic targeting of EMT induced by TGF- β may offer new inroads in ameliorating metastatic disease in breast cancer patients. Here we review the molecular, cellular, and microenvironmental factors that contribute to the pathophysiological activities of TGF- β during its regulation of EMT in normal and malignant MECs.

Keywords

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

Introduction

Epithelial-mesenchymal transition (EMT) is a complex process whereby polarized epithelial cells transition into apolar fibroblastoid-like cells, a phenomenon that underlies tissue morphogenesis and organogenesis in the embryo, as well as tissue remodeling and repair in adults (1–3). Moreover, the inappropriate reactivation of developmental EMT programs plays a significant role in the pathology of fibrotic diseases and cancer, including those of the breast. Epithelial cell sheets manifest as tightly packed cell monolayers that compose the

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skin and line the internal cavities (*e.g.*, airways and gastrointestinal tract), and in doing so, form a barrier that protects the host from environmental insults. In a similar fashion, mammary epithelial cells (MECs) exhibit a cobblestone appearance and are linked through the actions of numerous cell-cell complexes, including desmosomes, adherens, gap, and tight junctions (4). Collectively, these junctional structures provide MECs with their characteristic apical-basolateral polarity and cortical actin architecture. In stark contrast, mesenchymal cells lack cell-cell junctional complexes, leading to their apolar morphologies and enhanced migratory activities through the extracellular matrix (ECM). The plasticity of MECs enables them to dedifferentiate during EMT, and in doing so, transitioning MECs forego their cobblestone morphologies and instead acquire a spindle-shaped appearance characteristic of mesenchymal cells. In undertaking this phenotypic and morphologic transition, MECs first experience a disruption and delocalization of tight junction complexes (*e.g.*, zonula occludens-1 (ZO-1), claudin, and occludin), which is succeeded by the loss of E-cadherin expression and activity that results in the stabilization and nuclear accumulation of β -catenin. This process is also characterized by the dramatic remodeling of the cytoskeleton and its formation of actin stress fibers as transitioning cells acquire migratory mesenchymal phenotypes (1–3). Thus, EMT reflects the initiation of a complex cascade of genetic and epigenetic events that culminate in MECs discarding their expression of epithelial gene signatures (*e.g.*, E-cadherin, β 4 integrin, and ZO-1) and acquiring those of mesenchymal cells [*e.g.*, N-cadherin, vimentin, α -smooth muscle actin (α -SMA)]. Moreover, the process of EMT is highly metastable and is readily subject to phenotypic and morphologic reversion by mesenchymal-epithelial transition (MET), the molecular mechanisms of which are poorly understood and will not be discussed further herein (*see* (5, 6)). These general steps exhibited by transitioning MECs underlie both the biological and pathological episodes of EMT, which recently have been categorized into three distinct subtypes – *i.e.*, *a*) embryonic and developmental EMT, which is referred to as type 1 EMT; *b*) tissue regeneration and fibrotic EMT, which is referred to as type 2 EMT; and *c*) cancer progression and metastatic EMT, which is known as type 3 EMT (2).

Here we review recent findings that directly impact our understanding of the role transforming growth factor- β (TGF- β) plays in regulating the initiation and resolution of individual subtypes of EMT. In addition, we also discuss the clinical implications afforded by chemotherapeutic targeting of TGF- β effectors coupled to type 3 EMT and their potential to suppress breast cancer progression and the oncogenic activities of TGF- β , particularly its induction of EMT and metastasis in developing mammary carcinomas.

TGF- β and EMT Subtypes

TGF- β Signaling and Epithelial Plasticity

TGF- β is a multifunctional cytokine and a powerful tumor suppressor that governs essentially every aspect of the physiology and homeostasis of MECs, including their ability to proliferate, migrate, differentiate, and survive (7–9). During mammary tumorigenesis, a variety of genetic and epigenetic events conspire to circumvent the cytostatic and tumor suppressing activities of TGF- β , thereby enhancing the development and progression of evolving mammary neoplasms (1, 7, 8). Even more remarkably, neoplastic MECs that have acquired resistance to the cytostatic activities of TGF- β often exhibit oncogenic behaviors when stimulated by TGF- β . This phenotypic switch in TGF- β function during tumorigenesis is known as the “TGF- β Paradox,” which represents the most important and unanswered question concerning the pathophysiological actions of this pleiotropic cytokine (10). Interestingly, the differentiation and migration of mammary stem cells results in the production of both the outer myoepithelial and inner luminal layers that ultimately give rise to mature mammary glands (11–13), suggesting that the process of EMT is in some way linked to the generation and maintenance of stem cell populations. Numerous studies have

established TGF- β as a master regulator of EMT in normal and malignant MECs (1, 14, 15), while more recent findings have associated TGF- β stimulation of EMT with the acquisition of “stemness” in transitioning MECs (16, 17), and with the selection and expansion of breast cancer stem cells (18, 19). Along these lines, we and others have established EMT as being a vital component underlying the initiation of oncogenic TGF- β signaling in normal and malignant MECs (1, 14, 20). Thus, identifying the molecular mechanisms whereby EMT is induced by TGF- β is paramount to maintaining mammary gland homeostasis, and to suppressing the development and progression of mammary tumors.

The mechanisms through which TGF- β initiates its pathophysiological activities and initiation of EMT are shown schematically in Figure 1. Indeed, transmembrane signaling stimulated by TGF- β commences by its binding to the high-affinity transmembrane Ser/Thr receptor protein kinases, TGF- β type I (T β R-I) and type II (T β R-II). Mammals express three distinct TGF- β isoforms termed TGF- β 1, TGF- β 2, and TGF- β 3, which function analogously *in vitro*, but give rise to more than 30 distinct phenotypes upon their genetic deletion in mice (21). In addition, whereas TGF- β s 1 and 3 can bind directly to T β R-II, TGF- β 2 requires assistance from the accessory TGF- β receptor, TGF- β type III (T β R-III). In all of these scenarios, the ligation of TGF- β to T β R-II facilitates its transphosphorylation and activation of T β R-I, which subsequently phosphorylates and activates the latent transcription factors, Smads 2 and 3. Once phosphorylated, receptor-activated Smads 2 and 3 associate with the common Smad, Smad4, at which point these heteromeric complexes translocate en masse into the nucleus to regulate the expression of TGF- β -responsive genes. In addition, the amplitude and duration of Smad2/3-based signaling transpires through their physical interaction with a plethora of transcription factors, and with a variety of transcriptional activators and co-repressors in a gene- and cell-specific manner (*see* (22, 23)). The capacity of Smad2/3 to impact MEC behavior is also governed by their association with a number of adapter molecules, including SARA (24), Hgs (25), and Dab2 (26–28). Likewise, upregulated expression of the inhibitory Smad, Smad7, also limits the extent of Smad2/3 signaling by competitively inhibiting their phosphorylation by T β R-I (29–31), and by promoting the internalization and degradation of T β R-I (32, 33). Moreover, the anti-TGF- β activity of Smad7 is augmented by its interaction with the adaptor protein STRAP (34), and conversely, is attenuated by its association with either AMSH2 (35) or Arkadia (36–38). Collectively, TGF- β signals propagated through Smad2/3 are referred to as “canonical” TGF- β signaling, and their specific role in regulating EMT induced by TGF- β is discussed below.

Besides its ability to activate canonical Smad2/3-dependent pathways, TGF- β also regulates MEC behavior and the induction of EMT *via* the stimulation of numerous “noncanonical” effector systems, including (i) small GTP-binding proteins (*e.g.*, Ras, Rho, and Rac1); (ii) phosphoinositide-3-kinase (PI3K), AKT, and mTOR; (iii) MAP kinases (*e.g.*, p38 MAPK, ERK1/2, and JNK); and (iv) NF- κ B and Cox-2 (39–51). In addition, although inactivating mutations and decreased expression of T β R-I and T β R-II have been identified and characterized in human cancers, the occurrence of these mutagenic events is in fact quite rare. However, the loss of T β R-III expression does correlate with increased breast cancer tumorigenicity and decreased patient survival (52, 53), suggesting an important tumor suppressing function for this accessory receptor. Indeed, the functional loss of T β R-III coincides with EMT and enhances cell migration and invasion (54, 55). The coupling of TGF- β to these receptors and their noncanonical effectors is depicted schematically in Figure 1, as is our understanding of how integrins and proteins associated with focal adhesion complexes cooperate with TGF- β in regulating the behaviors and EMT status of normal and malignant MECs (45, 46, 56–60). The specific role of these noncanonical TGF- β effectors in regulating EMT in normal and malignant MECs is presented in the succeeding sections, while future studies clearly need to address the relative contribution of T β R-I,

T β R-II, and T β R-III in mediating activation of canonical and noncanonical TGF- β signaling systems.

EMT Subtypes Coupled to TGF- β

The process of EMT occurs in a variety of distinct biological and pathological settings, including during normal embryogenesis and tissue morphogenesis, during tissue remodeling and repair, and during fibrosis and cancer progression. Although the underlying molecular mechanisms that define the pathophysiological activities of EMT in distinct cellular contexts are likely to be overlapping and redundant, the diversity of biological outcomes engendered by EMT is nonetheless highly specialized and has resulted in the classification of three distinct subtypes of EMT (2). For instance, type 1 EMT is activated during embryogenesis and tissue morphogenesis, leading to the generation of mesenchymal cells that ultimately give rise to secondary epithelial structures. In contrast, type 2 EMT is normally activated during tissue regeneration and repair, and abnormally during tissue fibrosis resulting from dysregulated inflammatory reactions. Finally, type 3 EMT is activated by cancer cells, including those of the breast (1), to facilitate their acquisition of invasive and metastatic phenotypes, and ultimately to establish secondary sites of lethal tumor outgrowth. In the succeeding sections, we highlight the molecular mechanisms and biological settings whereby TGF- β participates in regulating individual EMT subtypes, particularly type 3 EMT during mammary tumorigenesis.

Type 1 EMT—Developmental or type 1 EMT is associated with embryogenesis and its accompanying organogenesis and tissue morphogenesis, both of which require epithelial-derived mesenchymal cells to undergo MET during the formation of new epithelial structures (Fig. 2). In stark contrast to types 2 and 3 EMT, this developmental mode of EMT results in the production of multipotent mesenchymal cells and is not associated with tissue fibrosis and inflammation, or with the aberrant migration of cancer cells. Interestingly, members of the TGF- β superfamily play important regulatory roles during type 1 EMT, particularly during the processes of gastrulation, palate fusion, and neural crest and endocardial cushion formation (61). Indeed, type 1 EMT is first initiated during embryogenesis to promote gastrulation, which results in the formation of the ectoderm, mesoderm, and endoderm from the invaginating primitive streak (2, 4). This process requires Wnt signaling inputs to render primitive streak cells competent to undergo EMT in response to the TGF- β superfamily members, Nodal and Vg1 (62–65), both of which are essential for primitive streak and mesoderm formation in gastrulating embryos (66–70).

Type 1 EMT is also initiated during neurulation and occurs in the neural plate to facilitate the formation of the neural tube, which ultimately gives rise to the spine and brain. As the neural tube fuses, EMT also occurs in neural crest cells and facilitates their migration and dissemination throughout the embryo where they ultimately contribute to the generation of numerous specialized tissues, including the adrenal medulla and the peripheral nervous and skeletal systems (4, 71). Bone morphogenic proteins (BMPs) belong to the TGF- β superfamily and are essential to the induction of EMT during neurulation (72, 73). Similarly, TGF- β signaling also oversees the latter stages of heart and secondary palate formation (4, 74). For instance, myocardial cells actively secrete an ECM that separates the endocardium from the myocardium, which also induces endocardial cells to undergo EMT and migrate into the endocardial cushion to facilitate atrioventricular valve formation (4). Experiments performed in chicks and mice have identified important roles during development for all three TGF- β isoforms, of which TGF- β 2 appears to play a dominant role in stimulating EMT in endocardial cells (75–77). Similarly, TGF- β 3 is essential in mediating secondary palate formation, which requires midline epithelial seam cells to undergo EMT to complete

oral palate fusion and closure (78, 79). Importantly, T β R-III expression is essential for EMT induced during the formation of both the heart and secondary palate (80, 81).

At present, a role for type 1 EMT in mediating mammary gland development has yet to be described; however, branching morphogenesis of normal MEC organoids in 3D–organotypic cultures showed that these structures exhibit a loss in polarity and acquire mesenchymal characteristics at invading branch tips, findings that point to a role of type 1 EMT in mammary gland development (82, 83). In addition, inappropriate reactivation of embryonic and type 1 EMT programs have been associated with the development and progression of mammary tumors (61). Indeed, aberrantly elevated expression of the Six1 homeoprotein not only elicits the formation of mammary tumors (84), but also stimulates breast cancer EMT and metastasis in part *via* a TGF- β -dependent mechanism (85). Thus, thoroughly defining the role of TGF- β during type 1 EMT will be critical to the development of novel chemotherapeutics capable of circumventing these activities during the inappropriate reactivation of type 1 EMT programs in mammary tumors.

Type 2 EMT—The initiation of type 2 EMT is essential in maintaining tissue homeostasis through its ability to induce wound healing and tissue remodeling in response to noxious insults. An important distinction between type 1 and type 2 EMT is that the latter is governed by inflammatory reactions, whose cessation resolves the EMT phenotype following wound repair (2). A corollary states that chronic inflammation underlies the initiation of tissue and organ fibrosis, which eventually elicits organ dysfunction and destruction if left unabated. As depicted in Fig. 3, the process of wound healing involves the orchestrated activities of numerous cell types to facilitate the re-epithelialization of denuded areas (86). In fact, signaling by TGF- β and the ECM are essential in promoting the activation and differentiation of myofibroblasts, which are the key cells involved in the repair of damaged epithelium and scar formation (86). Working in concert with epithelial and endothelial cells, activated myofibroblasts secrete matrix metalloproteinases (MMPs) that digest injured tissues and facilitate the synthesis of a provisional ECM (87, 88). Exposure of platelets and infiltrating immune cells to provisional ECM components elicits platelet degranulation, angiogenesis, and wound contraction, of which the latter response is mediated by myofibroblasts during the final stages of re-epithelialization (86, 87, 89). Under normal circumstances, the inflammatory reactions within healed wounds resolve, thereby terminating type 2 EMT and stimulating the elimination of myofibroblasts *via* apoptosis (88). However, sustained myofibroblast activation in conjunction with chronic inflammation underlies the initiation of fibrotic disorders due to unresolved type 2 EMT reactions (86, 87). Myofibroblasts represent a specialized cell type that exhibit traits reminiscent of smooth muscle cells, particularly the expression of α -smooth muscle actin (α -SMA; (90, 91)). As a group, myofibroblasts derive from fibroblasts, from circulating progenitor cells, and from epithelial cells following their completion of EMT, which is typically assessed by monitoring the extent of α -SMA expression in fully transitioned cells (1). Exacerbated α -SMA expression is also indicative of fibrotic states and fibroproliferative disorders (92–94), as well as correlates with increased tumor invasion and decreased survival rates in cancer patients (95). Not surprisingly, α -SMA expression is induced by TGF- β *via* the concerted signaling inputs of Smad2/3, RhoA/Rock, and ERK1/2 (96–99). Furthermore, integrin activation by laminin, fibronectin, and collagen also cooperates with TGF- β to induce EMT and myofibroblasts activation, an event coupled to the formation of β -catenin:Smad2 signaling complexes (100–102). Thus, in addition to its role in promoting type 2 EMT, aberrant TGF- β signaling also supports chronic inflammatory reactions that promote the establishment of fibroproliferative disorders in humans.

At present, the overall importance of fibrotic reactions in promoting mammary tumorigenesis remains to be determined definitively. However, mammographically dense

and fibrotic breast tissue have been linked to the increased incidence of mammary tumorigenesis (103, 104). Along these lines, radiation therapy of breast cancers is associated with the development of fibrosis (105), and with the initiation of EMT *via* a TGF- β -dependent mechanism (106). Moreover, mammary tumorigenesis is often accompanied by intense desmoplastic and fibrotic reactions, which elicit the formation of rigid tumor microenvironments that (i) enhance the selection and expansion of developing neoplasms, particularly that of late-stage metastatic tumors, and (ii) predict for poor clinical outcomes in patients with breast cancer (107–109). Interestingly, these aberrant cellular activities are highly reminiscent of those attributed to TGF- β (110), suggesting that TGF- β stimulation of EMT and fibrosis may promote the development and progression of mammary tumors. This supposition is bolstered by a recent study showing that inhibiting the cross-linking of collagen during mammary fibrosis reduces breast cancer progression in mice (111). Collectively, these findings suggest that chemotherapeutic targeting of the EMT inducing activities of TGF- β may offer a novel two-pronged approach to alleviate breast cancer progression – namely, the inactivation of pathologic type 2 (*i.e.*, fibrotic) and type 3 EMT (*see below*).

Mechanisms of Type 3 EMT Induced by TGF- β

The initiation of type 3 EMT is essential in facilitating cancer progression and metastasis, including that by mammary tumors (1, 2). In addition, type 3 EMT is primarily distinguished from its type 1 and 2 counterparts by the cellular context in which this phenotypic transition transpires – namely, type 3 EMT occurs in oncogenically transformed cells that house a variety of genetic and epigenetic abnormalities that conspire with the molecular cascade that underlies EMT to elicit metastatic dissemination. Even more remarkably, TGF- β stimulation of EMT has been associated with the selection and expansion of breast cancer stem cells (16–19), which by their nature exhibit robust resistance to traditional cancer chemotherapies (112). In the succeeding sections, we present our understanding of the molecular, cellular, and microenvironmental factors that contribute to the initiation of type 3 EMT by TGF- β in breast cancer cells.

Canonical TGF- β Signaling During EMT

Although the activities attributed to Smad2 and Smad3 during TGF- β signaling are commonly conjoined in the scientific literature, recent findings indicate that these latent transcription factors do in fact mediate distinct biological activities in response to TGF- β (113). These disparate functions mediated by Smads 2 and 3 also extend to EMT, which is inhibited by Smad2 and stimulated by Smad3. For instance, Smad2-deficiency enhances the development and progression of squamous cell carcinomas by elevating Snail expression and its induction of EMT (114), as well as elicits the acquisition of mesenchymal and fibrotic morphologies in hepatocytes (115). Interestingly, oncogenic Ras cooperates with Smads 2 and 3 to drive the formation of spindle cells and their subsequent acquisition of EMT and metastatic phenotypes (116). TGF- β stimulation of EMT in kidney cells results in the attenuated expression of SARA, an adapter molecule that facilitates the presentation of Smad2/3 to T β R-I (24). Moreover, depleting cells of SARA increased the ubiquitination and degradation of Smad2, leading to the acquisition of EMT phenotypes (117). In stark contrast, Smad3-deficiency (i) prevented TGF- β stimulation of EMT in lens (118) and renal (119) epithelial cells; (ii) reduced the EMT and migratory abilities of keratinocytes to TGF- β (120, 121); and (iii) preserved an epithelial phenotype in hepatocytes stimulated by TGF- β (115). Thus, these studies implicate a pro-EMT function associated with Smad3 activation, which is mirrored by the activation of Smad4 in pancreatic cells undergoing EMT induced by TGF- β (122). Consistent with its designation as an inhibitory Smad, over expression of Smad7 is sufficient to abrogate the ability of TGF- β to induce EMT in epithelial cells of the breast (123), liver (124), and lens (125). Collectively, these studies demonstrate that the

expression and activities of TGF- β -regulated Smads are critical to the initiation and resolution of EMT in diverse epithelial cell lineages.

Smad2/3 activation also figures prominently in mediating TGF- β stimulation of EMT in MECs. Indeed, TGF- β signaling through Smads 2, 3, and 4 induce an EMT transcriptional program in normal MECs, a physiological reaction that is blocked by overexpression of Smad7 (123). Interestingly, chronic TGF- β signaling elicits a metastable EMT phenotype in normal MECs that is characterized by reduced Smad2/3 signaling, leading to MEC resistance to the cytostatic and apoptotic activities of TGF- β (126). Thus, EMT may underlie the conversion of TGF- β function from that of a tumor suppressor to a tumor promoter in mammary tumors (*see below*). Along these lines, Smad4-deficiency not only prevented TGF- β stimulation of EMT in normal and malignant MECs, but also alleviated its induction of bone metastasis by breast cancer cells in mice (127). Furthermore, targeting and inactivating Smad2/3 signaling using aptamer technology was observed to neutralize the ability of TGF- β to induce EMT in normal MECs (128). Interestingly, Smad2 signaling has recently been shown to promote EMT in MECs by enhancing the DNA binding activity of the DNA methyltransferase, DNMT1, leading to chronic epigenetic silencing of epithelial-associated genes (129). Finally, a recent study established that the aberrant coupling of TGF- β to BMP-regulated Smads (*e.g.*, Smads 1 and 5) during mammary tumorigenesis confers a pro-migratory phenotype in breast cancer cells (130). Although a role for this unusual coupling event in mediating EMT by TGF- β was not investigated, it is nonetheless tempting to speculate that inappropriate cross-talk between TGF- β superfamily members may contribute to the pathophysiological outcomes of EMT initiated by TGF- β . Future studies will need to address this question, as well as define the underlying relationship between Smad-dependent and -independent signaling during TGF- β stimulation of EMT in MECs (*see below*).

Noncanonical TGF- β Signaling During EMT

The aberrant amplification of noncanonical TGF- β signaling systems plays a salient role in mediating the ability of TGF- β to induce EMT in normal and malignant MECs, and in underlying the initiation of the “TGF- β Paradox.” The function of noncanonical TGF- β effectors in coupling this cytokine to EMT and metastasis are discussed in the succeeding sections.

Rho-family GTPases—Oncogenic TGF- β signaling is often associated with the dysregulated activity of the Rho GTPase family, which includes RhoA/B/C, Rac1, and Cdc42 (1, 14, 20). This family of small GTP-binding proteins are anchored to the plasma membrane where they regulate dynamic changes in cell adhesion, morphology, and motility in part by modulating the formation of filopodia (*e.g.*, Cdc42), lamellipodia (*e.g.*, Rac1), and actin stress fibers (*e.g.*, RhoA) (131, 132). For instance, RhoA activation mediates the dissolution of adhesion complexes at cell-cell junctions, while that of Rac1 actually promotes the formation of these same complexes (133, 134). Additionally, constitutive activation of Cdc42 by T β R-III inhibits directional migration induced by TGF- β (135). Interestingly, the expression and activation of RhoC enhances the invasion and EMT of breast (136), prostate (137), and colon (138) cancer cells. The ability of TGF- β to induce EMT in MECs requires the activation of RhoA and its downstream effector, p160^{ROCK} (41). In addition, the coupling of TGF- β to RhoA and RhoC also correlates with altered expression of E-cadherin and α -SMA during EMT (96, 139). Recently, T β R-II-mediated phosphorylation of Par6 was shown to underlie the ubiquitination and degradation of RhoA, leading to the dissolution of tight junctions during the acquisition of EMT and metastatic phenotypes in breast cancer cells (140, 141). Along these lines, upregulation of miR-155 by canonical TGF- β signaling was observed to promote EMT in MECs by targeting the

destruction of RhoA mRNA (142). In stark contrast, reduced RhoA expression mediated by miR-31 was found to suppress, not promote, breast cancer metastasis (143, 144), suggesting an intricate and complex role for RhoA and its relatives in regulating the initiation of EMT and metastasis.

PI3K, AKT, and mTOR—Oncogenic TGF- β signaling and its stimulation of EMT also requires the activities of phosphoinositide 3-kinase (PI3K) and Akt, both of which confer proliferative and survival advantages to developing carcinomas (145). In addition, PI3K and Akt also mediate TGF- β stimulation of EMT in MECs (40), an event that arises directly from TGF- β receptor signaling inputs, or indirectly through their transactivation of the receptors for EGF (146) and PDGF (147). Interestingly, co-administration of TGF- β and EGF elicits an exaggerated EMT through the activation of ERK1/2 and PI3K/Akt (148). Somewhat surprisingly, pharmacological inhibition of PI3K/Akt had no effect on the morphologic and phenotypic characteristics of EMT; however, this same treatment regimen did alleviate the ability of TGF- β and EGF to induce cell migration and invasion (148), suggesting that the morphologic and motile features of EMT may in fact be distinct physiological entities. Along these lines, inactivating mTOR pharmacologically with rapamycin prevents TGF- β from increasing the physical size of MECs, as well as from stimulating their migration and invasion (42). As above, mTOR antagonism failed to impact the morphological features of EMT, which suggests that mTOR may facilitate the synergistic effects of TGF- β and EGF on EMT. Collectively, these studies highlight the essential function of the PI3K/Akt/mTOR pathway in promoting EMT and metastasis stimulated by TGF- β , while future studies need to clarify the underlying dissociation between “fibroblastoid-like” phenotypes and cell motility.

Nuclear Factor- κ B—Nuclear factor- κ B (NF- κ B) plays a prominent role in regulating the initiation and resolution of inflammatory reactions, and in promoting the growth, angiogenesis, invasion, and survival of developing carcinomas (149). In nontransformed tissues, TGF- β typically inhibits the activation of NF- κ B (49, 50, 150), presumably by inducing the expression of I κ B α (151), and by promoting the formation of T β R-III: β -arrestin2 complexes that prevent I κ B α degradation (152). In stark contrast, mammary tumorigenesis converts TGF- β from an inhibitor to a stimulator of NF- κ B activity by inducing the formation of T β R-I: α IAP:TAK1:IKK β complexes (49, 50, 153). Furthermore, activation of this noncanonical effector system by TGF- β was found to be essential for its induction of EMT in normal and malignant MECs (49, 50, 153, 154), and for its stimulation of mammary tumor development *via* activation of the innate immune system (49). Moreover, the coupling of TGF- β to NF- κ B activation underlies the ability for Ras-transformed breast cancer cells to colonize the lung (47, 155), and elicits the initiation of an autocrine Cox-2:PGE2:EP2 receptor signaling cascade that not only induces EMT in normal and malignant MECs, but also stimulates breast cancer metastasis (50, 51). Finally, TGF- β stimulation of NF- κ B in post-EMT cells stimulates their migration by establishing a SDF-1/CXCR4 signaling axis (156). Collectively, these studies highlight the importance of NF- κ B to the induction of EMT by TGF- β , while future studies need to assess the relative contribution of these events to enhanced survival and chemoresistance exhibited by post-EMT breast cancer cells.

MAP Kinases—The propensity of TGF- β to induce pathological EMT and metastasis is also associated with its stimulation of members of the MAP kinase family of protein kinases, including ERKs, JNKs, and p38 MAPKs (39, 157–159). For instance, pharmacological inhibition of MEK1/2 prevents TGF- β from stimulating EMT and its characteristic formation of actin stress fibers and delocalization of E-cadherin and ZO-1 from the cell surface in MECs (158). Similarly, methods that disrupt the coupling of TGF- β to JNK

activation prevent the morphological and transcriptional changes associated with EMT (160, 161). Along these lines, there exists a dynamic interplay between TGF- β and its production of various ECM components, which subsequently potentiate the activation of MAP kinase pathways during EMT. For instance, the expression of type I collagen activates PI3K, Akt, and JNK to induce EMT (162, 163). Moreover, TGF- β in conjunction with vitronectin signaling is necessary in mediating T β R-II phosphorylation by Src, a phosphotransferase reaction operant in activating p38 MAPK and EMT in MECs (23, 45). Likewise, the coupling of TGF- β to ERK1/2 and p38 MAPK activation is dependent upon the localization of TGF- β receptors to lipid rafts, not clathrin-coated pits, and as such, cholesterol-depleting methodologies are sufficient to block cell migration and EMT stimulated by TGF- β (164). Collectively, these studies implicate MAP kinases as crucial mediators linking the ability of TGF- β and ECM components to promote EMT.

Integrin-linked Kinase—The coupling of TGF- β to its noncanonical effectors during type 3 EMT is further exacerbated upon the activation of integrin-linked kinase (ILK), which is a Ser/Thr protein kinase coupled to the activation of small GTPases, PI3K/Akt, and MAP kinases (165–167). ILK activation also leads to the inhibition of GSK-3 β activity, which stabilizes β -catenin and facilitates its nuclear accumulation during EMT (166). Indeed, elevated expression of ILK in MECs is associated with their decreased expression of E-cadherin and increased invasion (168), and with their oncogenic transformation by hyperactive ERK1/2 and Akt (169). Along these lines, ILK-deficiency prevented TGF- β from stimulating cell migration and invasion in part by uncoupling this cytokine from regulation of the uPA/PAI-1 system (170). Finally, TGF- β stimulation of Smad2/3 induces PINCH-1 expression, which interacts physically with ILK during the initiation of EMT and its consequential loss of E-cadherin and ZO-1 expression (171). Collectively, these findings suggest that ILK interfaces integrin signaling with that of TGF- β , resulting in aberrant protease activation that drives the acquisition of EMT, invasive, and metastatic phenotypes.

Integrins and Focal Adhesion Signaling During EMT

Integrins and EMT Stimulated by TGF- β —Communication within cell microenvironments is controlled in part by integrins, which govern cell adhesion, migration, and invasion, as well as cell proliferation and survival (172, 173). Cells undergoing neoplastic transformation exhibit dramatically altered integrin expression profiles, as well as altered integrin affinities for ECM substrates, both of which enhance cancer cell invasion and metastasis (174). As a receptor family, integrins are unique in their capacity to physically link the ECM to cytoskeletal system within cells, thus enabling the efficient propagation of mechanotransduction in a bidirectional manner (111, 175). In addition, focal adhesion kinase (FAK) serves as a molecular bridge that links integrins to the receptors to EGF and PDGF, thereby conferring cell migration activities to these growth factors (176, 177). Integrins also play an important role in eliciting EMT and cell migration stimulated by TGF- β . For instance, α v β 6 and α v β 8 integrins bind latent TGF- β complexes and elicit the presentation of TGF- β to its cell surface receptors (178), presumably by promoting matrix metalloproteinase (MMP)-14 activation (27, 179). Along these lines, TGF- β induces the expression of α 3 β 1 and α v β 3 integrins, which confer migration and invasion properties to MECs (45, 46, 56, 157). Administering neutralizing β 1 integrin antibodies to MECs uncoupled TGF- β from the activation of p38 MAPK and the induction of EMT (157). In addition, genetic or pharmacologic inactivation of α v β 3 integrin in normal and malignant MECs prevented TGF- β from inducing EMT and pulmonary metastasis (45, 46, 56). Mechanistically, upregulated β 3 integrin expression stimulated by TGF- β results in the FAK-dependent formation of β 3 integrin:T β R-II complexes that promote the activation of Src and its phosphorylation of T β R-II at Y284 (56, 180). Upon its phosphorylation, Y284 coordinates the recruitment and binding of the SH2-domain proteins, Grb2 and ShcA, which

promote p38 MAPK activation and the initiation of EMT (45, 46, 56). Importantly, measures that disrupt this oncogenic TGF- β signaling axis completely abrogate the ability of TGF- β to induce EMT, and to promote the metastasis of breast cancer cells to the lung (46, 180, 181) and bone (182). Recently, we observed an interesting interplay between β 1 and β 3 integrins in regulating MEC response to TGF- β , such that genetic inactivation of β 1 integrin in MECs elicits a compensatory upregulation of β 3 integrin expression that impacts the coupling of TGF- β to EMT and cell motility (J.G. Parvani and W.P. Schiemann, *unpublished observation*). These findings implicate “integrin switching” as a potentially dangerous mechanism that may enable metastatic breast cancers to escape integrin-based chemotherapies. Future studies clearly need to investigate the validity of this hypothesis, as well as to identify the repertoire of integrins capable of regulating the diverse pathophysiological activities of TGF- β in normal and malignant MECs.

Nonreceptor Protein Tyrosine Kinases and Adapter Molecules of Focal Adhesion Complexes

As mentioned above, EMT and oncogenic TGF- β signaling transpires through a β 3 integrin:FAK:Src:phospho-Y284-T β R-II:Grb2:p38 MAPK signaling axis that forms constitutively in basal-like breast cancer cells (45, 46, 56, 180, 182). The importance of this α v β 3 integrin-based signaling axis in promoting the oncogenic activities of TGF- β in other genetically distinct breast cancer subtypes remains unexplored; however, a number of recent studies have identified essential functions for a variety of focal adhesion complex proteins in mediating the coupling of TGF- β to EMT and metastasis. Indeed, in addition to its ability to coordinate the formation of β 3 integrin:T β R-II complexes, FAK expression and activity are essential in coupling TGF- β to EMT and p38 MAPK activation, and to inducing pulmonary metastasis of breast cancer cells (180). Adjuvant FAK chemotherapy also inhibited breast cancer growth by suppressing the ability of macrophages to infiltrate the primary mammary tumor (180), suggesting that the tumor promoting activities of FAK manifest in carcinoma cells and their associated stromal compartment. Along these lines, FAK is essential in mediating TGF- β stimulation of E-cadherin redistribution and α -SMA expression during EMT (183, 184). In addition, the phosphorylation and activation of p130Cas functions as a molecular rheostat that governs the balance between canonical and noncanonical TGF- β signaling inputs. Indeed, activated p130Cas forms a heteromeric complex with Smad3 and T β R-I, which diminishes the phosphorylation of Smad3 and uncouples TGF- β from regulation of cell cycle progression (185). Interestingly, breast cancer patients whose tumors express abnormally high levels of p130Cas exhibit tamoxifen- and adriamycin-resistance, as well as diminished time to disease recurrence (186, 187). Likewise, elevated p130Cas expression significantly reduced the latency of mammary tumor formation driven by transgenic expression of Her2/Neu (188). We recently observed elevated p130Cas expression to mark the development of metastasis in breast cancers, and to skew the balance of TGF- β signaling from canonical to noncanonical effectors in metastatic MECs (189).

Similar to p130Cas, the adapter molecules Hic5 and Dab2 also promote the coupling of TGF- β to its noncanonical effectors during EMT. Indeed, Hic5 is a member of the paxillin superfamily that functions in the cytoplasm as a component of focal adhesion complexes (190), and in the nucleus as a transcriptional co-activator of the androgen receptor (191). Polarized MECs express low levels of Hic5, which are increased rapidly during EMT *via* a RhoA/ROCK-dependent pathway (190, 192). Interestingly, the LIM domain of Hic5 binds and inactivates Smad3 and Smad7 in prostate cancer cells (193, 194), which collectively diminishes Smad-dependent gene expression (*i.e.*, targeting Smad3) in the context of enhanced TGF- β receptor signaling (*i.e.*, targeting Smad7). Whether Hic-5 possesses similar anti-Smad activity in breast cancers remains unknown; however, it is tempting to speculate that Hic5 may cooperate with p130Cas in amplifying the coupling of TGF- β to its noncanonical effectors.

Finally, the adaptor molecule, Dab2 (Disabled-2) regulates the dynamics associated with the remodeling of the actin cytoskeleton during MEC adhesion and migration (195). In contrast to p130Cas and Hic5, Dab2 associates with TGF- β receptors and facilitates their activation of Smad2/3 (26), as well as that of TAK1 and JNK, which stimulate fibronectin expression and cell migration during EMT (160, 196). In addition, TGF- β stimulation of EMT assembles Dab2: β 1 integrin complexes that induce FAK activation. Mechanistically, translation of Dab2 mRNA is strongly repressed in polarized MECs by the actions of hnRNP E1, which binds structural elements in the 3'UTR of Dab2 transcripts. When activated by TGF- β , Akt2 phosphorylates and releases hnRNP E1 from Dab2 mRNA, thereby enabling the production of Dab2 and its initiation of EMT in MECs (197). Future studies need to identify other genes targeted by this novel post-transcriptional regulon, as well as to define their role in mediating the pathophysiological outcomes of EMT stimulated by TGF- β .

Gene Regulation-Coupled to EMT Induced by TGF- β

The ultimate phenotypic change associated with the activation of canonical and noncanonical TGF- β signaling inputs derives from altered patterns of gene expression and repression that transpire in a cell- and context-specific manner. In the succeeding sections, we highlight the important transcriptional mediators operant in driving and fine-tuning the EMT transcriptome targeted by TGF- β in normal and malignant MECs.

Nuclear Transcription Factors Targeted by TGF- β During EMT

Members of the Snail (SNAIL1 and SNAIL2/Slug), ZEB (ZEB1 and ZEB2/SIP1), basic helix-loop-helix (Twist), and Six family of homeobox (Six1) transcription factors are considered to be master regulators of EMT, including that stimulated by TGF- β (198, 199). As a group, these transcription factors play essential roles in mediating type I EMT during embryogenesis and tissue morphogenesis; however, their inappropriate reactivation of developmental EMT programs during tumorigenesis is considered a hallmark of disease progression and metastasis initiation (61). Indeed, activated Snail molecules readily form complexes with Smads 3 and 4 that collectively target conserved E-box sequences in the promoters for E-cadherin, occludin, and claudin, which strongly represses their expression and inactivates adherens (*i.e.*, E-cadherin) and tight junction (*i.e.*, occludins and claudin) complexes during EMT (200, 201). Similar targeting and inactivation of E-cadherin is associated with all of the aforementioned transcription factors, whose underlying roles in mediating the pathophysiology of EMT has been the subject of several recent reviews (*see* (198, 199)). Interestingly, dysregulated Myc expression has been observed to function cooperatively with Smad4 to induce an EMT-related transcriptional profile in normal and malignant MECs (202). Likewise, the reactivation of fibulin-5 expression in transitioning MECs initiated a positive-feedback loop that sensitized MECs to the EMT-promoting activities of TGF- β , an event dependent upon the synergistic induction of Twist expression by fibulin-5 and TGF- β (203). In addition, TGF- β stimulation of Smad3 results in upregulated Mdm2 expression, which destabilizes p53 during the initiation of breast cancer EMT and metastasis (204). Smad3/4 signaling also promotes the expression of HMGA2, which stimulates the EMT transcriptional program by inducing the expression of Snail, Slug, and Twist, while simultaneously repressing that of Id2 (205). Finally, members of the homeobox family of transcription factors have also been implicated in mediating EMT induced by TGF- β . For instance, inappropriate *LBX1* (ladybird homeobox 1) expression drives EMT and its expansion of breast cancer stem cells by stimulating the expression of TGF- β 2, Snail, and ZEBs 1 and 2 (206). Similarly, aberrant reactivation of the homeoprotein Six1 promotes the acquisition of EMT and metastatic phenotypes in breast cancer cells in part by upregulating the messages transduced through the TGF- β signaling

system (85). Collectively, these findings highlight the role of developmental EMT pathways that enhance the oncogenic activities of TGF- β during mammary tumorigenesis.

Estrogen Receptor- α

Estrogen receptor (ER) status has long been recognized as an important prognostic marker in developing breast cancers, particularly in terms of its diagnostic and therapeutic value. Indeed, the loss of ER- α expression during mammary tumorigenesis is associated with poor clinical outcomes, and with increased likelihood of breast cancer metastasis and disease recurrence (207). More recently, ER- α has been linked to the initiation of EMT through its ability to activate metastasis associated protein 3 (MTA3) in MECs (208, 209). Mechanistically, MTA3 serves as a subunit of the Mi-2/NuRD chromatin remodeling complex, which represses the expression of Snail (208). Thus, mammary tumors that have lost expression of ER- α exhibit reduced MTA3 activity that results in the inappropriate expression of Snail and its subsequent stimulation of EMT (208, 209). This process is further enhanced by the ability of Snail to repress ER- α expression, and to induce the expression of components of the TGF- β signaling system (*e.g.*, TGF- β 2 and T β R-II; (208)), thereby creating a powerful positive feedback loop to further potentiate the acquisition and stabilization of EMT phenotypes. Moreover, ER- α interacts physically with Smad3 and inhibits its ability to regulate gene expression (210). Thus, the loss of ER- α expression and activity may play a prominent role in engendering the initiation of oncogenic TGF- β signaling in normal and malignant MECs (8). In fact, aberrant cytoplasmic localization of ER- α has recently been proposed as a novel histopathological marker to identify sarcomatoid breast cancers *in vivo* (211). Future studies clearly need to elucidate molecular mechanisms that underlie the dynamic relationship between ER- α and TGF- β in regulating EMT, and to identify novel biomarkers capable of staging and stratifying breast cancer patients on the basis of their EMT, ER- α , and TGF- β status.

TGF- β and microRNAs

Recent studies have implicated the aberrant expression of numerous microRNA (miRs) in the initiation of EMT, and in the development and progression of mammary tumors (212–214). Indeed, members of the miR-200 family of microRNAs maintain polarized epithelial phenotypes by repressing the expression of the EMT-inducing transcription factors ZEB1 and ZEB2/SIP-1. Accordingly, monitoring the expression of miR-200 family members can be used to distinguish well-differentiated and immotile tumors that express E-cadherin from their poorly-differentiated and highly motile counterparts that express vimentin (215). In addition, TGF- β abrogates the expression of miR-200 family members, leading to the expression of ZEB1 and ZEB2 and their consequential downregulation of E-cadherin expression to initiate EMT (216, 217). Not surprisingly, miR-200 family member expression is frequently downregulated in invasive and metastatic mammary tumors, particularly those possessing mesenchymal-type breast cancer cells (216). Once expressed, ZEB1 can further repress the expression of miR-200 family members, thereby stabilizing the EMT phenotype in transitioning MECs (218).

The stimulation of EMT by TGF- β also transpires through its regulation of additional miRs. Indeed, TGF- β stimulation of normal MECs elicits their upregulated expression of miR-155 *via* a Smad4-dependent pathway. Once expressed, miR-155 participates in EMT by downregulating RhoA expression, leading to the dissolution of tight junctions (142). Along these lines, upregulated expression of miR-21 induced by TGF- β abrogates the expression of several tumor suppressors, including *a*) peroxisome proliferator-activated receptor; *b*) tissue inhibitor of metalloproteinase-3; *c*) programmed cell death 4; and *d*) AT-rich interactive domain 1A (219). Additionally, miR-21 expression also participates in the initiation of EMT by downregulating the expression of tropomyosin, leading to enhanced breast cancer

motility, invasion, and anchorage-independent growth (220, 221). Thus, the coupling of TGF- β to the regulation of miR expression and activity affords new avenues to potentially manipulate the pathophysiology associated with type 3 EMT in mammary tumors. As such, future studies need to rapidly and accurately define the cellular targets of EMT-associated miRs, and to determine the molecular mechanisms operant in regulating their expression in transitioning MECs.

DNA Hypermethylation

Hypermethylation and silencing of the E-cadherin promoter has been linked to the initiation of EMT, migration, and invasion in breast cancer cells (222–224). More recently, TGF- β signaling was observed to maintain DNA methylation patterns during EMT, resulting in the silencing of E-cadherin (*CDH1*), the tight junction genes *CGN* and *CLDN4*, and the protease *KLK10/NES1*. Mechanistically, overexpressing Smad7 in MECs or rendering them deficient in Smad2 inhibited the activity of the DNA methyltransferase, DNMT1, which suppressed EMT and cell motility by restoring E-cadherin expression (129). Along these lines, miR-200c and its relative, miR-141, normally inhibit the initiation of EMT and metastasis in MECs by suppressing the expression of ZEB1/2 (216, 217). However, during mammary tumorigenesis, aberrant DNA methylation inactivates expression of these miR-200 family members, leading to the acquisition of EMT and metastatic phenotypes in developing and progressing mammary tumors (225). Moreover, E-cadherin-deficiency that arises during EMT may in fact function as an initiating signal coupled to the expanded and directed hypermethylation of genes normally operant in suppressing mammary tumorigenesis (226). For instance, hypermethylation of the E-cadherin promoter marks Ras-transformed MECs that have undergone a stable EMT induced by serum *versus* a transient EMT induced by TGF- β (226). Collectively, these studies establish an essential role for DNA methylation in facilitating type 3 EMT stimulated by TGF- β , and in differentially stabilizing the EMT phenotype in response to varying stimuli.

Microenvironmental Inputs During EMT Stimulated by TGF- β

Tissue homeostasis in the breast is maintained by the balanced integration of signaling inputs derived from various tissue and cell architectures, and from their supporting microenvironment and ECM. Indeed, whereas normal mammary tissue specification requires reciprocal signaling inputs from distinct cell types and matrix proteins, the phenotype of developing mammary carcinomas is similarly dictated by the dynamic interplay between malignant cells and their accompanying stroma, which houses fibroblasts, endothelial cells, and a variety of infiltrating immune and progenitor cells (227–229). Along these lines, desmoplasia and fibrosis during mammary tumorigenesis can drive disease progression in a manner that mimics the oncogenic activities of TGF- β (110, 230), suggesting that the interactions between MECs and their supporting stromal constituents play pivotal roles in regulating EMT and metastasis stimulated by TGF- β . Recent findings pertaining to the connections between the microenvironment and EMT are summarized in the following sections.

Adherens Junctions and EMT

A hallmark of EMT is the dissolution of cell-cell junctions, particularly adherens junctions which derive from the homotypic interactions between adjacent E-cadherin molecules housed on neighboring MECs. Similar to integrins, the cytoplasmic domain of E-cadherin is tethered to the cytoskeleton through a heteromeric complex consisting of the α -, β -, and γ -catenins, which collectively serve in marking differentiated MECs and suppressing their tumorigenesis (231, 232). Interestingly, the process of EMT is often characterized by “cadherin switching,” a term referring to the ability of E-cadherin expression and activity to

give way to that of N-cadherin as MECs acquire mesenchymal phenotypes (233). Functional inactivation of E-cadherin transpires through a variety of mechanisms, including hypermethylation and epigenetic silencing of its promoter (234), as well as protease-mediated cleavage and shedding of its ectodomain (231). In rare cases, mutational inactivation of the E-cadherin gene, *CDH1*, has been observed and linked to increased risk for cancer development in affected individuals (235). However, transcriptional repression is by far the most common mechanism employed by transitioning MECs to downregulate their expression of E-cadherin. Indeed, TGF- β stimulation of EMT represses E-cadherin expression primarily by targeting the expression of Snail, ZEB, and bHLH family members (*see above*). Moreover, upregulated expression of the mesenchymal cadherins, N-cadherin and cadherin-11, occur concomitantly the loss of E-cadherin expression and correlate with increased tumor invasiveness and poor clinical outcomes (236–239). At present, the dynamic relationship between E- and N-cadherin in mediating TGF- β stimulation of EMT and metastasis remains unresolved, as does the manner through which bifurcated TGF- β signals coupled selectively to epithelial *versus* mesenchymal transcriptional programs influence the pathophysiological outcomes of EMT induced by TGF- β (50, 180).

Neuronal Cell Adhesion Molecule and EMT

Neuronal cell adhesion molecule (NCAM) belongs to the immunoglobulin superfamily and mediates calcium-independent cell-cell adhesion (240). Inappropriate NCAM expression has been associated with cancer progression and poor prognosis in cancers of the pancreas and colorectal system (233, 240). In addition, TGF- β stimulation of EMT in MECs significantly upregulates their expression of NCAM, a reaction that requires the activation of Smad4 and inactivation of E-cadherin. Mechanistically, NCAM translocates to lipid rafts and activates a p59^{l^yn}:FAK: β 1 integrin signaling axis that promotes EMT and cell invasion (241). Interestingly, EMT induced by TGF- β also activates matrix metalloproteinase (MMP)-28, which cleaves NCAM and latent TGF- β complexes (242). Thus, it remains unclear how upregulated NCAM expression and cleavage ultimately impact the ability of TGF- β to stimulate EMT in normal and malignant cells.

Protease Activation During EMT Induced by TGF- β

Matrix Metalloproteinases—One of the hallmarks of EMT is its propensity to bestow motile phenotypes in previously immotile cells (1–3). Matrix metalloproteinases (MMPs) are a superfamily of transmembrane and secreted endopeptidases that function in degrading a variety of ECM components, cytokines, and cell surface proteins and receptors. The net effect of these various MMP activities results in dramatic affects on cell differentiation, invasion, and EMT (243). Members of the MMP superfamily (*e.g.*, MMPs 2, 9, 13, and 14; (179, 244–246)) also function in mediating the cleavage of latent TGF- β complexes, which releases mature TGF- β and initiates transmembrane signaling in neighboring MECs, as well as mediates E-cadherin cleavage and breast cancer progression (243, 247, 248). Along these lines, TGF- β is a potent regulator of the expression of MMPs 2, 9, and 13 (60, 203, 249–252), thereby establishing a positive autocrine TGF- β signaling loop that (*i*) drives breast cancer EMT, invasion, and metastasis, and (*ii*) is readily suppressed by constitutive c-Abl activation in normal and malignant MECs (60). Future studies need to address how aberrant MMP activation contributes to the initiation of type 2 EMT (*see above*), and how these events ultimately impact the initiation of type 3 EMT and metastasis by TGF- β in mammary tumors.

Urokinase Plasminogen Activator and Plasminogen Activator Inhibitor-1—

Urokinase plasminogen activator (uPA) is serine protease that plays important roles in regulating the migration and invasion of breast cancer cells in part *via* its conversion of inactive plasminogen into active plasmin. Elevated expression of uPA correlates with

increased tumor aggressiveness and poor clinical outcomes for a variety of cancers, including those of the breast (253–255). The role of uPA in promoting breast cancer progression and metastasis has been recapitulated in a mouse model of mammary tumorigenesis (255), and in a chick chorioallantoic membrane model of breast cancer metastasis, which also associated upregulated uPA expression with hypoxia-induced EMT (256). TGF- β induces uPA expression by activating JNK- and ILK-dependent signaling systems that functionally converge to induce EMT and increased cell motility (161, 170). As noted previously, these findings point to a prominent role of noncanonical TGF- β effectors in mediating the stimulation of EMT by TGF- β . Accordingly, the expression and activity of FAK is essential in stimulating the production of uPA and its initiation of metastasis in 4T1 cells (255), which we (46, 49, 51, 60, 180, 189, 203) and others (257–259) established as a late-stage model of TGF- β -responsive breast cancer. In addition, hypoxia-induced EMT stimulates the expression of the uPA receptor, uPAR, which interacts physically with $\alpha 3 \beta 1$ integrin and promotes the activation of Src, Akt, Rac1, and GSK-3 β . The end product of these signaling inputs elicits Snail and Slug expression, which drive the acquisition of EMT phenotypes and loss of E-cadherin in transitioning cells (256, 260). Thus, future studies clearly need to define the connections linking the activation of these noncanonical TGF- β effectors to the formation of uPAR: integrin complexes.

Inappropriate activation of uPA is held in check by the expression of plasminogen activator inhibitor (PAI)-1 and PAI-2, which bind uPA:uPAR complexes and induce their internalization and degradation (253). Thus, elevated PAI-1/2 expression would be predicted to alleviate the EMT and metastasis promoting properties of uPA, an assumption that has been validated in a panel of breast, ovarian, endometrial, cervical, and osteosarcoma cell lines (261). Quite surprisingly, PAI-1 polymorphisms or its elevated expression has also been linked enhanced disease progression and metastasis development, and to decreased survival in breast cancer patients (253, 261, 262). TGF- β is a master regulator of PAI-1 expression, doing so through its stimulation of canonical and noncanonical effector systems (*see* (263)). In addition to binding and inactivating uPA:uPAR complexes, PAI-1 also interacts with vitronectin and prevents its activation of integrins (261), an event that may influence the coupling of integrin signaling to MEC migration. Thus, future studies need to clarify the tumor suppressing and promoting activities of PAI-1, particularly with respect to its role in mediating EMT and oncogenic TGF- β signaling in normal and malignant MECs.

Collagen

TGF- β has long been recognized for its ability to induce the expression of collagens (264), which function as important structural components of the ECM and serve as prominent ligands for integrins (243). In addition, activation of p38 MAPK by TGF- β upregulates the expression of MMPs 2 and 9, which cleave collagen to produce biologically active fragments that readily promote MEC migration and invasion (252). Along these lines, TGF- β stimulates breast cancer cells to upregulate their expression of the collagen receptor Endo180, which internalizes collagen and induces the growth of mammary tumors in mice (265). More recently, collagen binding to $\beta 1$ integrins has been shown to activate TGF- β receptor signaling independent of TGF- β ligands, leading to the activation of FAK and Src that culminate in the stimulation of Smad2/3 activity in MECs (266). Collectively, these findings establish that TGF- β and collagen engage one another in a reciprocal relationship, yet how these events impact the ability of TGF- β to promote the acquisition of EMT and metastatic phenotypes in mammary tumors remains an unresolved and interesting question.

Fibronectin

Fibronectin is an important component of the ECM and its expression is upregulated dramatically by TGF- β during EMT (264, 267). Functionally, fibronectin acts as a ligand for

integrins during cell adhesion and migration, particularly in Ras-transformed MECs which concomitantly upregulate $\alpha 5 \beta 1$ integrin (268). Importantly, administration of neutralizing antibodies against $\alpha 5$ integrin blocked the ability of fibronectin and TGF- β to stimulate EMT and cell motility in MECs (268). In addition, fibronectin expression has been shown to modulate the response of cells to TGF- β . For instance, the ability of TGF- β to induce anchorage-independent growth in fibroblasts could be recapitulated by administration of fibronectin, whose activation of cell signaling was dependent upon integrin ligation (264). Besides its ability to enhance TGF- β stimulation of EMT in bronchial epithelial cells (269), fibronectin expression has also been linked to the development of the “premetastatic niche,” which serves as a depot to recruit circulating progenitor cells and metastatic carcinoma cells to sites of secondary tumor growth (270, 271). Future studies need to assess the relative contributions of TGF- β and fibronectin in mediating EMT and its potential involvement regulating the formation of metastatic niches during breast cancer progression.

Conclusions and Future Directions

TGF- β is universally recognized as a master regulator of EMT, including that occurring during embryonic develop and tissue morphogenesis (*i.e.*, type 1 EMT), during wound healing and tissue fibrosis (*i.e.*, type 2 EM), and during invasion and metastasis (*i.e.*, type 3 EMT). Equally exciting are recent findings linking EMT stimulated by TGF- β to the acquisition of “*stem-like*” phenotypes in developing and progressing mammary tumors (16–19). Thus, pharmacological targeting of the TGF- β signaling system to alleviate EMT may elicit chemosensitivity in cancer stem cells previously resistant to standard treatment regimens, a supposition supported by recent findings obtained in a preclinical model of breast cancer progression (19). A corollary states that the phenomenon underlying selection and expansion of cancer stem cells *via* EMT may be “*druggable*” in clinical settings. Accordingly, high-throughput chemical screening technologies identified salinomycin as a novel agent capable of targeting breast cancer stem cells, thereby inhibiting mammary tumor growth in part by promoting epithelial differentiation (272). Future studies need to determine the efficacy of salinomycin and related compounds in antagonizing EMT stimulated by TGF- β in normal and malignant MECs, as well as investigate the relative contribution of cell microenvironments in mediating the various pathophysiological outcomes of EMT induced by TGF- β . Ultimately, these findings will form the foundation necessary to manipulate EMT and its initiation of the “TGF- β Paradox” in mammary tumors, and as such, to one day improve the clinical course of patients with metastatic breast cancer.

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Abbreviations

α-SMA	α -smooth muscle actin
BMP	bone morphogenic protein
ECM	extracellular matrix
EGF	epidermal growth factor
EMT	epithelial-mesenchymal transition

ERK	extracellular signal-regulated kinase
FAK	focal adhesion kinase
JNK	c-Jun N-terminal kinase
MAP kinase	mitogen-activated protein kinase
MEC	mammary epithelial cell
mTOR	mammalian target of rapamycin
MET	mesenchymal-epithelial transition
MTA3	Metastasis associated protein 3
NF-κB	nuclear factor- κ B
PAI	plasminogen activator inhibitor
PDGF	platelet-derived growth factor
PI3K	phosphoinositide-3-kinase
TβR-I	TGF- β type I receptor
TβR-II	TGF- β type II receptor
TβR-III	TGF- β type III receptors
TGF-β	transforming growth factor- β
uPA	urokinase plasminogen activator
uPAR	uPA receptor
ZO-1	zonula occluden-1

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Figure 1.

Schematic depicting the canonical and noncanonical TGF- β signaling systems coupled to EMT in MECs. Transmembrane signaling by TGF- β ensues through its binding and activation of the Ser/Thr protein kinase receptors, T β R-I and T β R-II. Indeed, TGF- β binding to either T β R-III or T β R-II enables the recruitment and transphosphorylation of T β R-I, resulting its activation and subsequent phosphorylation the receptor-activated Smads, Smad2 and Smad3. Once activated, Smad2/3 form heterocomplexes with common the Smad, Smad4, which collectively translocate en masse to the nucleus to regulate the expression of TGF- β -responsive genes in concert with an ever expanding list of transcriptional coactivators and repressors. This branch of the bifurcated TGF- β signaling system represents the “canonical” or “Smad2/3-dependent” TGF- β pathway (*left*). Alternatively, TGF- β also activates a variety of “noncanonical” or “Smad2/3-independent” effectors,

including Par6, NF- κ B, ILK, FAK, Src, a variety of small GTPases, members of the MAP kinase family (*e.g.*, ERK1/2, JNK, and p38 MAPK), and the PI3K:AKT:mTOR signaling axis (*right*). Activation of members of the Snail, ZEB, or bHLH family of transcription factors promote EMT by transcriptionally repressing the production of E-cadherin transcripts (*bottom*). Imbalances between canonical and noncanonical TGF- β signaling pathways have recently been identified and established as casual aberrancies underlying the initiation of oncogenic TGF- β signaling and its initiation of EMT in normal and malignant MECs. See text for details.

Figure 2.

Type 1 EMT in development and embryogenesis. Following fertilization, the zygote undergoes several rounds of cleavage to form a dense spheroid structure, which then undergoes blastulation to form a hollowed blastula. During gastrulation, TGF- β provides inductive EMT signals that elicits the formation of an invaginating structure that generates the three multipotent germ layers, namely the mesoderm, endoderm, and ectoderm. The mesoderm gives rise to muscle, skeleton, and connective tissue, while the endoderm gives rise to internal organs, such as the liver, colon, and stomach. The outer ectodermal layer generates the epidermis and ocular lens, as well as produces the mammary gland. The ectoderm also gives rise to the primordial nervous system *via* neurulation and neural crest

formation, a process that dependent upon EMT induced by TGF- β . Finally, TGF- β stimulation of EMT during organogenesis is essential for the faithful development of the kidney, and of the endocardial cushion and subsequent atrioventricular valve formation. The role of TGF- β in regulating type 1 EMT during mammary gland branching and invasion is discussed in the text.

Wound Healing

Figure 3.

Type 2 EMT in wound healing and tissue regeneration. Normal epithelia that arose from type 1 EMT during development experience a noxious event or injury that triggers endothelial and epithelial cells to produce factors that mediate coagulation and clot formation. These events, coupled with enhanced MMP production facilitate the recruitment of immune cells and platelets to denuded wounds, leading to the production of provisional ECM and activation of angiogenesis. TGF- β enhances the healing process by inducing EMT in myfibroblasts, which drives their differentiation, activation, and migration into denuded wounds to facilitate wound restitution, closure, and re-epithelialization. The role of TGF- β in regulating type 2 EMT during mammary gland fibrosis is discussed in the text.

Figure 4.

Type 3 EMT in cancer metastasis. Normal epithelia that arose from type I EMT during development experience a carcinogenic event that ultimately results in their oncogenic transformation and tumor formation. The development and progression of mammary tumors is accompanied by their acquisition of dysplastic and abnormal morphologies, and by their evolution in chronically inflamed tumor microenvironments, which further enhances their acquisition of EMT and fibrotic phenotypes. Through its ability to stimulate oncogenic EMT, TGF- β enables transitioned mammary carcinoma cells to invade the underlying basement membrane (BM) and escape the confines of the primary tumor. Once liberated, metastatic MECs undergo intravasation and traverse the blood stream prior to taking up

residence at distant locales, an event that ultimately leads to disease recurrence and poor clinical outcomes in breast cancer patients. See text for specific details on the molecular mechanisms whereby TGF- β promotes type 3 EMT and metastasis in breast cancer cells.