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Role of TGF-β and the Tumor Microenvironment During Mammary Tumorigenesis

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Transforming growth factor-β (TGF-β) is a multifunctional cytokine that functions to inhibit mammary tumorigenesis by directly inducing mammary epithelial cells (MECs) to undergo cell cycle arrest or apoptosis, and to secrete a variety of cytokines, growth factors, and extracellular matrix proteins that maintain cell and tissue homeostasis. Genetic and epigenetic events that transpire during mammary tumorigenesis typically inactivate the tumor suppressing activities of TGF-β and ultimately confer this cytokine with tumor promoting activities, including the ability to stimulate breast cancer invasion, metastasis, angiogenesis, and evasion from the immune system. This dramatic conversion in TGF-β function is known as the "TGF-β paradox" and reflects a variety of dynamic alterations that occur not only within the developing mammary carcinoma, but also within the cellular and structural composition of its accompanying tumor microenvironment. Recent studies have begun to elucidate the critical importance of mammary tumor microenvironments in manifesting the TGF-β paradox and influencing the response of developing mammary carcinomas to TGF-β. Here we highlight recent findings demonstrating the essential function of tumor microenvironments in regulating the oncogenic activities of TGF-β and its stimulation of metastatic progression during mammary tumorigenesis.

Key words: Mammary tumorigenesis; Metastasis; Microenvironment; Transforming growth factor-β (TGF-β)

INTRODUCTION

Transforming growth factor-β (TGF-β) is a multifunctional cytokine that suppresses tumorigenesis within the mammary epithelium by inhibiting cell cycle progression, by inducing apoptosis, and by maintaining cellular and tissue homeostasis. Although TGF-β possesses powerful cytostatic activity in normal mammary epithelial cells (MECs), its ability to do so in malignant MECs is frequently inactivated, an event that often gives rise to the acquisition of oncogenic activity by TGF-β in developing and progressing mammary tumors (72,130). This malicious switch in TGF-β function is referred to as the "TGF-β paradox" and is supported by a variety of genetic and epigenetic events that ultimately underlie the adverse prognosis associated with elevated TGF-β production in developing mammary carcinomas (132). At present, the precise sequence of events that manifest the TGF-β paradox remain to be fully elucidated, as does the manner in which these events dictate the extent to which TGF-β mediates its oncogenic activities across genetically distinct breast cancer subtypes (97,121,125). Genomic and proteomic technologies have identified a host of gene transcripts, microRNAs, and proteins that are differentially regulated by TGF-β in normal and malignant MECs. Although these analyses have yet to decipher the precise sequelae necessary to elicit oncogenic TGF-β signaling, these studies have nonetheless offered several unique in-

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sights into the role of TGF-β in mediating the development and progression of breast cancers. For instance, gene expression signatures associated with the TGF-β signaling system been linked to the acquisition of epithelial-mesenchymal transition (EMT) and stem cell-like phenotypes exhibited by breast cancer cells (112,118,123), as well as to their ability to disseminate to the bone (57,58,146) and lung (95) in response to TGF-β. Additional molecular profiling analyses have identified gene signatures capable of predicting the organotropic spread and clinical outcomes of patients with metastatic breast cancer (10, 77,78), thereby solidifying TGF-β as a major driver of metastatic breast cancer.

The mammary gland is comprised of two major compartments: (i) the epithelium, which consists of luminal and myoepithelial cells that make up the ductal structures, and (ii) the stroma, which houses fibroblasts, adipocytes, endothelial, and immune cells, as well as extracellular matrix (ECM) proteins and connective tissue elements. Collectively, both mammary gland compartments function in a coordinated manner to maintain cell and tissue homeostasis, and to suppress mammary tumorigenesis. In contrast, developing neoplasms harbor activated stromal compartments accompanied by inflammatory and fibrotic reactions that enhance tumor development and metastatic progression, as well as predict for poor clinical outcomes of breast cancer patients (11,19,133). In addition to its established functions in normal and malignant MECs, TGF-β is also recognized as a major player involved in regulating the composition and activation of tumor microenvironments, particularly during tumor progression and metastatic dissemination (11,133). Indeed, aberrant upregulation of TGF-β expression positively correlates with enhanced breast cancer progression, angiogenesis, and metastasis, all of which contribute to poor clinical outcomes in patients with late-stage disease (11). Likewise, tumor reactive stroma plays an essential role in dictating whether TGF-β functions as a tumor suppressor or a tumor promoter in developing mammary neoplasms (8,11,130,133). In the succeeding sections, we review recent findings detailing the complex and multifaceted role of TGF-β within mammary tumor microenvironments, including its regulation of (i) autonomous responses by carcinoma cells; (ii) angiogenesis by endothelial cells; (iii) immunosurveillance by infiltrating immune cells; and (iv) activation of cancer-associated fibroblasts (Fig. 1).

TGF-β SIGNALING

TGF-β is the prototypic member of a large family of evolutionary conserved cytokines that includes the activins, bone morphogenetic proteins, growth differentiation factors, Nodal, and inhibins (127). Mammals express three genetically distinct TGF-β ligands (e.g., TGF-β 1–3), whose mature and biologically active forms are ~97% identical and exhibit virtually indistinguishable actions in vitro (14,97). Individual TGF-β molecules play important roles during embryonic development and tissue morphogenesis, and in maintaining cellular and tissue homeostasis in adults (73). TGF-β signaling is initiated by its binding to three high-affinity receptors, TGF-β type I (TβR-I), type II (TβR-II), and type III (TβR-III or betaglycan). TβR-I and TβR-II both harbor Ser/Thr protein kinases in their cytoplasmic domains that are essential for the activation of intracellular signaling by TGF-β (35,73) (Fig. 2). Although TβR-III lacks intrinsic enzymatic activity, this polypeptide is typically the most abundant receptor for TGF-β and functions as an accessory molecule that modulates cellular responses to TGF-β. The expression of TβR-III is essential for the ability of TGF-β to suppress tumor formation, particularly in the breast, ovary, prostate, lung, pancreas, kidney, and endometrium (39). The binding of TGF-β to TβR-II allows for the subsequent recruitment, transphosphorylation, and activation of TβR-I by TβR-II. Activated TβR-I, in turn binds, phosphorylates, and stimulates the latent transcription factors, Smad2 and Smad3, which rapidly form higher order complexes with the common Smad, Smad4 (35,73). The resulting heteromeric Smad2/3/4 complexes accumulate in the nucleus where they regulate gene expression in a cell- and context-specific manner (35,73,117) (Fig. 2). The activation of Smads 2, 3, and 4 by TGF-β is referred to as "canonical TGF-β signaling" and these events are modulated in all subcellular compartments by numerous effector molecules (138). Besides its ability to activate canonical Smad2/3/4 signaling, TGF-β also regulates cell behavior through its activation of a variety of Smad2/3-independent pathways, which are collectively referred to as "noncanonical TGF-β signaling." Included in this ever expanding list of noncanonical TGF-β effectors are the (i) MAP kinases, ERK1/2, p38MAPK, and JNK; (ii) cell survival mediators, PI3K, AKT1/2, and mTOR; (iii) inflammatory mediators, NF-κB, Cox-2, and prostaglandins; (iv) small GTP-binding proteins, Ras, RhoA, Rac1, and Cdc42; and (v) nonreceptor protein tyrosine kinases, Src, FAK, and Abl (56,97).

Collectively, both branches of the TGF-β signaling system coalesce in generating the pleiotropic activities of TGF-β in distinct cell lineages. Importantly, imbalances between the canonical and noncanonical TGF-β signaling systems have been associated with disease development in humans, including cancers of the breast (130,132). Along these lines, early hypotheses to explain cancer development postulated tumors

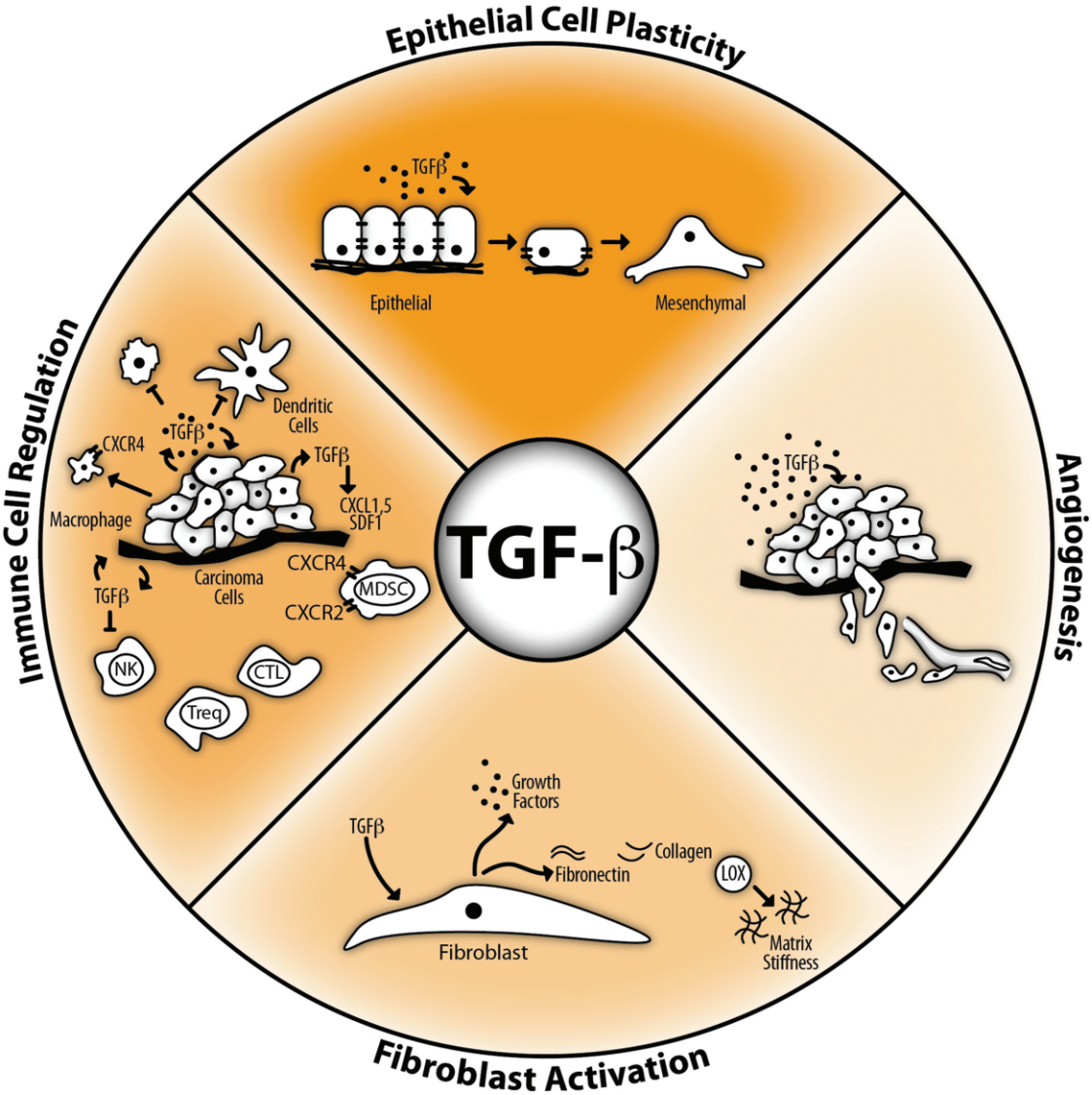

Figure 1. TGF-β is a master regulator of MEC plasticity and microenvironmental homeostasis. TGF-β induces malignant MECs to undergo EMT, leading to the acquisition of highly migratory, invasive, and metastatic phenotypes. TGF-β is also a potent inducer of tumor angiogenesis, which significantly enhances the growth and metastasis of late-stage mammary tumors. Through its ability to inhibit host immunosurveillance, TGF-β also plays an essential role in conferring immune privilege to developing and progressing breast cancers. Finally, TGF-β stimulates fibroblasts to synthesize and secret a variety of growth factors, cytokines, and ECM molecules that collectively create a tumor promoting microenvironment.

as being a collection of homogenous carcinoma cells, whose entire evolution and pathophysiology could be comprehended simply by elucidating the cell-autonomous properties of these clonal neoplasms. This idea has now given way to the view that tumor growth is in many respects reminiscent of that of developing organs, albeit in a highly dysfunctional and disorganized manner (108). Because virtually every cell in the human body is capable of both producing and responding to TGF-β (14), and because TGF-β is a major driver of metastatic progression in mammary tumors (125), it stands to reason that a true understanding of the "TGF-β paradox" will only be realized by first deciphering the functions of TGF-β in all specialized cell types within the tumor microenvironment, and by determining how these events collectively impact the development and progression of breast cancers in response to TGF-β. The role of TGF-β in regulating the activities of distinct stroma cell types is discussed in the succeeding sections (Fig. 1).

TGF-β AND MECs

TGF-β Expression and MECs

Examination of mice engineered to lack the expression of either TGF-β1, 2, or 3 suggest that the

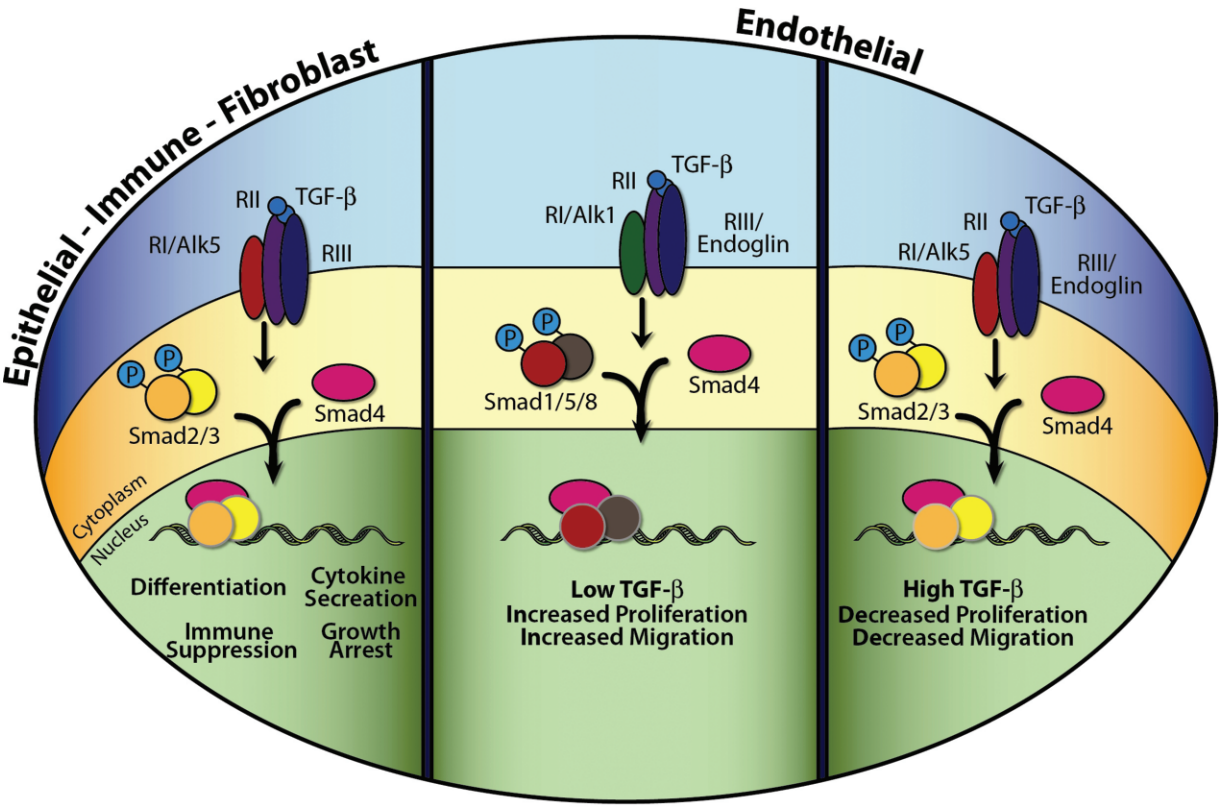

Figure 2. Schematic of canonical TGF-β signaling within distinct cell types located in tumor microenvironments. TGF-β predominantly activates a Smad2/3-based pathway in fibroblasts, epithelial, and immune cells (left panel), and in endothelial cells subjected to high TGF-β concentrations (right panel). In contrast, endothelial cells subjected to low TGF-β concentrations activate a Smad1/5/8-based pathway (middle panel). In general, TGF-β in the extracellular space binds either to TβR-III or endoglin, both of which present TGF-β to TβR-II. In some cells, TGF-β can bind directly to TβR-II independent of TβR-III or endoglin expression on the cell membrane. TβR-II bound to TGF-β then recruits, transphosphorylates, and activates the TβR-I isozymes, ALK-5 and ALK-1. Activated TβR-I/ALK-5 or TβR-I/ALK-1 then phosphorylate and activate Smad2/3 or Smad1/5/8, respectively, which then form heteromeric complexes with Smad4 that readily accumulate in the nucleus to regulate changes in gene expression in a cell- and context-specific manner.

activity of these cytokines are not required for embryonic development of the mammary gland. However, during the branching morphogenesis reactions that take place in postnatal mammary glands, all three TGF-β ligands are expressed and can suppress terminal end bud formation (102). During pregnancy, TGF-β2 and TGF-β3 are highly expressed in alveolar and ductal structures, while little-to-no TGF-β1 expression is detected in these same structures (109). After weaning, the expression of TGF-β3 is rapidly induced during the initial stages of mammary gland involution (34), which subsequently gives way to the elevated expression of TGF-β1 and TGF-β2 as glandular regression progresses and eventually resolves (34,109,114). Despite the fact that all TGF-β isoforms are functionally active in normal mammary tissues, the upregulated expression of TGF-β1 is most commonly associated with mammary tumorigenesis (27), and as such, the function of this TGF-β isoform will be highlighted throughout the remainder of this review.

The use of mouse models has greatly enhanced our understanding of the role of TGF-β and its signaling system in epithelial cells. For example, homozygous deletion of TGF-β1 elicits embryonic lethality in ~50% of the developing pups, while those that survive to term rapidly succumb to massive inflammatory reactions that develop in the heart, lungs, and salivary glands. Homozygous deletion of TGF-β2 or TGF-β3 both elicit perinatal lethality due to multiple developmental defects associated with aberrant EMT reactions during organogenesis and tissue morphogenesis. Along these lines, genetic inactivation of Smad2, Smad4, TβR-I, and TβR-II are all incompatible with life due to defects in mesoderm formation (Smad2), gastrulation (Smad4), and vascular development (TβR-I and TβR-II). In contrast, homozygous deletion of Smad3 and TβR-III result in viable mice that exhibit retarded growth rates and increased incidence of colon cancer due in part to altered immune function (Smad3), and osteoporotic lesions (TβR-III) (18,20). Collectively, these and numerous additional

studies have helped to define the essential role of TGF-β signaling in regulating organ development and immune privilege.

Transgenic mouse models have also played a valuable role in elucidating the functions of TGF-β during mammary tumorigenesis. For instance, mammary gland-specific expression of a constitutively active TGF-β1 mutant results in mammary ductal hypoplasia (64), as well as inhibits the formation of lobular-alveolar structures and the production of milk proteins (54). In addition, crossing MMTV-TGF-β1 mice onto a MMTV-TGF-α background significantly lengthens the latency of tumors induced by 7,12-dimethylbenz[*a*]anthracene (101). Taken together, these studies identify TGF-β as an inhibitory molecule coupled to the prevention of mammary tumorigenesis, particularly during the initial stages of neoplastic development. However, the paradoxical and tumor-promoting activities of TGF-β have also been observed in transgenic mouse models. Indeed, although crossing MMTV-TGF-β1 mice onto a MMTV-c-Neu background fails to alter the latency of tumor formation, this same genetic condition greatly enhances the acquisition of invasive and metastatic phenotypes due in part to the upregulated expression of vimentin in mammary carcinoma cells (82). Likewise, conditional expression of TGF-β1 in MMTV-PyMT-driven mammary tumors failed to alter their proliferative indices and size, but did elicit dramatic elevations in pulmonary metastasis (83). Collectively, these studies illustrate the dichotomy of TGF-β function between early and late-stage mammary tumors.

TGF-β Function in MECs

Our understanding of how MECs respond to TGF-β has also been aided by the transgenic expression constitutively active and dominant-negative versions of the receptors for TGF-β. For instance, mammary gland-specific expression of a truncated and nonfunctional TβR-II mutant (i.e., MMTV-DNIIR) elicits alveolar hyperplasia and excessive MEC differentiation in virgin animals (45), as well as accelerates glandular development and delays involution in their pregnant counterparts (44). Importantly, crossing MMTV-DNIIR mice onto either a MMTV-TGF-β or MMTV-Neu background significantly decreases tumor latency and reduces carcinoma cell invasion and pulmonary metastasis (44,119). Accordingly, crossing a constitutively active TβR-I receptor [i.e., MMTV-TβR-I(AAD)] onto a MMTV-Neu background significantly delays the rate of tumor formation and suppresses pulmonary metastasis (119). Thus, these findings reinforce the notion that TGF-β signaling is essential in both suppressing mammary tumor formation and promoting metastatic progression. Along these lines, conditional and specific deletion of TβR-II in the mammary epithelium (i.e., Tgfbr2MGKO) also elicits alveolar hyperplasia, as well as increased MEC apoptosis in hyperplastic tissues (37). Paradoxically, crossing Tgfbr2MGKO (i.e., TβR-II-deficient) mice onto a MMTV-PyMT background shortens tumor latency and, surprisingly, enhances the metastatic abilities of carcinoma cells rendered unresponsive to TGF-β (13,37). Finally, systemic administration (81) or transgenic expression (145) of a soluble Fc:TβR-II fusion protein, which antagonizes TGF-β signaling by binding and sequestering TGF-β, inhibits the survival, motility, and metastasis of mammary tumors in mice, thereby highlighting the differences between systemic and local actions of TGF-β in developing mammary tumors. Collectively, these intriguing findings demonstrate the plasticity present in the TGF-β signaling system as mammary carcinoma cells develop and progress to metastasis, events that are clearly dependent upon the differential activities of TGF-β in early versus late-stage carcinomas, as well as in the neighboring stromal compartment.

TGF-β and MEC Plasticity

TGF-β is well known for its ability to promote metastatic progression through the induction of EMT in MECs. This transdifferentiation process results in polarized MECs acquiring apolar and highly motile fibroblastoid-like phenotypes (125,138). The process of EMT is characterized by (i) changes in cytoskeletal architecture and intracellular organelle redistribution; (ii) loss of cell polarity due to downregulation of epithelial cell markers (e.g., E-cadherin, ZO-1, and β4 integrin); (iii) upregulation of fibroblastoid markers (e.g., vimentin, N-cadherin, α-smooth muscle actin); and (iv) elevated expression of invasion promoting factors [e.g., MMP-9, fibronectin; see (125,138)]. Recently, the process of EMT has been categorized into three distinct subtypes: (i) type 1 EMT, which represents the transdifferentiation process that occurs during embryogenesis and tissue morphogenesis; (ii) type 2 EMT, which is associated with tissue regeneration during wound healing, fibrotic reactions, and inflammation; and (iii) type 3 EMT, which represents the plasticity exhibited by carcinoma cells that enables them acquire invasive, metastatic, and stem cell-like phenotypes (55). In fact, EMT programs not only enhance the ability of carcinoma cells to invade locally as a means to exit the primary tumor, but also facilitate their survival in the circulation and ability to reinitiate proliferative programs at distant sites of metastasis (95,139,140). At present the contributions of the tumor microenvironment in coupling TGF-β

to EMT programs remains an important question for future research. Readers desiring additional information pertaining the molecular mechanisms whereby TGF-β induces EMT in normal and malignant MECs are directed to several recent comprehensive reviews (125,138).

TGF-β AND ENDOTHELIAL CELLS

TGF-β and Cell Junctions

Adhesive intercellular junctions between endothelial cells are formed by the actions of adherens junctions and tight junctions, which establish and maintain cell–cell contacts, as well as promote the transfer of intracellular signals between cells (90). Although the general organization of adherens and tight junctions in the endothelium is similar to those of epithelial cells, there are nonetheless some cell type-specific differences. For example, adherens junctions in epithelial cells are comprised primarily of the transmembrane protein, epithelial cadherin (E-Cad), which is connected to the actin cytoskeleton via α- and β-catenins (88). The associations between E-cad and TGF-β are well-studied during EMT programs (125,138), where TGF-β inactivates E-Cad function by (i) repressing the synthesis of E-Cad transcripts, and (ii) delocalizing and internalizing E-Cad proteins from the cell membrane, an event coupled to a loss of Rac1 activity (122). Actin cytoskeletal rearrangements engendered by TGF-β become apparent through the activation of RhoA, which reduces cell adhesion and elicits cell migration and invasion (7,107). In contrast to epithelial adherens junctions, those present in endothelial cells contain claudin-5, platelet/endothelial cell adhesion molecule (PECAM-1), and vascular endothelial (VE) cadherin (VE-Cad), which interacts directly or indirectly with multiple intracellular partners, including β-catenin, plakoglobin (γ-catenin), p120 catenin, and the endothelial-specific receptor protein tyrosine phosphatase, VE-PTP (3, 25). At present, the connections between TGF-β and VE-Cad in regulating endothelial cell biology remain to be fully elucidated. However, the VE-Cad has recently been shown to facilitate the maximal response of endothelial cells to TGF-β (111). Indeed, the expression and clustering of VE-Cad maximizes the coupling of TGF-β to antimigration and antiproliferation signals in endothelial cells. Mechanistically, VE-Cad interacts physically with and facilitates the assembly of TGF-β receptors into active signaling complexes, leading to enhanced Smad phosphorylation and gene transcription (111). Along these lines, tyrosine phosphorylation of VE-Cad regulates the ability of TGF-β to increase the paracellular permeability of vascular endothelial cells (116). Interestingly, malignant MECs that are undergoing EMT have been observed to upregulate their expression of VE-Cad. Indeed, elevated VE-Cad expression enhances the ability of breast cancer cells to proliferate in response to TGF-β, as well as to activate mammary tumor angiogenesis (62). Thus, these findings establish VE-Cad as a novel mediator of TGF-β signaling in mammary carcinoma cells and their supporting endothelial cells, suggesting that chemotherapeutic targeting of VE-Cad may provide a novel two-pronged approach to alleviate oncogenic TGF-β signaling in breast cancers.

In contrast to adherens junctions, tight junctions are formed by the actions of claudins, occludins, and junctional adhesion molecules (JAMs), which connect to the actin cytoskeleton by binding to a number of scaffolding proteins, including zonula occludens (ZO-1, -2, and -3), AF6/Afadin, PAR3 (partitioning-defective 3), and others (3,42). The association between TGF-β and Par6 (partitioning-defective 6) has been described in endocardial cells undergoing EMT (135). Par6 also plays an important role in controlling the formation of tight junctions, generation of apical-basolateral polarity, and the initiation of polarized cell migration (17). Par6 interacts physically with TGF-β receptors and can be phosphorylated by TβR-II, leading to the formation of Par6:Smurf1 complexes that promote the ubiquitination and degradation of RhoA (94). These results suggest that Par6 plays an important role in controlling the dynamics between tight junctions and TGF-β signaling in endothelial and epithelial cells.

TGF-β and Vascular Morphogenesis

Efficient tumor growth is absolutely dependent on its ability to secure a dependable supply of nutrients and oxygen, as well as a route to dispose of metabolic waste (2). To satisfy these essential needs, developing tumors synthesize a neovasculature system through the process of angiogenesis, which encompasses endothelial cell proliferation, migration, tubulogenesis, and anastomosis (53). Increased TGF-β expression has been positively associated with poor prognosis and increased tumor growth due the activation of angiogenic programs. Likewise, administration of anti-TGF-β agents has been shown to reduce tumor angiogenesis and, consequently, to inhibit tumor growth and progression (51). Thus, TGF-β likely plays a significant role in stimulating angiogenesis in late-stage mammary tumors. Along these lines, the engineering of mice that fail to express TGF-β or its receptors has revealed important roles for the TGF-β signaling system during vascular development (15,20). Indeed,

homozygous deletion of TGF-β1 in mice results in embryo lethality due to defective yolk sac vasculogenesis (26,46). Interestingly, the vascular abnormalities by TGF-β1 deletion were only observed in specific genetic backgrounds, suggesting the involvement of additional genetic modifiers coupled to vascular development in mice harboring defects in their TGF-β signaling systems. Additionally, genetic inactivation of the TGF-β receptors TβR-II, TβR-I (also called ALK-5), or ALK-1 results in embryonic lethality at E10.5 due to vascular defects, indicating an important role for these receptors in normal endothelial cell function (63,91,92). Along these lines, homozygous deletion in mice of the accessory receptor, endoglin, elicits embryonic lethality at E11.5 that reflects cardiovascular and angiogenic defects (66). Collectively, these findings implicate TGF-β as an essential mediator of vasculogenesis during embryonic development and tissue morphogenesis.

Consistent with the aforementioned conclusion, TGF-β also governs the expression of a variety of genes in endothelial cells, including collagens I, IV, and V, fibronectin, and the fibronectin receptor, integrin α5β1 (29,75,99). TGF-β also induces the expression of PDGF-B, which is important for the recruitment of pericytes during vessel maturation (41). Interestingly, whereas normal vessels are tightly associated with pericytes and benefit from their mechanical and physiological support, tumor vessels typically exhibit noticeably reduced levels of these auxiliary cells, leading to aberrant paracrine signaling networks between pericytes and their underlying endothelial cells (5,51,105). A proangiogenic function for TGF-β and its activation of ALK-1 is further supported by their induction of the transcription factor, Id1, which mediates endothelial cell proliferation and migration (47,48,89). Additionally, activation of ALK-1 readily promotes the angiogenesis, growth, and progression of tumors (24), and, as such, pharmacological inactivation of ALK-1 signaling (e.g., ALK-1-Fc fusion protein) significantly reduces the angiogenesis and growth of pancreatic and breast carcinomas, including that induced by TGF-β, VEGF, and bFGF (24,79). Thus, targeted chemotherapies against ALK-1 may represent a novel class of antitumor agents capable of inhibiting tumor progression by alleviating tumor angiogenesis.

The aforementioned findings clearly implicate TGF-β as a potent inducer of tumor angiogenesis; however, this designation remains controversial given the findings in the scientific literature that link TGF-β signaling to angiostatic programs that transpire in context-specific manner (48,96). For example, whereas expression of constitutively active ALK-1 induces angiogenesis in mouse embryonic endothelial cells (48), similar expression of constitutively active TβR-I (ALK-5) inhibits angiogenesis in human umbilical vein endothelial cells (93). Along these lines, the ability of TGF-β to differentially regulate endothelial cell proliferation and migration may reflect changes in the microenvironmental balance between TGF-β and additional angiogenic factors. In fact, low TGF-β concentrations are known to promote bFGF- and VEGF-mediated endothelial cell proliferation and sprouting, while administration of high TGF-β concentrations prevents these events from occurring (48, 99,115) (Fig. 2). In addition, inhibiting ALK-5 activity readily uncouples TGF-β from activating canonical Smad2/3 signaling. However, a recent study observed Smads 2 and 3 to mediate diametrically opposed activities in developing mammary tumors. Indeed, whereas the activation of Smad2 was found to inhibit mammary tumor angiogenesis, growth, and metastasis, Smad3 activation was linked to the oncogenic activity of TGF-β and its stimulation of mammary tumor angiogenesis and metastasis (100). Thus, the selective inactivation of Smad3 may provide a novel and effective means to prevent tumor angiogenesis stimulated by TGF-β. Likewise, administering neutralizing antibodies against endoglin inhibits VEGF-mediated endothelial cell sprouting in vitro, and mammary tumor growth in mice (52,128,136).

Collectively, these findings emphasize the delicate balance that fine tunes and orchestrates the signaling systems that determine whether TGF-β couples to the induction or suppression of tumor angiogenesis. Future studies need identify the microenvironmental factors that govern the angiogenic or angiostatic activities of TGF-β, as well as assess their clinical relevance as potential therapeutic targets or diagnostic biomarkers for breast cancer patients.

TGF-β AND THE IMMUNE SYSTEM

An essential function of TGF-β within tumor microenvironments lies in its ability to suppress immunosurveillance by inhibiting the functions of infiltrating immune cells operant in mediating tumoricidal activities, and to facilitate the recruitment of macrophages and monocytes that enhance metastatic progression (12). Along these lines, we defined a novel TAB1:xIAP:TAK1:IKKβ:NF-κB signaling axis coupled to the production of proinflammatory cytokines in breast cancer cells (85–87). A major effect of this noncanonical TGF-β effector system results in the elevated expression of Cox-2 and its synthesis of PGE2, which promotes breast cancer progression, EMT, and metastasis via autocrine activation of EP2 receptors (131). The overall importance of TGF-β in

regulating immune cell function is underscored by the fact that mice deficient in TGF-β1 expression readily develop lethal multifocal inflammatory disease (60,61). Likewise, genetic inactivation of Smad3 impairs T-cell responsiveness, as well as elicits chemotaxis defects in neutrophils, T cells, and B cells (144). Here we highlight the specific activities of TGF-β on T cells, macrophages, and myeloid-derived suppressor cells (MDSCs) that enhance the development and progression of mammary tumors.

TGF-β Suppresses T Cell Immunosurveillance

$CD8^+$ cytolytic T lymphocytes (CTLs) play a critical role in mediating the clearance of tumor cells. Tumor development and progression is bolstered by the ability of TGF-β to suppress the proliferation, immunosurveillance, and cytolytic activities of $CD8^+$ CTLs. In fact, engineering mouse fibrosarcoma cells to overexpress TGF-β1 enhanced tumor growth by suppressing CTL-mediated tumor rejection (134). Additionally, specific abrogation of TGF-β signaling in T cells mediated by their enforced expression of a nonfunctional TβR-II mutant (i.e., truncated TβR-II) enabled mice to mount an effective immune response capable of eradicating melanoma growth and metastasis (43). Mechanistically, TGF-β inhibits the production of IL-2, represses the expression of c-Myc and cyclins D2 and E, and stimulates the expression of the CDK inhibitors, p15, p21, and p27. The net effect of these events results in a significant decrease in T-cell proliferation and response (67,126,141). Along these lines, TGF-β also represses the ability of T cells to transcribe a variety of apoptosis inducing factors, including perforin, granzymes A and B, FAS ligand, and interferon-γ (1,16,129). Moreover, TGF-β stimulation of $CD8^+$ T cells enhances their production and secretion of IL-17, which activates survival signaling in carcinoma cells (84). Unlike $CD8^+$ T cells, TGF-β has no effect on the proliferation of $CD4^+$ T cells, but instead functions to inhibit their differentiation (43). TGF-β also inactivates the tumoricidal activities of CTLs by inducing the selection and expansion of Tregs, which suppress granule release by activated CTLs (69,74). Similar to $CD8^+$ CTLs, natural killer (NK) cells play an essential role in suppressing tumor formation by targeting tumor cells for destruction. The ability of NK cells to kill carcinoma cells depends upon the activation of NKp30 and NKG2D receptors, whose expression is readily downregulated by TGF-β as a means to inactivate the cytolytic activities of NK cells (4,80,110). Indeed, systemic attenuation of TGF-β signaling increases immune-mediated clearance of tumor cells in vivo, presumably due to the unveiling of normal CTL and NK cell tumoricidal activity (59,129).

Besides its ability to directly inhibit the functions of CTLs and NK cells, TGF-β also suppresses T-cell activity via an indirect mechanism involving the actions of neutrophils and dendritic cells. For instance, TGF-β functions as a potent chemoattractant for neutrophils (106) and inhibits their ability to recognize and destroy Fas ligand (FasL) that is abundantly expressed on carcinoma cells. As such, tumor-infiltrating CTLs undergo apoptosis upon contacting FasL-expressing carcinoma cells, an event that confers these cells immune privilege and promotes their metastatic progression (50). Dendritic cells function in initiating immune responses by presenting antigens to T cells, B cells, and NK cells (12). Interestingly, administering TGF-β to dendritic cells inhibits their maturation and production of the proinflammatory cytokines, IL-1 and IL-12, thereby failing to mount an effective antitumor CTL response (40). Taken together, these studies suggest that measures capable of reducing TGF-β levels within tumor microenvironments will significantly improve the CTL activity and tumor clearance by T cells.

TGF-β and Monocytes and Macrophages

Generally speaking, the recruitment of monocytes and macrophages to tumor microenvironments is associated with enhanced tumor progression (22,70,71). TGF-β is a potent inducer of IL-1 and IL-6 expression by monocytes, as well as a powerful stimulator of their differentiation into macrophages (36). Likewise, TGF-β readily attenuates the effector and cytotoxic functions of macrophages that normally target carcinoma cells for destruction (49). Finally, the activation of resting monocytes by TGF-β stimulates their chemotaxis and infiltration into tumor microenvironments where they (i) promote carcinoma progression by stimulating ECM degradation necessary for tumor angiogenesis, invasion, and metastasis, and (ii) create an immunosuppressive environment through their release of TGF-β (68,103,137).

TGF-β and MDSC Recruitment

The preceding sections highlighted the importance of tumor-associated macrophages, monocytes, and neutrophils in promoting tumor development and metastatic progression (12,23). More recently, immature $Gr\text{-}1^+CD11b^+$ myeloid cells, which are also known as myeloid-derived suppressor cells (MDSCs), have been shown to possess robust immunosuppressive activities (38,142). Indeed, genetic inactivation of TβR-II in mammary carcinoma cells elicits tumor infiltration of $Gr\text{-}1^+CD11b^+$ cells in part via the activation of SDF-1/CXCR4 and CXCL5/CXCR2 chemokine signaling axes. Upon gaining entry into mammary tumor

microenvironments, MDSCs readily inhibit the function of dendritic cells, NK cells, and B and T lymphocytes (12,143). Additionally, MDSCs also secrete high levels of (i) matrix metalloproteinases, which aids breast cancer cell dissemination from the primary tumor, and (ii) TGF-β, which further suppresses host immune response and promotes acquisition of invasive and metastatic phenotypes by breast cancer cells (142). Because MDSCs localize to the invasive front of breast cancers (13,142) and aid in establishing premetastatic niches during breast cancer dissemination (30), it stands to reason that chemotherapeutic targeting of MDSCs may provide a novel opportunity to improve the clinical course of breast cancer patients by simultaneously improving host immune surveillance and inhibiting metastatic progression.

TGF-β AND STROMAL FIBROBLASTS

TGF-β and Cancer-Associated Fibroblasts

Besides its ability to govern the activities and behaviors of epithelial, endothelial, and hematopoietic cell lineages, TGF-β is also a master regulator of the proliferation and differentiation status of fibroblasts, including those in the mammary tumor microenvironment (9,104). Indeed, TGF-β present in tumor microenvironments induces fibroblasts to secret a variety of growth factors, cytokines, and ECM proteins that act in a coordinated fashion to either suppress or promote tumor development in the adjacent epithelium (11,132). Similar to the differential gene expression profiles exhibited between normal and malignant MECs, recent microarray analyses have identified distinct gene expression signatures that readily distinguish normal mammary fibroblasts from their cancer-associated counterparts (120). Importantly, TGF-β was identified as a prominent protein downregulated in tumor-derived fibroblasts, suggesting that diminished TGF-β production by stromal cells engenders a tumor-promoting microenvironment (120). In support of this supposition, conditional deletion of TβR-II specifically in fibroblasts significantly expands the stromal compartments of the prostate and forestomach due in part to disruptions in paracrine signaling networks between fibroblasts and epithelial cells. Ultimately, these aberrant events culminate in the generation intraepithelial neoplasia in the prostate and invasive squamous cell carcinoma in the forestomach (6). Similar genetic inactivation of TβR-II specifically in mammary fibroblasts also expands their abundance in the mammary gland, as well as increases the turnover of adjacent ductal epithelial cells. Interestingly, transplanting TβR-II-deficient fibroblasts under the renal capsule with mammary carcinoma cells greatly increases their growth and invasion relative to that mediated by TβR-II-expressing fibroblasts. The elevated malignancy exhibited by transplanted mammary carcinoma cells reflects their activation of several receptor tyrosine kinases (RTKs), including EGFR, ErbB2, RON, and c-Met. Importantly, TβR-II deficiency in mammary fibroblasts increases their production and secretion of the cognate ligands for these RTKs (e.g., TGF-β, MSP, and HGF) (21). Recently, loss of a single TβR-II allele was determined to enhance the accumulation of fibroblasts, as well as increase the aggressiveness and metastasis of MMTV-PyMT tumors in mice (33). Collectively, these findings indicate that TGF-β signaling in fibroblasts functions to suppress the activation of paracrine signaling networks that promote tumorigenesis in adjacent MECs epithelial cells.

TGF-β and Fibroblast Transdifferentiation

Besides its ability to regulate the activation and proliferation of fibroblasts, TGF-β also promotes the transdifferentiation of fibroblasts into myofibroblasts, which is defined immunohistochemically by their expression of α-smooth muscle actin (α-SMA) and vimentin (76). Fibroblast transdifferentiation is reminiscent of EMT reactions that occur in normal and malignant epithelial cells stimulated with TGF-β, and, as such, transdifferentiated myofibroblasts are highly abundant in invasive breast cancers compared to their in situ counterparts. Moreover, myofibroblasts typically localize to the invasive front of mammary tumors, suggesting an important role for transdifferentiated fibroblasts during metastatic progression (28,113). In fact, tumor-associated myofibroblasts are the predominant cell type responsible for eliciting desmoplastic reactions in mammary tumors as they become palpable. Moreover, desmoplastic reactions lead to the formation of mechanically rigid tumor microenvironments that drive metastatic progression and predict for poor clinical outcomes in breast cancer patients (28,30–32,65). Future studies need to fully characterize the autocrine and paracrine signaling systems that exist between myofibroblasts and their reactive stromal constituents in promoting the oncogenic activities of TGF-β and its stimulation of metastatic progression.

TGF-β, Fibroblasts, and ECM Protein Production

Fibroblasts and myofibroblasts within the tumor microenvironment are the largest producers of ECM components, of which collagen I and fibronectin are the most abundant proteins. TGF-β stimulates the expression and secretion of both of these ECM components (97,125), particularly during fibrotic reactions

coupled to desmoplasia and increased mechanical tension within tumor microenvironments. The exaggerated rigidity exhibited in tumor microenvironments contributes to metastatic progression in mammary tumors and reflects the elevated crosslinking of a variety of ECM components, most notably collagen and elastin (19,32,65,98). Indeed, lysyl oxidases (LOXs) comprise a five-member gene family of copper-dependent amine oxidases that catalyze the crosslinking of collagens and elastin in the ECM, leading to increased tissue tension and stiffness (19,32,65, 98). We observed TGF-β to induce LOX expression in normal and malignant MECs, and in triple-negative breast cancers produced in mice (124). LOX also functions in recruiting Gr-1$^+$CD11b$^+$ cells to premetastatic niches where they produce MMPs, thereby enhancing the invasion and recruitment of bone marrow derived cells (BMDCs) and metastatic breast cancer cells to these secondary sites of metastasis (30). Importantly, we observed differences in ECM tension to alter the response of MECs to TGF-β, such that exposing metastatic breast cancer cells to compliant microenvironments can partially reestablish the cytotoxic activities of TGF-β even in late-stage mammary tumors (124). Future studies need to identify the effectors of mechanotransduction operant in mediating the oncogenic activities of TGF-β in cancers of the breast, as well as to determine the potential of these molecules to serve as novel chemotherapeutic targets and diagnostic markers of mammary tumor development.

CONCLUSIONS

Studies performed over the last 30 years have clearly established TGF-β as a potent tumor suppressor in normal MECs and early stage mammary tumors, whose progression to aggressive disease states is accompanied by the acquisition of oncogenic activity by TGF-β (130,132). This incredible duality in TGF-β function represents a significant challenge to the development of targeted TGF-β chemotherapeutics designed to accentuate the cytostatic functions of TGF-β, while simultaneously attenuating its oncogenic activities in neoplastic mammary tissues. This challenge is further complicated by the fact that TGF-β exerts tumor cell autonomous activities, as well as induces cell- and context-specific activities within individual cell types housed in adjacent tumor microenvironments. Overcoming this challenge will require concerted efforts to map the genetic and epigenetic events that confer TGF-β with oncogenic activities, and to determine the relative extent to which these events derive from aberrancies within mammary carcinoma cells, from within their stromal compartment, or from within both cellular compartments. The emerging evidence presented here highlights the essential role played by tumor microenvironments to influence the pathophysiology of cancer cells and their response to TGF-β. As such, developing novel chemotherapeutics aimed at targeting specific cell types within reactive tumor microenvironments may provide an effective means alleviate the oncogenic activities of TGF-β in patients harboring metastatic breast cancers.

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