Chapter 7
The Multifunctional Roles of TGF-β in Navigating the Metastatic Cascade

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Abstract The role of TGF-β during tumorigenesis is best characterized by the diverse functions this multifunctional cytokine exhibits in early-stage versus late-stage cancers. For instance, during the initial stages of tumorigenesis, TGF-β uniformly acts as a potent tumor suppressor, even in low-grade carcinomas capable of evading the cytostatic activities of TGF-β. However, as carcinoma cells continue to evolve and progress towards more aggressive disease states they typically acquire the ability to surmount the last vestiges of the tumor suppressing activities of TGF-β, ultimately gaining a selective advantage that enables TGF-β to promote their metastatic progression and production of recurrent secondary tumor lesions that are refractory to standard chemotherapies. The molecular, cellular, and microenvironmental mechanisms that permit metastatic carcinoma cells to usurp and commandeer TGF-β for oncogenic activities are highly diverse and remain incompletely understood. Here we review recent advances that provide new insights into how aggressive carcinoma cell populations are selected to respond to the oncogenic activities of TGF-β, focusing specifically on its essential functions coupled to metastatic outgrowth and the acquisition of chemoresistance.

Keywords Chemoresistance • Epithelial-mesenchymal transition • Metastasis • Signal transduction • TGF-β

Abbreviations

ECM	Extracellular matrix
EGF	Epidermal growth factor

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EMT	Epithelial-mesenchymal transition
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase
JNK	c-Jun N-terminal kinase
LAP	Latency-associated peptide
MAP kinase	Mitogen-activated protein kinase
MEC	Mammary epithelial cell
MET	Mesenchymal–epithelial transition
NF-κB	Nuclear factor-κB
PI3K	Phosphoinositide-3-kinase
TGF-β	Transforming growth factor-β
TβR-I	TGF-β type I receptor
TβR-II	TGF-β type II receptor
TβR-III	TGF-β type III receptors

7.1 Introduction

The major causes of cancer mortality are the processes of tumor recurrence and secondary tumor metastasis. Indeed, the 5-year survival rates for patients diagnosed with localized versus disseminated disease drops from (1) 99 to 23 % for breast cancers, (2) 90 to 12 % for colorectal cancers, (3) 98 to 15 % for melanomas, (4) 92 to 27 % for ovarian cancers, and (5) 100 to 29 % for prostate cancers (Siegel et al. 2012). Unfortunately, the multistep complexity of metastasis and its associated cascade of gene expression and repression has stymied our understanding of this inefficient and deadly process. Interestingly, recent findings have clearly established the TGF-β pathway as being an important mediator of carcinoma recurrence and metastasis (Taylor et al. 2010), which contrasts dramatically from its ability to suppress cellular transformation by preventing normal cells from proliferating uncontrollably, as well as by inducing their differentiation and apoptosis (Tian et al. 2010). Numerous studies over the last three decades have investigated the mechanisms that underlie these dichotomous functions of TGF-β in normal and malignant cells, a phenomenon that is collectively known as the "TGF-β Paradox." Although a single unifying sequelae operant in eliciting the "TGF-β Paradox" has yet to be defined, a significant body of work has demonstrated that aberrations within the TGF-β signaling system figure prominently in its acquisition of oncogenic activity in developing carcinomas (Rahimi and Leof 2007; Tian and Schiemann 2009; Wendt et al. 2012b). The propagation of TGF-β messages across plasma membranes is initiated by its binding to the TGF-β type II receptor (TβR-II), a Ser/Thr protein kinase that recruits, transphosphorylates, and activates the Ser/Thr protein kinase activity of the TGF-β type I (TβR-I) (Parvani et al. 2011; Tian et al. 2010). Once activated, TβR-I rapidly phosphorylates and activates the latent transcription factors, Smad2 and Smad3, which subsequently accumulate in the nucleus as part of heterocomplexes that contain Smad4 and govern gene expression in a cell- and context-specific manner (Feng

and Derynck 2005; Heldin and Moustakas 2012; Massague and Gomis 2006). The Smad2/3 pathway is commonly referred to as "canonical" TGF-β signaling, which is complemented by the ability of TGF-β to activate an ever expanding list of "non-canonical" signaling pathways, including (1) integrins and focal adhesion proteins; (2) PI3K and its effectors, AKT and mTOR; (3) NF-κB and inflammatory mediators; and (4) MAP kinases and their downstream effectors (Parvani et al. 2011; Taylor et al. 2010; Wendt et al. 2009a). Interestingly, a number of studies have demonstrated that genetic or epigenetic disruptions that elicit imbalances between these two core branches of TGF-β signaling represent the crux of the "TGF-β Paradox" and its ability to confer oncogenic functions to TGF-β in developing and progressing carcinomas (Tian and Schiemann 2009; Wendt et al. 2009b). Readers desiring additional information related to specific effector molecules and pathways of the TGF-β signaling system are directed to Chap. 1 herein, as well as to several recent reviews (Parvani et al. 2011; Taylor et al. 2010; Wendt et al. 2009a). Here we review recent findings that depict the function of TGF-β and its effector molecules as potent promoters of carcinoma progression and metastasis.

7.2 Epithelial–Mesenchymal Transition

One cannot address the role of TGF-β in mediating metastasis without first exploring the topic of epithelial–mesenchymal transition (EMT), which at its very core reflects the transdifferentiation of polarized epithelial cells into genetically and phenotypically distinct fibroblastoid-like cells (Kalluri and Weinberg 2009; Taylor et al. 2010; Wendt et al. 2009a). The epithelium is comprised of a monolayer of tightly packed epithelial sheets that not only form the skin but also line the internal cavities (e.g., ductal structures of the mammary gland, lung airways, and gastrointestinal tract), thereby providing a protective barrier to ward off environmental insults. Fully differentiated and polarized epithelial cells also exhibit a variety of specialized secretory, sensory, and glandular functions, many of which are regulated by TGF-β. In stark contrast, the mesenchyme functions to provide structural support for the epithelium, doing so via the production and secretion of the extracellular matrix (ECM), particularly collagens and fibronectins (Hay 2005). Moreover, although epithelial cells are highly confined and immobile, their mesenchymal counterparts are exceedingly mobile and invasive and thus are capable of exiting their associated epithelium and traversing through the underlying basement membrane and ECM (Micalizzi et al. 2010). As a single entity, the process of EMT is insufficient to drive carcinoma metastasis. In fact, EMT programs are a normal physiological process that has been hijacked by carcinoma cells to enable their dissemination to distant locales. In clarifying this issue, Kalluri and Weinberg recently subcategorized EMT into three distinct programs, including (1) Type 1 EMT, which occurs embryonically and developmentally during the formation of the endocardial cushion, neural crest formation, and palate closure and fusion; (2) Type 2 EMT, which occurs during tissue remodeling and repair, as well as during

inflammation-driven fibrotic reactions; and (3) Type 3 EMT, which occurs during the metastatic progression of carcinomas (Kalluri and Weinberg 2009). TGF-β is clearly a master regulator of all EMT subtypes and readers desiring a more thorough summary of the role of TGF-β in regulating EMT programs are directed to several comprehensive reviews (Heldin et al. 2012; Taylor et al. 2010; Wendt et al. 2009a). In the succeeding sections, we highlight the mechanisms whereby TGF-β induces EMT reactions, as well as discuss the utility of these events to identify and diagnose metastatic carcinomas.

7.2.1 Mechanisms of EMT Induced by TGF-β

Metastatic carcinoma cells that have emerged from EMT programs induced by TGF-β are endowed with several important characteristics, including heightened invasiveness, resistance to apoptosis, and the appearance of stem-like behaviors (Taylor et al. 2010; Wendt et al. 2009a; Yang and Weinberg 2008). The ability of TGF-β to induce EMT programs requires signaling inputs from both the canonical and noncanonical branches of the TGF-β signaling system, which converge in the nucleus to regulate the expression and activity of a variety of master transcriptional regulators of EMT (Taube et al. 2010). Included in this essential group of EMT transcription factors are members of the Snail (SNAI1 and SNAI2/Slug), ZEB (ZEB1 and ZEB2/SIP1), basic helix-loop-helix (Twist1 and Twist2), Six family of homeobox (Six1), and Forkhead (FOXC2), as well as members of the High Mobility Group proteins (HMGA2), which modify DNA structure to enhance transcription factor binding (Wendt et al. 2012b) (Fig. 7.1, *Mechanisms*). Interestingly, these transcription factors play essential roles during the performance of developmental Type 1 EMT programs, as well as during the initiation of oncogenic Type 3 EMT programs, suggesting that the inappropriate reactivation of embryonic EMT programs underlies the acquisition of metastatic phenotypes in response to TGF-β-driven EMT (Micalizzi et al. 2010). For instance, the basic helix-loop-helix transcription factor Twist1 plays a critical role in regulating developmental cell fate determination and differentiation reactions (Leptin 1991). However, consistent with the notion that metastatic carcinoma cells hijack physiological EMT programs during their systemic dissemination (Kang and Massague 2004), Twist1 expression is dramatically upregulated in metastatic breast cancer cells, a reaction that is regulated by TGF-β and essential for their metastasis in mice (Yang et al. 2004). Moreover, heterologous expression of Twist1 also empowers mammary epithelial cells with stem-like characteristics and increases their tumor-initiating capacity (Mani et al. 2008), thereby introducing the concept that EMT plays a number of diverse roles during carcinoma metastasis ranging from their increased invasiveness to their enhanced survival to their heightened chemoresistance (Fig. 7.1, *Consequences*). We recently added to this growing list of EMT functions by demonstrating that recombinant expression of Twist1 was sufficient to initiate the

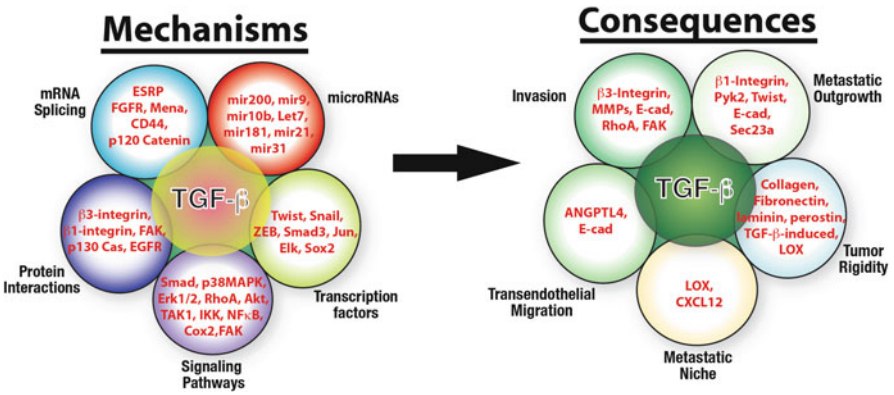

Fig. 7.1 The mechanisms and consequences of oncogenic TGF-β signaling during tumor progression and metastasis. TGF-β is a multifunctional cytokine that suppresses tumor formation by inhibiting cell proliferation and by stimulating cell differentiation or apoptosis. During carcinoma development and progression, TGF-β function is readily converted to that of a tumor promoter, leading to enhanced carcinoma cell EMT, invasion, and metastasis. Several of the established mechanisms of oncogenic TGF-β signaling are shown (*left*). The consequences of these events in relation to specific steps of the metastatic cascade are shown (*right*). At present, the precise "cause and effect" link between specific mechanisms and their associated consequences is expanding at a rapid rate; however, understanding how the consequences and pathophysiological readouts of oncogenic TGF-β signaling relate to multiple mechanistic events remains a daunting challenge to basic and translational researchers. See text for details

pulmonary outgrowth of disseminated breast cancer cells that otherwise succumb to systemic dormancy (Wendt et al. 2011b) (Fig. 7.1, *Consequences*). Along these lines, recent findings indicate that transcription factors coupled to the induction of EMT programs work in concert both directly and indirectly to bring about EMT reactions. For instance, Casas and colleagues (Casas et al. 2011) recently demonstrated that the ability of Twist1 to repress E-cadherin expression during the EMT and metastasis of breast cancer cells was absolutely dependent upon its stimulation of Snail2 (Slug) (Fig. 7.1, *Mechanisms*). Similar to the response of Twist1 to TGF-β, members of the Snail family of zinc finger transcription factors (SNAI1-3) are also induced in cells stimulated by TGF-β (Horiguchi et al. 2008), leading to the direct transcriptional repression of a variety of epithelial genes, including E-cadherin (Hajra et al. 2002) (Fig. 7.1, *Consequences*). Finally, TGF-β readily stimulates the expression of high mobility group A2 (HMGA2), which promotes EMT programs by upregulating the expression of Snail1, Snail2, and Twist, which collectively converge in downregulating that of E-cadherin (Thuault et al. 2006) (Fig. 7.1). Future studies need to define spatiotemporal regulation of these EMT transcription factors by TGF-β in developing and progressing carcinomas and, more importantly, establish the specific steps in the metastatic cascade that are dependent upon these transcription factors.

7.2.2 *Defining the Metastatic EMT Morphology*

To date, EMT programs have been predominantly studied in the context of traditional 2D-culture systems, whose artificial nature imposes significant cellular and genetic adaptations necessary for cells to thrive in this biomechanically rigid microenvironment. Indeed, the prominent feature of transitioning epithelial cells in 2D-cultures is their formation of actin-rich filopodia and actin stress fibers as they acquire fibroblastoid-like morphologies. Teleologically, these morphological features are in keeping with the acquisition of motile, invasive, and metastatic phenotypes by carcinoma cells. Unfortunately, these actin-based architectures are an artifact of propagating mesenchymal cells on rigid 2D-cultures and, in fact, are not observed in mesenchymal cells in vivo (Hay 2005). Similarly, the advent of 3D-organotypic culture systems and the propagation of post-EMT cells in these systems have also called into question the precise morphology exhibited by fully transitioned carcinoma cells in vivo, particularly in tumor microenvironments that possess elevated expression and activity of TGF-βs. Indeed, we recently established that applying breast cancer cells to 3D-organotypic cultures results in the formation of intricate branched structures that readily assume dense multicellular spherical structures in response to TGF-β (Wendt et al. 2010, 2011a, b, 2012a). Importantly, the formation of these "invasospheres" transpires through an EMT-dependent mechanism and reflects the ability of TGF-β to promote the invasion and metastasis of these same breast cancer cells in mice (Wendt et al. 2010, 2012a). Collectively, these findings suggest that the histological association of sarcomatoid morphologies with EMT phenotypes may be misleading and misguiding when attempting to identify post-EMT carcinoma cells in vivo. In an effort to alleviate the current controversies surrounding EMT phenotypes in vivo, future studies clearly need to identify the morphologies and features of post-EMT cells at specific steps of the metastatic cascade, as well as to determine their ability to predict the overall survival and clinical outcome of patients with metastatic disease.

7.2.3 *The Diagnostic Value of EMT*

The crosstalk and linkages between the aforementioned EMT transcription factors have greatly increased our understanding of how TGF-β regulates epithelial plasticity in normal and malignant cells. However, while the list of "master regulators" of EMT programs continues to expand at an alarming rate (Fig. 7.1; *Mechanisms*), the utility of these transcription factors to function as diagnostic or predictive biomarkers of metastatic progression, as well as novel therapeutic targets to alleviate carcinoma dissemination is compromised at an equally alarming rate. Indeed, single cell analysis of circulating tumor cells has shown that these cells simultaneously express epithelial and mesenchymal markers, suggesting the existence of metastable and dynamic EMT phenotypes in carcinoma cells that have escaped the primary tumor

(Armstrong et al. 2011). Given the difficulty to pathologically identify EMT in vivo and the plasticity of EMT programs in highly malignant cells, the overall clinical utility and effectiveness of detecting and targeting of post-EMT carcinoma cells harboring mesenchymal characteristics remains to be determined. For instance, EMT and its counterpart, mesenchymal–epithelial transition (MET), are transient processes that likely take place numerous times during the evolution of a tumor and its dissemination to vital organs. Indeed, several recent studies that examined the plasticity of a variety of breast and other carcinoma cell progression series indicate that the maintenance of a perpetual mesenchymal-state limits the overall tumorigenicity and aggressiveness of fully transitioned cells in response to TGF-β (Korpal et al. 2011; Wendt et al. 2011a, b). As such, evolving the capacity to undergo successive cycles of EMT:MET selects for carcinoma cells that possess the highest metastatic potential. Moving forward, future studies need to identify and validate novel biomarkers capable of differentiating primary, secondary, and tertiary EMT states, which may provide novel diagnostic and therapeutic regimens to alleviate metastatic disease.

7.3 Emerging Paradigms in TGF-β-Mediated Gene Regulation

7.3.1 Diversity in Smad Signaling

The specific set of genes targeted by canonical TGF-β signaling varies greatly in a cell- and context-specific manner. For example, the expression of a particular gene may be induced by TGF-β in fibroblasts, whose proliferation is stimulated by TGF-β; however, this same gene may be potently downregulated by TGF-β in epithelial cells, whose proliferation is inhibited by TGF-β. This diversity in TGF-β signaling has historically been believed to reflect the differential ability of TGF-β to couple to its canonical and noncanonical effectors in distinct cells types, as well as the differential influence of heterologous signals that impinge upon TGF-β effector molecules. However, recent findings have demonstrated an important alternative mechanism, namely the differential and physical interactions between Smad3 with specific "master" transcription factors, including Oct4 in embryonic stem cells (ESCs), Myod1 in myotubes, and PU.1 in pro-B cells (Mullen et al. 2011), thereby enabling Smad3 and TGF-β to direct cell-fate outcomes during tissue morphogenesis and remodeling (Fig. 7.1, *Mechanisms*). Canonical TGF-β signaling is also impacted by the direct phosphorylation of Smad2/3 by a variety of Ser/Thr protein kinases, including members of the CDK and MAP kinase families, GSK-3β, PKC, CK1γ2, and several others (Hayashida et al. 2003; Matsuura et al. 2005; Matsuzaki 2011; Wrighton et al. 2009) (Fig. 7.1, *Mechanisms*). Secondary phosphorylation of Smad2/3 by these protein kinases can alter the subcellular localization, stability, and transcriptional activity of these canonical TGF-β effectors, particularly their

regulation of the expression of metastasis-associated genes (Hayashida et al. 2003). Along these lines, the amplified activation of oncogenic signaling systems (e.g., receptor tyrosine kinases, Ras, PI3K, etc.), which modulate the activation status of Smad2/3-directed protein kinases, further dysregulates the repertoire of canonical TGF-β signaling and enables developing carcinoma cells to evade the tumor suppressing activities of TGF-β (Fig. 7.1, *Mechanisms*).

7.3.2 Regulation of MicroRNAs

In addition to traditional modes of gene expression regulated by canonical and non-canonical TGF-β signaling systems, recent studies have established TGF-β as a potent regulator of microRNAs in developing carcinomas, particularly during their acquisition of metastatic phenotypes. The linkage of microRNAs to oncogenic TGF-β signaling was initially described by its regulation of the miR-200 family of microRNAs, which function in suppressing the expression of the EMT transcription factors, ZEB1 and ZEB2 (SIP1) (Gregory et al. 2008; Korpal et al. 2008) (Fig. 7.1, *Mechanisms*). During EMT programs and metastatic progression, TGF-β promotes the downregulation of miR-200 family members, which stabilizes expression of ZEB1 and ZEB2 and enables their binding to and repression of the *Cdh1* (E-cadherin) promoter, resulting in a loss of E-cadherin expression in transiting carcinoma cells (Fig. 7.1, *Consequences*). Intervening studies have since expanded the repertoire of gene transcripts that are targeted and regulated by the miR-200 family, which are remarkably correlated with those targeted and regulated by TGF-β (Howe et al. 2011; Schliekelman et al. 2011). Interestingly, a significant portion of genes targeted by members of the miR-200 family are coupled to the production of proteins operant in producing or sensing alterations in the tumor microenvironment. Included in this growing list of microenvironmental sensors are the ECM molecules fibronectins, collagens, and laminins, as well as cell surface receptors that connect the extracellular and intracellular milieu, such as integrins and growth factor receptors (Fig. 7.1, *Consequences*). Importantly, we recently demonstrated that aberrant expression of these factors underlies the initiation of the "TGF-β Paradox" and promotes the acquisition of oncogenic TGF-β signaling in developing and progressing carcinomas (Galliher and Schiemann 2006, 2007; Galliher-Beckley and Schiemann 2008; Taylor et al. 2011; Wendt and Schiemann 2009; Wendt et al. 2009b). Additionally, recent findings implicate a powerful feed-forward signaling loop between TGF-β and miR-200 family members in driving oncogenic EMT and carcinoma metastasis (Gregory et al. 2011). Collectively, these findings establish miR-200 family members as tumor suppressors capable of preventing oncogenic TGF-β signaling and its induction of EMT in normal epithelial cells (Schliekelman et al. 2011). Surprisingly, two recent studies demonstrated that highly metastatic 4T1 breast cancer cells are more epithelial-like as compared to their isogenic and non-metastatic 4T07 counterparts (Korpal et al. 2011; Wendt et al. 2011a, b). Amongst the differences between these two cell types is the reestablishment of miR-200 in

4T1 cells, an event that aids in the production and secretion of metastasis-promoting proteins necessary for the outgrowth of macroscopic metastases (Korpal et al. 2011) (Fig. 7.1, *Consequences*).

Besides regulating the expression of miR-200 family members, TGF-β also dictates the activities of microRNAs by promoting their processing. For instance, Smad2/3 interacts physically with DROSHA, which enhances the conversion of primary miR-21 transcripts into their pre-miR-21 counterparts in a Smad4-independent manner (Davis et al. 2008) (Fig. 7.1, *Mechanisms*). The coupling of TGF-β to EMT programs also proceeds via its ability to upregulate the expression of miRs 21 and 31, resulting in the downregulation of Tiam1 (Cottonham et al. 2010; Zavadil et al. 2007), and miR-155, leading to the repression of RhoA expression (Kong et al. 2008) (Fig. 7.1, *Consequences*). Finally, we have identified miR-181a as a TGF-β-inducible "metastamir" that enhances breast cancer metastasis by facilitating the downregulation of the pro-apoptotic molecule, Bim, thereby conferring miR-181a-expressing carcinoma cells resistance to anoikis (Taylor et al. 2013) (Fig. 7.1, *Consequences*).

7.3.3 Alternative Splicing

The splicing of mRNAs is a post-transcriptional modification that functions to dramatically increase the diversity of gene products produced from a static genome. In fact, transcription of the vast majority of the genes in the genome (~90 %) gives rise to two or more protein products due to the process of alternative splicing. Recently, TGF-β has been suggested to play a major role in mRNA splicing, particularly in developing carcinomas through its ability to suppress the expression of epithelial splicing regulatory proteins (ESRPs), which are a class of RNA-binding proteins that govern the splicing of epithelial gene transcripts (Horiguchi et al. 2012; Warzecha et al. 2010). Indeed, the ability of distinct protein variants to impact cancer development, EMT, and metastasis is rapidly coming to the forefront of cancer biology, including that regulated by TGF-β. For instance, the initiation of alternative gene splicing programs that transpire during EMT reactions driven by TGF-β results in part from its ability to downregulate the expression of ESRP2 via a ZEB1/δEF1- and ZEB2/SIP1-dependent manner (Horiguchi et al. 2012) (Fig. 7.1, *Mechanisms*). Perhaps the best studied example of differential gene splicing governed by TGF-β relates to its regulation of the four member fibroblast growth factor receptor (FGFR) family, of which FGFRs 1–3 are subject to alternative splicing that greatly enhances isoform diversity and function (Fig. 7.1, *Mechanisms*). TGF-β regulates two major splicing events in FGFRs. First, alternative splicing of the extracellular Ig-like domain I (α-loop) results in either the inclusion or the exclusion of the first Ig-like domain. Importantly, exclusion of Ig-like domain I drastically increases the affinity of the FGFR to bind members of the FGF ligand family, which currently comprises 22 individual cytokines (Jain and Turner 2012; Wesche et al. 2011). Second, alternative splicing of the distal portion of Ig-like domain III dictates the specificity of

FGFRs to bind specific FGF ligands (Holzmann et al. 2012; Jain and Turner 2012; Werner et al. 1992). Inclusion of exon 8 results in the production of FGFR-IIIb isoforms capable of binding to FGF7 (KGF) and FGF10, while inclusion of exon 9 results in the production of FGFR-IIIc isoforms that selectively bind FGF2 and FGF4 (Holzmann et al. 2012; Jain and Turner 2012; Werner et al. 1992). Interestingly, FGFR-IIIb and -IIIc isoforms are preferentially expressed in epithelial and mesenchymal cells, respectively, and the induction of EMT programs by TGF-β is sufficient to switch epithelial FGFR-IIIb expression to that of its -IIIc counterpart in post-EMT cells (Shirakihara et al. 2011) (Fig. 7.1, *Mechanisms*). It should be noted, however, that the metastatic cells that exhibit epithelial morphologies and characteristics retain FGFR-IIIc expression (Chaffer et al. 2006), suggesting the presence of a metastable phenotype in metastatic cells that have emerged from successive rounds of EMT:MET. Future studies need to further address the consequences of FGFR splicing during TGF-β-driven EMT and metastasis, particularly during the latter stages of metastatic progression and emergence from micrometastatic dormancy (Korpal et al. 2011; Wendt et al. 2011a, b), and as a potential predictive biomarker and therapeutic target for recurrent metastatic tumors.

7.4 TGF-β and the Metastatic Cascade

7.4.1 Primary Tumor Local Invasion

The initial step of the metastatic cascade requires tumor cells to exit the confines of a primary tumor and invade into the surrounding tissue. TGF-β plays a critical role in stimulating tumor cell invasion through its ability to upregulate the expression and activity of extracellular proteases charged with degrading tumor ECM and supporting basement membrane. For instance, TGF-β robustly induces the expression of several matrix metalloproteinases (MMPs), including MMPs 2, 3, 7, 13, MT1, and especially MMP-9 (Wendt et al. 2009a) (Fig. 7.1, *Consequences*). Although the signaling mechanisms that couple TGF-β to MMP expression remain to be determined definitively, recent studies have implicated the noncanonical effector, TAK1 and its downstream targets as mediators of MMP-9 expression stimulated by TGF-β (Safina et al. 2008) (Fig. 7.1, *Mechanisms*). In general, genetic depletion or pharmacologic inactivation of MMPs drastically decreases the ability of TGF-β to stimulate the invasion of metastatic carcinoma cells (Wendt et al. 2009a). Besides their ability to degrade ECM during carcinoma invasion, MMPs also cleave LAP (latency-associated peptide) that permits the liberation of biologically active TGF-β from inactive ECM depots, thereby initiating a positive feed-forward loop coupled to elevated autocrine TGF-β signaling (Rifkin 2005; Taylor et al. 2010).

TGF-β also enhances carcinoma invasiveness by altering their production of and response to ECM molecules within the developing tumor microenvironment (Stover

et al. 2007). Indeed, when stimulated by TGF-β, metastatic carcinoma cells elevate their production of a wide array of ECM proteins, including collagens (Ignotz and Massague 1986), fibronectin (Ignotz and Massague 1986), periostin (Wen et al. 2010), and laminins (Giannelli et al. 2005; Kumar et al. 1995), as well as a newly described protein known as TGF-β-induced [TGFBI; (Nummela et al. 2012)] that limits cell adhesion to structural ECM proteins (Fig. 7.1, *Consequences*). Integrins are heterodimeric transmembrane receptors that function as conduits capable of linking the ECM to intracellular signaling and cytoskeletal systems (Hall 2009; Zutter 2007). Principle players involved in TGF-β-mediated response to ECM proteins are αvβ3 integrins, whose expression is robustly induced by TGF-β via a Smad2/3-independent mechanism (Galliher and Schiemann 2006; Pechkovsky et al. 2008). Amplified expression of αvβ3 integrin results in its indirect interaction with TβR-II, a reaction mediated by focal adhesion kinase (FAK); (Wendt and Schiemann 2009). Importantly, the incorporation of αvβ3 integrin within activated TGF-β receptor complexes enables Src to phosphorylate TβR-II at Tyr284 (Galliher and Schiemann 2007). This phosphorylation event facilitates the binding of Grb2 and ShcA to TβR-II leading to amplified noncanonical TGF-β signaling through MAP kinases (Galliher et al. 2006; Galliher and Schiemann 2007; Galliher-Beckley and Schiemann 2008). Additionally, αvβ3 integrin:TβR-II complexes couple TGF-β to FAK (Wendt and Schiemann 2009), p130Cas (Wendt et al. 2009b), and Pyk2 (Wendt et al. 2012a), which further enhance the coupling of TGF-β to several noncanonical effector molecules, including NF-κB (Neil and Schiemann 2008), Cox-2 (Neil et al. 2008), and PGE2 (Tian and Schiemann 2010). In fact, pharmacologic or genetic inactivation of FAK effectively blocks the invasion and metastasis of breast cancer cells (Wendt and Schiemann 2009), while similar approaches against the FAK target, p130Cas, only inhibit breast cancer invasion and primary tumor dissemination without affecting the overall extent of pulmonary metastasis (Wendt et al. 2009b). Thus, these findings point to the existence of novel rate-limiting steps during initiation of the metastatic cascade in response to TGF-β.

Finally, β3 integrin also governs oncogenic TGF-β signaling by interacting physically with EGFR, a reaction that enables fibronectin to transactivate EGFR in the absence of EGF ligands (Balanis et al. 2011). Along these lines, we showed that FAK serves as a bridge that links EGFR with TβR-II in post-EMT cells, leading to their acquisition of invasive phenotypes when stimulated with EGF (Wendt et al. 2010). Finally, β3 integrin binds FGF and the co-expression of both molecules is highly predictive for lung cancer recurrence (Massabeau et al. 2009; Mori et al. 2008). Collectively, these studies establish the unique ability of integrins, particularly β3 integrin, to influence both the mechanisms of TGF-β signaling and the metastatic consequences of these events (Fig. 7.1). Moreover, these findings highlight the ability of oncogenic TGF-β signaling to cooperate with and activate a cascade of aberrant growth factor signaling events that coalesce to promote several aspects of the metastatic cascade.

7.4.2 Establishment of the Metastatic Niche

The pathophysiology of TGF-β in developing and progressing carcinomas in many respects reflects changes in its expression and bioavailability within the tumor microenvironments, as well as changes within the biomechanics of the tumor microenvironment. For instance, we recently established that TGF-β upregulates the expression of lysyl oxidase (LOX) (Taylor et al. 2011), a secreted copper amine oxidase that enzymatically crosslinks collagen to elastin (Erler et al. 2009; Erler and Giaccia 2006). The outcome of these LOX-mediated crosslinking reactions produces hydrogen peroxide as a by-product that serves as a novel second messenger for TGF-β (Taylor et al. 2011), as well as generates the rigid, palpable characteristics of carcinomas as compared to their surrounding normal tissues (Butcher et al. 2009) (Fig. 7.1, *Consequences*). Collectively, these events drive the oncogenic activities of TGF-β, including its stimulation of EMT, invasive, and metastatic phenotypes (Taylor et al. 2010, 2011) in part by amplifying integrin-mediated signaling events (Levental et al. 2009) and establishing the premetastatic niche (Erler et al. 2009). The latter activity reflects the ability of crosslinked collagens to serve as homing and docking sites during the recruitment of myeloid progenitors to future metastatic sites. Importantly, the recruitment of myeloid progenitors and formation of a premetastatic niche at peripheral sites is controlled in part by TGF-β via its regulation of stromal cell derived factor-1 [SDF-1; (Yang et al. 2008)] (Fig. 7.1, *Consequences*). Taken together, these studies suggest a model wherein aberrant TGF-β signaling upregulates primary tumor expression of LOX, while simultaneously downregulating that of SDF-1, leading to the homing of myeloid progenitors and premetastatic niche formation at peripheral sites of high SDF-1 expression, such as the lungs, brain, and bone marrow.

7.4.3 Transendothelial Cell Migration

Upon exiting the primary tumor, carcinoma cells must intravasate and extravasate the blood or lymphatic vessels before they can invade into a secondary tumor site (Nguyen et al. 2009). The processes of intravasation and extravasation are reminiscent of those employed by immune cells, whose ability to perform these tasks remain incompletely understood. Not surprisingly, even less is known mechanistically with regard to how metastatic cells emulate these events. Recently, TGF-β was shown to induce angiopoietin-like 4 (ANGPTL4) expression, which enables breast cancer cells to disrupt the vasculature and transit through the pulmonary endothelium (Padua et al. 2008) (Fig. 7.1, *Consequences*). Along these lines, the downregulated expression of E-cadherin in post-EMT prostate cancer cells facilitates their migration through endothelial cell layers (Drake et al. 2009) (Fig. 7.1, *Consequences*). Taken together, these findings highlight two consequences of TGF-β signaling that are operant in facilitating transendothelial migration of carcinoma cells during their dissemination. Future studies need to bolster our understanding of transendothelial

cell migration and its regulation by TGF-β, as well as to determine the therapeutic potential of targeting the effectors operant in this process as a novel means to prevent carcinoma cell seeding at secondary tumor sites.

7.4.4 Secondary Tumor Outgrowth

Following their dissemination and invasion into secondary tumor sites, metastatic carcinoma cells must surmount the tendency to undergo systemic dormancy, which may represent the most significant obstacle of the metastatic cascade (Brackstone et al. 2007). Indeed, estimates indicate that even small tumors (~1 cm) can release more than one million cells each day into the blood stream (Chiang and Massague 2008; Talmadge and Fidler 2010), signifying that the metastatic cascade is a highly inefficient process that is subject to two major limitations, namely the inability of disseminated cells to (1) escape the lethality of vascular shear stress and (2) circumvent metastatic dormancy. With respect to the latter phenomenon, it is important to remember that metastatic cells typically exit biomechanically rigid primary tumors and subsequently take up residence in new organs that are noticeably more compliant (Butcher et al. 2009). Indeed, using 3D-organotypic cultures that recapitulate rigid primary tumor and compliant pulmonary microenvironments, we observed that a compliant microenvironment could restore some of the tumor suppressing activities of TGF-β to highly metastatic breast cancer cells (Allington et al. 2009; Taylor et al. 2011), indicating that the extent to which TGF-β exhibits oncogenic activities is directly proportional to the degree of microenvironmental rigidity. Interestingly, metastatic cells that have completed an EMT program readily circumvent the suppressive activities of compliant microenvironments and instead utilize TGF-β as a means to promote their outgrowth (Wendt et al. 2011a, b, 2012a). Mechanistically, increased tissue compliance inhibits the coupling of TGF-β to Smad4 (Korpal et al. 2009; Wendt et al. 2011a, 2012a) (Fig. 7.1, *Mechanisms*), which prevents the expression of Pyk2 necessary to mediate escape from metastatic dormancy (Wendt et al. 2012a) (Fig. 7.1, *Consequences*). These events are also regulated by the reciprocal expression patterns of E-cadherin and β1 integrin, such that dormant cells are typically E-cadherin high, β1 integrin low, while outgrowth proficient cells are typically β1 integrin high, E-cadherin low (Barkan et al. 2008, 2010; Shibue and Weinberg 2009; Wendt et al. 2011a, 2012a). Importantly, we determined that EMT programs represent the molecular switch between metastatic dormancy and proficiency due to the downregulation of E-cadherin expression, which interacts in a heterotypic manner with the extracellular domain of β1 integrin (Whittard et al. 2002), downregulating its expression and that of Pyk2, collectively preventing the initiation of proliferative programs (Wendt et al. 2011a, b, 2012a). Likewise, the expression and activity of FAK has also been implicated in the initiation of metastatic outgrowth (Shibue and Weinberg 2009), and as such, FAK and Pyk2 represent essential TGF-β effectors that link altered integrin expression and ECM biomechanics with the micrometastatic machinery operant in driving secondary tumor formation (Fig. 7.1, *Consequences*).

7.5 Conclusions

In years past, cancer biologists have analyzed tumor development and progression primarily using a "tumoricentric" approach—i.e., focusing on studies that determine the molecular and genetic basis of how tumors are initiated, progress, and ultimately impact their surroundings. New studies emphasizing translational and tissue-based research approaches have ushered in a new arena of cancer biology aimed at understanding how clinical regimens and targeted therapies impact the aforementioned processes of tumor development and metastatic progression. A primary focus of these analyses deals with the development of chemoresistance, which typically reflects three distinct subtypes of acquired resistance: (1) development of secondary mutations within the drug target; (2) compensatory activation and/or amplification of additional growth promoting pathways; and (3) acquisition of EMT phenotypes following drug treatment (Baum et al. 2008; Sharma et al. 2010; Singh and Settleman 2010). With respect to the third mechanism, recent studies suggest that chemoresistant cells do in fact display a mesenchymal phenotype indicative of EMT programs. Thus, the principal question moving forward relates to whether chemoresistant carcinoma cells are actually derived from a low-frequency resident population that was selected out of the bulk tumor, or whether these chemoresistant variants emerged from their performance of an EMT program? Although additional experimentation is clearly warranted, the chemoresistance of metastatic carcinoma cells has been associated with the acquisition of EMT programs (Creighton et al. 2009; Farmer et al. 2009; Sharma et al. 2010; Taube et al. 2010), particularly that driven by TGF-β (Mani et al. 2008; Shipitsin et al. 2007). Likewise, TGF-β has also been observed to drive an EMT program coupled to radioinsensitivity (Barcellos-Hoff and Akhurst 2009). Collectively, these findings suggest that co-administering TGF-β antagonists with targeted chemotherapies may provide novel inroads to reverse this consequence of EMT programs, and in doing so, may sensitize late-stage carcinoma cells not only to newly developed targeted chemotherapies but also to traditional genotoxic and cytotoxic agents. Future studies need to address this hypothesis and determine the most effective drug and dosing combinations needed to eradicate these recalcitrant tumor subpopulations. Likewise, future studies need to continue elucidating the molecular mechanisms whereby TGF-β drives carcinoma development and metastatic progression, paying particular attention at determining how the flux through the TGF-β signaling system changes at distinct steps of the metastatic cascade, and at defining novel TGF-β-based biomarkers capable of predicting clinical outcomes, directing treatment regimens, and serving as novel drug targets.

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