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The complexities of TGF-β action during mammary and squamous cell carcinogenesis

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Abstract

Many advanced tumors produce excess amounts of Transforming Growth Factor-β (TGF-β), which is a potent growth inhibitor of normal epithelial cells. However, in tumors its homeostatic action on cells can be diverted along several alternative pathways. Thus, TGF-β signaling has been reported to elicit a preventative or tumor suppressive effect during the earlier stages of tumorigenesis, but later in tumor development, when carcinoma cells become refractory to TGF-β-mediated growth inhibition, response to TGF-β signaling elicits predominantly tumor progressing effects. This is not a simple switch from suppression to progression, but more like a rheostat, involving multiple complementary and antagonizing activities that slowly tip the balance from one to the other. This review will focus on the multiple activities of TGF-β in regulation of two epithelial tumor types, namely squamous cell carcinoma and breast cancer. Basic findings in current mouse models of cancer are presented, as well as a discussion of the complicating issue of outcome of altered TGFβ signaling depending on genetic variability between mouse strains. This review also discusses the role TGF-β within the tumor microenvironment particularly its ability to polarize the microenvironment towards a pro-tumorigenic milieu.

Keywords

TGF-β; tumor microenvironment; Breast cancer

1. INTRODUCTION

Transforming Growth Factor β (TGF-β) is the most potent growth inhibitor known for normal epithelial, hematopoietic and immune cells, and plays an important function in normal tissue homeostasis. However, in pathological situations its homeostatic action on epithelial cells can be diverted along several alternative routes, particularly during cancer progression when loss of tumor suppressors and mutation of oncogenes disrupt the intracellular signaling networks of the tumor cell. The current consensus view, based on a large body of literature, is that TGF-β signaling elicits a preventative or tumor suppressive effect during the earlier stages of tumorigenesis but that later in tumor development, when carcinoma cells become refractory to TGF-β-mediated growth inhibition, the TGF-β signaling pathway is diverted to elicit tumor progressing effects, acting via an array of cellular and molecular mechanisms [1]. This review will focus on the multiple activities of

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TGF-β in regulation of two epithelial tumor types, namely squamous cell carcinoma and breast cancer.

2. Mouse Models of Tumorigenesis

Much of our knowledge on TGF-β action during tumorigenesis has come from studies of mouse models. The authors' lab and those of others have focused on the use of the mouse model of chemically-induced skin carcinogenesis in order to dissect the multiple functions of TGF-β in tumorigenesis. The strength of the model is that multiple stages of carcinogenesis; initiation, promotion, malignant conversion, epithelial-mesenchymal transition, even metastasis, may be studied at the cellular, genetic and molecular levels, and tumor formation is easily followed without the use of sophisticated imaging technology [2]. Moreover, this experimental system is an excellent model for spontaneous human tumorigenesis because tumors arise *de novo* and *in situ* and are not necessarily driven by transgenic oncogenes. Consequently, the model has been finely dissected with respect to major cytogenetic, genetic, molecular and cellular changes that occur throughout the long progression towards the development of a full blown malignant carcinoma.

The mouse skin model of chemical carcinogenesis begins by tumor initiation, undertaken with a single topical application of the mutagen 9,10-dimethylbenz[*a*]anthracene (DMBA). In virtually all DMBA-induced tumors there is a specific mutation of an adenine to thymidine residue within codon 61 of the H-ras gene that results in constitutive activation of this oncogene and which drives tumor outgrowth [3]. This H-ras mutation rate is low [3] and tumor outgrowth depends on a tumor promoting step, which is provided by a twice-weekly topical treatment with a tumor promoter, such as 12-*O*-tetradecanoylphorbol-13-acetate (PMA). Over the first 6–20 weeks of tumor progression, up to 40 benign and highly differentiated papillomas may appear on the dorsal skin. Only a small percentage of these papillomas (0–10%) undergo malignant transformation to full blown Squamous Cell Carcinoma (SCC) which are more heterogeneous in character and range from low to high grade in terms of differentiation and invasive status. A fraction of these SCCs undergo an epithelial to mesenchymal transformation (EMT) to form Spindle Cell Carcinoma (SpCC) in which the spindle cells lose epithelial markers and become fibroblastoid in character [4]. Metastasis is uncommon in the DMBA/PMA model of carcinogenesis, as is the case for human SCC, nevertheless metastasis does occur at low frequency and tumor cells home to draining lymph nodes, liver and lung. Cell lines derived from such metastases have been used in syngeneic allograft studies to investigate the metastatic properties of these carcinomas [5]. Importantly, cell lines have been established from each step of the neoplastic progression from normal keratinocyte through to spindle cell carcinoma [6]. These cell lines have been used as surrogates for the cellular analysis of molecular events that occur during this *in vivo* multi-stage tumorigenesis model.

This model is an excellent tool with which to dissect the mechanisms of mammalian tumor initiation and progression, nevertheless, it is a model and not a perfect representation of human SCC development. Human SCCs, for example, generally arise following UV mutagenesis and are driven by p53 mutations, rather than by H-ras. Indeed, H-ras is not commonly mutated in human cancers, apart from bladder cancer. There are many molecular parallels between the mouse skin cancer model and that of breast cancer. The same signaling pathways are frequently mutated. Indeed, many of the genes and molecular cancer mechanisms discovered using the mouse skin cancer models have also been shown to play similar roles in human breast cancer [7]. Part of this similarity may result from the fact that the mammary gland is a derivative of epidermis, considered to be a modified and highly specialized type of apocrine (sweat) gland. Molecular parallels between the mouse skin

model and human breast cancer are particularly true with respect to the cancer biology of TGF-β.

Mouse models of breast cancer include both a chemically-induced (DMBA) model specifically within the B6D2F1 mouse strain which is particularly susceptible to mammary tumorigenesis [8] or use of transgenics in which an oncogene, normally HER2/neu or Polyoma virus middle-T (PyMT), is driven by a mammary-specific gene promoter such as Mouse Mammary Tumor Virus (MMTV), or the whey acidic protein (WAP) [9]. The HER2/neu gene is amplified in about 15–20% of human breast cancers, and the MMTV-driven HER2/neu mouse model results in the development of multifocal adenocarcinomas with lung metastasis roughly 15 weeks post pregnancy [9b]. Similarly, mammary-specific expression of PyMT leads to development of mammary adenocarcinomas with metastatic lesions in both lymph nodes and lungs [9a].

Using mouse models of breast and skin cancer, many labs have demonstrated the biphasic role of TGF-β during tumorigenesis. The signaling pathway is tumor suppressive at early stages of tumorigenesis but, as the tumor sequentially acquires more numerous mutations, including oncogene activation and loss of tumor suppressor genes, TGF-β1 loses its cell autonomous tumor suppressive activity and gradually acquires tumor promoting activity [1b, c, 1e]. There does not appear to be one “switch” from tumor suppressor to promoter, but a multitude of genetic, epigenetic and cellular events involving both the tumor cell *per se* and the tumor microenvironment that tips the balance from suppression to progression in a stepwise fashion.

3. Tumor suppressing activity of TGF-β in mouse models

In various mouse models in which TGF-β1 was over-expressed, this ligand had a tumor suppressive role at early stages of tumorigenesis [8, 10]. Expression of TGF-β1 from an MMTV gene promoter suppressed mammary tumor outgrowth induced by MMTV-TGF-α expression and inhibited mammary tumor formation in DMBA-treated B6D2F1 mice [8]. In the mouse skin model, TGF-β1 over-expression acts in a tumor suppressive manner [10–11] and *Tgfb1* genetic haplo-insufficiency results in enhanced papilloma numbers [7b]. Although these studies clearly demonstrate the tumor suppressive role of TGF-β, the mechanism(s) of tumor suppression may be multifold. In addition to the obvious cytostatic effects of TGF-β, [12] in some epithelial cell types this ligand can also induce apoptosis [13] as well as senescence [14]. Indeed, Boulanger and Smith postulated that TGF-β acted as a tumor suppressor by inducing senescence of the mammary stem cell population [15] since this ligand was able to diminish the self-renewing capability of pluripotent epithelial cells of the mammary gland [16].

In further exploration of the issue of TGF-β function in cancer, the converse approach, namely knock out of TGF-β signaling, also supports a tumor suppressing role for this signaling pathway in tumorigenesis. Expression of dominant-negative TβRII (MMTV-ΔTβRII) in mouse mammary epithelium led to an increase in mammary tumor incidence after DMBA treatment [17], and decreased the latency of MMTV-neu driven mammary tumors [18]. In the skin model, use of a keratinocyte-targeted dominant-negative TβRII or targeted genetic ablation of the endogenous *Tgfbr2, Smad2* or *Smad4* genes increased tumorigenesis and promoted tumor progression [10, 19].

Loss of TGF-β signaling, as seen by genetic knock out, has also revealed a role for TGF-β as “a guardian of the genome”. Glick and Yuspa [20], demonstrated that genetic loss of TGF-β1 or expression of ΔTβRII in keratinocytes resulted in enhanced genome instability, with increased rates of aneuploidy and chromosome breaks that preceded enhanced malignant transformation. A similar phenomenon was seen in a murine model of head and neck SCC

(HNSCC) induced by targeted genetic loss of *Smad4−/−* within epithelial cells [19a]. The *Smad4−/−* genotype correlated with reduced expression of genes of the DNA repair pathway, such as *Brca1* and *Fanc.*

Barcellos-Hoff's group has demonstrated a more proximal role for TGF-β in maintenance of genomic stability. The DNA damage response, specifically phosphorylation of ATM and downstream targets, p53, Chk2 and Rad17, is impaired by genetic loss of *Tgfb1* or pharmacological inhibition of TβRI [21]. Clearly, genetic loss of TGF-β signaling early during tumorigenesis, as several mouse models show [19c, 22] will provide not only a growth advantage and a consequent increase in benign target for subsequent mutational events, but also provide an environment conducive to accumulation of further mutations by down-regulating the DNA repair pathway. Depending on the extent of signaling knockdown and the stage during carcinogenesis when this genetic loss occurs, the ablation of TGF-β signaling might therefore stimulate further tumor progression due to activation of pro-oncogenic signaling pathways consequent to the general elevation in mutation rate and chromosomal instability.

Because of the complex actions of TGF-β *in vivo*, findings from mouse model studies sometimes appear contradictory. The variety of outcomes observed from seemingly similar experiments provides strong support for the highly context-dependent action of this growth factor. Dissecting the cellular and molecular mechanisms responsible for these disparate experimental outcomes teaches us more about the intricacies of this signaling pathway, allowing more informed choices for drug regimen and more refined drug targets.

Variable outcomes have been seen in tumorigenesis studies using transgenic TGF-β1-over-expression models. These differences might result from subtle differences in expression level of the ectopically expressed ligands. Targeting high levels of TGF-β1 to keratinocytes is lethal at birth, possibly due to the potent epithelial growth inhibitory effects of TGF-β, or due to induction of inflammation [23]. In contrast, targeting physiological levels of TGF-β1 may have no overt phenotypic effect until mice are challenged, for example, by carcinogenesis [10–11, 24]. The target cell for TGF-β over-expression will also be critical. Within the epidermis, targeting TGF-β to the stem, progenitor or differentiated keratinocyte populations would also result in distinct phenotypic outcomes [10–11, 25]. In particular, the inflammatory response within the dermis or tumor stroma might vary dependent on the source and concentration of ligand, with expression adjacent to the basement membrane more likely to have inflammatory effects in the dermis, than targeting to differentiated keratinocytes at distal locations.

Studies that have undertaken tissue specific knock down of the TGF-β signaling pathway can also give variant results dependent on the technical approaches taken. Down-regulation of TβRII using transgenic ΔTβRII driven from an MMTV gene promoter [19b] resulted in a divergent outcome in the MMTV-neu driven mammary tumorigenesis model *versus* complete deletion of the endogenous *Tgfbr2* gene in the MMTV-PyMT expressing mouse [26]. Although both MMTV-*Tgfbr2−/−* and MMTV-ΔTβRII mice both developed more mammary carcinomas following loss of TβRII, those carcinomas that expressed MMTV-ΔTβRII were less invasive and less metastatic than control carcinomas, consistent with the view of biphasic activity during tumorigenesis [18]. In contrast, MMTV-*Tgfbr2−/−* targeted gene knock out in PyMT-induced mammary carcinomas developed more aggressive and metastatic carcinomas than mice expressing the functional *Tgfbr2* gene [22a]. The difference between these two studies, apart from the oncogene utilized, might be due to the fact that the ΔTβRII protein may not be completely effective in knocking out all TGF-β signaling. Low level TGF-β signaling might allow certain cell autonomous process(es), such as DNA repair, whilst being insufficient to fire others, such as migration or survival. Tobin

et al, demonstrated that *in vitro* MDA-MB-231 cells still respond to TGF-β regardless of the expression of a dominant-negative receptor [27]. It has also been reported that over-expression of the ΔTβRII receptor can interfere with other TGF-β-related signaling pathways, such as BMPs [28], whereas complete knock out of the *Tgfbr2* gene should not have a direct effect on other pathways. Finally, when over-expressing a dominant negative receptor the potential to produce a soluble receptor by shedding might also sequester the binding of ligand to adjacent stromal and immune cells [29].

5. Mutational and epigenetic inactivation of the TGF-β signaling pathway in human breast carcinoma and SCC

In general, the most commonly mutated TGF-β pathway genes in cancer are *TGFBR2, TGFBR1, SMAD4* and *SMAD2*[30]. In breast and skin cancer, TGF-β pathway mutations are uncommon. *TGFBR2* is infrequently mutated in breast or SCC, although a 10-base pair poly-adenine repeat within its coding sequence is a common mutational target in the minor fraction of colo-rectal and gastric carcinomas that have mutations in the recombination error repair system. In breast cancer, a study of 34 matched primary and recurrent tumors, Lucke et al, demonstrated that, despite no detection of *TGFBR2* mutations in primary tumors, 12% of recurrent breast tumors contained receptor activity-attenuating point mutations suggesting that in the minority of breast tumors that mutate *TGFBR2* this is a late event [31]. Similarly, mutations in *TGFBR1* are relatively rare in breast cancer or SCC [30].

Loss of heterozygosity (LOH) on chromosome 18q that harbors *SMAD4* is seen in 30% of breast tumors, but specific *SMAD4* mutations within this region of LOH are only seen in 12% of these tumors [32]. It was recently reported that in over 80% of 17 human skin SCC specimens that were examined there was LOH at either *SMAD2* and/or *SMAD4,* which are genetically linked at 18q. However, in this study it is not clear which gene(s) were driving the rather large regions of LOH on 18q, since mutational studies were not undertaken [33]. Nevertheless, the authors reported down-regulation of Smad proteins in many skin SCC tumors. Whether this was due to mutation, epigenetic or transcriptional down-regulation of the genes remains to be elucidated. In conclusion, in the majority of breast carcinoma and SCC the TGF-β signaling pathway remains genetically intact, with notable exceptions.

6. TGF-β tumor suppression to tumor promotion: EMT and stem cells

Once the tumor cell has undergone certain genetic and/or epigenetic changes that attenuate the growth suppressive pathway of TGF-β, targeted over expression of TGF-β1 can drive malignant progression and metastasis [1e]. This has been seen in both the mouse mammary and skin tumor models [10, 25, 34], and is consistent with the fact that many advanced human and mouse tumors secrete this ligand in abundance [5b]. Even once the growth inhibitory pathway is down-modulated or attenuated, both breast carcinoma cells and SCC cells can still respond to TGF-β in other ways, such as phenotypic changes that result in enhanced migration, invasion, extravasation and cell survival [1c, 1e, 35] as well as by changes in the profile of cytokines that the tumor cell secretes [36].

In some cases, the epithelial tumor cell can undergo certain aspects of EMT in a TGF-β-stimulated Smad-independent manner, such as re-organization of the cytoskeleton and loss of epithelial tight junctional complexes [37]. However, the SMAD pathway is required for a complete phenotypic switch in the transcriptional program from an epithelial to a mesenchymal cell type. This EMT is characterized by down regulation of E-cadherin, and up regulation of snail, slug, vimentin and fibronectin, and which has been associated with enhanced invasion and metastasis [10, 38]. There are many changes that occur during EMT, and some cell types will undergo a limited number of molecular or cellular changes that

contribute to invasion and metastasis whilst others, especially oncogene-activated carcinomas of mouse skin, can undergo an overt EMT with complete loss of epithelial molecular markers to form fibroblastic SpSC. This variable extent of EMT in different systems and in response to different stimuli has resulted in some confusion in the literature as to how to define EMT [39]. Most cells require a number of oncogenic changes, upregulating the raf/ras and PI3K pathways. Numerous studies have indicated a synergy between the raf and TGF-β signaling pathways in eliciting EMT. But, although EMT is often dependent on TGF-β, other growth factors, particularly HGF acting through the Met receptor, can also elicit EMT independent of TGF-β. Most importantly, although TGF-β-mediated EMT can contribute to a more invasive and metastatic tumor cell phenotype [11–12], EMT is not an essential component for invasion or metastasis because these two processes can each occur without EMT [11], often being supported by co-migratory bone marrow-derived cells of the tumor stroma [40], (see later). Just as the old English idiom states: “there are many ways to skin a cat”, so there are many molecular routes to achieve tumor invasion and metastasis, and the tumor cell will try them all.

Some believe that EMT is irrelevant to cancer progression, partly as many metastases tend to be epithelial rather than mesenchymal. However, EMT is known to be plastic and reversible until or unless the mesenchymal phenotype becomes fixed by epigenetic changes or further gene mutation. EMT might indeed occur transiently to promote cancer cell intravasation into lymphatics and blood vessels. The phenotype of the tumor at the secondary metastatic site is most likely determined by the stromal components at that site, rather than innate properties of the tumor cell, as has been seen in a metastatic skin carcinoma cell line, E4. This carcinoma line reversibly transforms from fully epithelial to fully mesenchymal in culture dependent on the addition of TGF-β. When injected subcutaneously into a mouse, it grows as a TGF-β-dependent spindle tumor, but if injected intra-peritoneally it grows with a squamous phenotype on the mesothelial lining of the abdomen [5b]. This notion that the carcinoma phenotype can be dictated by underlying support tissue aligns with classical evidence from tissue recombination studies undertaken in chick and mouse embryos, and by similar tissue recombination studies with tumor material, in which normal fibroblasts can “normalize” carcinoma cells, whereas carcinoma-associated fibroblasts (CAFs) promoted tumorigenesis [41]. Regardless of its role in migration and invasion, EMT might be even more important in supporting a transition towards a more “stem cell-like phenotype”, with accompanying molecular changes towards cell survival and self renewal – which is equally, if not even more important for metastasis (see later).

Mani et al, demonstrated that induction of EMT by either TGF-β1, Snail or Twist in immortalized human mammary epithelial cells (HMECs) promoted the expression of cell surface markers associated with cancer stem cells [42]. Moreover, TGF-β is able to further polarize cancer stems cells (CSCs) towards a more multi-potential phenotype. Mesenchymal Stem Cells (MSCs) were first reported in the hematopoietic system, but have more recently been described in many solid tumors, such as breast, colon and brain [42]. Battula et al, demonstrated that human mammary epithelial cells (HMECs) stably expression TGF-β1, Snail or Twist exhibited a cell surface marker profile very similar to that of MSCs. Along with stem cell surface markers TGF-β-induced HMECs showed strong (70%) similarity in gene expressing profile to bone marrow derived MSCs. Indeed, these cells were more similar to MSCs than to other mammary tumor cell types [43].

Battula et al, also presented proof that EMT-induced HMECs were multipotent. HMECs that had undergone EMT could differentiate along the three major mesodermal lineages: osteoblasts, adipocytes and chondrocytes [43]. Additionally, in line with the observation that MSCs have a homing capacity towards wounds and tumors [44], the EMT-induced HMECs were able to invade towards PDGF-β and towards breast cancer cells (MDA-MB-231 cells)

in vitro at similar rates to that of bone marrow-derived MSCs and they were able to home to wounded tissue *in vivo* in a similar fashion to MSCs [43]. This TGF-β induced stem-like phenotype supports tumor progression and metastasis.

7. TGF-β tumor suppression to tumor promotion: Molecular switch or rheostat?

The question of which molecular players "switch" the tumor cell response to TGF-β from growth inhibition and apoptosis towards tumor migration, invasion and metastasis, has been the research subject of numerous labs over many years, each searching for the magic bullet that will target TGF-β-stimulated tumor progression whilst keeping the tumor suppression arm intact – or indeed even reactivating tumor suppression. There have been many papers demonstrating different molecular mechanisms for this "switch". In reality, there are many sequential molecular changes in several pathways that can lead down the road from tumor suppression to progression effects, including Ras, Raf, Rock, Akt, Ski-SnoN, CEBP, Six1 to name a few (see [1b, 45] for review).

C/EBPβ, a central transcription factor that binds within the Smad transcription factor complex, elicits various TGF-β cytostatic responses, including transcriptional induction of p21 and p15 CDKs and repression of c-Myc. The C/EBPβ gene drives expression of three distinct isoforms by differential gene promoter usage. LAP1 and LAP2 stimulate transcriptional activity of this complex whereas LIP is an inhibitory component. In many human breast tumors there is an imbalance in LAP/LIP ratio leading to excessive production of LIP, and a consequent down-regulation in the cytostatic transcriptional program elicited by TGF-β [12b]. Other transcription factors that have been found to stimulate the tumor progressing arm of TGF-β signaling at the expense of the growth inhibitory pathway include the homeo-domain transcription factor, Six1, which is elevated in expression in many tumor types in addition to that of breast [46]. Breast cancer patients whose tumors over-expressed Six1 had a shortened time to relapse and metastasis and an overall decrease in survival[46].

A recent study indicates that DAB2 may also be a pivotal player in the balance between tumor suppressing and promoting activities of TGF-β in human SCC. This multifunctional adaptor protein, that has been shown to play a role in clathrin-coated endocytosis and down-regulation of many oncogenic signaling pathways, is often found to be epigenetically inactivated during HNSCC tumor progression. Its expression within SCCs was inversely correlated with that of Smad2 and with poor patient prognosis [47]. Hannigan et al, showed that down-regulation of DAB2 *in vitro* was capable of blocking TGF-β–mediated inhibition of cell proliferation, but switched the response of SCC to TGF-β towards promotion of cell motility, anchorage-independent growth, and tumor growth *in vivo*[47]. Thus, DAB2 is required for growth inhibitory responses to TGF-β, and its down-regulation, which occurs epigenetically, is permissive for execution of the tumor progressing response to TGF-β.

Many other signaling pathways have been shown to interface with the TGF-β pathway to tip the balance between growth inhibition and tumor progression including Wnt, Notch, Rock and PI3K. At any one time there are both tumor suppressive and progressing activities within the same cell [6, 48], with accumulating mutations pushing this equilibrium towards malignant progression and ultimately to complete loss of the cytostatic response. In the skin model we have shown that sequential elevation in both ras and TGF-β signaling are required for the progression from papilloma to SCC to SpCC [5a]. Thus the transformation from normal epithelial cell to metastatic tumor might be considered a rheostat rather than a switch.

8. Molecular interplay between Smads within the cancer cell: TGF-β-mediated tumor suppression *versus* progression

Since the discovery of receptor-associated Smads, Smad2 and Smad3, as immediate downstream targets of the TGF-β receptor complex, there has been considerably focus on the relative role of these transcription co-factors and their partner, the ubiquitous nuclear shuttling Smad4, in the balance between tumor suppression and progression. Several groups have shown that Smads are required for both growth inhibition and EMT. Phosphorylation of the Smad2/3 linker region by the Ras Raf Erk pathway has been variously proposed to attenuate the growth inhibitory responses to Smad [45b], or to stimulate the tumor progressing arm [5a, 49].

There has also been debate as to the relative importance of Smad2 versus Smad3 in EMT and growth inhibition. Once again, experimental findings have been contradictory, much of which might depend on experimental approach. Various labs have used dominant negative and dominant active mutant Smads [5a, 50], mutant TβRI receptors that can signal to non-Smad but not to Smad pathways [51], and stable knock down and genetic knock out of Smads [19a, 33, 52] to address these issues. Dominant negatives may not be fully effective, and may exert non-specific effects by binding and inactivating other Smad partners. Genetic knock out experiments are very clean and give definitive results, but do not reflect the situation in the cancer cell which rarely has homozygous inactivation of Smads.

Using targeted genetic knock out of the *Smad2* gene in basal keratinocytes, Xiao-Jing Wang's lab, elegantly showed that Smad2 inhibits rather than mediates EMT in the mouse skin carcinogenesis model *in vivo*[33]. This outcome was attributed to its normal role in inhibiting Smad3/4 binding to, and transcriptional activation of, the *Snail* gene promoter. Thus an imbalance between Smad2 and Smad3 in favor of the latter would drive the TGF-β response from growth inhibition towards EMT. A similar finding was made in breast MDA-MB-231 cells, whereby stable knock down (KD) of Smad2 enhanced Smad3 activity, resulting in the increased expression of VEGF and other metastasis genes in the Smad2 KD cells, culminating in a more aggressive tumor phenotype as well as stimulation of metastasis when injected into nude mice [52].

In contrast to the above findings, several years ago our own lab demonstrated that a constitutively activated form of Smad2 could drive tumor metastasis of SpCCs in tail vein injection mouse [5a]. Additionally, more recent reports implicate PSmad2 signaling in promoting EMT or a more aggressive cancer in both breast carcinoma and SCC. Papageorgis et al, [53] showed that inhibiting Smad signaling either by over-expressing the inhibitory Smad, Smad7 or by knocking down Smad2, resulted in a mesenchymal to epithelial transformation (MET) of a tumorigenic MCF10A breast cell derivative (MIII). The MET effect was not attributed to transcriptional regulation by Smads, but due to widespread demethylation and consequent de-repression of genes that are characteristically silenced by Dnmt1-dependent methylation during tumor progression and EMT. Thus Smad2 was implicated in epigenetic silencing of genes [53].

Several lines of evidence implicate active Smad2 signaling in tumor progression of human SCC. First, Smad2 is clearly phosphorylated and presumably active in most human cutaneous SCC [48, 54]. Organ transplant recipients that have an extremely elevated risk of developing multiple highly invasive SCC following long term treatment with immunosuppressive drugs, have higher P-Smad2 levels than those observed in spontaneous SCC [54]. Intriguingly, in a panel of 18 spontaneous HNSCC tumors, it was found that P-Smad2 localization was mutually exclusive with DAB2 [47], that DAB2 was epigenetically silenced in HNSCC, and that DAB2 methylation correlated significantly with poorer

prognosis. These data, together with the observations of Papageorgis et al, raise the possibility that Smad2 is functionally implicated in epigenetic DAB2 silencing in HNSCC, which correlates with poor patient outcome [53].

9. The problem of a tumor microenvironment bathed in excess TGF-β

Paget's "seed and soil" hypothesis of metastasis suggests a mutual interaction between the cancer cell and the specific host microenvironment that may encourage tumor progression [55]. The tumor microenvironment describes all non-epithelial components of the tumor and immediate surroundings, including stromal and immune cells, extracellular matrix (ECM) and blood vessels. A major factor that can help explain the conundrum of tumor promoting versus inhibiting activities of TGF-β is the action of TGF-β on cells of the tumor microenvironment. It is clear from early studies [56] and a slew of papers over the last five years, that TGF-β is a major cytokine involved in modulating tumor stroma, recruiting immune cells to the tumor and subsequently polarizing these various cell types towards a pro-metastatic tumor-supportive microenvironment.

TGF-β and the tumor stroma -Instructive signals from host to tumor

Integrin activation on both the tumor cells and supporting stomal cells polarizes the tumor microenvironment to support tumor progression. Integrins are a family of transmembrane receptors which bind the cytoskeleton and ECM, thereby acting as a link between the interior of the cell and its microenvironment. Integrins regulate cytoskeleton organization and consequently influence proliferation, adhesion and migration. TGF-β signaling influences tumor cell integrin signaling through transcriptional regulation of genes encoding many integrins and their binding partners, including the up-regulation of fibronectin and laminins [57]. Integrins reciprocally induce expression by TGF-β creating a cooperative signaling and a feed-forward loop [57]. Integrins can activate latent TGF-β via recruitment of MMP-14 which activates TGF-β through proteolytic cleavage [57]. Changes in the integrin profiles of tumor cells promote tumor progression by allowing the tumor cell to escape anoikis and facilitating tumor cells to home to specific tissues [57]. Integrin α3-deficient keratinocytes have elevated expression of the inhibitory Smad7, suggesting that the expression of α3β1 integrins may down-regulate Smad7, thereby enhancing TGF-β signaling [58]. TGF-β signaling has also been shown to cause the *de novo* expression of integrins not normally expressed in epithelial cells, enhancing the migratory invasive properties of the tumor cells [7b]. TGF-β induced changes in the tumor cell integrin profile and encourages tumor cells to interact with the stroma to promote tumor progression. Metastatic breast tumor cells show differential integrin hetero-dimerization and activation compared to non-metastatic tumor cells possibly playing a role in tumor cell homing to the bone [59]. Furthermore, it has been demonstrated that in breast cancer αvβ3 binding of the stromal protein, ostepontin, is necessary for tumor cell colonization of bone. This binding promotes adherence to the bone matrix [60].

Integrin expression on the tumor cell is only half the story. Whilst TGF-β stimulated integrins on the tumor cell allow the tumor to interact with its associated stroma, the altered integrin profile of tumor stromal cells supports tumor cell survival and invasion. Stromal cell integrins support seeding of tumor cells at metastatic sites, as well as tumor growth at these sites. Stromal expression of vascular cell adhesion molecule-1 (VCAM-1), supports bone remodeling and tumor growth. Myeloma cells expressing integrin α4β1 have been shown to bind VCAM-1-expressing bone marrow stroma. This interaction contributes to bone tumor growth and osteoclast (OC) recruitment. The over-expression of integrin αvβ3 in metastatic breast tumor cells also leads to recruitment of OCs resulting in increased osteolysis [61], which in turn releases more TGF-β. Bone metastasis, but not metastasis to other sites, was inhibited by antibodies against VCAM-1 [60a]. Stromal integrin profiles may

also support the sequestering and maintenance of a “dormant” tumor cell population within the bone marrow. Korah et al, demonstrated that the interaction of breast tumor cell integrin α5β1 with stromal fibronectin contributes to the survival of growth–arrested tumor cells. Stimulation with the FGF2 growth factor lead to breast cancer cell growth arrest and up-regulation of integrin α5β1 resulting in cell death. However, those tumor cells which bound fibronectin via α5β1 initiated a cell survival program [62].

Lastly, neovascularization is essential for tumor cell growth and metastasis. Many integrin heterodimers have been implicated in tumor-associated angiogenesis. The αvβ3 integrin is expressed at high levels on tumor-associated vasculature. Anti-β3 neutralizing antibodies can inhibit tumor-associated angiogenesis [63]. TGF-β induction of *de novo* expression of several integrin not normally expressed in epithelial cell (α5β1, αvβ3, αvβ5 and αvβ6) [57]permits the tumor cells to use stroma integrin signaling to promote tumorigenesis.

Tumor immunity -The hijacking of acquired and innate immune responses by TGF-β

TGF-β can suppress or modulate activation of both innate and adaptive immune cells. Through dampening the adaptive immune response, TGF-β is able to suppress tumor immune-surveillance. Broadly speaking, many of the effects of TGF-β on both adaptive and innate immune cells of the tumor microenvironment result from the ability of this cytokine to polarize these cells towards a determination state that reflects a relatively immature state. Put another way, TGF-β attenuates the full differentiation program of these cell types. This has a two-fold effect on tumor progression, both blunting the normal anti-tumor functions of type I differentiated T-cells, macrophages and neutrophils, and stimulating the release of pro-tumorigenic cytokines (including Il-11 and yet more TGF-β), from “immature” type 2 undifferentiated immune cells [64].

Adaptive immunity

TGF-β signaling within the tumor microenvironment suppresses the fully differentiated anti-tumor “cytotoxic” arm of the adaptive immune system in several manners, acting via both CD4+ and CD8+ T cells. Transgenic mice expressing a dominant negative TβRII under the regulation of a T-cell specific gene promoter (CD4-ΔTβRII), exhibited spontaneous T-cell differentiation, consequently developing an autoimmune-like disease [65]. More importantly, this T cell-specific attenuation of TGF-β signaling indirectly enhanced the differentiation of $CD8^+$ cytotoxic T lymphocytes (CTLs), such that the CD4-ΔTβRII transgenic mice, when challenged with a tumor cell line, were more effective in tumor eradication due to an increased tumor-specific CTL response compared to their wild type littermates [66]. The inhibition of TGF-β signaling by monoclonal antibodies also led to increased cytotoxic activity of CTLs [67].

In addition to cytotoxic $CD8^+$ T cell regulation, TGF-β signaling also variously affects the differentiation of $CD4^+$ effector T cells, additional central players in adaptive immunity. TGF-β has effects on all subsets of CD4+ effector T cells by influencing the expression of master transcriptional regulators, such as FoxP and Tbet. There are four major $CD4^+$ T cell lineages Th1, Th2, Treg and Th17. $CD4^+$ T helper cell types, Th1 and Th2, have opposing roles in tumorigenesis, Th1 being the helper cell for cytotoxic T cells and the Th2 promoting a non-cytotoxic inflammatory cascade and associated tumor progression associated with the release of cytokines from these cells. Th2 cells are also under the control of the early T cell development transcription factor GATA-3, whereas Th1 cells are not, indicating that Th2 cells have a more immature differentiation state than Th1 cells [68]. TGF-β signaling within the tumor microenvironment is able to polarize $CD4^+$ T helper cells toward the Th2 tumor promoting phenotype by inhibiting the expression of T-bet which attenuates Th1 cell differentiation [64].

Interestingly the effect of TGF-β signaling on T regulatory cells (Tregs) mirrors the dual role of TGF-β signaling in tumor progression. TGF-β signaling can contribute to the differentiation of T regulatory cells either into classical activated FOXP3-expressing cells (Tregs) or, in combination with Il-6, along an alternative differentiation pathway of IL-17-expressing Tregs (Th17). Classical Tregs are considered tumor suppressing and large numbers of Treg cells in cancer patients is inversely correlated with survival [69]. Conversely, Th17 cells are thought to have a tumor promoting role.

Su et al, reported that the number of Th17 cells were elevated in tumor-infiltrating lymphocytes (TILs) from melanoma and breast cancers [70]. However, other studies have shown a decrease of Th17 cells in ovarian cancer and non-Hodgkin's lymphomas [71]. This dichotomy may be linked to the TGF-β-altered fate of CD4+ precursors, a tumor suppressing Treg versus a tumor-promoting of Th17, dependent on the relative levels of TGF-β and Il-6 in the tumor vicinity. The transcription factors RORγt and STAT3 are known to be critical for the development of Th17 cells, and Smad2 is able to bind and synergize with RORγt to generate Th17 cells [72]. One mechanism through which Th17 cells may promote tumorgenesis and/or progression is by promoting angiogenesis. IL-17 is a well-established angiogenic cytokine that stimulates migration and cord formation of endothelial cells *in vitro* and of blood vessel formation *in vivo*[73].

Innate Immunity

TGF-β signaling within the tumor microenvironment blocks monocytes from full differentiation into an anti-tumor (M1) macrophage phenotype. Within the tumor, TGF-β thus polarizes these cells towards a more immature M2 cell phenotype that secrets inflammatory and angiogenic cytokines and growth factors. M2 macrophages also secrete more TGF-β *per se* into the tumor microenvironment, creating a positive feedback loop that leads to infiltration of yet more M2 macrophages, also known as tumor associated macrophages (TAMs), into the tumor microenvironment [74]. In some breast tumors, TAMs can comprise up to 80% of the tumor mass, making this cellular population the largest component of the tumor microenvironment and exceedingly important in driving tumor progression [75]. Indeed, clinical studies have demonstrated a correlation between high TAM expression in tumors and poor prognosis [76].

TAMs tend to accumulate in areas of low oxygen tension and they support angiogenesis through increased expression of proteins such as hypoxia-inducing factor 1 α (HIF-1α) and cyclooxgenase-2 (COX-2) [77]. In the absence of blood supply tumors are only able to develop to the maximal volume of 1 to 2mm^3. TGF-β is chemotactic towards macrophages and elevated expression of this cytokine may contribute indirectly to angiogenesis through recruitment and polarization of TAMs. TGF-β has also been shown to polarize tumor associated neutrophils (TANs) away from a differentiated tumor suppressing (N1) phenotype, towards a tumor promoting immature (N2) cell type. The gene expression profiles of N2 neutrophils are characterized by gene signatures associated with the promotion of angiogenesis and metastasis [78]. Furthermore, TGF-β signaling blockade re-polarized TANs from an immature N2 phenotype towards the more cytotoxic, tumor suppressing N1 phenotype and resulted in significantly inhibited tumor growth in mice. In this model, activation of CD8+ T cells by mature N1 neutrophils was reported to be the major mechanism for mediating this anti-tumor effect [78].

Lastly myeloid-derived suppressor cells (MDSCs) may be the definitive example of how TGF-β signaling blockade of immune cell differentiation promotes tumor progression. MDSCs also known as myeloid immune suppressor cells (MISCs) are immature myeloid cells that are Gr-1$^+$CD11b$^+$. MDSCs in healthy mice are present in low numbers in the bone marrow and spleen however; tumor-bearing mice show increased numbers of MDSCs in the

blood, spleen and lymph nodes [79]. Yang et al, demonstrated that the deletion or alteration of TGF-β signaling in the mammary epithelial results in the recruitment of MDSCs [40]. MDSCs within the tumor microenvironment have been shown to express high levels of TGF-β and MMP-9 [40], possibly creating a positive feedback loop upon where MDSCs are recruited to the tumor microenvironment and once there remain in an immature state.

10. TGF-β and metastasis: TGF-β is a good fertilizer for "tumor soil"

The bone is a common site of dissemination for breast cancer and a microenvironment rich in TGF-β reserves. Upon reaching the bone, metastatic cells release pro-metastatic cytokines which activate osteoclast differentiation; this activation leads to osteoclastic degradation of bone matrix and release of TGF-β [35]. Additionally, histological examination of human bone metastasis biopsies showed that 75% of the biopsies were positive for nuclear phosphorylated-Smad2 (PSmad2); indicating active TGF-β signaling in human breast cancer samples [80]. Massague's group identified a set of genes that mediate osteolytic bone metastasis by the breast cancer cell line MDA-MB-231. Included in this signature was the TGF-β inducible *IL-11* gene. The *IL-11* gene is suggested to be a mediator of osteolysis in breast cancer bone metastasis [81]. Moreover, an *in vivo* metastasis assay employing MDA-MB-231 cells transduced with a TGF-β reporter indicated active TGF-β signaling within the bone metastasis [80]. The genetic manipulation of the TGF-β pathway through knock down of Smad4, ectopic expression of inhibitor Smad7 or expression of dominant-negative TGFBR2 dramatically decreased bone metastasis in both breast cancer and melanoma models [80, 82].

Evidence supporting the role of TGF-β signaling in priming bone for metastasis is the TGF-β induced homing of mesenchymal stem cells (MSCs) and pro-tumor immune cells. MSCs have been shown to be localized around vascular areas of bone marrow [83]. Regions the bone marrow stromal compartment close to the endosteum are also colonized by quiescent breast cancer cells (BCCs) [84]. However, the mechanism(s) by which these BCCs evade the host immune response is still unclear. It has been postulated that MSCs through TGF-β induced T cell recruitment may confer immune protection to BCCs [83]. Thus, TGF-β expression at the site of metastasis primes the microenvironment allowing tumor homing as well as escape from immunosurveillance of the newly arrived tumor cells.

11. Personalized responses to TGF-β action

It is no wonder that the outcome of TGF-β signaling in any particular tumor type or at any stage is so highly context-dependent, since tumor cell responses are determined by co-activation/inactivation of other signaling pathways, and because of the vast array of TGF-β-sensitive cell types in the tumor microenvironment that can each respond in context-dependent manners. However, superimposed on this complexity is the fact that there are clearly innate genetic differences between individuals that strongly influence the TGF-β outcome. Once again this is exemplified by studies in mouse models. In 2006, we demonstrated that loss of a single allele of the *Tgfb1* gene in NIH mice resulted in enhanced susceptibility to tumorigenesis. *Tgfb1+/−* mice developed significantly more papillomas than their wild type littermates in response to the skin chemical carcinogenesis induction protocol – clearly demonstrating relief of the tumor suppressive arm of TGF-β action [7a]. Yet recent reports, studying the exact same *Tgfb1+/−* knock out line but bred into a BalbC genetic background, showed that *Tgfb1+/−* mice developed fewer papillomas than their wild type littermates using the same tumor induction protocol [48], suggesting that in this case, *Tgfb1* hemizygosity resulted in loss of the tumor promoting arm of TGF-β signaling. Clearly, the genetic background of the mice appears to dictate these diametrically opposed outcomes of carcinogenesis.

Similarly, Li et al demonstrated on the 129Sv/EV genetic background, that Smad3 was required for efficient tumor formation in the mouse skin chemical carcinogenesis model [85]. Our own studies on NIH/Ola mice (unpublished), showed no difference in papilloma or carcinoma development between *Smad3−/−, Smad3+/−* and wild type littermates. Clearly, there are strain specific differences in response to loss of TGF-β signaling components *in vivo*. Indeed, we have shown that different mouse strains exhibit different levels of basal phospho-Smad2, a marker of active TGF-β signaling [7b]. Further, genome-wide genetic linkage analysis for tumor susceptibility genes in several large mouse F1 backcrosses between different mouse strains identified a genomic locus, *Skts16/Tgfbm3,* that can determine the magnitude of TGF-β1-dependent tumor susceptibility. This locus was independently found as a *Tgfb1*-interacting locus in a genome-wide mouse embryo screen, and encodes a cluster of genes some known to interact with the TGF-β signaling pathway and to show variant expression and/or bio-activity between the different mouse strains [7b].

Certainly, in humans, several components of the TGF-β signaling pathway are known to be genetically and functionally polymorphic, including *TGFB1, TGFB2, TGFB3, TGFBR1, TGFBR2*[86] and these have been genetic associated with altered risk of cancer. Intriguingly, a hypermorphic variant of *TGFB1* has been genetically associated with protection from low grade breast cancer, but with risk of high grade breast and prostate cancer, exemplifying the biphasic action of TGF-β in cancer in humans [7c].

The issue of natural genetic variation between individuals altering the biological outcome of TGF-β signaling is a further challenge to be tackled in this field, especially if considering the use of TGF-β inhibitors as drugs. Not only might the tumor response to TGF-β inhibitors vary between individuals due to the mutation spectrum of the carcinoma itself which will dictate innate responsiveness to excess TGF-β but the tumor response will also depend on host responses to TGF-β inhibition, such as angiogenesis and immunity. Similarly, adverse effects to treatment by this biological class of drugs, either immune or cardiovascular, might also vary considerably dependent on germ-line genetic variation.

Understanding the complexities of TGF-β action in carcinogenesis will lead to better diagnostic tools as well as therapeutic strategies. The goal of pharmacological inhibition of TGF-β signaling is to target the tumor promoting properties in both the cell and the tumor microenvironment and concurrently avoid inhibition of TGF-β tumor suppression properties. There have been several preclinical and clinical reports on the use of both large and small molecule inhibitors of the TGF-β pathway for various oncology applications [87]. However, clearer insight on how TGF-β signaling by acting as a rheostat to polarize the tumor and tumor microenvironment will aid in the next generation of drug design.

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