

# TGF- $\beta$ Signaling in Breast Cancer Cell Invasion and Bone Metastasis

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**Abstract** The contribution of transforming growth factor  $\beta$  (TGF- $\beta$ ) signaling to breast cancer has been studied for more than two decades. In an early phase TGF- $\beta$  may act as a tumour suppressor, while later, when cells have become resistant to its anti-mitogenic effects, the role of TGF- $\beta$  switches towards malignant conversion and progression. TGF- $\beta$  stimulates cell invasion and modifies the microenvironment to the advantage of cancer cells. Studies have shown that TGF- $\beta$  promotes bone and lung metastasis via different mechanisms. The therapeutic strategies to target the TGF- $\beta$  pathway in breast cancer are becoming increasingly clear. This review will focus on the role TGF- $\beta$  in breast cancer invasion and metastasis.

**Keywords** EMT · Invasion · Metastasis · Microenvironment · Stroma · TGF- $\beta$

## Abbreviations

ANGPTL4 Angiopoietin-like 4  
AnxA1 Annexin A1  
BLBC Basal-like breast cancers

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BMP Bone morphogenetic protein  
CAF Carcinoma-associated fibroblasts  
CAR Cocksackie- and adenovirus receptor  
CDK Cyclin-dependent kinase  
Cre Cre recombinase  
CTGF Connective tissue growth factor  
CXCR4 C-X-C chemokine receptor type 4  
ECM Extracellular matrix  
EGF Epidermal growth factor  
EMT Epithelial-mesenchymal transition  
FARP FERM RhoGEF (ARHGEF) and pleckstrin domain protein  
FGF Fibroblast growth factor  
GR-1 Granulocyte differentiation antigen 1  
HDM2 Human double minute 2  
HMEC Human mammary epithelial cells  
HIF-1 $\alpha$  Hypoxia-inducible factor-1 $\alpha$   
IL-11 Interleukin 11  
MAP Mitogen activated protein  
MDM2 Murine double minute 2  
MET Mesenchymal-epithelial transition  
MMP Matrix metalloproteinase  
MMTV Mouse mammary tumour virus  
M-RIP Myosin phosphatase Rho interacting protein  
Nedd9 Neural precursor cell expressed developmentally down-regulated 9  
PTHrP Parathyroid hormone-related protein  
RANKL Receptor activator of nuclear factor Kappa-B ligand  
SDF-1 Stromal-derived factor-1  
shRNA Short hairpin RNA  
siRNA Small interfering RNA  
TGF- $\beta$  Transforming growth factor- $\beta$   
T $\beta$ RI TGF- $\beta$  type I receptor  
T $\beta$ RII TGF- $\beta$  type II receptor  
uPAR Urokinase receptor

|      |                                    |
|------|------------------------------------|
| VEGF | Vascular endothelial growth factor |
| WAP  | Whey acidic protein                |

## Introduction

Breast cancer is the most common cancer in women and a major cause of morbidity or mortality. Worldwide, approximately 350,000 women die from breast cancer each year [1]. Many factors are currently studied to understand the causes of breast cancer. These include lifestyle [reviewed in 2], environmental [for example 3], genetic and biological factors. One of the genetic and biological factors in breast cancer biology that is widely studied is the secreted cell to cell signaling molecule, transforming growth factor- $\beta$  (TGF- $\beta$ ).

TGF- $\beta$  is part of a large family of polypeptide growth factors that includes activins, inhibins, and bone morphogenetic proteins (BMPs). Three human TGF- $\beta$  isoforms, which are structurally and functionally closely related, have been described. All three isoforms are secreted as latent precursor molecules. Proteolytic cleavage, interaction with integrins, or pH changes in the local environment activate latent TGF- $\beta$  [4]. Classic TGF- $\beta$  signaling involves the binding of TGF- $\beta$  to TGF- $\beta$  type II receptors (T $\beta$ RIIs), recruitment of type I receptors (T $\beta$ RI), transphosphorylation by T $\beta$ RII kinase, and the subsequent phosphorylation of receptor regulated (R-)Smad2 and Smad3. Bone morphogenetic proteins (BMPs) signal specific BMP type I and type II receptors, and stimulate the activation of R-Smad1, 5 and 8. Phosphorylated Smads form heteromeric complexes with common mediator (co-)Smad4 that then accumulate into the nucleus. The Smad complexes interact with transcription factors, co-activators, and co-repressors where they participate in the regulation of target gene expression [reviewed in 5]. Besides the canonical TGF- $\beta$ /Smad pathway, TGF- $\beta$  can directly activate non-Smad signaling pathways [6, 7], including the mitogen activated protein (MAP) kinases. Erk was found to be phosphorylated via a direct T $\beta$ RI-induced phosphorylation of Shc [8]. In addition, small GTPases such as Ras, Rho, Rac and CDC42, have been implicated in non-Smad TGF- $\beta$  signaling [9–11].

TGF- $\beta$  is a very potent growth inhibitor of primary human mammary epithelial cells. Loss of TGF- $\beta$  growth inhibition and increased expression of TGF- $\beta$  have been associated with malignant conversion and progression in breast cancer. Specific mutation of TGF- $\beta$  signaling components occurs only occasionally in breast cancers. Rather, TGF- $\beta$  growth response is abrogated by changes in the profile of other active signaling networks or the relative availability of transcriptional co-repressors or co-activators

that bind to and modulate the canonical Smad pathway [12]. Estrogens also appear to negatively regulate TGF- $\beta$  signaling in breast cancer [13] and there is evidence that many pathway components may be epigenetically regulated during critical transitions in malignant progression [14]. Moreover, a large number of reports indicate that TGF- $\beta$  can turn into a promoter of progression at later tumour stages [15]. In support of this notion is clinical evidence that indicates a correlation between expression of the TGF- $\beta$  ligands and poor patient outcome [16–19].

TGF- $\beta$  supports tumour progression by stimulating the transdifferentiation of epithelial cancer cells into migratory mesenchymal cells [20, 21], by promoting cell invasion, and dissemination to distant sites [22], enhance angiogenesis [23] and mediating immune evasion of tumour cells [24]. Thus, besides direct effects on tumour cells, TGF- $\beta$  influences the tumour microenvironment to stimulate local movement and survival of neoplastic cells. The metastasis of breast cancer cells to remote tissues is not a random process. For example, decreased BMP7 in primary breast cancer is significantly associated with the formation of bone metastasis. It is theorised that BMP7 supports MET and inhibits TGF- $\beta$ -induced metastasis to the bone [25]. The mechanisms that underlie the choice of residence at distant organs, such as bone and lung, are emerging [26].

The mechanisms of TGF- $\beta$ -induced growth arrest via the induction of cyclin-dependent kinase (CDK) inhibitors and repression of c-Myc protooncogene, and how cells become refractory to its cytostatic effects by mutations or epigenetic mechanisms are well understood [reviewed in 22] and will not be discussed here. The signaling cascades involved in its pro-oncogenic roles are emerging from recent studies and are the focus of this review. Here we will discuss recent advances into the molecular mechanisms that control EMT and tumour invasion of breast cancer, the interaction of tumour cells with neighbouring stromal cells and metastasis of breast cancer cells to bone and lung and specifically focus on the role of TGF- $\beta$  in these processes.

## Epithelial Plasticity

EMT is a highly coordinated process that involves a complex series of events [27]. EMT starts with apico-basal polarity loss and the dissolution of tight junctions, which permit the intermingling of apical and basolateral membrane components [28]. In addition, other cell-cell junctions disassemble and degrade the underlying basement membrane. The cell surface protein that mediates epithelial connection to neighbouring cells and the basement membrane, E-cadherin, is replaced by N-cadherin. The transient adhesive properties of N-cadherin prime the cell for the mesenchymal phenotype. Cytoskeletal elements are then

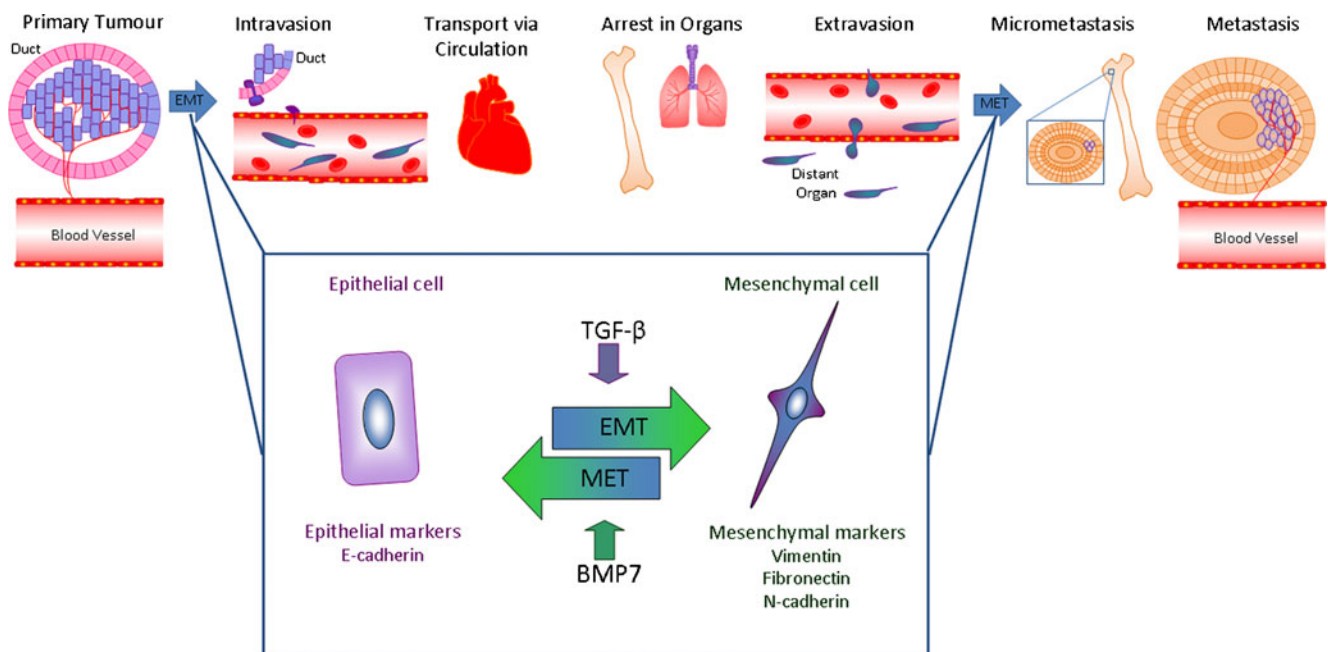
reorganized and stress fibers replace the peripheral actin cytoskeleton, and vimentin replaces cytokeratin intermediate filaments. These changes cause the cell to change from a cuboidal to a spindle shape. It is then that the cell acquires the ability to move and invade into the extracellular matrix (ECM) [29, 30].

TGF- $\beta$  treatment has been shown to cause delocalization and downregulation of cell-cell contact proteins (such as ZO-1, E-cadherin,  $\beta$ -catenin), cytoskeleton reorganization (stress fiber assembly, myosin light chain phosphorylation), and robust  $\alpha$ -smooth muscle actin synthesis [31]. Frequently, Namru Murine Mammary Gland (NMuMG) breast cancer cells have been used for studies involving TGF- $\beta$ -induced EMT [32, 33]. More recently, TGF- $\beta$  has been shown to be involved with the early stage changes of EMT in Human Mammary Epithelial Cells (HMEC). The epithelial cell polarity marker ZO-1 was repressed with the addition of TGF- $\beta$ . Furthermore, a rapid increase in expression of mesenchymal markers Vimentin and Fibronectin is seen after TGF- $\beta$  treatment of HMECs [34].

While initially the occurrence of EMT in cancer was received with skepticism, the concept of epithelial cancer cell plasticity contributing to cancer progression is now gaining acceptance [27]. Evidence for the role of EMT in cancer is complicated by the fact that at the distant site the

metastatic cells most likely need to undergo a reversion or mesenchymal to epithelial transition (MET), permitting colonization of the distant site [29]. EMT is a transient and reversible process in the course of cancer progression. This was recently shown by Jo et al., who could reverse EMT by targeting the urokinase receptor (uPAR) [35]. The first direct evidence of EMT in the local invasion of tumour cells was obtained by cell fate mapping of epithelial tumour cells in Whey Acidic Protein (WAP)-Myc transgenic mice [36].

EMT rarely occurs homogenously across the whole tumour. The exceptions include diffuse lobular carcinoma [37] and sarcomatoid tumour of the breast, also referred to as spindle-cell carcinoma tumours [31]. Based on the expression of EMT markers, EMT appears to occur at the invasive front of the tumour [38, 39]. These tumour cells are primed to undergo EMT by genetic and epigenetic changes. Extracellular inputs, such as the activation of TGF- $\beta$  and Wnt signaling [40] at the leading edge of the tumour are coupled with the expression of EMT regulators such as Snail/Slug/Twist, Cripto-1 and Six1 [34, 41–46], causes cells to acquire a mesenchymal phenotype that allow them to invade locally and escape from the primary tumour (Fig. 1). This was discussed in detail in the review by Micalizzi et al. [38] on EMT in breast cancer.



**Fig. 1** The metastasis cascade. Epithelial cells at the edge of the primary tumour, within a duct in the mammary gland, when triggered by interactions with the underlying stroma, will breach the basal membrane, undergo EMT as they invade into the stroma and become mesenchymal. The newly acquired mesenchymal state allows local invasion and intravasation within nearby vessels, resulting in circulation of the tumour cells. The circulating tumour cells will extravasate into the tissue of distant organs. The microenvironment of

the distant organ has a normal stroma and lacks the signals that induced EMT. The new microenvironment triggers the tumour cells to undergo MET and establish within the tissue. Although most of the tumour cells that shed from the primary tumour site will die either during transport or at the site of landing, some will create micrometastases. While most of these micrometastases may remain dormant, some will proliferate forming a full blown metastasis

TGF- $\beta$  was identified as one of the main inducers of EMT. TGF- $\beta$ -induced EMT of NMuMG breast cancer cells was found to be mediated via Smad3 and Smad4 [47]. Activated Smads mediate EMT by inducing the expression of transcriptional repressors, such as Snail, Slug and HMGA2. The Smads also make complexes with these repressors to enhance their transcriptional effects [41, 48]; a SNAIL1-SMAD3/4 complex was shown to repress Coxsackie- and adenovirus receptor (CAR), occludin and E-cadherin transcription. A strong correlation was found between loss of CAR and E-cadherin and nuclear co-expression of SNAIL1 and SMAD3/4 at the invasive front of breast carcinomas [40].

Smad proteins downregulate the expression of the miRNA-200 family to induce EMT [49]. Also non-Smad mediated signaling has been implicated in TGF- $\beta$ -induced EMT. P38 and Rho kinase inhibitors attenuate TGF- $\beta$ -induced stress fiber formation and the subsequent relocalization of E-cadherin [9]. TGF- $\beta$ -induced phosphatidylinositol 3-kinase and Akt activation was required for TGF- $\beta$ -induced ZO-1 relocalization from tight junctions and change in cell morphology [50]. Furthermore, TGF- $\beta$  receptor-induced phosphorylation of Par6 leads to a loss in tight junctions and contributes to EMT in the transplantable mouse mammary tumour cell line, EMT6 [42].

A recent paper investigated the hypothesis whether an actin regulatory protein, Annexin A1 (AnxA1), is functionally involved in breast cancer progression [51]. AnxA1 was found to be highly expressed in basal-like cancers (BLBC) compared to luminal-like breast cancer cells. BLBC-like cells converted from a mesenchymal to an epithelial morphology upon AnxA1 knockdown, and that ectopic expression of AnxA1 in the luminal-like MCF-7 human breast cancer cell line increased cell scattering and Smad3/4 transcriptional reporter activity. These latter effects were mediated by TGF- $\beta$ -like activity as they could be blocked by the TGF- $\beta$  type I receptor kinase inhibitor SB-431542 [51]. Moreover, AnxA1 knockdown in the highly invasive 4T1 mouse breast cancer cells reduced the number of surface metastases in the lungs, but had no effect on primary tumour growth [51].

Araki et al. studied the Smad-dependent TGF- $\beta$  signaling in the context of breast cancer progression [52]. In this study, the authors reported that TGF- $\beta$  increased the expression of the E3 ubiquitin ligase human double minute 2 (HDM2) in a Smad3/4-dependent manner. Similar changes were seen in murine double minute 2 (MDM2) expression during murine EMT. The identification of HDM2 as a downstream target of TGF- $\beta$  represents a critical pro-survival mechanism in cancer progression and provides a potential therapeutic intervention target in late-stage cancer. A recent paper investigated the properties of EMT induced by TGF- $\beta$  in cooperation with fibroblast

growth factors (FGFs) [43]. Moreover, the cells generated through EMT mediated by FGF-2 and TGF- $\beta$  facilitated cancer cell invasion, when the cells undergoing EMT were mixed with cancer cells. Therefore, the results of this paper show that TGF- $\beta$  and FGF-2 cooperate with each other and may regulate EMT in the cancer microenvironment.

It has also been shown that TGF- $\beta$  stimulation of EMT elicits a fundamental change in the coupling of EGFR to its downstream effectors. Furthermore, Wendt et al. show that in 3D-organotypic culture post-EMT mammary epithelial cells manifest as dense cellular aggregates that are characteristic of highly metastatic breast cancer cells [44]. Also, Sabbah et al. have demonstrated that CCN5, an estrogen-inducible gene in estrogen receptor-positive cell lines, acts as a transcriptional repressor. CCN5 was shown to regulate tumour progression by repressing expression of genes associated with EMT as well as expression of key components of the TGF- $\beta$  signaling pathway, prominent among them T $\beta$ RII receptor [53].

## Invasion

Invasion into neighbouring tissue and ectopic survival are required for cancer progression and are a requirement to form metastasis [54]. It is known that the movement of neoplastic cells is not a random process. However, the mechanisms controlling the neoplastic cells movement, survival in foreign tissue environments, and choice of residence at a final destination are not clear [38]. Invasion and metastasis are the cause of malignancy and responsible for treatment failure [55].

Molecular profiles of isolated luminal epithelial and myoepithelial cells have identified a complex network involving TGF- $\beta$ , Hedgehog, cell adhesion, and p63 to be required for myoepithelial cell differentiation, the elimination of which resulted in loss of myoepithelial cells and progression to invasion [56]. Recent investigations using invasive mouse breast tumour cells have shown that Fra-1, a member of the FOS family of transcription factors, is involved in breast tumour invasion. This Fra-1 initiates activation of the IL-6/JAK/Stat3 signaling pathway, which creates a malignant switch in breast tumour cells. The subsequent increased release of proangiogenic factors MMP-9, VEGF, and TGF- $\beta$  from tumour cells causes an intensified invasion and progression of breast cancer [57].

Tumour cells often form related structures called invadopodia that are thought to promote invasion and metastasis. Organization of the invadopodia requires signaling through phosphatidylinositide 3-kinase and Src kinase. Furthermore, degradation of the ECM requires extracellular signal-regulated kinase signaling, and each of

these pathways is activated by TGF- $\beta$  in CA1D human breast cancer cells [58].

An elegant TGF- $\beta$ -dependent invasion assay system, consisting of spheroids of MCF10A1 normal breast epithelial cells and RAS-transformed (pre-)malignant derivatives embedded in collagen gels, has recently shown that the TGF- $\beta$ /Smad pathway induces breast cancer cell invasion through the up-regulation of matrix metalloproteinase (MMP) 2 and 9 [59]. Both basal and TGF- $\beta$ -induced invasion of these cell lines was found to correlate with their tumorigenic potential. Furthermore, basal invasion was strongly inhibited by the TGF- $\beta$  receptor kinase inhibitor SB-431542, indicating the involvement of autocrine TGF- $\beta$  or TGF- $\beta$ -like activity. TGF- $\beta$ -induced invasion in pre-malignant and highly malignant breast cells was also inhibited upon specific knockdown of Smad3 or Smad4.

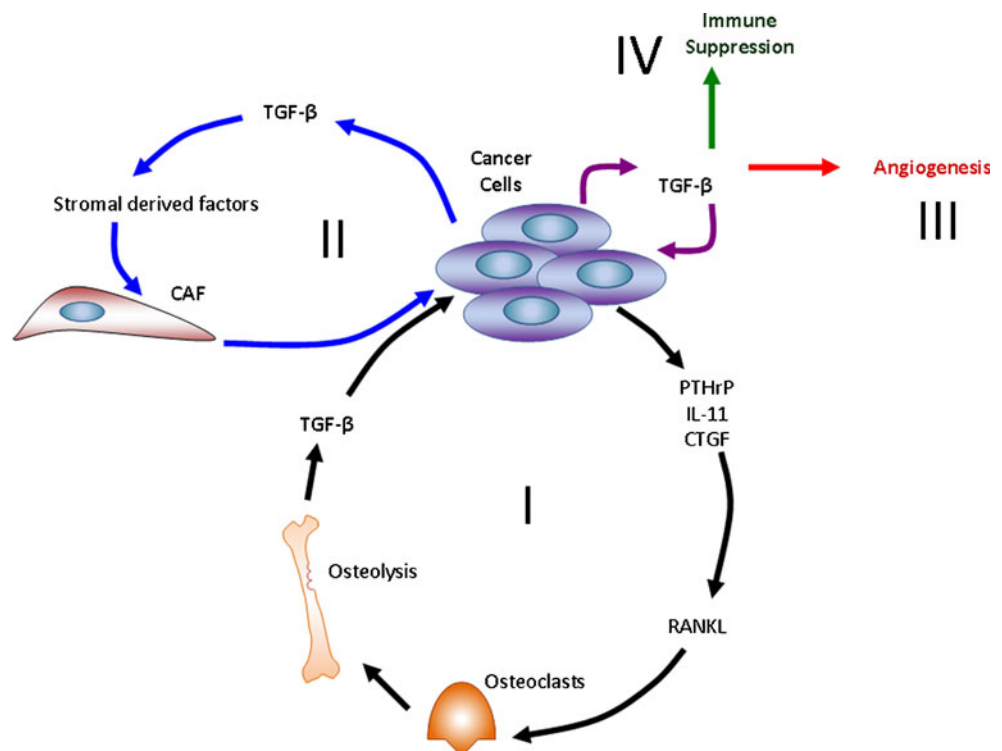
Intravital imaging has been used to demonstrate a reversible transition to a motile state as breast cancer cells spread. Giampieri et al. were able to demonstrate that transient TGF- $\beta$  signaling is essential for blood-borne metastasis. TGF- $\beta$  was shown to be capable of switching cells from cohesive to single cell motility through a transcriptional program involving Smad4, EGFR, neural precursor cell expressed, developmentally down-regulated 9 (Nedd9), myosin phosphatase Rho interacting protein (M-RIP), FERM, RhoGEF (ARHGEF) and pleckstrin domain protein (FARP) and Rho C. Furthermore, they showed that a blockade of TGF- $\beta$  signaling prevented cells moving singly *in vivo*, but did not inhibit cells moving collectively.

In fact, the cells restricted to collective invasion were capable of lymphatic invasion, but not blood-borne metastasis [60]. Thus, although TGF- $\beta$  is known to suppress epithelial cell proliferation and therefore primary tumorigenesis, it has been shown to promote breast cancer progression via the induction of EMT and tumour cell invasion.

## Tumour Stroma Interactions and the Microenvironment

Tumour progression may be a product of an evolving crosstalk between different cell types within the tumour and its surrounding supporting tissue, or tumour stroma (Fig. 2). The tumour compartment is defined by genetically abnormal cells. It is the surrounding and interwoven stroma that can provide a connective-tissue framework of the tumour tissue. This framework includes the ECM as well as cellular components such as fibroblasts, immune and inflammatory cells, and blood vessel cells [61, 62]. Its constitution resembles that of the granulation tissue formed during wound healing [63]. In fact, stromal alterations during wound healing, induced by TGF- $\beta$ , can be an important determinant of tumour growth [64]. Similarly to the development and function of normal organs, it is the interaction between cancer cells and their microenvironment that can largely determine the phenotype of the tumour [65]. For example, recent studies have shown that the establishment of human breast tumour xenografts in

**Fig. 2** The microenvironment. A vicious cycle, where tumour cells alter the bone microenvironment by stimulating osteoclast factors, such as PTHrP, IL-11 and CTGF. These factors act on osteoblasts to stimulate the release of RANKL, which promote osteoclast formation and function (I). CAFs secrete stroma-derived factors, such as SDF-1, which directly interacts with its receptor on breast cancer cells, CXCR4, and directs tumour cell migration (II). TGF- $\beta$  contributes to the regulation of angiogenesis through direct and indirect mechanisms (III). In the microenvironment TGF- $\beta$  has immunosuppressive effects on immune cells involved in the antitumour response following its secretion by stromal cells and various tumour cells (IV)



mice depends on the presence of human tumour-derived stromal fibroblasts [66–68].

Frequently present in the stroma of human breast carcinomas are carcinoma-associated fibroblasts (CAFs). The precise cellular origins of CAFs and the molecular mechanisms by which these cells evolve into tumour-promoting myofibroblasts remain unclear [69]. Using a co-implantation breast tumour xenograft model, Kojima et al. show that resident human mammary fibroblasts progressively convert into cancer-associated myofibroblasts during the course of tumour progression [70].

TGF- $\beta$ -mediated carcinoma suppression is not limited to cell-autonomous signaling. Recent results that highlight the role of stromal–epithelial crosstalk in the regulation of cancer have shown that TGF- $\beta$  signal transduction in stromal fibroblast can be important for the suppression of tumorigenesis in the adjacent epithelium [71–73]. Conditional inactivation of the T $\beta$ RII gene in mouse fibroblasts resulted in intraepithelial neoplasia in the prostate and invasive squamous cell carcinoma of the forestomach, and both were associated with an increased abundance of stromal cells [71]. These T $\beta$ RII-deficient fibroblasts promoted growth and invasion of co-transplanted mammary carcinoma cells [72, 73].

In addition, CAFs can increase the tumorigenic ability of cancer cells. Tumorigenicity of normal mammary epithelial cells was indeed shown to be significantly enhanced by the irradiated fibroblasts in vivo [74]. The enhancement was due to the overexpression of TGF- $\beta$  from the irradiated stroma [75]. In another study, CAFs were isolated from patients with invasive breast cancer. It was shown that TGF- $\beta$  significantly increased the myofibroblast percent and invasion rate in CAF cultures. In fact, the CAFs were measurably different from normal fibroblasts in response to TGF- $\beta$ , suggesting that TGF- $\beta$  stimulates changes in CAFs that foster tumour invasion [76].

It has also been demonstrated that the tumour microenvironment facilitates metastatic spread by eliciting reversible changes in the phenotype of cancer cells. Bone-marrow-derived human mesenchymal stem cells, when mixed with otherwise weakly metastatic human breast carcinoma cells, cause the cancer cells to increase their metastatic potency greatly when this cell mixture is introduced into a subcutaneous site and allowed to form a tumour xenograft [68]. The breast cancer cells stimulate *de novo* secretion of the chemokine CCL5 (also called RANTES) from mesenchymal stem cells, which then acts in a paracrine fashion on the cancer cells to enhance their motility, invasion and metastasis.

It is well known that tumour associated macrophages, monocytes and neutrophils can promote tumour progression [77, 78]. However, evidence now supports a

significant role for immature myeloid cells as promoters of tumour progression and metastasis [79]. In mouse models, these cell populations are often identified by their cell surface expression of granulocyte differentiation antigen 1 (GR-1) and CD11b proteins. Within the GR-1+ CD11b+ cell population it has been shown that the GR-1<sup>high</sup> populations enriches for the polymorphonuclear cells whereas the GR1<sup>int/low</sup> population enriches for the mononuclear cells. Importantly, the authors suggest that the mononuclear fraction is better able to suppress CD8+ T-cell mediated immunity than the polymorphonuclear fraction [80]. Another study has demonstrated that Gr-1+ CD11b+ myeloid cells are recruited into mammary carcinomas with T $\beta$ RII deletion and directly promote tumour metastasis [81].

It is becoming clear that the crosstalk between the cancer cells and the microenvironment plays a key role in the progression of cancer, and understanding this mutual relationship would eventually enable better treatment of patients, potentially by targeting CAFs.

## Metastasis

Metastasis is a complex, multi-step process by which primary tumour cells invade adjacent tissue. These cells enter the systemic circulation (intravasate), translocate through the vasculature, and arrest in distant capillaries where they extravasate into the surrounding tissue parenchyma, and these microscopic growths (micrometastases) proliferate into macroscopic secondary tumours [82]. Metastasis is the result of several sequential steps and represents a highly organized, non-random and organ-selective process [83] that involves interactions from a variety of proteolytic enzymes, growth factors, and cell-cell and cell-substrate adhesion molecules [84].

In numerous models of breast cancer associated invasion and metastasis, activated TGF- $\beta$  signaling induces increased aggressiveness. For example, in mice overexpressing the Neu oncogene, activated TGF- $\beta$  signaling increases the number of lung metastases even while decreasing the growth of the primary tumour [35]. Likewise, ablation of TGF- $\beta$  signaling in the same model decreases lung metastasis while also decreasing the latency of primary tumour growth, again emphasizing the dual functions of TGF- $\beta$  signaling in tumorigenesis [37]. Additionally, clinical evidence suggests a correlation between expression of the TGF- $\beta$  ligands and poor patient outcome [16–18, 39]. Furthermore, activated TGF- $\beta$  signaling has been observed in breast cancer bone metastases and contributes to the establishment of these lesions [19, 84, 85]. There have also been many specific studies to analyse the role of TGF- $\beta$  in tumour metastasis to lung [29].

## Breast to Bone Metastasis

Bone metastases are common in patients with advanced breast cancer. Tumour cells co-opt bone cells to drive a feed-forward cycle which disrupts normal bone remodeling to result in abnormal bone destruction or formation and tumour growth in bone [86, 87]. There is abundant evidence to support the role of TGF- $\beta$  as a major bone-derived factor. TGF- $\beta$  promotes a feed-forward cycle responsible for tumour growth and (Fig. 2) in bone. Osteolytic bone destruction is caused when tumours in bone secrete osteolytic factors, such as parathyroid hormone-related protein (PTHrP) and interleukin 11 (IL-11) [88]. TGF- $\beta$  is released and activated from the mineralized bone matrix by osteoclastic resorption and further induces tumour production of osteolytic and prometastatic factors including PTHrP and IL-11 [89]. Human breast cancer bone metastases have increased PTHrP expression, more so than primary breast cancers [90]. PTHrP is a central mediator of TGF- $\beta$  induced osteolytic metastases; PTHrP neutralizing antibodies blocked the development and progression of breast cancer bone metastases in mouse models [91]. Another paramount study in 1999 showed that a dominant negative T $\beta$ RII stably expressed in the breast cancer cell line MDA-MB-231 rendered the cells unresponsive to TGF- $\beta$ , inhibited PTHrP secretion induced by TGF- $\beta$  and suppressed bone metastases in a mouse model [92]. TGF- $\beta$  increases PTHrP secretion from MDA-MB-231 cells via Smad and p38 MAP kinase pathways [93]. Furthermore, TGF- $\beta$  released during bone resorption is likely to have direct effects on bone cells, stimulating osteoclastic bone resorption and inhibiting osteoblast differentiation.

The complexity in the origin of bone metastases has been exemplified by recent transcriptional profiling of subpopulations of human breast cancer cells with an aggressive bone metastases phenotype [94, 95]. Many of these genes, such as IL-11, connective tissue growth factor (CTGF), C-X-C chemokine receptor type 4 (CXCR4), and MMP1 have effects on bone cells [96], which could promote bone metastases. Bone resorption is stimulated by IL-11 and MMP-1 causing an increase in osteoblast production of receptor activator of nuclear factor kappa-B ligand (RANKL) [97]. CXCR4, a chemokine receptor that binds to the osteoblast product stromal-derived factor-1 (SDF-1) produced by osteoblasts to promote homing of cancer cells to bone [98–100]. CTGF stimulates osteoblast proliferation as well as angiogenesis [101]. These genes act cooperatively when expressed together, to cause osteolytic metastasis by promoting homing to bone, angiogenesis, and invasion [102]. Among the bone metastasis genes identified, Kang et al. showed that IL-11 and CTGF were regulated by TGF- $\beta$  via the classical TGF- $\beta$ /Smad pathway

in metastatic cells [84]. Other studies indicate that CXCR4 and MMP-1 are also regulated by TGF- $\beta$  [98, 99].

Since these first studies into TGF- $\beta$  and the bone microenvironment, there have been many advances. Recent evidence has suggested that Gli2, a Hedgehog signaling molecule, is required for TGF- $\beta$  to stimulate PTHrP expression and that blocking Hedgehog-independent Gli2 activity will inhibit tumour-induced bone destruction [103]. Using a murine syngeneic model that mimics osteolytic changes associated with human breast cancer, one laboratory has examined the role of tumour-bone interaction in tumour-induced osteolysis and malignant growth in the bone microenvironment [104]. T $\beta$ RII was identified as a commonly upregulated gene at the tumour-bone interface. Moreover, nuclear localization of phospho-Smad2 was higher in tumour cells and osteoclasts at the tumour-bone interface as compared to the tumour-alone area [104]. A mouse model using Cre/LoxP technology, with the WAP promoter driving transgenic expression of Cre recombinase (Cre), ablated the T $\beta$ RII expression specifically within mouse mammary alveolar progenitors [105]. Transgenic expression of the polyoma virus middle T antigen, under control of the mouse mammary tumour virus (MMTV) enhancer/promoter, was used to produce mammary tumours in the absence or presence of Cre or T $\beta$ RII. The loss of TGF- $\beta$  signaling was significantly correlated with increased tumour size and enhanced carcinoma cell survival.

Human breast cancer bone metastases show active Smad signaling in bone metastasis by accumulation of phosphorylated Smad2 in the nucleus of tumour cells [84].

Knockdown of Smad4 expression in breast cancer cells reduced growth of bone metastases in a mouse model [47, 84]. Different studies in mouse models of bone metastases, using live imaging of tumour cells by bioluminescence, have shown that TGF- $\beta$  signaling is activated in the bone metastases, but not in metastases to adrenal glands [12, 84, 106]. In this preclinical model, either anti-TGF- $\beta$  therapy with a small molecule inhibitor of T $\beta$ RI kinase activity or a bisphosphonate inhibitor of bone resorption was effective to decrease TGF- $\beta$  signaling activity in these bone metastases [106]. These data indicate that TGF- $\beta$  signaling is prominent in bone metastases compared with other metastatic sites and that inhibiting either the TGF- $\beta$  pathway or osteoclastic bone resorption can impair this activity.

Further investigation of the specific functions of Smad2 and Smad3 in TGF- $\beta$ -induced responses in breast cancer cells in vitro and in vivo for breast cancer metastasis have recently been undertaken. Studies have shown that Smad2 and Smad3 differentially affect breast cancer bone metastasis formation in vivo [107]. Knockdown of Smad3 in breast cancer cells in vivo resulted in prolonged latency and delayed growth of bone metastasis. However, Smad2 knockdown resulted in a more aggressive phenotype

compared with controls. Furthermore, the data suggest that bone-derived TGF- $\beta$ , released as a consequence of osteoclastic bone resorption, is the major source of TGF- $\beta$  to act on tumour cells in bone. Overexpression of BMP-7 in breast cancer cells decreased the development of bone metastases in mice, but had no effect on orthotopic tumours [25, 108]. BMP7 was found to antagonize TGF- $\beta$ /Smad signaling. Therefore, BMP-7 may be useful as an inhibitor of bone metastases [109–111].

Another unique aspect of the bone microenvironment is hypoxia. Bone is a hypoxic microenvironment and hypoxia has also been implicated to enhance tumour growth and metastasis [112]. TGF- $\beta$  and hypoxia signaling pathways in breast cancer cells were additive to induce vascular endothelial growth factor (VEGF) and CXCR4, via hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) in vitro. HIF-1 $\alpha$  and TGF- $\beta$  pathways were inhibited in breast tumour cells using shRNA against HIF-1 $\alpha$  and dominant negative T $\beta$ RII approaches [99, 113]. In vivo, inhibition of either pathway decreased bone metastasis, but there was no additional effect on the development of bone metastasis with a double blockade. In contrast, treatment with pharmacologic inhibitors targeting both pathways decreased bone metastases more than either alone [99].

Preclinical studies have indicated that tumour cells express a number of genes which encode for proteins that act at different sites of the metastatic cascade as well as at the bone site. For example, several studies have shown that tumours cells produce adhesive molecules that promote binding to marrow stromal cells and bone matrix. These adhesive interactions increase tumour production of angiogenic and bone resorbing factors that enhance tumour growth in bone [114, 115].

#### Breast to Lung Metastasis

There is evidence that TGF- $\beta$  can prime breast cancer cells for metastasis to the lungs. This is based on the study by Padua et al. which showed that the process is dependent on the induction of angiopoietin-like 4 (ANGPTL4) by TGF- $\beta$  via the Smad signaling pathway [29]. TGF- $\beta$  induction of Angptl4 in cancer cells that are about to enter the circulation enhances their subsequent retention in the lungs, but not in the bone. Tumour cell-derived Angptl4 disrupts vascular endothelial cell-cell junctions, increases the permeability of lung capillaries, and facilitates the trans-endothelial passage of tumour cells [116]. It is suggested that the primary breast tumour microenvironment induces the expression of cytokines in departing tumour cells, enabling these cells to disrupt lung capillary walls and seed pulmonary metastases [29].

Functional studies have demonstrated that Id1 and its closely related family member Id3 are required for tumour

initiating functions, both in the context of primary tumour formation and during metastatic colonization of the lung microenvironment [117]. In vivo characterization of lung metastatic progression revealed that Id1 and Id3 facilitate sustained proliferation during the early stages of metastatic colonization, subsequent to extravasation into the lung parenchyma. Sadej et al. have shown that attenuation of TGF- $\beta$ 1-induced responses correlated with reduced retention in the lung vascular bed, inhibition of pneumocyte-induced scattering of breast cancer cells in three-dimensional Matrigel, and decrease in experimental metastasis to the lungs. These results identify CD151 as a positive regulator of TGF- $\beta$ 1-initiated signaling and highlight the important role played by this tetraspanin in TGF- $\beta$ 1-induced breast cancer metastasis [118].

The role of TGF- $\beta$  coreceptor endoglin has also been studied in breast lung metastasis. Ectopic expression of endoglin in a breast cancer cell line blocked TGF- $\beta$ -enhanced cell motility and invasion and reduced lung colonization in an in vivo metastasis model [119]. Endoglin does not modulate Smad-mediated TGF- $\beta$  signaling in breast cells but attenuates the cytoskeletal remodeling to impair cell migration and invasion [120].

#### Perspectives

In numerous models of breast cancer associated invasion and metastasis, activated TGF- $\beta$  signaling induces increased aggressiveness. Activated TGF- $\beta$  signaling has been observed in breast cancer bone metastases and there have also been many specific studies to analyse the role of TGF- $\beta$  in tumour metastasis. The role that TGF- $\beta$  plays in this complex, multi-step process is becoming clearer. New methods of research, such as the collagen-embedded spheroid system [59] and other three-dimensional coculture assays [121], offer a valuable method to study the crucial microenvironmental cues that may be lost in two-dimensional culture assay with a plastic substrata.

In order to dissect the TGF- $\beta$ /Smad pathway, which underlies the complex biological responses in the mammary epithelium, a new method to generate conditionally immortalized mammary epithelial cells was developed. The method involves the intercrossing of the “Immortomouse”, which expresses a temperature-sensitive mutant of the simian virus-40 large T-antigen, with mice of different Smad genotypes. Thus, conditionally immortalized mammary epithelial cell cultures can be derived from the mammary glands of offspring from these crosses [122]. Further dissection of the signaling pathways involved and elucidation on which signaling components are shared and distinct between tumour suppression and tumour promoting role of TGF- $\beta$ , may lead to novel pharmacological targets



for the intervention of breast cancer progression, while leaving the tumour suppressive effects of TGF- $\beta$  intact.

Stroma cells, together with ECM components, provide the microenvironment that is pivotal for cancer cell growth, invasion and metastatic progression. Crucial in this process are fibroblasts that are located in the vicinity of the neoplastic epithelial cells. They are able to modify the phenotype of the epithelial cells by direct cell-to-cell contacts, through soluble factors or by modification of extracellular matrix components. Seminal functional studies in various cancer types, including breast, colon, prostate and lung cancer, have confirmed the concept that fibroblasts can determine the fate of the epithelial cell, since they are able to promote malignant conversion as well as to revert tumour cells to a normal phenotype. The study by Kojima et al. suggests that the autocrine-signaling mechanism of TGF- $\beta$  in the tumour-stroma interactions may prove to be an attractive therapeutic target to block the evolution of tumour-promoting CAFs [70].

Clear definitions of the molecular and cellular contexts that are permissive for the tumour suppressor versus oncogenic activities of TGF- $\beta$  are allowing new therapeutic opportunities to emerge. The most obvious therapeutic challenge in targeting the TGF- $\beta$  signaling pathway in breast cancer is: how to restore the lost tumour suppressor function while either eliminating or preventing acquired pro-oncogenic effects [123]. Unfortunately late-stage invasive, metastatic breast cancer is typically characterized by locally or systemically elevated TGF- $\beta$  levels. This elevation of TGF- $\beta$  is coupled with diminished responsiveness of tumour cells to its suppressor functions, as discussed throughout this review. TGF- $\beta$  antagonists could be efficacious. However, given the roles of TGF- $\beta$  on normal tissue homeostasis, the antagonist design and/or delivery mode will have to be optimized to minimize adverse effects.

In 2010, Ganapathy et al. investigated the possible clinical utility of TGF- $\beta$  antagonists in a human metastatic basal-like breast cancer model [124]. Two TGF- $\beta$  pathway antagonists, 1D11 (a mouse monoclonal pan-TGF- $\beta$  neutralizing antibody) and LY2109761 (a chemical inhibitor of T $\beta$ RI and T $\beta$ RII kinases) were tested on MDA-MB-231. 1D11 and LY2109761 were shown to effectively block TGF- $\beta$ -induced phosphorylation of receptor-associated Smads in vitro. Moreover, both antagonists inhibited TGF- $\beta$  stimulated in vitro migration and invasiveness. In addition, both antagonists significantly reduced the metastatic burden to either lungs or bones in vivo. These studies not only support the notion that TGF- $\beta$  plays an important role in both bone-and lung metastases of basal-like breast cancer, but also that targeting of the TGF- $\beta$  pathway holds promise as a novel therapeutic approach for metastatic basal-like breast cancer.

TGF- $\beta$ -specific inhibitors based on blockade of synthesis, ligand/receptor binding or receptor kinase signaling are in clinical trials [125]. Clearly, further research can refine the therapeutic rationale by focusing on drug scheduling and delivery, identifying patients who will benefit most from such therapy, and combining therapeutic modalities such that cancer is eliminated without normal tissue toxicity or long term health effects.

### Concluding Remarks

The role of TGF- $\beta$  signaling in breast cancer cell invasion and metastasis involves multiple factors. Expression of TGF- $\beta$  promotes a more aggressive tumour phenotype. TGF- $\beta$  is known to be involved in the process of EMT, invasion and metastasis, and can influence the microenvironment. Crosstalk between the breast tumour cells and the microenvironment can promote a bone or lung metastasis. TGF- $\beta$  is involved in the crosstalk by recruiting and regulating the activity of multiple cell types. Whereas much is understood about the effects of these factors in cancer cells at the primary tumour site, continued research is necessary to clearly understand metastatic breast cancer. Understanding how TGF- $\beta$  allows the tumours to progressive to the metastatic phenotype may help to identify potential targets for therapeutic intervention to halt tumour growth and bone metastasis.

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