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ORIGINAL PAPER

TGF-β in the Bone Microenvironment: Role in Breast Cancer Metastases

Jeroen T. Buijs · Keith R. Stayrook · Theresa A. Guise

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Abstract Breast cancer is the most prevalent cancer among females worldwide. It has long been known that cancers preferentially metastasize to particular organs, and bone metastases occur in ~70% of patients with advanced breast cancer. Breast cancer bone metastases are predominantly osteolytic and accompanied by bone destruction, bone fractures, pain, and hypercalcemia, causing severe morbidity and hospitalization. In the bone matrix, transforming growth factor-β (TGF-β) is one of the most abundant growth factors, which is released in active form upon tumor-induced osteoclastic bone resorption. TGF-β, in turn, stimulates bone metastatic cells to secrete factors that further drive osteolytic destruction of the bone adjacent to the tumor, categorizing TGF-β as a crucial factor responsible for driving the feed-forward vicious cycle of cancer growth in bone. Moreover, TGF-β activates epithelial-to-mesenchymal transition, increases tumor cell invasiveness and angiogenesis and induces immunosuppression. Blocking the TGF-β signaling pathway to interrupt this vicious cycle between breast cancer and bone offers a promising target for therapeutic intervention to decrease skeletal metastasis. This review will describe the role of TGF-β in breast cancer and bone metastasis, and pre-clinical and clinical data will be evaluated for the potential use of TGF-β inhibitors in clinical practice to treat breast cancer bone metastases.

Keywords Transforming growth factor-beta · TGF-β · Breast cancer · Bone metastasis · Bone · Small molecule inhibitors · Antibodies · Bone resorption

J. T. Buijs · K. R. Stayrook · T. A. Guise (✉)
Department of Medicine, Division of Endocrinology,
Indiana University School of Medicine,
980 West Walnut Street, Walther Hall R3, #C132,
Indianapolis, IN, USA
e-mail: tguise@iupui.edu

List of Abbreviations

ALK	Activin receptor-like kinase
ASO	Antisense oligonucleotides
BMP	Bone morphogenetic protein
BSP	Bone sialoprotein
CAT	Cambridge antibody technology
CSC	Cancer stem cell
DN	Dominant negative
EMT	Epithelial-to-mesenchymal transition
GM-CSF	Granulocyte macrophage colony stimulating factor (a.k.a. CSF2)
Hfg	Halofuginone
HIF	Hypoxia inducible factor
HMEC	Human mammary epithelial cells
i.v.	Intravenous
i.p.	Intraperitoneal
IGF	Insulin growth factor
IL	Interleukin
JNK	JunN-terminal kinase
MAPK	Mitogen-activated protein kinase
MET	Mesenchymal-to-epithelial transition
MMTV	Mouse mammary tumor virus
OPG	Osteoprotegerin
OPN	Osteopontin
PDGF	Platelet-derived growth factor
PTHrP	Parathyroid hormone-related protein
R-Smads	Receptor-regulated Smads
RANK	Receptor activator of nuclear factor κB
RANKL	Receptor activator of nuclear factor κB ligand
s.c.	Subcutaneous
SDF-1	Stromal derived growth factor-1
TβRI	Transforming growth factor-β type I receptor (a.k.a ALK5)
TβRII	Transforming growth factor-β type II receptor
TGF-β	Transforming growth factor-β
VEGF	Vascular endothelial growth factor

Introduction

Cancer is the leading cause of death in economically developed countries [1]. Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death in females worldwide, accounting for 23% (1.38 million) of the total new cancer cases and 14% (458,400) of the total cancer deaths in 2008 [1]. Breast cancer frequently metastasizes to the skeleton, and approximately 70% of patients with advanced breast will develop bone metastases [2–4]. These bone metastases are predominantly osteolytic in nature, and cause bone pain, fractures, hypercalcemia and nerve compression syndromes diminishing quality of life [4, 5]. Perhaps the most adverse aspect is that once cancer metastasizes to bone, it is incurable. Standard antiresorptive treatments decrease skeletal morbidity, but do not cause regression or cure disease [4, 5]. Patients with cancer metastases to bone, particularly those with breast and prostate cancer, can survive for many years, during which they will suffer significant morbidity. Thus, better treatments are needed to achieve the long-term goal of curing bone metastases.

The bone microenvironment is unique and may provide a fertile soil for cancers to thrive. The mineralized bone matrix is embedded with various growth factors and cytokines, such as transforming growth factor-β (TGF-β), which are released and activated upon tumor-induced osteoclastic bone resorption [6]. High local levels of liberated TGF-β results in increased invasion, angiogenesis and immunosuppression. In addition, TGF-β stimulates tumor production of osteolytic factors that stimulate further bone resorption [7, 8]. This categorizes TGF-β as a crucial factor responsible for driving the feed-forward vicious cycle of tumor growth in bone. Therefore blocking TGF-β release, its production and/or signaling is a promising strategy to treat bone metastasis. Over the past two decades, several therapeutic strategies have been developed to inhibit TGF-β, including TGF-β neutralizing antibodies, soluble receptor decoy-Fc fusions, TGF-β antisense oligonucleotides and TGF-β receptor kinase inhibitors [9]. Many of these are now in early-stage clinical trials for various disease indications with particular emphasis as potential cancer therapies, including bone metastases. In this review, we will focus on the role of TGF-β in breast cancer and bone metastasis and subsequently discuss the potential use of novel TGF-β inhibiting compounds and biologics in clinical practice to treat bone metastases.

TGF-β

TGF-β is a ubiquitously expressed, multifunctional cytokine that controls tissue homeostasis by regulating cellular processes such as apoptosis, proliferation and differentiation [10]. TGF-β orchestrates the response to tissue injury and mediates repair by inducing epithelial-to-mesenchymal transition (EMT) and cell migration, and it is a critical regulator of the immune response. Dysregulation of TGF-β actions has been associated with many disorders, including chronic fibrosis, cardiovascular diseases and cancer [11, 12].

TGF-β Structure and Signaling

TGF-β is a member of the TGF-β superfamily, along with bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs), activins, inhibins, nodal and anti-Müllerian hormone [13, 14]. TGF-β is secreted as a latent precursor, which is then proteolytically activated into a homodimer of two 12.5 kd polypeptides joined by a disulfide bond [13, 15]. In humans, three isoforms of TGF-β have been described, TGF-β_1, TGF-β_2 and TGF-β_3. The signaling of these isoforms is comparable but the expression levels differ across tissues [16]. Active TGF-β isoforms bind with high affinity and selectivity to the membrane-spanning serine/threonine kinase receptor TGF-β receptor type II (TβRII), which then recruits and activates TGF-β receptor type I (TβRI, a.k.a. ALK5) (Fig. 1) [15]. Activated TβRI/ALK5 can then phosphorylate the receptor-associated Smads (R-Smads), Smad2 and Smad3 [17]. The activated R-Smads form a heterodimeric stable complex with the common mediator Smad, Smad4, and translocate to the nucleus [15, 17]. The Smad complex associates with DNA binding transcription factors to achieve high affinity for binding to the Smad-binding elements in the promoter of TGF-β target genes [18, 19]. Various families of transcription factors, such as forkhead, homeobox, zinc finger, AP1, Ets and basic helix-loop-helix, are Smad partners [19]. Moreover, the Smad complex recruits co-activators, such as p300 and CREB binding protein, or co-repressors, such as retinoblastoma-like 1, to regulate gene transcription [15, 17, 19]. So, while Smad proteins are intrinsically transcriptional activators, the transcriptional outcome of their target genes often depends on the transcriptional partners Smads interact with.

More recently, it has become apparent that TβRI/ALK5 can also activate the R-Smads Smad1 and −5, required for anchorage-independent growth and cell migration [20, 21]. Furthermore, TGF-β can also activate the R-Smads, Smad1, -5 and −8 via another receptor, ALK1, which is mainly expressed by endothelial cells [22]. In fact, TGF-β/ALK1 signaling potentiates and TGF-β/ALK5 inhibits endothelial cell proliferation and migration [23, 24]. TGF-β can also activate non-Smad signaling pathways including extracellular signal regulated kinases (ERK-1, ERK-2 and p38), c-Jun amino-terminal kinase (JNK) and

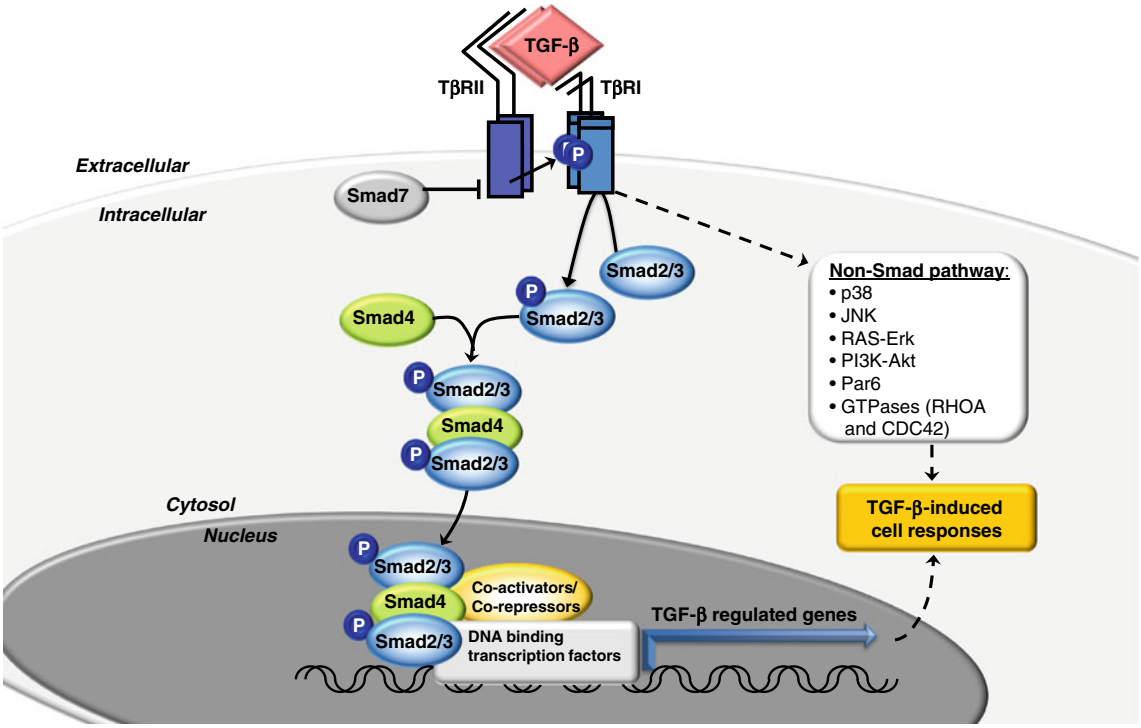

Fig. 1 TGF-β signaling pathway. Bioactive TGF-β binds to the TGF-β type II receptor (TβRII), recruiting the TGF-β type I receptor (TβRI a.k.a. ALK5). TβRII phosphorylates and activates TβRI/ALK5, which in turn phosphorylates and activates the intracellular mediators, the receptor-associated Smad (R-Smads) proteins (Smad2 and Smad3). The activated R-Smads form a complex with Smad4 and translocate to the nucleus, binding to DNA binding transcription factors, co-activators and co-repressors regulating TGF-β/Smad target gene expression. In addition, TGF-β is also known to regulate non-Smad pathways, including, Erk, p38 MAPK, Jun N-terminal kinase (JNK), PI3K-Akt and small GTPases

mitogen activated protein kinases (MAPKs) in various cell types [25] (Fig. 1).

TGF-β in Breast Cancer

TGF-β plays an essential role in maintaining homeostasis in many tissues through its ability to induce cell cycle arrest, differentiation and apoptosis, thereby preventing uncontrolled proliferation of epithelial, endothelial and hematopoietic cells [26, 27]. However, many cancers often become refractory to this growth inhibition either because of genetic loss of TGF-β signaling components or, more commonly, because of downstream perturbation by other signaling pathways [28]. At this time, pro-tumorigenic actions of TGF-β may prevail, including immunosuppression, induction of angiogenesis and promotion of the EMT, facilitating cancer migration and invasion (reviewed in [22, 29, 30]).

Dual Role of TGF-β in Breast Cancer Progression

Transgenic mouse models have been particularly informative to understand the roles of TGF-β in mammary gland development and tumor progression. In three independent studies, the mammary gland selective mouse mammary tumor virus (MMTV) promoter was used to drive the expression of either a soluble TβRII:Fc fusion protein [31], a dominant negative TβRII (DNTβRII) [32] or full length TβRII antisense [33] as a means to potently inhibit TGF-β signaling in this tissue. Consistent with the homeostatic role of TGF-β, a proliferative mammary gland phenotype was observed in all models. In addition, spontaneous mammary tumors developed in the DNTβRII transgenic model, but these were mostly carcinoma in situ, and arose after a prolonged latency [32].

In two additional studies, transgenic mice expressing the activated *neu* gene in the mammary gland were crossed with strains that expressed either active TGF-β_1 or constitutively active TβRI/ALK5 [34, 35]. Consistent with the tumor suppressive role of TGF-β, primary tumor development was marked delayed in both cases, and tumor growth was slower than in single *neu* transgenics [34, 35]. However, the carcinomas that did arise were more metastatic than those occurring in MMTV-*neu* single transgenics.

These and other [36, 37] studies have provided strong support for a tumor-suppressive role for epithelial TGF-β

signaling in mammary gland tumorigenesis. However, while TGF-β and its signaling may suppress early stages of mammary cancer formation and growth in these models, it also appears to enhance the metastatic potential of the carcinomas that do develop once they have broken through the growth suppressive barrier provided by TGF-β signaling.

TGF-β Expression Levels in Human Breast Cancer

When the TGF-β suppressive effects are lost, TGF-β overproduction is commonly observed in many solid tumors. In breast cancer, higher levels of TGF-β are often detected in tumors when compared to corresponding normal mammary gland tissue, and it appears even higher in the most advanced stages of tumor progression [38–40]. Moreover, TGF-β expression levels are correlative with angiogenesis and the prognosis of breast cancer patients [41]. Plasma TGF-β_1 levels have also been increased in breast cancer patients, and found to be correlative with disease stage [42–45]. Patients whose plasma TGF-β_1 levels normalized after tumor resection were found to have a favorable prognosis, whereas patients with persistently elevated plasma TGF-β_1 levels had an increased risk of lymph node metastases and disease progression [44]. These data may suggest an important causal role for TGF-β in metastases and disease progression.

Plasma TGF-β_1 levels have also recently been determined in 49 bone metastasis patients, including 23 breast cancer patients, and were reported to be elevated in more than half of the cancer patients and positively correlated with TGF-β signaling related markers, including parathyroid thyroid hormone-related peptide (PTHrP) and interleukin (IL)-10 [46]. TGF-β plasma levels may be indicative of TGF-β-dependent metastatic disease and may be useful biomarkers to predict the success of treatment with TGF-β antagonists in metastatic disease. These questions are currently tested in ongoing clinical trials. In addition, there is a highly significant association between TβRII expression and reduced survival of patients with estrogen receptor negative breast cancer [47].

TGF-β and Breast Cancer Stem Cells

An increasing body of basic and clinical studies have provided evidence of self-renewing, stem/progenitor-like cells within solid tumors, which have also been referred to as cancer stem cells (CSCs) [48–54]. CSCs are believed to constitute a small minority of neoplastic cells within a given tumor and are defined by their ability to propagate a tumor and potentially seed new metastases [51]. The concept of CSCs underscores the importance of targeting the correct cells for cancer therapy, since eliminating only the more differentiated, rapidly dividing cells by chemo- or radiation therapy is not likely to result in successful long term remission, despite any short-term palliative effects in patients, if the less differentiated and slower proliferating CSCs remain to repopulate the tumor.

By sorting breast cancer cells for a normal mammary stem cell phenotype ($CD44^+/CD24^{-/low}$), Al-Hajj et al. was the first to isolate the breast CSC fraction [48]. More recently, Shipitsin et al. demonstrated that genes that were co-expressed with CD44 included vimentin, connective tissue growth factor (CTGF), PAI-1, osteonectin, as well as TβRII [55]. In fact, many of the genes actively transcribed by $CD44^+$ cells were associated with a mesenchymal phenotype and many were known TGF-β target genes. Moreover, the presence of this gene signature correlated with poor prognosis [55]. Recently, Mani et al. demonstrated with a series of elegant studies that EMT generates cells with properties of stem cells [56]. TGF-β-induced EMT in immortalized human mammary epithelial cells (HMEC) was associated with the acquisition of the $CD44^+/CD24^{-/low}$ phenotype and mesenchymal traits, and increased ability to form mammospheres, a property associated with mammary epithelial stem cells. In addition, forcing EMT by overexpressing the EMT transcription factors (and TGF-β target genes) SNAI1 or TWIST also resulted in a $CD44^+/CD24^{-/low}$ phenotype that displayed enhanced tumorigenic potential when injected in mice. Taken together, these studies provide evidence that TGF-β is important in regulating the dynamics of cancer cell populations by favoring CSC self renewal and inhibiting the commitment to differentiation.

TGF-β in Bone Homeostasis

Adult bone is continuously remodeled by the coordinated activities of bone-resorbing osteoclasts and bone-forming osteoblasts. TGF-β_1 is one of the most abundant growth factors in bone matrix [57, 58], and the effects of TGF-β on osteoblast, osteoclasts and bone remodeling are complex and are both temporal- and spacial-dependent [59].

Evidence is accumulating that TGF-β is a key mediator in coupling bone resorption to bone formation [60–62]. Osteoblasts secrete TGF-β, where it is embedded into the mineralized bone matrix [59]. Osteoclastic bone degradation releases and activates TGF-β_1, which will result in recruitment of osteoblast precursors to sites of bone resorption [60–62]. The exposed bone mineral matrix and release of osteotropic factors, such as bone morphogenetic proteins (BMPs), insulin growth factor (IGF)-I and -II, and platelet derived growth factor (PDGF), may then promote differentiation of the osteoblast precursor to osteoblasts [63]. In later phases of osteoblastic differentiation, TGF-β

has been shown to block osteoblast differentiation and bone mineralization [64, 65], and induce osteoblast survival during transdifferentiation into osteocytes [66].

The effects of TGF-β on osteoclastogenesis and bone resorption are equally complex. Although TGF-β appears to have biphasic effects in osteoclastogenesis and bone resorption in vitro [67, 68], studies with genetically-modified mice showed that increased levels of TGF-β in bone microenvironment promoted osteoclastogenesis and bone resorption [69, 70].

Mohammad et al. studied the effects of systemic TGF-β inhibition on normal bone in adult mice using the orally active TβRI/ALK5 kinase inhibitor SD-208 [71]. Treatment with SD-208 increased osteoblast differentiation and bone formation as well as reduced osteoclast formation and bone resorption, resulting in increased trabecular bone volume [71]. These data should be interpreted carefully since all known TβRI/ALK5 kinase inhibitors described to date are also equipotent inhibitors of ALK4 [72–75], which is utilized by the bone matrix-embedded TGF-β superfamily member, Activin. Activin inhibition within bone has been shown in several contexts to promote bone formation and mass [76, 77]. However, use of a highly selective pan-neutralizing TGF-β antibody 1D11 recently by Edwards et al. resulted in significant gain of bone mass corroborating SD-208 studies and validating the critical importance of TGF-β specifically in bone homeostasis [78].

TGF-β in Bone Metastasis

Metastasis to bone is a multistep process of events [2, 4, 5]. First, cancer cells must detach from the primary tumor, enter into the systemic circulation (intravasation), evade the immune system, arrest in bone marrow capillaries, extravasate into bone marrow and form a micrometastasis. Eventually some micrometastases may grow into an overt bone metastatic lesion.

Numerous studies have shown the importance of the TGF-β signaling pathway for the development of bone metastases (Fig. 2). Yin et al. were the first to demonstrate that blocking TGF-β-signaling by stably transfecting a dominant negative TβRII (DNTβRII) in MDA-MB-231 breast cancer cells inhibited TGF-β-induced expression of PTHrP production in tumor cells and suppressed the formation of osteolytic bone metastases in an intracardiac injection mouse model [8]. In a subsequent study, TGF-β was demonstrated to induce the expression of PTHrP via both Smad-dependent and -independent (p38 MAP kinase) pathways [79].

PTHrP plays a major role in the development of the osteolytic features of bone metastatic lesions [80], and is considered to be responsible for the humoral hypercalcemia of malignancy (reviewed in [81, 82]). PTHrP stimulates osteoclast activation by inducing RANKL and downregulating OPG in cells of the osteoblast lineage [83]. In breast cancer 90% of metastases in bone were found to express PTHrP, compared to only 17% at non-bone sites and 60% of the primary tumors [84, 85]. Initially, expression of PTHrP in the primary tumor appeared to be associated with formation of bone metastases [86, 87]. In contrast, a large prospective study of 526 consecutive patients with operable breast cancer demonstrated that PTHrP expression in primary breast cancer was significantly associated with fewer (bone) metastases [84, 88–90]. Therefore, the most likely explanation for the observed increased PTHrP expression in breast cancer bone metastases [84, 90], is that TGF-β in the bone microenvironment induces cancer cells to express PTHrP rather than cancer cells that metastasize to the bone having an intrinsically higher PTHrP expression.

Transcriptional profiling of subpopulations of human breast cancer cells in a mouse model using intracardiac injection of MDA-MB-231 cells further illustrated the complexity of bone metastases development. Using serial passaging of MDA-MB-231 cell, Kang et al. isolated clones, which cause more aggressive bone metastases when compared to the parental line. Microarray analysis identified a number of genes that were selectively upregulated in these aggressive bone metastatic clones. Many of these proteins, including IL-11, CXCR4, CTGF and MMP-1 have all demonstrable effects on bone cells. IL-11 stimulates bone resorption by increasing osteoblast production of RANKL [7]. CXCR4 is a chemokine receptor that binds to stromal-derived factor-1 (SDF-1) produced by osteoblasts, and its expression promotes homing of cancer cells to bone. CTGF stimulates osteoblast proliferation as well as angiogenesis, and MMP-1 promotes bone metastasis by activating an EGFR-dependent paracrine signaling cascade suppressing the expression of OPG by osteoblasts [91, 92]. When expressed together, these proteins act cooperatively to cause osteolytic metastasis by promoting homing to bone, angiogenesis and invasion. Among the various bone metastasis genes identified, Kang et al. showed that two of these genes, IL-11 and CTGF, were directly regulated by TGF-β via the canonical TGF-β/Smad pathway in metastatic cells [7]. Other studies indicate that CXCR4 and MMP-1 are also regulated by TGF-β [93, 94]. Using the same mouse model, knockdown of the TGF-β signaling molecule Smad4 inhibited the formation and growth of bone metastases [95, 96]. Very recently, is was shown that active TGF-β stimulates Jagged1 expression in breast cancer bone metastases, which in turn stimulates Notch signaling in osteoclasts and osteoblasts after direct contact [97]. This results in increased osteoclastogenesis and the production of the cytokine IL-6 by osteoblast, acting as a potent inducer of

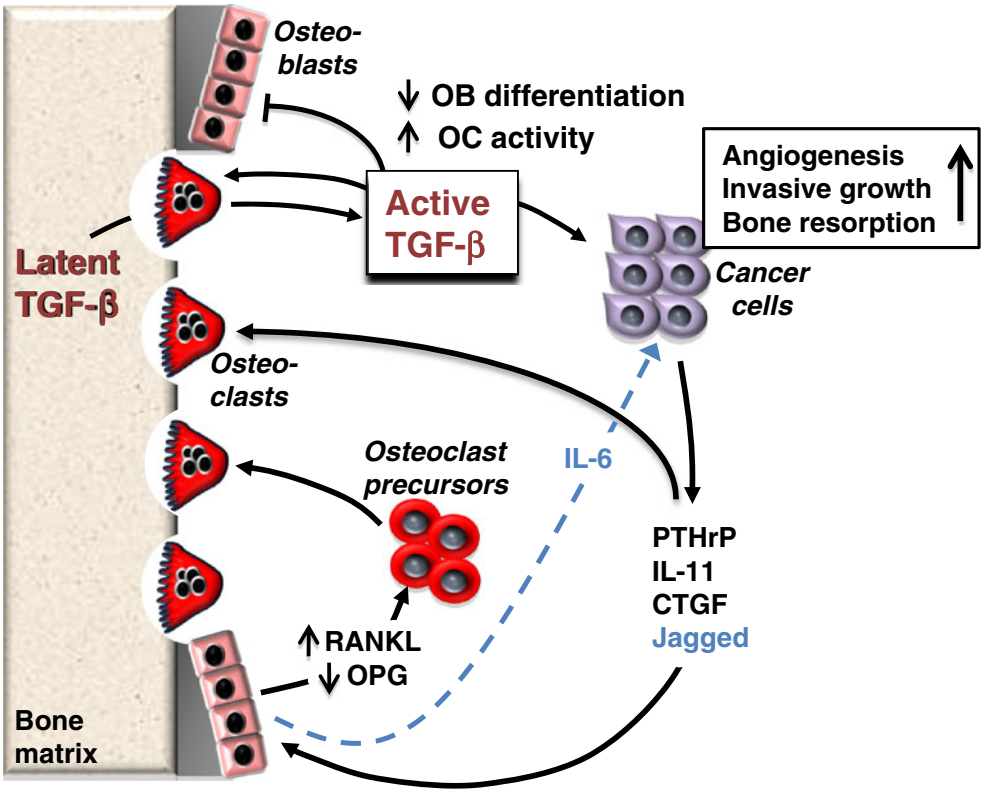

Fig. 2 TGF-β in osteolytic breast cancer bone metastasis. Bone is a preferential site for breast cancer metastases. In these bone metastases, TGF-β is released by osteoclasts from the bone matrix and acts on breast cancer cells to stimulate the production of osteolytic factors, such as parathyroid hormone-related protein (PTHrP), connective tissue growth factor (CTGF) and interleukin-(IL)6 and −11. These factors increase the RANKL/OPG expression ratio in osteoblasts, and other stromal cells, resulting in osteoclastogenesis. Additionally, active TGF-β stimulates Jagged1 expression in tumor cells, which in turn stimulates Notch signaling in osteoclasts and osteoblasts after direct contact. This results in increased osteoclastogenesis and production of the cytokine IL-6 by osteoblast (*blue dotted line*), acting as a potent inducer of proliferation of tumor cells. TGF-β itself has also direct effects on bone cells by stimulating osteoclast activity and inhibiting osteoblast differentiation. Collectively, these effects of TGF-β perpetuate the feed forward cycle to increase tumor growth in bone

proliferation of tumor cells. Collectively, these effects of TGF-β perpetuate the feed forward cycle to increase tumor growth in bone.

These preclinical data also extend to human studies. Bone metastases samples from breast cancer patients displayed enhanced Smad-dependent TGF-β-signaling as there is accumulation of phosphorylated Smad2 in the nucleus of tumor cells and cells of the surrounding stroma [96, 98]. In mouse models of bone metastases, using live imaging of tumor cells by thymidine kinase activity or dual-bioluminescence, it was also demonstrated that TGF-β signaling is activated in bone metastases, but not in metastases to adrenal glands [6, 96, 99]. Furthermore, treatment with a TβRI kinase inhibitor could effectively reduce TGF-β signaling in bone metastasis as could the bisphosphonate, pamidronate, a potent inhibitor of bone resorption [6]. These data underscore the central role of TGF-β in the pathogenesis of bone metastases as well as the role of bone resorption as the driving source to provide active TGF-β in the bone microenvironment. Furthermore, bone sialoprotein (BSP) and osteopontin (OPN), two secreted glycoproteins regulated by TGF-β, play important roles in bone turnover and were found to be highly expressed in malignant prostate and breast cancer tissue and correlated with tumor grade. In these patients, serum and mRNA levels of BSP and OPN were identified to be prognostic indicators for bone metastases [100–103].

Hypoxia is observed in most solid tumors and is caused by reduced or inadequate oxygen supply [104]. Due to already low oxygen levels (1%–7% O_2) in the bone marrow microenvironment, hypoxia and increased expression of hypoxia inducible factor 1α (HIF-1α) are particularly observed in bone metastases [93, 105]. HIF-1α was shown to promote formation of osteolytic MDA-MB-231 bone metastases, by stimulating angiogenesis, osteoclastogenesis and inhibition of differentiation of osteoblasts [105]. Multiple interactions exist between hypoxia and TGF-β biology. TGF-β stabilizes HIF-1α by inhibiting its degradation [106], and additive responses in the induction of vascular endothelial growth factor (VEGF) and CXCR4 are observed in vitro [93, 106]. In the MDA-MB-231 bone metastasis model, inhibition of either pathway (using HIF-1α

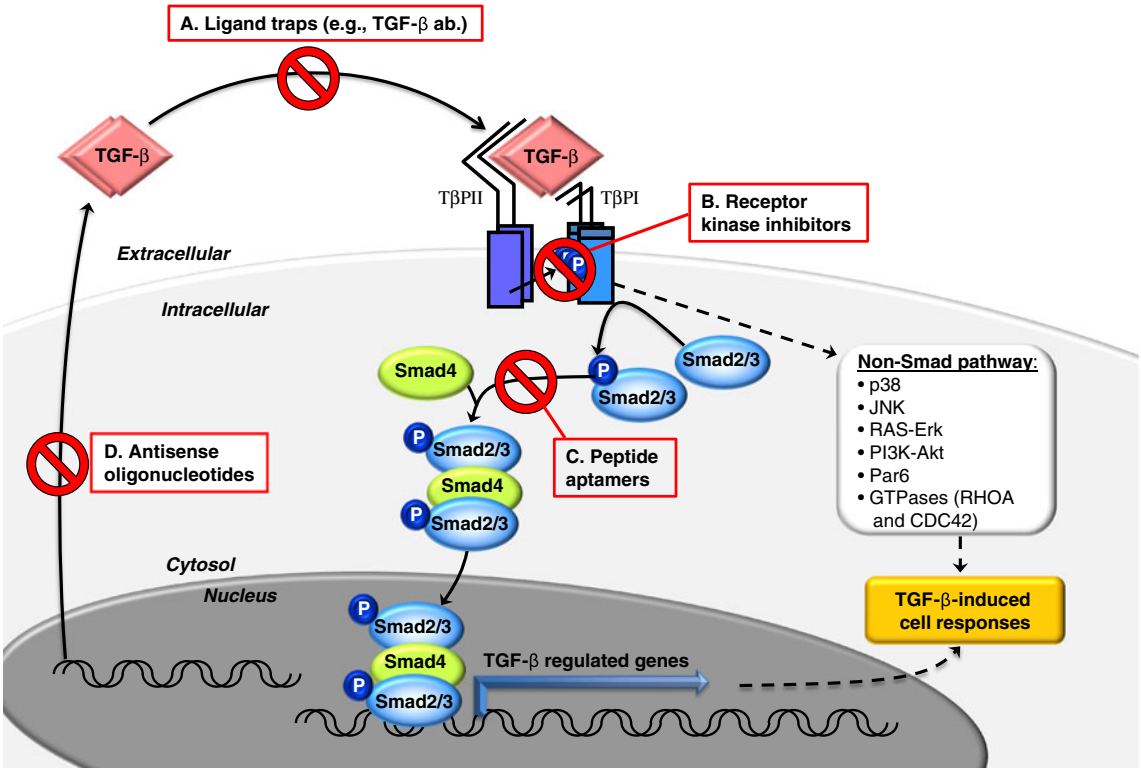

Fig. 3 Therapeutic strategies to target TGF-β in (pre)-clinical development. Several strategies to inhibit the TGF-β signaling have been pre-clinically tested, and are currently in clinical trials. The different classes of TGF-β inhibitors used include: **a** Ligand traps, including monoclonal neutralizing TGF-β antibodies and soluble decoy receptor proteins that bind and neutralize TGF-β ligand, preventing TGF-β ligand-receptor interactions; **b** Receptor kinase inhibitors, which are small molecules that inhibit TβRI (and TβRII) kinase activity, preventing the activation TGF-β R-Smads; **c** Peptide aptamers, which bind to and inhibit R-Smads, preventing complex formation with Smad4 and transcription of Smad-dependent TGF-β regulated genes; and **d** Antisense oligonucleotides, which inhibit TGF-β expression at transcriptional/translational level, preventing the production and release of TGF-β in microenvironment

knockdown or DNTβRII) in these cancer cells decreased the formation of bone metastases, while additive inhibitory effects were not observed when both pathways were blocked simultaneously [93]. In contrast, combined treatment with pharmacologic inhibitors of these pathways, targeting both the tumor and the bone microenvironment, decreased bone metastatic growth more than either treatment alone [93]. These data indicate that hypoxia and TGF-β signaling drive in parallel tumor bone metastases and that small molecule inhibitors, by acting on both tumor cells and the bone microenvironment, can additively decrease tumor burden [93].

Lu et al. further substantiated the important role of hypoxia in bone metastasis by showing that hypoxia stimulated the expression of CXCR4 and DUSP1 [107], two genes previously recognized among 11 genes that are most upregulated in MDA-MB-231 bone metastatic clones [7].

TGF-β as Therapeutic Target

As a result of its wide variety of effects, blockade of TGF-β or its signaling has provided therapeutic opportunities for the treatment of various different diseases, including fibrotic disease and cancer [9, 108, 109]. Many of these agents under development for disease indication other than breast cancer will be reviewed in this section as well, as these might be indicative of the potential tolerability and potential success of TGF-β-signaling antagonists in breast cancer patients with bone metastases. While many of the therapeutic agents targeting TGF-β or its signaling pathway have not moved beyond pre-clinical development, there are an increasing number of agents that are currently undergoing human clinical trials. The different classes of TGF-β inhibitors used include: (A) Ligand traps, including monoclonal neutralizing TGF-β antibodies and soluble decoy receptor proteins that trap TGF-β ligands thereby preventing TGF-β ligand-receptor interactions; (B) Receptor kinase inhibitors, which inhibit TβRI/ALK5 (and TβRII) kinase activity and prevent the activation TGF-β R-Smads; (C) Peptide aptamers, which bind to and inhibit R-Smads, preventing transcription of TGF-β regulated genes; and (D) Antisense oligonucleotides, which inhibit TGF-β expression at the transcriptional/translational level, thereby preventing the production and release of TGF-β in the microenvironment (Table 1; Fig. 3).

Table 1 Current pre-clinical and clinical compounds targeting TGF-β. BM = Preclinically tested for inhibitory effect on breast cancer bone metastases

Agent/ Target	Target	Company	Phase	Bone Mets	References
Trap Ligands					
Soluble decoy receptors					
sTβRII:Fc	TGF-$\beta_{1\text{-}3}$	Biogen	Pre-clinical		[110, 174]
sTβRIII (betaglycan)	TGF-$\beta_{1,3}$		Pre-clinical		[130]
P144	TGF-β_1	ISDIN /Digna Biotech	Pre-clinical		[131]
Antibodies					
1D11	TGF-$\beta_{1\text{-}3}$	Genzyme/CAT	Pre-clinical	BM	[175, 176]
2G7	TGF-$\beta_{1\text{-}3}$	Genentech	Pre-clinical		[113, 177]
Fresolumimab/GC1008	TGF-$\beta_{1\text{-}3}$	Genzyme	Phase I/II		[124]
Lederlimumab/CAT152	TGF-β_2	Genzyme/CAT	Phase I/II		[119]
Metelimumab/CAT192	TGF-β_1	Genzyme/CAT	Phase I/II		[120]
Antisense Oligonucleotides					
AP11014	TGF-β_1 mRNA	Antisense Pharma	Pre-clinical		[139]
Trabedersen (AP12009)	TGF-β_2 mRNA	Antisense Pharma	Phase II/III		[136, 178, 179]
Peptide Aptamers					
Trx-SARA	Smad3,4		Pre-clinical		[158]
Trx-xFoxH1b	Smad2,4		Pre-clinical		[157]
Receptor Kinase Inhibitors					
A-83-01	ALK5 (TβRI) + TβRII	Kyoto University	Pre-clinical		[75]
GW788388	ALK5 + TβRII	NextBio	Pre-clinical		[74]
IN-1130	ALK5	In2Gen	Pre-clinical		[180]
Ki 26894	ALK5	Kirin Brewery Company	Pre-clinical	BM	[146]
LY2109761	ALK5 + TGFRII	Eli Lilly & Co.	Pre-clinical	BM	[6, 154, 155]
LY2157299	ALK5	Eli Lilly & Co.	Phase I	BM	[156, 181]
LY364947	ALK5	Eli Lilly & Co.	Pre-clinical	BM	[147, 182]
LY550410	ALK5	Eli Lilly & Co.	Pre-clinical		[183]
LY580276	ALK5	Eli Lilly & Co.	Pre-clinical		[183]
SB-431542	ALK5	GlaxoSmithKline	Pre-clinical		[73, 184, 185]
SB-505124	ALK5	GlaxoSmithKline	Pre-clinical		[72]
SD-093	ALK5	Scios, Inc. / Johnson&Johnson	Pre-clinical		[148, 149]
SD-208	ALK5	Scios, Inc. / Johnson&Johnson	Pre-clinical	BM	[148, 150, 152, 186]
Sm16	ALK5	Biogen	Pre-clinical		[187, 188]
SM305	ALK5	Biogen	Pre-clinical		[189]
SX-007	ALK5	Scios, Inc. / Johnson&Johnson	Pre-clinical		[190]
Antibody targeting ALK1					
PF-03446962	ALK1	Pfizer	Phase I		[127]
Combined Vaccine/Antisense					
2G7 + IL-2	TGF-$\beta_{1\text{-}3}$ + IL-2	Genentech	Pre-clinical	BM	[191]
Lucanix™ (Belagenpumatucel-L)	TGF-β_2	NovaRx Corp	Phase II/III		[192, 193]
Glionix™	TGF-$\beta_{1,2}$	NovaRx Corp	Phase I		[9]
TGF-β_2 antisense	TGF-β_2 mRNA	Gradalis Inc	Phase I		[194]
+GMCSF expression vector	+GMCSF expression				
Other Molecules					
Antagonizing Effects of TGF-β					
Halufiginone	TGF-β effects	Collgard Biopharmaceuticals	Pre-clinical/ phase II	BM	[169]
BMP7	TGF-β effects	Stryker	Pre-clinical/ phase III	BM	[164, 166]

Neutralizing Antibodies and Soluble Decoy Receptor Proteins

TGF-β production by tumor cells and fibroblasts is often increased during cancer progression and in many types of cancer it has been shown that TGF-β expression is correlated with aggressiveness and grade/stage of the tumor [29, 35, 110, 111]. One strategy to reduce excessive amounts of TGF-β is to trap TGF-β ligand using soluble decoy receptors encompassing the ectodomains from either TβRII or TβRIII/betaglycan protein or via neutralizing TGF-β antibodies. The pan-neutralizing mouse monoclonal antibodies, 1D11 and 2G7, bind and reduce biological activity of all three TGF-β isoforms and have demonstrated therapeutic potential in mouse tumor models. While TGF-β overexpression in MCF-7 breast cancer cells could overcome estrogen-receptor dependence and promote tumorigenesis, treatment with the 2G7 antibody totally abrogated tumor growth [112]. In another study, treatment with 2G7 suppressed the growth of established MDA-MB-231 s.c. tumors and lung metastases in athymic mice by stimulating host natural killer cell function [113]. Similarly, treatment of mice with 1D11 following orthotopic injection of 4T1 breast cancer cells suppressed metastasis to lungs by sensitizing tumor cells for complement mediated lysis, inhibiting angiogenesis in the primary tumor, and strongly enhancing the CD8+ T-cell-mediated antitumor immune response [114–116]. Data from Biswas et al., presented in abstract form, also showed that 1D11 reduced skeletal tumor burden and osteolytic bone lesions caused by MDA-MB-231 cells in mice while also increasing the bone volume [117].

For use in patients, fully humanized TGF-β monoclonal neutralizing antibodies have been developed, including Lerdelimumab/CAT-152 [118, 119], Metelimumab/CAT-192 [120] and GC-1008 [9]. The TGF-β_2 neutralizing antibody Lerdelimumab was developed by Cambridge Antibody Technology (CAT) and has been successfully used in phase I/II clinical trials to reduce scarring after glaucoma surgery [121]. However, its development was stopped after unsuccessful subsequent clinical trial results. Metelimumab is a human TGF-β_1 antibody developed by CAT for the treatment of scleroderma. While Phase I/II clinical trials showed no significant adverse effects, Metelimumab clinical development was dropped from further development in favor of GC-1008/fresolumimab after subsequent unsuccessful clinical trial results [9]. GC-1008, currently being developed by Genzyme, is a pan-neutralizing antibody directed against all three isoforms of TGF-β, and completed phase I dose-escalation studies in 22 patients with renal cell carcinoma and metastatic melanoma who had failed at least one prior therapy [122–124]. No dose-limiting toxicities were observed in these patients. Intriguingly, among patients with malignant melanoma, one patient had a partial response, three had a mixed response and one had stable disease [124]. Phase I trials for the use of GC-1008 in focal segmental glomerulosclerosis and idiopathic pulmonary fibrosis have also been completed [125, 126]. In 16 patients with primary focal segmental glomerulosclerosis, single-dose levels of GC-1008 (up to 4 mg/kg) were well tolerated with pustular rash the only adverse event in two patients [125]. The half-life was determined ~14 days [125]. PF-03446962 generated by Pfizer, is an antibody directed against ALK1 and displays potent anti-angiogenic effects in vitro [127]. Currently, this antibody is in phase I clinical trials to evaluate optimal pharmacokinetic parameters for this agent in patients with advanced solid tumors [128].

Another approach to prevent binding of TGF-β to its receptors is the use of recombinant Fc-fusion proteins containing the soluble ectodomains of TβRII or TβRIII/betaglycan. Systemic administration of soluble TBRII:Fc in MMTV-PMT transgenic mice increased apoptosis in primary tumors, and reduced tumor cell motility, intravasation and lung metastases [110]. Similarly, transgenic mice that systemically expressed an antagonistic soluble type II receptor fragment were protected against experimental metastasis without any apparent side effects [31]. Ectopic expression of a truncated soluble ectodomain of betaglycan in human MDA-MB-231 breast cancer cells inhibited their metastasis-initiating capability [129]. Furthermore, treatment with the recombinant soluble ectodomain of betaglycan inhibited metastasis and angiogenesis in the MDA-MB-231 xenograft model as well [130].

The TGF-β antagonistic Peptide 144 (P144) has recently been generated by using the betaglycan sequence and 15-mer phage display technology. P144 blocked TGF-β signaling and displayed potent inhibition of angiogenesis in several in vitro assays [131]. Phase I clinical trials have been completed and P144 is currently in phase II clinical trials sponsored by ISDIN in collaboration with DigNa Biotech, to assess efficacy and safety of topical application of P144 in the treatment of skin fibrosis in patients with systemic sclerosis [132].

Antisense Oligonucleotides

Another feasible therapeutic strategy to minimize excessive levels of TGF-β in the local tumor microenvironment is to reduce TGF-β synthesis and secretion by using antisense oligonucleotides (ASOs). ASO's are single-stranded polynucleotide molecules 13–25 nucleotides in length that are designed to hybridize to complementary RNA sequences. ASO's inhibit mRNA function and protein synthesis through modulation of splicing and inhibition of translation by disrupting ribosome assembly. They accelerate mRNA degradation by RNase H. RNase H

cleaves the mRNA strand and leaves the ASO intact allowing it to continue to bind to other targets [133, 134]. Several limitations need to be considered when using ASO's, including the stability, RNA binding affinity, efficiency of delivery to the target cells, and associated non-specific effects.

So far, numerous studies have demonstrated the potential of TGF-β ASO's. To address the role of autocrine TGF-β in metastasis formation in breast cancer, Muraoka-Cook et al. used a model of orthotopic implantation of PyMT mammary tumors [111]. While PyMT tumors overexpressing TGF-β resulted in increased metastasis and survival, overexpression of a TGF-β ASO reduced metastasis and survival, suggesting that at least in part, autocrine TGF-β produced by cancer cells might be responsible for these actions [111].

Antisense Pharma has developed AP-12009 (Trabedersen), an ASO specific for the mRNA of human TGF-β_2. AP 12009 was well tolerated in its first three clinical Phase I/II-studies for the treatment of patients with recurrent or refractory malignant (high-grade) glioma [135]. In a phase IIB study, the efficacy and safety of two intratumoral treatment doses (10 and 80 μM) with AP 12009 were tested and compared to standard chemotherapy (temozolomide) in patients with recurrent/refractory glioblastoma multiforme or anaplastic astrocytoma [136]. Superior efficacy and safety for the lower dose AP-12009 over the higher dose and chemotherapy were observed and the positive risk-benefit assessment suggests this lower dose as the optimal dose for further clinical development in high grade glioma [136]. Currently, patients with anaplastic astrocytoma are being recruited for a phase III study to evaluate the efficacy and safety of intratumoral treatment with AP-12009 compared with temozolomide [137]. In addition, a phase I dose-escalation clinical study is currently ongoing to evaluate the safety and tolerability of i.v. administration of AP 12009 in patients with advanced tumors known to overproduce TGF-β_2, such as melanoma, pancreatic and colorectal carcinomas [138]. Antisense Pharma also has an ASO for TGF-β_1 (AP 11014) that is currently tested in preclinical studies for the treatment of non-small-cell lung, colorectal and prostate cancers [139].

TGF-β ASO's can also be used to generate ASO-modified tumor vaccines. In an early study by Fakhrai et al., gliomasarcoma cells were genetically modified to express TGF-β_2 ASO to block TGF-β_2 expression [140]. Rats with established intracranial gliomas were then immunized s.c. with these modified 9L gliosarcoma cells and showed reduced TGF-β-mediated immune suppression, resulting in tumor rejection of the intracranial tumor and increased survival [140]. In an early-phase clinical trial, the effects of TGF-β ASO modified tumor cells and vaccine regimen (NovaRx) were tested in patients with grade IV astrocytoma. It was well tolerated and resulted in improved survival and enhanced cellular antitumor immunity in some patients [141].

Recently, Gradalis Inc. is sponsoring a phase I clinical trial for the treatment of advanced metastatic carcinoma using a combination of a TGF-β_2 ASO and a granulocyte/ macrophage colony stimulating factor (GM-CSF) expression vector plasmid. While GM-CSF transgene is expected to directly stimulate the expression of tumor antigens and enhance dendritic cell migration, the TGF-β_2 ASO should reduce immune inhibiting activity at the vaccine site. Both agents in separate trials, have demonstrated similar beneficial effects without any evidence of significant toxicity in advanced cancer patients [142].

Receptor Kinase Inhibitors

While ligand traps and ASOs limit the bio-availability of active TGF-β ligands to directly interact with its cognate receptor, TGF-β receptor kinase inhibitors are a group of small molecule inhibitors that act via ATP-competitive inhibition of the kinase catalytic activity of TβRI/ALK5 upon its recruitment, phosphorylation and activation by TGFβ-bound TβRII. While there may be several advantages to the development and scalability of small molecule inhibitors including potentially optimized pharmacokinetic/ pharmacodynamic properties, the potential lack of selectivity of kinase inhibitors continues to be a challenge. Currently, all known small molecule TβR1/ALK5 inhibitors described in the literature to date, where tested, display equipotent inhibition against ALK4 kinase activity and less inhibition against ALK7 [73–75, 143, 144]. Interestingly, activin(s) have been shown to require ALK4 for their signaling and recent animal studies using activin-inhibiting soluble extracellular domain ACVR2A-Fc fusions also display significant bone formation activities in various animal models [76, 77]. Whether the combined inhibition of TGF-β and activin signaling by ALK4/5 inhibitors is advantageous for the treatment of breast cancer remains to be determined.

SB-431542 (GlaxoSmithKline) is a small molecule inhibitor of TβRI/ALK5, activin type I receptor, and nodal type I receptor [73]. SB-431542 blocks TGF-β-regulated gene expression of fibronectin and collagen in renal epithelial carcinoma cells [145] and inhibits the proliferation of human osteosarcoma and glioma cells [145]. Ki26894, a TβRI/ALK5 kinase inhibitor developed by Kirin Brewery Company (Gunma, Japan), was shown to block TGF-β signaling, invasion and motility in MDA-MB-231-D bone tropic cells, including suppression of PTHrP and IL-11 [146]. Systemic treatment with Ki26894 initiated 1 day before intracardiac inoculation of MDA-MB-231-D into nude mice results in decreased bone metastasis and prolonged mouse survival [146]. Similarly, the TβRI/ALK5 kinase inhibitor [3-(pyridine-2yl)-4-(4-

quinonyl)]-1H pyrazole/ LY364947 also reduced the formation of early bone and lung metastases in a xenograft model using intracardiac injection [147].

SD-208 and SD-093 (Scios Inc) are TβRI/ALK5 kinase inhibitors that were shown to inhibit migration, invasion and EMT in murine mammary epithelial (NMuMG) and carcinoma (4T1, R3T) cells [148]. SD-093 has been shown to inhibit TGF-β-induced Smad2 phosphorylation, invasion and EMT in pancreatic carcinoma cells [149] as well. SD-208 is orally active and has been used as a treatment in a wide variety of animal models to treat different type of cancers. Treatment with SD-208 blocked the biological effects of TGF-β_1 and TGF-β_2 in glioma cells and prolonged the median survival of SMA-560 glioma-bearing mice [150]. In an orthotopic xenograft model of pancreatic adenocarcinoma, SD-208 attenuated growth and metastasis of established tumors [151]. In syngeneic models, treatment with SD-208 inhibited the growth and metastasis of R3T or 4T1 mammary carcinoma tumor bearing mice. Dosing with SD-208 inhibited primary tumor growth as well as number and size of metastases in these mice [148]. In a xenograft model of intracardiac injected MDA-MB-231 human breast cancer cells in the left heart ventricle of athymic mice, preventive treatment with SD-208 significantly inhibited the size of osteolytic lesions, bone metastatic growth and survival. Furthermore, SD-208 treatment in mice with already established bone metastases inhibited further tumor growth and formation of osteolytic lesions [93]. In a similar xenograft model using PC3 human prostate cancer cells, preventive SD-208 treatment was also able to inhibit bone metastatic growth and formation of osteolytic lesions [152]. However, SD-208 treatment was unable to inhibit tumor growth in bone of LuCaP23.1 prostate cancer cells that form osteoblastic bone metastases [152]. TGF-β blockade may not be effective in osteoblastic bone metastases where tumor cells already reciprocally interact with and highly stimulate osteoblasts [153]. Although pre-clinical results in animal studies using SD-208 appear promising, no plans for clinical development have been forthcoming (Johnson & Johnson/Scios Inc.)

LY2109761 (Eli Lilly & Co.), described as an inhibitor of both the TβRI/ALK5 and TβRII, was shown to inhibit the formation of metastases in several models, including colon [154], breast [6] and pancreatic [155] cancer. Using an engineered xenograft model system with conditional control of the TGF-β-Smad signaling pathway and a dual-luciferase reporter to measure metastatic burden and TGF-β signaling status in mice, it was shown that preventive treatment with LY2109761 results in significantly less TGF-β signaling and a concomitant reduction in the number of bone metastases. Interestingly, the same effect was observed with the anti-resorptive bisphosphonate pamidronate. However, when either LY2109761 or pamidronate were given in a therapeutic setting to mice where bone metastases were already established (21 days after intracardiac injection MDA-MB-231 cells), TGF-β signaling was still inhibited, but tumor progression was not significantly affected [6]. In contrast, SD-208 was able to inhibit the growth of already established MDA-MB-231 bone metastases in a similar type of xenograft model [93], suggesting that SD-208 may be either more potent or mechanistically distinct vs. LY2109761 in inhibiting TGF-β signaling in vivo.

LY2157299, also being developed by Eli Lilly & Co, is a recently described TβRI/ALK5 kinase inhibitor that reduced primary tumor growth induced by the Calu6 non-small lung cancer line and the MX1 breast cancer line in nude mice. LY2157299 entered phase I clinical trials to determine the safety and pharmacokinetics in patients with metastatic malignancies who had exhausted current standard of care. Daily doses of 40 mg and 80 mg LY2157299 has currently been shown to be well tolerated in patients with various cancer types [156].

Peptide Aptamers

Another innovative strategy is targeting intracellular TGF-β signaling molecules, such as Smad molecules. Potentially, this approach provides targeting of only very specific TGF-β responses. Aptamers are small peptide molecules designed to bind to a target and interfere with its function. They are constructed with a target binding domain and a scaffolding domain that stabilize the molecule. Several peptide aptamers have been designed to bind to Smad2 and Smad3, disrupting the binding of Smad4 and hence, TGF-β signaling. The Trx-xFoxH1b aptamer uses a thioredoxin A scaffold and an xFoxH1b transcriptional domain that binds to the Smad2/4 heterocomplex. This aptamer has been shown to significantly reduce TGF-β-induced expression from a FoxHl-dependent reporter gene [157]. The Trx-SARA aptamer specifically bound to Smad2 and Smad3 and reduced the levels of Smad2 and Smad3 complexed with Smad4 after TGF-β_1 stimulation, and consequently inhibited TGF-β-induced reporter gene expression and EMT in NMuMG murine mammary epithelial cells [158].

Other Molecules that Antagonize TGF-β

Numerous additional biologically active molecules that not necessarily directly bind and inactivate TGF-β, its receptor or its signaling molecules, may still antagonize the effects of TGF-β. Here, two examples of molecules that antagonize TGF-β will be shown that have been extensively tested in breast cancer bone metastasis models.

BMP7 is another member of the TGF-β superfamily and signals via different type I and II receptor kinases activating the R-Smads, Smad1, -5 and −8 [159, 160]. It has long been recognized that BMP7 can counteract TGF-β-induced EMT, and induce the opposite process, a mesenchymal-to-epithelial transition (MET) in embryonic kidney and eye development [161–163]. More recently, it was demonstrated that BMP7 could also counteract the TGF-β-induced EMT in breast and prostate cancer. In addition, forced overexpression or systemic administration of BMP7 inhibited the formation of bone metastases from osteolytic breast and prostate cancer [98, 164–166]. It is tempting to speculate that BMP7, by counteracting TGF-β-induced EMT, may act as a differentiation-inducing agent that targets the CSC fraction in breast and prostate cancer. More research is certainly warranted to address whether BMP7 is a valid novel approach in the treatment of breast and prostate cancer. As a potent inducer of bone formation, BMP7 is currently approved for clinical use in open fractures of long bones, non-unions and spinal fusion [167].

Halofuginone (Hfg) is a natural product derivative that is known to inhibit TGF-β signaling. It recently completed phase II clinical trials for the treatment of sarcoma [168]. Unpublished data from our laboratory show that Hfg inhibits TGF-β signaling in vitro in several cell types, and that systemic daily treatment of Hfg in mice significantly inhibits the formation of osteolytic lesions and bone metastases after intracardiac inoculation of MDA-MB-231 breast and PC3 prostate cancer cells [169]. Although the exact mechanism remains to be investigated, Hfg treatment represents a novel agent to inhibit TGF-β signaling in bone metastasis.

Combination Therapy

An attractive approach to increase treatment efficacy for patients with bone metastases is to combine treatments that antagonize the effects of TGF-β with other therapies. For example, targeting TGF-β signaling can enhance the therapeutic efficacy of various cytotoxic agents as was recently shown for rapamycin [170] and doxorubicin [171, 172]. Unpublished studies in our laboratory show that SD-208 dosed in combination with an inhibitor of bone resorption, zoledronic acid, reduces the progression of established osteolytic metastases from breast cancer more effectively than either therapy alone [173]. Using the same bone metastasis model of intracardiac inoculation of MDA-MB-231 breast cancer cells, we tested the effects of a combined treatment of SD-208 and 2-methoxyestradiol, an inhibitor of HIF-1α, the key mediator of hypoxia. Combined treatment with these agents reduces osteolytic lesions, tumor burden and improves survival of mice more effectively than either treatment alone [93].

Risks, Limitations and Opportunities

As a result of its biological importance and wide variety of effect, blockade of TGF-β or its signaling provides intriguing therapeutic opportunities for the treatment of many different disease indications. However, potent and/or chronic inhibition of this wide-spread biologically important molecule may also potentially result in a variety of undesirable side effects.

Potential Risks

Knock-out mice for TGF-β_1 display reduced numbers of regulatory T-lymphocytes, uncontrolled activation of the immune system and loss of immune tolerance resulting in generalized inflammation and autoimmunity [195–198]. TGF-β blockade may also paradoxically increase the risk of tumorigenesis [199, 200], inhibit wound healing [201], and is likely to be teratogenic to the fetus [202]. However, severe toxicity has not been observed when TGF-β is blocked in animal models. Lifetime expression of a soluble TβRII, under the regulation of the mammary-selective MMTV-LTR promoter, protected against metastasis without any obvious adverse side effects [31]. In addition, chronically administering the TGF-β neutralizing antibody 1D11 showed minimal effects on immune parameters and no evidence of increased inflammation in any of the peripheral tissues examined [203]. In contrast to the severe chronic deficiency of TGF-β observed in knockout mouse models, neutralizing antibodies and soluble receptor decoys typically achieve only a partial deficiency of TGF-β, and may only interfere with the excessive TGF-β activity observed in the setting of cancer without significantly altering normal homeostatic TGF-β signaling. The first human dose escalation studies with the small molecule TGF-β inhibitor, LY2157299, in patients with advanced metastatic malignancies like melanoma, colon cancer, prostate cancer, adrenal gland cancer and breast cancer demonstrated that the drug is well tolerated and no drug-related grade 3 or 4 toxicities were observed [156]. Additional clinical studies are underway in patients with bone metastasis and should provide useful information on possible toxicities of the various agents described in this review. For the small molecule TβRI/ALK5 kinase inhibitors, it may be challenging to dissect TGF-β-dependent toxicity from the off-target effects of these compounds. Biologic-based inhibitors may possibly be more indicative of true TGF-β-dependent toxicities due to their highly selective nature. Of note, in a clinical trial testing a TGF-β antibody from Genzyme, one patient developed premalignant skin lesion. However, this effect was reversible upon discontinuation of the drug. Clinical trials with the Antisense Pharma compound AP12009 showed very good safety and tolerability upon intratumoral treatment of patients

with high grade glioma [136]. The use of anti-TGF-β_2 antibodies and ASO for reduction of scarring after postoperative ocular surgery was also well tolerated with no obvious adverse side effects [204].

The effects of blocking TGF-β on angiogenesis can be dependent on what type of inhibitor is used. TGF-β can indirectly stimulate angiogenesis by upregulating tumor production of VEGF, which can be blocked with treatment with TGF-β antibodies, TGF-β ASO or TβRI/ALK5 kinase inhibitors. Interestingly, while TGF-β antibodies and TGF-β ASO block both the pro-(TGF-β/ALK1-mediated) as well as the anti-(TGF-β/ALK5-mediated) angiogenic effects in endothelial cells, TβRI/ALK5 kinase inhibitors block only the anti-angiogenic effects in endothelial cells. A, several studies have demonstrated that treatment with TβRI/ALK5 kinase inhibitors can even promote angiogenesis [22, 205, 206]. Therefore, the therapeutic strategies to target TGF-β must be carefully considered.

Limitations and Opportunities

It is imperative to keep in mind that the different therapeutic strategies to target TGF-β described in this review have fundamentally different properties regarding to mechanism of action, pharmacokinetic properties and delivery challenges. Besides specificity, the main challenge with ASO has been its mode of administration, which so far has been overcome for AP12009 in brain tumors with continuous intracerebral and intrathecal infusion [135, 207]. Drug delivery challenges may also be faced with TGF-β antibodies and other large molecules such as TβRII/III:Fc fusion proteins. The generation of small molecules such as TGF-β receptor kinase inhibitors overcome the necessity of injectable delivery, loss of efficacy due to neutralizing antibody generation and/or tissue penetration issues commonly observed with biologic-based agents [9]. Moreover, most of them are suitable for oral dosing. The list of newly generated TGF-β receptor kinase inhibitors has steadily grown resulting in over 20 patent applications in the last 5 years alone [208]. The wide variety in structures and binding modes may eventually result in selective kinome profiles that may prove safe and effective for their designed purpose. [9, 208]. However, TGF-β receptor kinase inhibitors used so far are less selective than the current TGF-β ASO's or biologic-based TGF-β-directed therapies. For example, the TβRI/ALK5 small molecule kinase inhibitors can also potently inhibit ALK4 and less so ALK7 due to their very close catalytic domain homology to TβRI/ALK5 [182]. Furthermore, it is anticipated that the TGF-β receptor kinase inhibitors may have considerable shorter durations off-target exposure/activity due to inferior pharmacokinetic profiles when compared to long lived systemic TGF-β antibodies [9].

Although speculative at present, some of the tumor-inhibiting effects of anti-TGF-β compounds described in this review may also be contributed to inhibitory effects on breast CSCs. To this end, it was shown as proof-of-principle that treatment with the TβRI/ALK5 inhibitor LY2109761 inhibited TGF-β signaling in $CD44^{+}/CD24^{-/low}$ breast CSC-like cells and reversed their mesenchymal stem cell-like phenotype to a more epithelioid $CD44^{-/low}/CD24^{+}$ phenotype [55]. More research will be needed to further understand the importance of the currently controversial CSC hypothesis and their contribution to tumor behavior and chemotherapeutic resistance.

Another promising novel approach to overcome off-target tissue toxicity and poor drug exposure to tumor cells in bone metastatic disease are the use of bisphosphonate-coated liposomes, which may be useful as a targeting device to sites of high bone turnover, including sites with bone metastatic disease [209]. Potentially, these bone-targeted liposomes may allow for a more prolonged local exposure to higher concentrations of the bioactive compounds described in this review, thereby enhancing therapeutic efficacy and minimizing systemic side effects. Additionally, these bioactive compounds of interest could be delivered to bone metastatic sites in combination with other anti-cancer agents with synergistic or mechanistic action.

Evidence is accumulating that Smad3 is a tumor promoter, while Smad2 has mainly a tumor suppressive function [210]. Therefore, molecules such as peptide aptamers that specifically target Smad3 may hold great promise as novel therapeutic strategies to target the tumor-promoting effects of TGF-β, without affecting other pathways regulated by TGF-β [156, 157]. However, the effects of most of these compounds will first need thorough validation in in vitro and in vivo models.

Immunohistochemical studies have demonstrated that increased expression of TGF-β_1 and phosphorylated Smad2/3 in clinical samples from primary tumors are often correlated with a high incidence of metastasis [211, 212]. However, immunohistochemical analyses only provide static snapshots of TGF-β signaling activity and fail to provide any information on the eventual gene expression changes induced by TGF-β. It is possible that mutations found downstream of the Smad proteins may not allow for appropriate TGF-β-induced gene responses. To circumvent this limitation, Padua et al. applied bioinformatic tools to define a TGF-β response (TBRS) that can be used to more accurately assess the TGF-β response status of human breast tumors [213]. Analysis of clinical samples revealed that ~40% of human breast tumors responded actively to TGF-β signals. In addition, a validated signature of TGF-β upregulated genes is clearly associated with poor prognosis subsets of basal-like- and Her2-positive breast cancers [39].

These findings suggest that the presence of a TBRS might drive tumor progression in estrogen-independent cancer, but may reflect a suppressive host cell response in estrogen-dependent luminal cancers. Taken together, patient stratification based on the presence of the TBRS signature in primary tumor samples might help identify a rationally tailored subset of patients that are likely to respond to anti-TGF-β therapies.

Conclusion

TGF-β is a pluripotent cytokine with a prominent role in breast cancer progression and bone metastasis. TGF-β is a major component of the bone, and a central mediator in driving a feed-forward cycle of tumor growth in bone. This has provided a powerful rationale to develop agents for the purpose of inhibiting TGF-β activity to prevent and/or treat bone metastases arising from primary breast cancers. Currently, three therapeutic modalities targeting TGF-β have been pursued and are presently being tested in clinical trials in cancer patients (incl. bone metastatic disease): TGF-β antibodies, TGF-β receptor kinase inhibitors and TGF-β antisense oligonucleotides (Table 1). All three modalities have fundamentally different pharmacokinetic/pharmacodynamic properties and mechanisms of actions, providing a broad range of limitations and/or opportunities, particularly with respect to specificity and toxicity. TGF-β has many other functions in normal physiology, and may also act as a tumor suppressor in certain malignancies. Therefore concerns will remain that long-term blockade of this pathway may have other off-target effects. One possible benefit is that blockade of TGF-β signaling may increase bone mass in cancer patients. This may be particularly beneficial in a breast cancer patient population that may be receiving or has previously received a therapeutic regime including radiation- or chemo-therapy resulting in significant bone loss. However, concerns about immune function and growth promoting effects remain. The next decade promises new and exciting clinical data that will determine which TGF-β therapeutic strategies are most effective, alone or in combination with other therapies, for the treatment of patients suffering from breast cancer to prevent and/or cure bone metastatic disease with minimal side effects.

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