REVIEW

The role of TGF-β in bone metastasis: novel therapeutic perspectives

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The skeleton is a preferred site for cancer metastasis. These bone metastases cause dysregulated bone remodeling and the associated morbidity of fractures, pain, hypercalcemia and catastrophic nerve compression syndromes. Transforming growth factor-β (TGF-β) is stored in mineralized bone matrix, and released and activated by osteoclastic bone resorption. Once activated, TGF-β stimulates nearby metastatic tumor cells within the bone microenvironment to secrete factors that further drive osteolytic destruction of the bone. Therefore, TGF-β and its signaling constitute a critical component driving the feed-forward vicious cycle of cancer growth in bone. Moreover, additional pro-tumorigenic activities attributed to TGF-β include activation of epithelial-to-mesenchymal transition, increased tumor cell invasion, enhanced angiogenesis and various immunomodulatory properties. Blocking the TGF-β signaling pathway to interrupt this vicious cycle and manipulate the bone microenvironment offers a promising area for therapeutic intervention to decrease skeletal metastasis and normalize bone homeostatic mechanisms. In this review, preclinical and clinical data are evaluated for the potential use of TGF-β pathway inhibitors in clinical practice to treat bone metastases and its associated comorbidities.

Introduction

The skeleton is a preferred site for cancer metastasis. Up to 80% of patients with advanced breast or prostate cancer will develop bone metastases as will 30–40% of patients with lung, renal or thyroid cancer.[1,2] This devastating complication of cancer causes bone pain, fractures, hypercalcemia and nerve compression syndromes severely diminishing the quality of life.[3,4] Bone metastases are classified as osteolytic or osteoblastic, based on radiographic appearance. Solid tumors such as breast, lung and renal cancer are typically associated with osteolytic lesions as is the hematologic malignancy multiple myeloma while prostate cancer is associated with osteoblastic bone metastases. Despite these classifications, most patients with solid tumor metastases to bone have components of both bone destruction and new bone formation. Multiple myeloma differs in that it is almost always associated with profound bone destruction and suppressed bone formation.

Perhaps the most devastating consequence is that once cancer metastasizes to bone, it is incurable. Current standard of care to treat bone metastases include antiresorptive therapy to decrease skeletal morbidity; clearly beneficial, but without regression of disease or cure.[3,4] 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 preventing or curing bone metastases.

The bone microenvironment is unique and provides a special milieu that metastatic cancer cells can colonize. The mineralized bone matrix is embedded with abundant growth factors and cytokines during the bone formation phase, such as transforming growth factor-β (TGF-β), activins and insulin-like growth factors, which are released and activated upon tumor-induced osteoclastic bone resorption.[5] High local levels of active TGF-β cause increased invasion, chemotaxis, angiogenesis and immunomodulation. In addition, TGF-β stimulates tumor production of osteolytic factors that further stimulate bone resorption (**Figure 1**).[6,7] 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.

TGF-β

TGF-β is a ubiquitously expressed, pluripotent cytokine that controls tissue homeostasis by regulating cellular processes such

as apoptosis, proliferation and differentiation.[8] Almost all cells secrete TGF-β and express TGF-β receptors. Therefore, it is not surprising that dysregulation of TGF-β actions has been associated with many disorders, including impaired wound healing, chronic fibrosis, cardiovascular diseases and cancer.[9,10] The pluripotent nature of TGF-β and its ubiquitous expression provides both opportunities and challenges to neutralize its effects.

TGF-β structure and signaling. Three highly homologous isoforms of TGF-β have been described in humans, TGF-β_1, TGF-β_2 and TGF-β_3. The signaling of these isoforms is comparable but the messenger RNA (mRNA) expression levels and/or protein presence differ across various tissues.[11] Generally, all of the 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) (**Figure 2**).[12] The activated TβRI/ALK5 complex can then phosphorylate the receptor-associated Smads (R-Smads), Smad2 and Smad3.[13] These activated R-Smads form a stable heterodimeric complex with the common mediator Smad, Smad4, and translocate to the nucleus.[12,13] Ultimately, the transcriptional outcome on their target genes depends on a host of transcriptional partners (co-activators or corepressors) that Smads interact with. This repertoire of Smad coregulatory proteins is variable, based on the cell type and physiological response being regulated by TGF-β in each cellular niche.

Non-canonical TGF-β signaling can also activate non-Smad signaling pathways including extracellular signal-regulated kinases (ERK-1, ERK-2 and p38), c-Jun amino-terminal kinase and mitogen-activated protein kinases (MAPKs) in various cell types[14] (**Figure 2**).

TGF-β in Bone Homeostasis

Adult bone is continuously remodeled by the coordinated and balanced activities of bone-resorbing osteoclasts and bone-forming osteoblasts. TGF-β_1 is one of the most abundant growth factors found in bone matrix,[15,16] and the effects of TGF-β on osteoblast, osteoclasts and bone remodeling are both temporal-dependent, spacially and context-dependent.[17]

Significant evidence has demonstrated that TGF-β is a key mediator in the dynamic coupling of bone resorption and bone formation.[18–20] Osteoblasts secrete predominantly TGF-β_1, where it is embedded as a latent form into the mineralized bone matrix.[17] Osteoclastic bone degradation releases and activates TGF-β, which will result in recruitment of osteoblast precursors to sites of bone resorption.[18–20] The exposed bone mineral matrix and release of osteotropic factors, such as bone morphogenetic proteins (BMPs), insulin-like growth factor-I and -II, and platelet-derived growth factor, may then promote differentiation of the osteoblast precursor to osteoblasts.[21] In later phases of osteoblastic differentiation, TGF-β has been shown to block osteoblast differentiation, impede bone mineralization[22,23] and induce osteoblast survival during transdifferentiation into osteocytes.[24]

Although TGF-β appears to have complex biphasic effects in osteoclastogenesis and bone resorption *in vitro*,[25,26] studies with genetically modified mice showed that increased levels of TGF-β in bone microenvironment promoted osteoclastogenesis
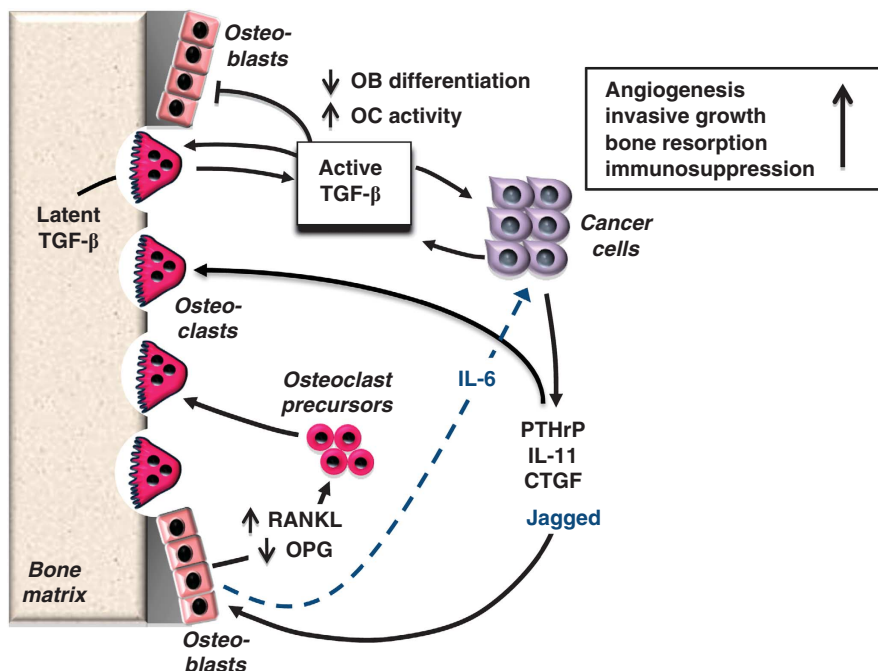

Figure 1 TGF-β in osteolytic bone metastasis. The skeleton is a preferred site for cancer metastasis. In these skeletal metastases, TGF-β is released by osteoclasts from the bone matrix and acts on cancer cells to stimulate the production of osteolytic factors, such as PTHrP, connective tissue growth factor (CTGF) and IL-6 and -11. These factors increase the RANKL/OPG expression ratio in bone stromal cells such as osteoblasts, resulting in osteoclastogenesis. Moreover, active TGF-β stimulates Jagged1 expression in cancer 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. In aggregate, the effects of TGF-β perpetuate the feed-forward cycle to increase tumor growth in bone.

and bone resorption, and inhibited osteoblast differentiation and bone formation.[27,28]

In support of these data, it has been shown recently that systemic TGF-β inhibition—either by using the orally active TβRI/ALK5 kinase inhibitor, SD-208,[29] or the highly selective pan-neutralizing TGF-β antibody, 1D11—resulted in increased trabecular bone volume. This was accompanied by enhanced osteoblast differentiation and subsequent bone formation as well as reduced osteoclast formation and bone resorption.[29]

TGF-β in Cancer

TGF-β has an essential role in maintaining physiological 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 within the various cellular microenvironment niche(s).[30,31] However, many cancers often become refractory to this growth inhibition either due to genetic loss of TGF-β signaling components or, more commonly, because of downstream perturbation by other integrated signaling pathways.[32] During this time, the pro-tumorigenic actions of TGF-β may prevail, including immunomodulatory properties, induction of angiogenesis and/or promotion of the epithelial-to-mesenchymal transition (EMT) facilitating cancer migration and invasion (reviewed in refs [33–35]). When TGF-β-suppressive effects are lost, TGF-β overproduction and signaling are also commonly observed in many solid tumors.[36,37]

TGF-β in Bone Metastasis

Metastasis to bone is a multistep process of events.[3,4,38] First, cancer cells must detach from the primary tumor, enter into the systemic lymphatic or vascular circulation (intravasation), evade the immune system, home and arrest in the bone marrow capillaries or sinusoids, extravasate into the bone marrow and form a micrometastasis. Eventually, some micrometastases may grow into overt bone metastatic lesions.

Numerous studies have shown the importance of the TGF-β signaling pathway for the development of osteolytic bone metastases (**Figure 1**). Yin *et al.*[7] were the first to demonstrate that blocking TGF-β signaling by stably expressing a dominant-negative TβRII (DNTβRII) in MDA-MB-231 breast cancer cells inhibited TGF-β-induced expression of parathyroid hormone-related protein (PTHrP) production in tumor cells and suppressed the formation of osteolytic bone metastases in a mouse model. *GLI2* appeared to be a TGF-β/Smad target gene[39,40] that appears, independent of the canonical Hedgehog, to be one of the important mediators in the upregulation of PTHrP.[41,42] In addition, PTHrP is also increased via Smad-independent pathways (p38 MAPK and to a lesser extend TGF-β activated kinase).[43,44]

PTHrP has a major role in the development of the osteolytic features of bone metastatic lesions,[45] and is considered to be responsible for the humoral hypercalcemia of malignancy syndrome (reviewed in refs [46,47]). PTHrP stimulates osteo-
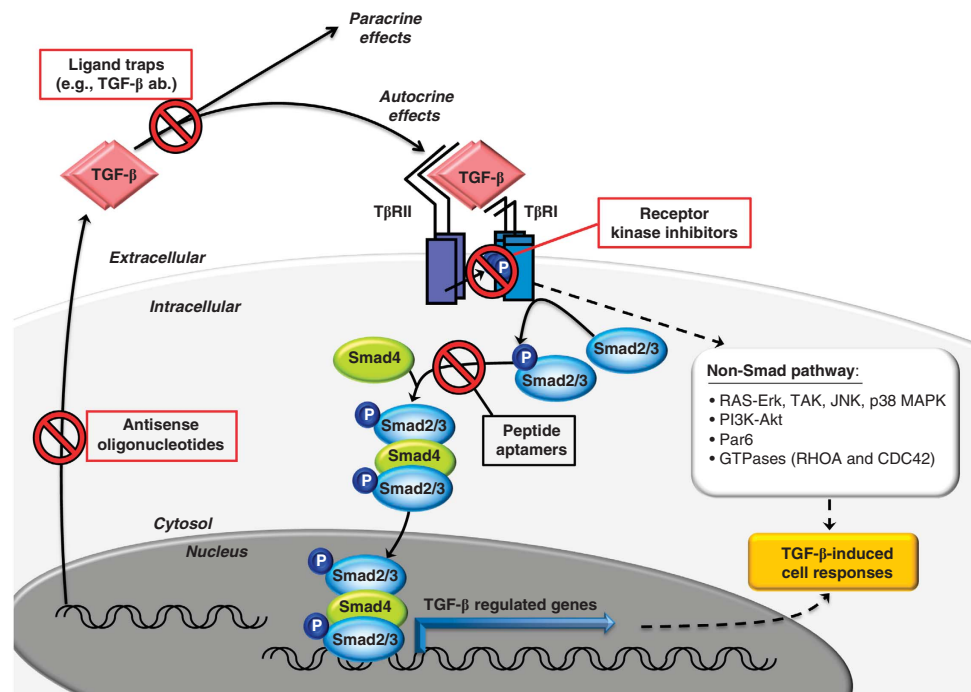

Figure 2 The TGF-β signaling pathway and several therapeutic strategies to intervene. Upon binding of active TGF-β to the TGF-β type I (ALK5) and type II (TβRII) receptor, ALK5 is phosphorylated and activated by TβRII. ALK5, in turn, phosphorylates and activates the R-Smads, Smad2 and Smad3, which form a complex with Smad4. This complex translocates to the nucleus, binds 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. Several strategies to inhibit the TGF-β signaling have been preclinically tested, and are currently in clinical trials (see red boxes). Three of these classes of TGF-β inhibitors are tested in the clinic and include the following: ligand traps, including monoclonal-neutralizing TGF-β antibodies that bind and neutralize TGF-β ligand, preventing TGF-β ligand–receptor interactions; receptor kinase inhibitors, which are small molecules that inhibit TβRI (and TβRII) kinase activity, preventing the activation TGF-β R-Smads; and antisense oligonucleotides (ASOs) which inhibit TGF-β expression at transcriptional/translational level, preventing the production and release of TGF-β in microenvironment. Peptide aptamers are a class of TGF-β inhibitors that are not yet tested *in vivo*. They can specifically bind to R-Smads of interest and prevent complex formation with Smad4 offering targeting of specific TGF-β responses.

clast activation by inducing receptor activator of nuclear factor-κB ligand and downregulating osteoprotegerin in cells of the osteoblast lineage.[48] In breast cancer, 90% of metastases in bone were found to express PTHrP, compared with only 17% at non-bone sites and 60% of the primary tumors.[49,50] A large prospective study demonstrated that PTHrP expression in primary breast cancer was significantly associated with fewer (bone) metastases.[51] Therefore, the most likely explanation for the observed increased PTHrP expression in breast cancer bone metastases[49,50] is that factors in the bone microenvironment, such as TGF-β, induces cancer cells to express PTHrP rather than the possibility that primary breast cancer cells that express PTHrP have a greater propensity to metastasize to bone.

Transcriptional profiling of various selected subpopulations of human breast cancer cells in a mouse model of MDA-MB-231 bone metastases further illustrated the complexity of bone metastases pathophysiology. Using serial passaging of MDA-MB-231 cells, Kang *et al.* isolated clones that cause more aggressive bone metastases when compared with its parental line. Microarray analysis identified a number of genes that were selectively upregulated in these aggressive bone metastatic clones. Many of these proteins, including interleukin (IL)-11, CXCR4, connective tissue growth factor and matrix metalloproteinase (MMP)-1, have well-described effects on bone cells. IL-11 stimulates bone resorption by increasing osteoblast production of receptor activator of nuclear factor-κB ligand.[6] CXCR4 is a chemokine receptor that binds to stromal-derived factor-1 produced by osteoblasts, and its expression promotes homing of cancer cells to bone. Connective tissue growth factor 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 osteoprotegerin by osteoblasts.[52,53] When expressed and cosignaling is allowed to occur, 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.*[6] showed that two of these genes, IL-11 and connective tissue growth factor, were directly regulated by TGF-β via the canonical TGF-β/Smad pathway in metastatic cells. Other studies indicate that CXCR4 and MMP-1 are also regulated by TGF-β.[54,55] Using the same mouse model, knockdown of the TGF-β signaling molecule Smad4 inhibited the formation and growth of bone metastases.[56,57] Recently, it 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.[58] This results in increased osteoclastogenesis and the production of the cytokine IL-6 by osteoblast, acting as a potent inducer of proliferation of tumor cells. Collectively, these effects of TGF-β perpetuate the feed-forward cycle to increase tumor growth in bone in a complex and multifaceted way.

These preclinical data also extend to human studies. Plasma TGF-β_1 levels were shown to be elevated in more than half of the 49 (including 23 breast and 15 prostate cancer patients) bone metastasis patients and positively correlated with TGF-β signaling-related markers, including PTHrP and IL-10.[59] Bone metastases samples from breast cancer patients also 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.[57, 60] 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.[5,57,61] 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.[5] 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 and osteopontin, two secreted glycoproteins regulated by TGF-β, have 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 bone sialoprotein and osteopontin were identified to be prognostic indicators for bone metastases.[62–65]

Hypoxia is observed in most solid tumors and is caused by reduced or inadequate oxygen supply.[66] Owing to already low oxygen levels (1–7% O_2) in the bone marrow microenvironment, hypoxia and increased expression of hypoxia-inducible factor 1α are particularly prevalent in bone metastases.[54,67] Hypoxia also stimulates the expression of *CXCR4* and *DUSP1*,[68] two genes previously recognized among 11 genes that are most upregulated in MDA-MB-231 bone metastatic clones.[6] In addition, TGF-β stabilizes hypoxia-inducible factor 1α by inhibiting its degradation,[69] and additive responses in the induction of vascular endothelial growth factor and CXCR4 are observed *in vitro*[54,69] and *in vivo*.[54] Therefore, the significant interplay of TGF-β signaling and hypoxia may be a key cooperative determinant for various mechanisms of bone metastasis.

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.[70–72] For bone metastatic disease, most compounds still remain at the preclinical stages (**Table 1**). As multiple agents under development for the various disease indications other than bone metastatic disease have been described, this might be indicative of the potential tolerability and potential success for safe TGF-β signaling antagonism (**Table 2**). A few of these novel chemical entities will be discussed here in detail while others have been extensively reviewed elsewhere.[72,73]

Neutralizing antibodies and soluble decoy receptor proteins. One strategy to reduce signaling by the excessive amounts of TGF-β in the (bone) microenvironment is to physically neutralize or trap TGF-β ligand using soluble decoy receptors encompassing the ectodomains from either TβRII or TβRIII/betaglycan protein or via neutralizing TGF-β antibodies (**Figure 2**). The pan-neutralizing mouse monoclonal antibody 1D11 binds and reduces biological activity of all three TGF-β isoforms and has demonstrated therapeutic potential in both syngeneic and xenograft mouse tumor models, including preclinical bone metastasis models[74,75] (**Table 1**).

For use in patients, a fully humanized TGF-β monoclonal-neutralizing antibody, GC-1008, is currently being developed by Genzyme (Cambridge, MA, USA)[72] (**Table 2**). GC-1008 is a pan-neutralizing antibody directed against all three isoforms of

Table 1 Anti-TGF-β compounds successfully used in preclinical bone metastasis models

Agent/target	Target	Company	References
Trap ligands			
Antibodies			
1D11	TGF-β$_{1-3}$	Genzyme/CAT	74, 75, 113
Receptor kinase inhibitors			
Ki 26894	ALK5	Kirin Brewery Company	114
LY2109761	ALK5+TGFRII	Eli Lilly and Co.	5, 93–95
LY2157299	ALK5	Eli Lilly and Co.	96, 115
LY364947	ALK5	Eli Lilly and Co.	116, 117
SD-208	ALK5	Scios, Inc./Johnson & Johnson	54, 90–92, 118–120
Combined vaccine/antisense			
2G7+IL-2	TGF-β$_{1-3}$+IL-2	Genentech	121
Other molecules antagonizing effects of TGF-β			
Halofuginone	TGF-β effects	Collgard Biopharmaceuticals	108
BMP7	TGF-β effects	Stryker	102, 104

Abbreviation: TGF-β, transforming growth factor-β.

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 previous therapy.[76] 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.[76] PF-03446962 generated by Pfizer (New York City, NY, USA), is an antibody directed against ALK1 and displays potent anti-angiogenic effects *in vitro*.[77] Currently, this antibody is in phase I clinical trials to evaluate optimal pharmacokinetic parameters for this agent in patients with advanced solid tumors (**Table 2**).

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). ASOs inhibit mRNA function and protein synthesis through modulation of splicing and/or inhibition of translation by disrupting ribosome assembly.[78,79] Several limitations need to be considered when using ASOs, including stability, RNA-binding affinity, efficiency of delivery to the target cells and associated off-target effects. While TGF-β ASOs should, in theory, not interfere with activated TGF-β proteins released from bone, it is possible that ASO treatment could result in lower concentrations of TGFβ being deposited in bone.

Antisense Pharma (Regensburg, Germany) has developed AP-12009 (Trabedersen), an ASO specific for the mRNA of human TGF-β$_2$,[80,81] for which patients with anaplastic astrocytoma are currently being recruited for a phase III study to evaluate the efficacy and safety of intratumoral treatment with AP-12009 compared with standard chemotherapy (temozolomide) (**Table 2**). In addition, a phase I dose-escalation clinical study is currently ongoing to evaluate the safety and tolera-

bility of intravenously administration of AP-12009 in patients with advanced tumors known to overproduce TGF-β$_2$, such as melanoma, pancreatic and colorectal carcinomas (**Table 2**).

Furthermore, TGF-β ASOs are also currently being tested in early-phase clinical trials to generate ASO-modified tumor vaccines with the goal of reducing the immune inhibiting activity at the vaccine sites.[82]

Receptor kinase inhibitors. While ligand traps and ASOs limit the bioavailability 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 TβRI/ALK5 inhibitors described in the literature to date, where tested, display equipotent inhibition against ALK4 kinase activity and less inhibition against ALK7.[83–87] 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 anti-resorptive and bone formation activities in various animal models.[88,89] Whether the combined inhibition of TGF-β and activin signaling by ALK4/5 inhibitors is advantageous for the treatment of cancer remains to be determined. Of all the different classes of TGF-β antagonists, the TβRI/ALK5 kinase inhibitors are the ones most extensively tested in both syngeneic and xenograft preclinical bone metastasis models (**Table 1**). For example, the orally active TβRI/ALK5 kinase inhibitor SD-208 inhibited progression and development of melanoma and osteolytic breast cancer bone metastases.[90,91] In addition, it inhibited bone metastases from osteolytic PC3 prostate cancer cells, but was unable to inhibit tumor growth in bone of LuCaP23.1 prostate cancer cells that form osteoblastic bone metastases.[92] Therefore, TGF-β blockade may be more effective to treat osteolytic than osteoblastic disease, perhaps owing to the fact that inhibition of TGF-β may result in an additional osteoblastic response.[29]

LY2109761 (Eli Lilly and Co., Indianapolis, IN, USA), an inhibitor of both the TβRI/ALK5 and TβRII, inhibits the formation of metastases in several mouse models, including colon,[93] breast,[5] prostate[94] and pancreatic[95] cancer. While SD-208 successfully inhibited the growth of established MDA-MB-231 bone metastases,[54] LY2109761 did not,[5] and suggest that SD-208 may be a more potent or mechanistically distinct versus LY2109761 in inhibiting TGF-β signaling *in vivo*.

LY2157299, under development by Lilly and Co, has entered phase I clinical trials to determine the safety and pharmacokinetics in patients with advanced metastatic malignancies including melanoma, colon cancer, prostate cancer, adrenal gland cancer and breast cancer who have exhausted current standard of care. Daily doses of 40 mg and 80 mg LY2157299 have currently been well tolerated and no drug-related grade 3 or 4 toxicities were observed.[96] Additional clinical studies are underway in patients with bone metastasis.

Other molecules that antagonize TGF-β. Numerous additional biologic-based molecules, that do not necessarily directly bind

Table 2 Anti-TGF-β compounds currently in clinical trials

Class (compound)	Target	Company	Phase	Disease	References
Trap ligands					
Antibodies					
Fresolumimab/ GC1008	TGF-β$_{1-3}$	Genzyme	Phase I (c)	RCC + Melanoma	NCT00356460
			Phase I (r)	Metastatic breast cancer	NCT01401062
			Phase I (c)	Advanced melanoma	NCT00923169; 76
Antibody targeting ALK1					
PF-03446962	ALK1	Pfizer	Phase I (r)	Advanced solid tumors	NCT00557856; 77
Antisense oligonucleotides					
Trabedersen (AP12009)	TGF-β$_2$ mRNA	Antisense Pharma	Phase I (c)	Pancreatic + colorectal neoplasms, melanoma	NCT00844064; NCT00431561 NCT00761280
			Phase IIb (c)	Glioma	
			Phase III (r)	AA, glioblastoma	
Receptor kinase inhibitors					
LY2157299	ALK5	Eli Lilly and Co.	Phase Ib/IIa (r)	Glioma	NCT01220271
			Phase II (r)	Hepatocullar carcinoma	NCT01246986
			Phase Ib/II (r)	Pancreatic cancer	NCT01373164
Combined TGF-β AOS with a vaccine					
Lucanix™ (Belagenpumatucel-L)	TGF-β$_2$	NovaRx Corp	Phase II (c)	NSCLC	NCT01058785; 122 – 123
			Phase III (r)	NSCLC	NCT006765
TGF-β$_2$ ASO + GMCSF expression vector	TGF-β$_2$ + GMCSF	MCMRC	Phase I (r)	Advanced cancer	NCT00684294

Abbreviations: AA, anaplastic astrocytoma; CRC, colorectal carcinoma; (c), study completed; MCMRC, Mary Crowley Medical Research Centre; NCT#, clinical trial identifier (www.clinicaltrial.gov); NSCLC, non-small-cell lung cancer; (r), study currently recruiting patients; RCC, renal cell carcinoma; TGF-β, transforming growth factor-β.

and inactivate TGF-β, its receptor or its signaling molecules, may still antagonize the effects of TGF-β indirectly. Here, two examples of molecules that antagonize TGF-β function are described that have been extensively tested in 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.[97,98] It has long been recognized that BMP7 can counteract TGF-β-induced EMT, and induce the opposite process, a mesenchymal-to-epithelial transition in embryonic kidney and eye development.[99–101] More recently, it was demonstrated that BMP7 could also counteract the TGF-β-induced EMT in breast and prostate cancer. In addition, pretreatment, forced overexpression or systemic administration of BMP7 inhibited the formation of bone metastases from osteolytic breast and prostate cancer.[60,102–105] It is tempting to speculate that BMP7, by counteracting TGF-β-induced EMT, may act as a differentiation-inducing agent that targets the cancer stem cell (CSC) fraction in breast and prostate cancer.[105,106] However, 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.[107]

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 (**Table 2**). Unpublished data from our laboratory show that Hfg inhibits TGF-β signaling *in vitro* in several cell types, and that the systemic daily treatment of Hfg in mice significantly inhibits the formation of osteolytic lesions and bone metastases after intracardiac inocu-

lation of MDA-MB-231 breast, PC3 prostate cancer and 1205Lu melanoma cells.[108] Although the exact mechanism remains to be investigated, Hfg treatment represents another novel agent that may 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 such as rapamycin[109] and doxorubicin.[110,111] 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.[112] Using the same bone metastasis model of MDA-MB-231 human breast cancer cells, we tested the effects of a combined treatment of SD-208 and 2-methoxyestradiol, an inhibitor of hypoxia-inducible factor 1α. Combined treatment with these agents reduced osteolytic lesions and tumor burden, and improved survival of mice more effectively than either treatment alone.[54]

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.

Risks. Knockout 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.[124–127] TGF-β blockade may also paradoxically increase the risk of tumorigenesis,[128,129] inhibit wound healing[130] and is likely to be teratogenic to the fetus.[131] However, severe toxicity has not been observed in animals when TGF-β is blocked by lifetime expression of a soluble TβRII, under the regulation of the mammary-selective MMTV-LTR promoter,[132] or when TGF-β-neutralizing antibodies are chronically administered for long periods of time.[133] 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. In clinical trials, TGF-β-neutralizing antibodies, TGF-β ASOs and TGF-β receptor kinase inhibitors have been documented to be generally well tolerated. One patient developed premalignant skin lesion testing a TGF-β antibody from Genzyme, but upon discontinuation of the drug, this effect resolved with time. Furthermore, acquired resistance to the TGF-β receptor kinase inhibitor LY2109761 has recently been observed in a mouse skin model of *de novo* chemically induced carcinogenesis. The resultant carcinomas were more aggressive and inflammatory in nature, with de-localized and elevated expression of MMPs.[134] In addition, some toxicities may differ in rodent compared with primate models.

Limitations and opportunities. TGF-β receptor kinase inhibitors are most suitable for oral dosing, have no loss of efficacy due to neutralizing antibody generation and better tissue penetration than observed with biologic-based agents.[72] The wide variety of chemical pharmacophores and binding modes may eventually yield selective kinome profiles that may prove safe and effective for their designed purpose.[72,135] However, the TGF-β receptor kinase inhibitors described to date are less selective than the current TGF-β ASOs or the biologic-based TGF-β-directed therapies. For example, the TβRI/ALK5 small-molecule kinase inhibitors can also potently inhibit ALK4 and less so ALK7 owing to their very close catalytic domain homology to TβRI/ALK5.[117] In line with these observations, it may be challenging to dissect TGF-β-dependent toxicity from the off-target effects of these compounds. For this, biologic-based inhibitors may prove to be more indicative of true TGF-β-dependent toxicities owing to their highly selective nature.

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 vascular endothelial growth factor, which can be blocked with treatment with TGF-β antibodies, TGF-β ASOs or TβRI/ALK5 kinase inhibitors. Interestingly, while TGF-β antibodies and TGF-β ASOs 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. Indeed, several studies have demonstrated that treatment with TβRI/ALK5 kinase inhibitors can even promote angiogenesis.[34,136,137] Therefore, the therapeutic strategies to target TGF-β must be carefully considered. Furthermore, it is interesting to speculate to what extend the mechanistic distinction may be due to the inhibition

of canonical versus non-canonical TGF-β signaling by these various agents.

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 the mechanism of action, pharmacokinetic properties and delivery challenges. Besides specificity, one of the main challenges with TGF-β ASOs will be its mode of administration to bone metastases. In addition, drug delivery challenges are also faced with neutralizing TGF-β antibodies and other large molecules such as TβRII/III:Fc fusion proteins.

Although speculative at present, some of the tumor-inhibiting effects of anti-TGF-β compounds described in this review may also be attributed to inhibitory effects on CSCs. To this end, as a proof-of-principle, treatment with the TβRI/ALK5 inhibitor LY2109761 inhibited TGF-β signaling in CD44$^+$/CD24$^{-/low}$ breast CSC-like cells and reversed their mesenchymal and tumorigenic stem cell-like phenotype to a more epithelioid CD44$^{-/low}$/CD24$^+$ phenotype.[140] New studies are warranted to address whether TGF-β inhibitors also affect this CSC subpopulation in primary tumors and bone metastasis in both clinical and preclinical models.

A 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 or nanoparticles, which may be useful as a targeting device to sites of high bone turnover, including sites with bone metastatic disease.[138] 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. In addition, these bioactive compounds of interest could be delivered to bone metastatic sites in combination with other anti-cancer agents with synergistic action.

Another approach to target bone metastases was recently shown by using an oncolytic adenovirus expressing soluble TβRII-Fc.[139] This resulted in inhibition of established MDA-MB-231 bone metastases, but whether this activity may extend into the clinical situation remains to be studied and is unknown.

Biomarkers and patient stratification. Clinical trials are currently addressing whether plasma TGF-β levels in bone metastasis patients may be indicative of TGF-β-dependent metastatic disease and may be useful as a biomarker to predict the success of treatment with TGF-β antagonists in metastatic disease.

Immunohistochemical studies have also demonstrated that increased expression of TGF-β$_1$ and phosphorylated Smad2/3 in clinical samples from primary tumors are correlated with a high incidence of metastasis.[140,141] 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 bioinformatics tools to define a TGF-β response signature that can be used to more accurately assess the TGF-β response status of human breast tumors.[142] Analysis of clinical samples revealed that ~40% of human breast tumors actively responded to TGF-β signals. In addition, a validated signature of TGF-β upregulated

genes is clearly associated with poor prognosis of the subsets of basal-like- and Her2-positive breast cancers.[143] These findings suggest that the presence of a TGF-β response signature might drive tumor progression in estrogen-independent cancer, but may also reflect a suppressive host cell response in estrogen-dependent luminal cancers. Taken together, patient stratification based on the presence of the TGF-β response signature signature in primary tumor samples might help to identify a rationally tailored subset of patients that are likely to respond to anti-TGF-β therapies.

Conclusion

TGF-β is a multifunctional cytokine with a prominent role in 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 that inhibit TGF-β activity to prevent and/or treat bone metastases. Three therapeutic modalities targeting TGF-β have been pursued and are currently being tested in clinical trials in cancer patients (including bone metastatic disease): TGF-β antibodies, TGF-β receptor kinase inhibitors and TGF-β ASOs. All three modalities have fundamentally different pharmacokinetic/pharmacodynamic properties and mechanisms of actions, providing a broad range of limitations and opportunities, particularly with respect to specificity and toxicity. One benefit is that blockade of TGF-β signaling may increase bone mass in cancer patients. This may be particularly beneficial in a cancer population with already a low bone mineral density baseline resulting from either gonadal status or due to an oncolytic therapeutic regime including hormone-, radiation- or chemo-therapy. However, concerns about the impact on immune function and/or growth-promoting effects of these agents continue to remain. The next decade promises new and exciting clinical data that will determine which TGF-β therapeutic strategies will be most effective to prevent and/or cure bone metastatic disease coupled with a tolerable side-effect profile.

Conflict of Interest

TAG is a consultant for Amgen, Roche and Novartis, and has stock ownership of Amgen. KRS is an employee of Eli Lilly & Company. JTB has no potential conflict of interests.

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