Published in final edited form as:
Curr Opin Oncol. 2007 November ; 19(6): 579–585. doi:10.1097/CCO.0b013e3282f0ad0e.

Targeting transforming growth factor-β signaling

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Abstract

Purpose of review—Most cancers are characterized by excessive transforming growth factor-β production by tumors, which can promote tumor growth and mediate epithelial-to-mesenchymal transition. Transforming growth factor-β also has the ability to overproduce extracellular matrix components in response to injury and other stimuli. There are many strategies undergoing current evaluation for inhibiting the deleterious biological effects of transforming growth factor-β by disrupting its signaling at various levels. The current review focuses on the recent advances made in this area, and the potential of these strategies in the clinical treatment of cancer and fibrosis.

Recent findings—Four main strategies used most recently for disrupting transforming growth factor-β signaling are brought into focus in this review: inhibition or sequestration of the transforming growth factor-β protein ligands, inhibition of transforming growth factor-β receptor kinase activity, inhibition of SMAD signaling downstream of transforming growth factor-β kinase activity and restoration of antitumor immunity upon transforming growth factor-β inhibition. Various techniques currently used to employ these four strategies are discuussed.

Summary—Several lines of evidence suggest that altered transforming growth factor-β signaling contributes to tumor progression and metastasis as well as development of fibrosis. Accumulating data from preclinical and clinical studies indicate that antagonizing aberrant transforming growth factor-β signaling is a promising novel therapeutic approach in cancer and fibrotic disorders.

Keywords

cancer; fibrosis; SMAD; transforming growth factor-β receptor 1; transforming growth factor-β

Introduction

Transforming growth factor (TGF)-β is a ubiquitously expressed cytokine that belongs to a large superfamily of related polypeptides such as bone morphogenetic proteins and activins. This universally expressed cytokine displays an array of pleiotropic effects that have an active role in cellular functions such as proliferation, homeostasis, angiogenesis and wound healing [1,2]. Aberrant regulation of TGF-β function in these processes contributes to cancer progression and fibrotic disorders (Fig. 1, upper right panel), making TGF-β an attractive candidate for treatment of these conditions. The purpose of this review is to illustrate the current state of TGF-β signaling antagonism for the treatment of cancer and fibrosis by pointing out the current methodology in this field.

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Inhibiting/sequestering transforming growth factor-β ligand proteins

One popular method of TGF-β signaling inhibition in both cancer and fibrosis models is targeting the expression and function of TGF-β ligands. Strategies such as RNA interference (RNAi), neutralizing monoclonal antibodies and drug treatment are among the most commonly used recent approaches to prevent TGF-β ligand activation of downstream signaling (Fig. 1, lower left panel and Fig. 2, upper left panel).

RNAi is a relatively new technology that is becoming an active subject of investigation in the context of inhibiting aberrant TGF-β cytokine expression. Short hairpin RNA vectors, which contain small interfering RNA sequences that serve as guides for enzymatic cleavage of complimentary mRNAs, can be stably transfected into cells and used for inhibiting TGF-β ligand translation, thereby reducing TGF-β secretion (Fig. 2, upper left panel). Several preclinical studies support effective short hairpin RNA inhibition of TGF-β mRNA and protein expression producing desirable effects in induced models of fibrosis *in vitro* and *in vivo* [3–5]. One particular study showed the promise of a small interfering RNA strategy for prevention of induced liver cirrhosis in a mouse model. The investigators demonstrated a decrease in type I collagen and α-smooth muscle actin expression in mouse livers upon a small interfering RNA-targeted decrease of TGF-β expression, which subsequently resulted in enhanced liver regeneration after induced liver damage [4]. Another study was able to show type I collagen inhibition in a rat model of renal fibrosis resulting in decreased kidney damage, thus showing the promise of this method in treating various types of fibrotic disorders [3]. One challenge of this method is effective delivery of short hairpin RNA vectors in a clinical setting, warranting additional studies in this particular area.

Antagonizing TGF-β ligand activity is proving to be useful for treating increased levels of circulating TGF-β in cancer models. One group observed that an increase of TGF-β in the bloodstream induced by radiation and chemotherapy had a causative effect on the amount of lung metastasis occurring in a MMTV/PyVmT mouse model. With the use of 2G7, a neutralizing pan-TGF-β monoclonal antibody, investigators were able to significantly reduce radiation-induced surface lung metastasis and circulating tumor cells in these mice [6•]. This particular study highlights the potential benefits of screening patients for increased levels of TGF-β in the bloodstream for diagnostic, therapeutic and prognostic purposes. It also justifies further investigation into the use of anti-TGF-β antibodies for the treatment of TGF-β responsive disease.

One interesting novel approach to reduce elevated circulating levels of TGF-β in the bloodstream was performed by Yamamoto *et al.* [7••] who used a specially constructed immunosuppressive substance adsorption column with the ability to adsorb the latent form of TGF-β for direct hemoperfusion treatment (Fig. 1, lower left panel). A single treatment was able to decrease rat hepatocellular carcinoma tumor volume and was shown to significantly increase survival in tumor-bearing rats.

Other methods of inhibiting TGF-β expression include the use of antiinflammatory drugs that target the transcription of TGF-β (Fig. 1, lower left panel). The drug pirfenidone inhibits human glioma cell proliferation *in vitro.* In addition to an observed decrease of cell proliferation in multiple cell lines, pirfenidone also has the ability to prevent the upstream activation of TGF-β by decreasing the enzymatic activity of furin, a TGF-β activating protease [8].

These observations show that controlling excessively expressed TGF-β protein ligands can reduce tumor cell proliferation and block the progression of fibrotic disorders. One potential advantage of cytokine inhibition is the opportunity to only partially inhibit TGF-β biological effects instead of totally abrogating its response. Treatment with moderate to low levels of TGF-β ligand inhibitors have the potential to halt the effects of excess TGF-β signaling while

allowing normal levels of signaling to occur. This would theoretically prevent toxicity due to TGF-β signaling abrogation and could improve the efficacy of other treatments found to be previously ineffective due to the effects of excess TGF-β expression. In contrast to the severe toxicity due to TGF-β signaling abrogation observed in *Tgfb1* knockout mice [9], long-term exposure of mice either to a TGF-β antibody [10] or a TGF-β soluble antagonist [11] is well tolerated. The results from these two studies suggest thatTGF-β blockade in post-embryonic animals yields acceptable toxicities and may be considered for therapeutic applications.

Inhibiting transforming growth factor-β receptor kinase activity

Inhibiting TGF-β signaling at the receptor kinase level is dominated by the use of small-molecule inhibitors. These inhibitors typically function by binding to the ATP-binding domain of the TGF-β receptor (TGFBR) 1 kinase and prevent its phosphorylation upon association with TGFBR2. This keeps TGFBR1 in an inactive configuration, rendering it incapable of activating down-stream targets such as SMAD2 and SMAD3 (Fig. 1, lower right panel). Inhibition of SMAD signaling by way of small-molecule inhibitors has shown to be sufficient for inhibiting tumor growth and proliferation of tumor cells, decreasing progression of cells into an epithelial-to-mesenchymal transition-like phenotype, inhibiting TGF-β-mediated transcriptional responses, and decreasing migration and invasion of tumor cells [12•,13–16].

SD-208 is a small-molecule inhibitor that has been used recently in several animal studies for treating different types of cancer. Specifically, one group showed inhibition of mouse mammary tumor growth *in vivo* as well as reduced tumor-associated microvessel density upon oral administration of SD-208. The investigators also noted that SD-208 was well tolerated in mice, observing little to no clinically relevant toxicity upon prolonged treatment [13]. More recently, SD-208 was used in a study showing its ability to reduce tumor growth and metastasis in a mouse orthotopic xenograft model of pancreatic adeno-carcinoma. This group also showed SD-208-mediated attenuation of gene responses involved in tumor progression via gene arrays. Using the gene array analysis in conjunction with SD-208 allowed the discovery of novel TGF-β-mediated protein regulation in PANC-1 cells involving angiogenesis and lymphangiogenesis, indicating that small-molecule inhibitors like SD-208 can be useful in the molecular characterization of tumors [12•].

There are efforts underway to create a structurally ideal TGFBR1 small-molecule inhibitor that will reduce kinase activity more efficiently than those currently developed. Using already existing TGFBR1 inhibitors as a template, some groups are synthesizing slightly modified structures of these molecules to find a more structurally compatible isoform that will exhibit increased TGFBR1 binding efficiency. One recent example is a study performed by Tojo *et al.* [16] where 17 different small-molecule variants of a pyrazole-based inhibitor were developed. A screening process determined that one of them was superior in its ability to inhibit progression of epithelial-to-mesenchymal transition. Another recent study described by Ishida *et al.* [17] also utilized this approach for the development of SM305, a derivative of the previously described inhibitor, HTS466284 [18]. SM305 induced a decrease of TGF-β-dependent reporter activity and extracellular matrix protein expression *in vitro* and *in vivo* using primary fore-skin fibroblasts as a fibrosis model [17]. An advantage of screening inhibitor variants in this manner is the possible elimination of prospective inhibitor molecules that can cross-react with TGFBR1-like kinases, causing undesirable signaling alterations in other pathways.

TGFBR1 small-molecule inhibitors are also showing promise for treating fibrotic disorders with advancements made in studies utilizing induced fibrosis models. One particular molecule named IN-1130 was investigated in a comprehensive fibrosis study and was able to inhibit the overproduction of extracellular matrix proteins in the presence of induced renal fibrosis by

unilateral ureteral obstruction in rats. This study tested a wide range of fibrosis-associated markers using fairly sensitive assays that underline the efficacy of this small molecule in the inhibition of fibrosis [19]. Another study used a liver fibrosis model in rats to study the effects of GW6604, a TGFBR1 small-molecule inhibitor, which was shown to decrease induced liver damage and increase levels of liver regeneration after partial hepatectomy [20].

The use of small molecules can circumvent some technical challenges of clinical treatment in disease, such as delivery. The potential of oral administration of these inhibitors makes them attractive candidates for future clinical studies; however, the cross-reactivity of most small-molecule inhibitors with other kinases presents challenges that need to be assessed thoroughly in the clinical trials that are underway.

Direct inhibition of SMAD signaling

As it is well accepted that a majority of TGF-β-mediated responses occur through the action of SMAD signaling downstream of TGF-β kinase activity, it is not surprising that several groups are investigating ways to antagonize SMAD signaling in order to abolish the effects of TGF-β in disease. Many strategies have been utilized to accomplish this task including phosphatase inhibition, targeting receptor-regulated SMADs (R-SMADs) for ubiquitin-dependent degradation and sequestration of R-SMADs (Fig. 2, upper right panel) [21].

Overexpression of R-SMADs physiological inhibitors is a potentially interesting approach for treating TGF-β-responsive disease. SMAD7, an inhibitory SMAD, acts in normal cellular processes to prevent phosphorylation of R-SMADs and recruits ubiquitin ligases for degradation of the latter. A recent study used SMAD7 in a mouse model that mimicked melanoma-mediated bone metastasis. In mice overexpressing SMAD7 there was less total osteolytic lesion area observed at metastatic lesion sites coupled with an extended survival time when compared to controls [22].One downfall of this approach, however, is the fact that SMAD7 engages in cross-talk with important cellular response proteins including CDC42, mitogen-activated protein kinase-1 and β-catenin, which may lead to undesirable side effects.

Another group successfully inhibited R-SMAD activity by using a specific targeting and sequestration strategy. This was done by utilizing the *Escherichia coli* thioredoxin A protein (Trx) as a protein scaffold harboring different SMAD-binding protein elements that were able to efficiently bind and sequester R-SMAD proteins, therefore inhibiting their activity (Fig. 2, upper right panel). Two reports from the same group showed that four different R-SMAD-binding peptide elements were able to inhibit R-SMAD activity and block TGF-β-mediated transcriptional reporter activity [23,24••]. One specific study demonstrated inhibition of epithelial-to-mesenchymal transition progression as a result of decreased R-SMAD activity using the Trx–SMAD anchor for activation complex, which is made up of the SMAD anchor for activation peptide recognition sequence harbored by the Trx protein scaffold [24••]. The possibility of Trx–SMAD anchor for activation binding to other important transcriptional regulators cannot yet be ruled out, but this approach is a novel proof of concept demonstrating the potential of SMAD signaling interference in the treatment of cancer and fibrosis.

The main benefit of direct SMAD inhibition is the ability to target SMAD-specific responses without affecting other pathways regulated by TGF-β such as mitogen-activated protein kinase-1 and c-Jun N-terminal protein kinase proteins which reside downstream of TGF-β activated kinase protein activation.

Restoring immunity through transforming growth factor-β inhibition

TGF-β is the most potent naturally occurring suppressor of immune function. Restoration of immunity is a direct result of targeting the TGF-β signaling pathway in conditions associated

with excessive production of TGF-β ligands. In many studies, it is the primary desired outcome upon administration of TGF-β inhibitors and is worth mentioning in the context of treating cancer (Fig. 2, lower panel). Immune responses are occasionally depressed in patients with advanced cancer, and one potential explanation of this phenomenon is the excessive TGF-β secretion by tumors and fibroblasts, which may have both systemic and local immunosuppressive effects. This concept has led to the development of immunotherapeutic approaches for treating disease.

Suzuki *et al*. [15] observed that treatment with SM16, a TGFBR1 small-molecule inhibitor, was able to induce a $CD8^+$ antitumor response in a murine mesothelioma model, resulting in the inhibition of malignant mesothelioma tumor growth. They justify their observation by noting a diminished antitumor response to SM16 treatment in the immunodeficient SCID mouse model.

Perhaps one of the more interesting approaches in this area involves an adoptive cell transfer technique that modifies immune cells isolated from the host *ex vivo* to render them insensitive to TGF-β signaling. By transfecting $CD8^+$ T cells with a TGFBR2 dominant-negative green fluorescent protein construct, one group produced immune cells with the ability to kill tumor cells in the presence of excess TGF-β By coinjection of these TGF-β-insensitive $CD8^+$ T cells with CD8-depleted splenocytes, investigators were able to induce a significant decrease in tumor volume and weight in a murine prostate cancer model [25••].

The use of tumor vaccines in combination with TGF-β2 antisense oligonucleotides is another immunotheraputic approach that has proven useful in treating preestablished and advanced glioma. Liu *et al*. [26] were able to show a reduction of implanted glioma tumor growth in rats receiving subcutaneously administered tumor cell vaccine and local anti-TGF- β2 oligonucleotide injection. A similar method described below has been used in humans yielding promising results in patients with advanced glioma [27].

Clinical trials of transforming growth factor-β signaling antagonists

The large amount of preclinical data generated to date provides a strong rationale to assess the potential clinical benefits of TGF-β signaling blockade in humans. Results from a phase I trial performed by NovaRx in San Diego using an antisense modified tumor vaccine indicate a reduction of tumor size in patients with advanced glioma. Increased immune cell infiltration of tumors was observed as well, presumably due to an increased antitumor immune response [27]. A study at Baylor College of Medicine in Houston, Texas is currently recruiting patients for a phase I trial to test another type of immunotherapeutic strategy for treatment of relapsed Epstein–Barr virus-positive lymphoma with administration of TGF-β-resistant cytotoxic T lymphocytes, with the likely intent to induce an antitumor immune response [28].

Antisense Pharma of Germany is utilizing an antisense-based therapy against TGF-β2 overexpression in an international phase I/II study restricted to patients with malignant glioma as well as another phase I/II study investigating treatment of colorectal carcinoma, pancreatic carcinoma and malignant melanoma. A recent report indicated encouraging results regarding the efficacy and safety of this treatment in the glioma trials [29].

Pirfenidone was tested in a randomized, double-blind, placebo-controlled multicenter trial in patients with idiopathic pulmonary fibrosis. Pirfenidone resulted in a significant improvement of pulmonary function and prevented episodes of acute exacerbation [30].

Following the promising results of TGF-β neutralizing antibodies in preclinical studies, genzyme is currently recruiting patients into phase I clinical trials for the treatment of advanced

renal cell carcinoma/malignant melanoma and pulmonary fibrosis utilizing a pan-TGF-β neutralizing antibody, GC-1008 [31,32].

Conclusion

The current review of the recent literature shows that TGF-β signaling inhibition is becoming an important tool in elucidating the distinct roles of TGF-β in disease as well as the development of therapies. Much of our current knowledge regarding TGF-β antagonism derives from preclinical work with limited input from clinical studies. As TGF-β antagonists continue to demonstrate encouraging application for treatment of cancer and fibrosis, the field should experience an increased shift into clinical research in the next few years. Consequently, TGF-β signaling inhibition has the potential to emerge as novel effective therapy for a wide variety of conditions.

Abbreviations

RNAi, RNA interference; R-SMAD, receptor-regulated SMAD; TGF, transforming growth factor; TGFBR, transforming growth factor-β receptor; Trx, thioredoxin A.

Acknowledgement

This work was supported by grants CA112520 and CA108741 from the NCI, the Jeannik M. Littlefield-AACR Grant in Metastatic Colon Cancer Research and the Walter S. Mander Foundation Chicago, IL.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

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Additional references related to this topic can also be found in the Current World Literature section in this issue (p. 684).

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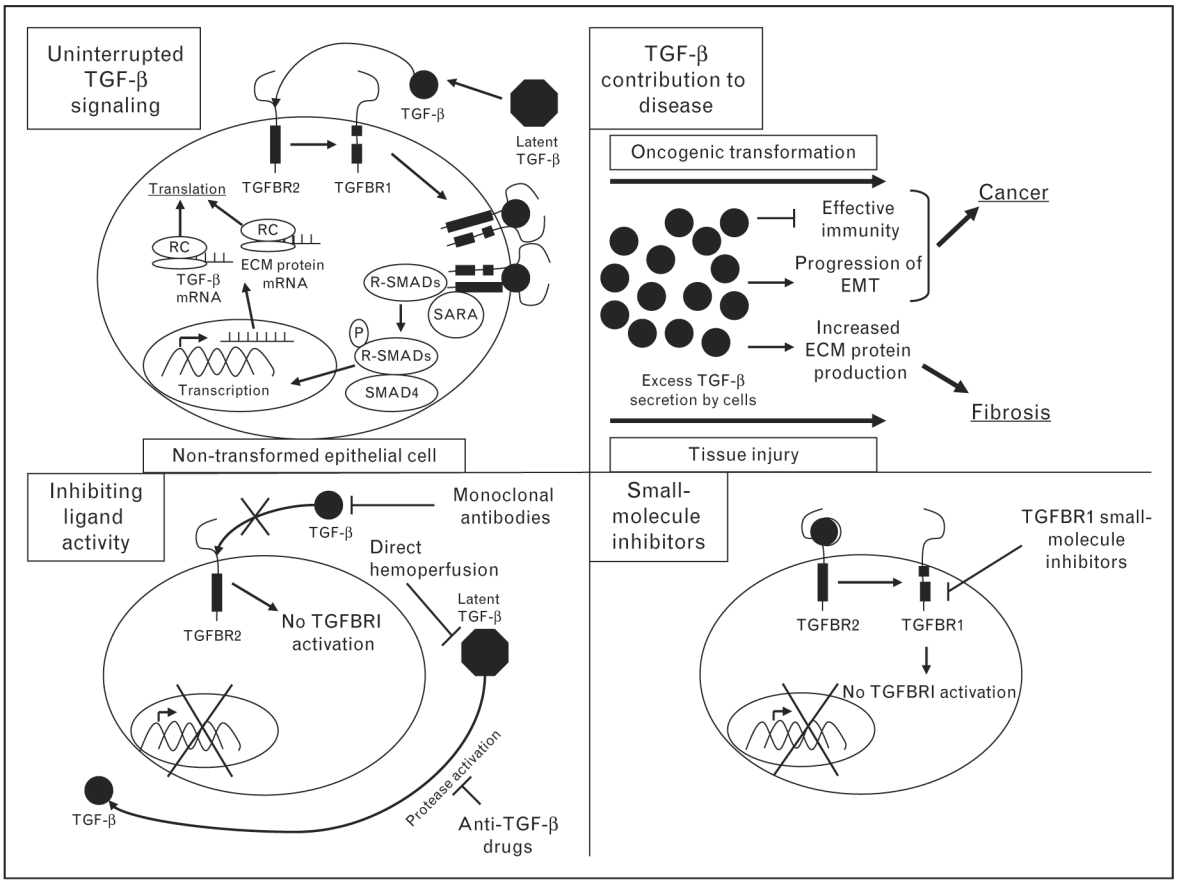

Figure 1. TGF-β signaling, its role in disease progression and methods of inhibition

(Upper left panel) A brief example of uninterrupted TGF-β signaling in nontransformed epithelial cells is shown. The major components of TGF-β signaling affected by the various signaling inhibitors described in the text are illustrated. Latent TGF-β is proteolytically activated outside of the cell and binds to TGFBR2. This results in TGFBR1 and TGFBR2 heterotetrameric complex formation, which leads to R-SMAD phosphorylation/activation. Activated R-SMADs then bind to SMAD4, translocate to the nucleus, and promote transcription of TGF-β-responsive genes. (Upper right panel) Excessive TGF-β production leads to local tumor progression by decreasing antitumor immunity, enhancing EMT. Excessive TGF-β also leads to development of fibrosis. (Lower left panel) Monoclonal antibodies and anti-TGF-β drugs can prevent ligand binding to TGFBR2 and the proteolytic activation of TGF-β. (Lower right panel) TGFBR1 small-molecule inhibitors prevent activation of TGFBR1 by preventing its ability to enter its active conformational structure. ECM, extracellular matrix; EMT, epithelial-to-mesenchymal transition; RC, ribosome complex; R-SMAD, receptor-regulated SMAD; SARA, SMAD anchor for activation; TGF, transforming growth factor; TGFBR, transforming growth factor-β receptor; Trx, thioredoxin.

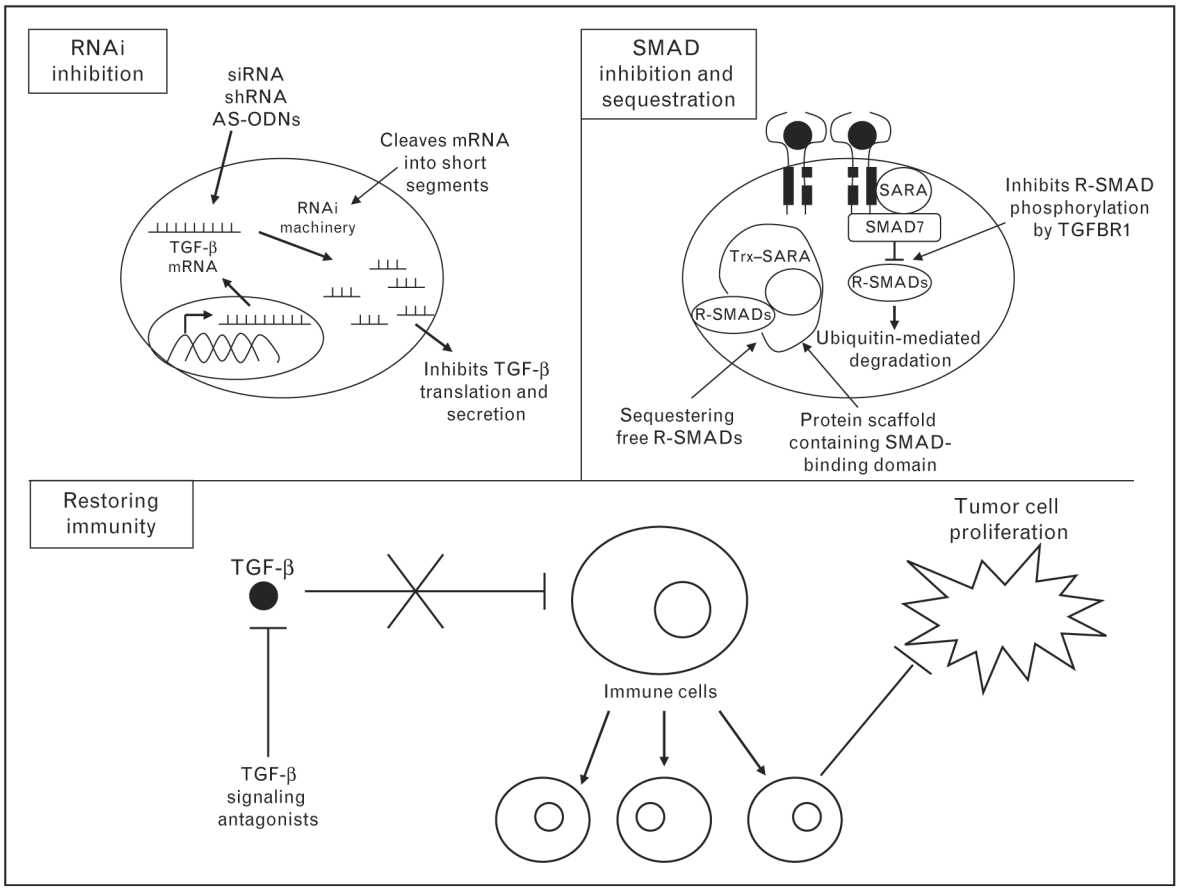

Figure 2. Additional methods of TGF-β inhibition

(Upper left panel) RNAi techniques used for inhibiting TGF-β protein translation/expression are shown. Antisense nucleotide sequences that recognize and bind to TGF-β mRNA act in concert with proteins that make up the RNAi machinery complex in order to enzymatically cleave the mRNA, thereby preventing translation. (Upper right panel) Physiological R-SMAD inhibitors, such as SMAD7, prevent phosphorylation of R-SMADs to prevent their activation and can also recruit ubiquitin ligases to target R-SMADs for degradation by the proteasome. Peptide aptamer scaffolds harboring an R-SMAD peptide-binding region (Trx–SARA in this example) are able to sequester R-SMADs in the cytoplasm to prevent their activation. (Lower panel) Various TGF-β signaling antagonists are able to prevent TGF-β-mediated immunosuppression, allowing an antitumor immune response to arise. AS-ODNs, antisense oligonucleotides; RNAi, RNA interference; R-SMAD, receptor-regulated SMAD; shRNA, short hairpin RNA; siRNA, small interfering RNA; TGF, transforming growth factor; Trx–SARA, thioredoxin A–SMAD anchor for activation complex.