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Targeting the Transforming Growth Factor- β Signaling Pathway in Human Cancer

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Abstract

The transforming growth factor- β (TGF- β) signaling pathway plays a pivotal role in diverse cellular processes. TGF- β switches its role from tumor suppressor in normal or dysplastic cells to a tumor promoter in advanced cancers. It is widely believed that Smad-dependent pathway is involved in TGF- β tumor suppressive functions, whereas activation of Smad-independent pathways coupled with the loss of tumor suppressor functions of TGF- β is important for its pro-oncogenic functions. TGF- β signaling has been considered as a very suitable therapeutic target. The discovery of oncogenic actions of TGF- β has generated a great deal of enthusiasm for developing TGF- β signaling inhibitors for the treatment of cancer. The challenge is to identify the group of patients where targeted tumors are not only refractory to TGF- β -induced tumor suppressor functions but also responsive to tumor promoting effects of TGF- β . TGF- β pathway inhibitors including small and large molecules have now entered clinical trials. Preclinical studies with these inhibitors have shown promise in a variety of different tumor models. Here we emphasize on the mechanisms of signaling and specific targets of the TGF- β pathway that are critical effectors of tumor progression and invasion. This report also focuses on the therapeutic intervention of TGF- β signaling in human cancers.

1. Introduction

Transforming growth factor- β (TGF- β) is a multifunctional polypeptide that controls proliferation, differentiation, embryonic development, angiogenesis, wound healing and other functions in many cell types (1). TGF- β acts as a tumor suppressor in normal epithelial cells and in early stage of tumor progression. In advanced cancers the growth inhibitory function of TGF- β is selectively lost, and TGF- β induces many activities that lead to growth, invasion and metastasis of cancer cells (2,3). TGF- β is a prototypic member of a large superfamily of secreted proteins that include three TGF- β isoforms (TGF- β 1, TGF- β 2 and TGF- β 3), activins, growth and differentiation factors, bone morphogenetic proteins (BMPs), inhibins, nodal, and anti-Mullerian hormone.

Cancer cells, in general, secrete larger amounts of TGF- β than their normal counterparts. The association of TGF- β with cancer is strongest in the most advanced stages of tumor progression. TGF- β is highly protective against cancer in the early stages and genetic or epigenetic loss of TGF- β signaling would lead to tumor outgrowth and progression, and to dramatically impact the ability of tumor cells to spread throughout the body (4). The actions of TGF- β are complex and the members of the TGF- β family produce different effects that depend on the type and state of the cells. TGF- β protein-receptor interaction promotes processes such as immune

suppression, tissue remodeling and formation of blood vessels that lead to the growth and metastasis of cancer cells.

TGF- β is very selective in its interactions with its receptors, due to the fact that four receptor subunits bind in an interdependent manner, interacting not only with TGF- β but with each other as well. Understanding the detailed nature of the interactions between TGF- β and its receptors represents a critical new step forward, and presents potential opportunities to find new drugs that mimic these interactions or develop more effective therapies. Such drugs or inhibitors could be designed to block the assembly of the TGF- β signaling complex and in turn eliminate its tumor-promoting activities.

2. TGF- β and receptors

The TGF- β ligands are synthesized as homodimeric pro-proteins that are cleaved by furin-type proteases. The trans-Golgi generates the mature TGF- β , 25-kDa dimer which is non-covalently associated with the latency associated protein (LAP). Latent TGF- β -binding protein (LTBP) is frequently linked to LAP through disulfide bonds before the entire latent complex is secreted (5). TGF- β is stored in the extracellular matrix as a complex of TGF- β , the pro-peptide, and LTBP (6). The attachment of TGF- β to LTBP prevents it from binding to its receptors. LTBP must be activated to release the mature bioactive TGF- β 1 protein, which binds to TGF- β receptors to elicit a response. TGF- β receptors are type 1 receptor (TGF β RI), type 2 receptor (TGF β RII) and type 3 receptor (TGF β RIII) (Figure 1). The first receptor discovered for TGF- β family members was a type II receptor for activin and thus was named ActRII. A second type II receptor for activin, ActRIIB, was then identified, as were type II receptors for TGF- β (TGF β RII), BMP-2 (BMPRII), and MIS (MISRII). Type I receptors were discovered and found to be transmembrane serine-threonine kinase receptors that were structurally related to type II receptors but had distinguishing features such as a GS (Glycine-Serine) region (7). TGF β RII is also a transmembrane-spanning serine-threonine kinase TGF- β receptor. The serine-threonine kinase receptor family represents the only known signaling receptors for TGF- β family members, and only TGF- β family members have been shown to signal through these receptors. TGF β RIII is a transmembrane proteoglycan with no intrinsic signaling capacity which binds with high affinity to all three TGF- β isoforms. It is thought that binding of TGF- β to the type III receptor increases the local concentration of ligand and enhances presentation of ligand to the type II receptor that has a lower intrinsic binding affinity for TGF- β . TGF β RIII functions by first binding to TGF- β and then transferring the cytokine to its signaling receptors TGF β RI and TGF β RII. .

TGF β RI and TGF β RII contain an extracellular ligand binding domain, a transmembrane domain, and a cytoplasmic serine-threonine kinase domain. Following binding of the ligand to TGF β RII, TGF- β forms TGF β RI/TGF β RII heteromeric complex at the cell surface, which mediates biological activities in cells (8). The TGF- β receptors discriminate between different TGF- β s and bind the different ligands with different affinities. For example, TGF- β 1 and TGF- β 3 bind to TGF β RII leading to the recruitment of TGF β RI into a signaling complex. TGF- β 2 has a stronger affinity to betaglycan, which can interact with TGF β RII/I. In the absence of TGF β RIII, an alternatively spliced variant of the TGF β RII binds to TGF- β 2 and signals without the recruitment of TGF β RIII (9). After binding with the ligand, TGF β RII autophosphorylates and then transphosphorylates TGF β RI through the GS domain (SGSGSG). Phosphorylation of the TGF β RI GS domain leads to the activation of its kinase and the L45 loop in the kinase domain confers a high degree of specificity in Smad interaction with TGF β RI. Once activated by the TGF β RII, the TGF β RI phosphorylates Smads through its SSXS motif (10). Smad2 and Smad3 associate with Smad4 as they are translocated to the nucleus (Figure 1).

3. TGF- β signaling pathway in tumor suppression

TGF- β is recognized for its tumor suppressor as well as tumor promoter activity. This dual role has been a major concern in deciding if an inhibitor of TGF- β and/or its downstream signaling pathway would be beneficial in the treatment of cancer.

In epithelial cells, TGF- β regulates cell proliferation, apoptosis, differentiation, senescence, cell shape, cell-cell and cell-extracellular matrix adhesion and cell motility as a tumor suppressor. The resistance may be due to loss of expression of the genes for one or more known components of the TGF- β signaling pathway. TGF- β inhibits the proliferation of epithelial cells in both developing organs and in adult organs (11). The inhibition of epithelial cell proliferation by TGF- β has been extensively studied in normal human and mouse epithelial cell lines (12). TGF- β can induce apoptosis of many epithelial cell types, including thyroid cells, hepatocytes and colon and mammary epithelial cells (3). Tissue specific inactivation of TGF β RII in mouse models rarely leads to spontaneous tumor formation with little to no pathology in many organs (13,14), suggesting that TGF- β elicits its antiproliferative effects in specific contexts.

Loss of TGF- β induced growth arrest may also result from either the aberrant expression of positive regulators, such as the cyclins and cyclin-dependent kinases (cdks), or the negative regulators, such as the cdk inhibitors. The downregulation of c-Myc expression by TGF- β has been observed to be lost in epithelial cells and in various cancer cell lines concomitant with the loss of the growth inhibitory response to TGF- β (15). TGF- β 1 can inhibit the ability of cells to enter S phase when the inhibitory peptide is added to cultures at both early and late points during the pre-replicative G1 period. In most epithelial, endothelial and hematopoietic cells, TGF- β mediates G1 cell cycle arrest by inducing or activating cdk inhibitors p16^{INK4A}, p15^{INK4B}, p21^{CIP1} and/or p27^{Kip1}, depending on the cell type (16) and downregulates Myc, Id1 and Id2. These three transcription factors are involved in proliferation and are inhibitors of differentiation. In most tumor cells, TGF- β is unable to induce p15^{INK4B} and p21^{CIP1} as well as the inability to downregulate Myc and Id proteins. In certain cases, this is due to the disruption of TGF- β signaling caused by somatic mutations in components of the TGF- β pathway (16).

The role of TGF- β as a tumor suppressor is also supported by functional inactivation of receptors and Smads (TGF- β receptor substrates), downregulation of receptors, and enhanced expression of TGF- β signaling inhibitors in human carcinoma and in tumor development in mouse models following genetic manipulation of signaling molecules (17). The primary target for TGF- β mediated tumor suppression is the tumor cell itself, which responds to its own TGF- β in an autocrine fashion to activate tumor suppressive biological responses. Inactivation of certain Smads appears to impart a distinct advantage in tumor progression. Although the mechanism of TGF- β -induced apoptosis *in vivo* remains to be established, candidates include several Smad-dependent and -independent mechanisms.(18).One function of the Smads as downstream effectors of TGF- β signaling is to act as tumor suppressors. The identification of somatic mutations in the genes for TGF β RI and TGF β RII, and in the Smad genes has provided strong support for their roles as tumor suppressors. Finally, the direct effects of TGF- β on tumor cells and its indirect effects on tumor growth suggest that the inhibition of TGF- β signaling may result in beneficial responses, especially in advanced stages of cancer. TGF β RIII has also been considered as a suppressor of cancer progression due to the following reasons: 1) the frequent loss of TGF β RIII expression in human cancers (19), 2) loss of expression correlation with disease progression and poor patient prognosis and 3) restoration of expression establishing a direct role for TGF β RIII in regulating cancer cell migration and invasion *in vitro* and angiogenesis and metastasis *in vivo*. There is compelling evidence supporting the concept that *TGF β RI* is a tumor suppressor gene, and *TGF β RI* mutations are

associated with various human cancers, including head and neck cancers, cervical and ovarian carcinomas (20–23). Inactivating mutations in *TGFβRI* and *TGFβRII* have also been reported in human lymphoma. Mutations in *TGFβRII* have been reported in colon and gastric cancers (24).

4. Tumor promoting roles of TGF-β

The most important signal transducers for the transmission of TGF-β intracellular signaling are the Smads. It has been clearly demonstrated that Smads have the ability to propagate signals from the activated receptor complex to the nucleus (25) (Figure 1). The receptor-activated (R-) Smads (Smad1, Smad5, Smad8 and Smad9 for BMPs, and Smad2 and Smad3 for TGF-βs and activins) are phosphorylated by type I receptors and then form hetero-oligomeric complexes with the common mediator Smad4, which are translocated to the nucleus where they regulate the transcription of specific genes (26). SARA (Smad Anchor for Receptor Activation) was described as recruitment factor for R-SMAD to the Type I receptor (27). SARA contains both a Smad-binding domain (SBD), which interacts with Smad2 and Smad3, and a C-terminal, TGFβR complex interacting region (27). SARA was therefore proposed to play a role in presenting R-Smads to the receptor for phosphorylation. The inhibitory (I-) Smads, Smad6 and Smad7, can inhibit signaling via heteromeric serine-threonine kinase receptor complexes in a feedback mechanism but also, , promote certain non-Smad signaling pathways. In the Smad pathway, the activated TGFβRI phosphorylates receptor-activated Smads (R-Smads), for example, Smad2 and Smad3 in response to TGF-β. Following phosphorylation by the TGFβRI kinase, R-Smads bind to the related Co-Smad (Smad4) and translocates to the nucleus, where the R-Smad-Smad4 complexes associate with other transcription factors to regulate the transcription of target genes (27). The phosphorylation of TGFβRI by TGFβRII kinase is required for I-Smads to bind to the TGFβRI. Structurally, the R-Smads and Smad4 share two homologous protein domains, MH1 and MH2. The carboxyterminal MH2 domain mediates Smad-receptor, Smad-Smad, and Smad-transcription factor interactions (27). Serine-threonine kinase receptor-associated protein (STRAP, a WD-40 repeat protein) can associate with Smad7 and activated type I and type II receptors. Thus, STRAP stabilizes the Smad7 association with the receptor complex and inhibits TGF-β-induced transcription, probably through blocking the access of Smad and Smad3 to the receptor complex (28,29).

Using the mouse model of squamous cell carcinoma that depends on the cooperation of Ras and TGF-β signaling, Oft and colleagues convincingly showed that tumor cell invasiveness and metastasis required the activity of H-ras and Smad2 downstream of TGF-β. Elevated H-ras levels, however, are required for nuclear accumulation of Smad2, both of which are essential for the EMT (30). A mouse model that is haploinsufficient for Smad4 and carries mutant Apc allele (Smad4^{+/-}/Apc^{Δ716}) develops invasive colon adenocarcinoma unlike the Apc^{Δ716} mouse, which only develops small intestinal adenomas (31). This model suggests that Smad4 inactivation may have a role in the progression of colon cancers as opposed to cancer initiation. K5-Cre;Smad4^{Co/Co} mice that are null for Smad4 in the skin spontaneously develop well differentiated squamous cell carcinomas, squamous papillomas, and cell carcinomas (32). Recently, Graff and colleagues observed a high frequency of invasive colon carcinoma in Smad3^{-/-} mice, suggesting that Smad3 is a tumor-suppressor gene in the colon (33).

I-Smads, Smad6 and Smad7, have been shown to be upregulated in a variety of tumors. Overexpression of Smad7 has been shown to induce carcinogenesis (12,34). Pancreas specific expression of Smad7 in mice results in development of premalignant ductal lesions with characteristics of pancreatic intraepithelial neoplasia and increased fibrosis (35), suggesting an important role of increased Smad7 expression in pancreatic carcinogenesis. Smad7 overexpression inhibits TGF-β induced growth inhibition and apoptosis in the colorectal cancer cell line by interfering with the formation of the Smad2/3-Smad4 complex (34). It is possible

that elevated expression of Smad7 in the early stages of cancer may result in acceleration of carcinogenesis through the loss of inhibition of tumor growth. Thus, blocking TGF- β signaling by Smad7 may provide new therapeutic strategies for control of advanced cancers.

TGF- β family proteins also signal through Smad-independent pathways, such as those mediated by the Ras-Raf-Erk mitogen-activated protein (MAP) kinase, Phosphatidylinositol-3 (PI3) kinase-Akt, Rho like GTPases, JNK and p38 MAP kinase pathways (36,37) (Figure 1). These receptor activated non-Smad pathways, regulate the cellular response to TGF- β either alone in conjunction with Smad signaling, or converge on to Smads to control Smad activity (38). The balance of direct Smad activation and activation of MAP kinase pathways may have a key role in the coordinated cellular response to TGF- β . TGF- β also activates RhoA, Rac1 and Cdc42 in the epithelial-mesenchymal transdifferentiation (39). Phosphatidylinositol-3 kinase (PI3 kinase) has a role in TGF- β signaling and TGF- β can activate PI3 kinase, as indicated by phosphorylation of its downstream effector Akt (40). TGF- β -induced activation of protein kinase A (PKA) requires formation of the Smad3 and Smad4 complex. The Smad3-Smad4 complex interacts with the regulatory subunit of PKA thereby releasing the catalytic subunit from the PKA holoenzyme (41). Although Akt does not phosphorylate members of the Smad family, it interacts with Smad3 resulting in changes of the sensitivity of various cell lines to TGF- β mediated apoptosis, suggesting that the balance between these inputs is critical for determining downstream responses (42). Similarly, TGF- β can stimulate the activation of the prosurvival factor NF- κ B, A correlation between TGF- β and activity of NF- κ B has been seen in tumor cell lines (43). TGF- β treatment was also demonstrated to induce a marked alteration in integrin expression in several cell types, resulting in modulation of cell binding to matrix components (44). Investigations into how these unique receptors control non-smad pathways promise to continue providing rich new insights into an astounding diversity of biological and disease processes. TGF- β is also a potent regulator of T-cell, neutrophil, monocyte, macrophage, natural-killer (NK)-cell, carcinoma-associated fibroblast and carcinoma-cell-autonomous signaling in the tumor microenvironment (45). TGF- β stimulates the migration of fibroblasts, T cells, neutrophils and monocytes, and influences their behavior to suppress or promote tumor progression. TGF- β attracts immune components to the tumor microenvironment, thereby enabling the expression of additional tumor promoting factors (45). Thus, TGF- β produced by tumor cells can suppress a functional immune response and inhibition increases the recognition and destruction of tumor cells. Recently TGF- β 's role was highlighted in stromal-epithelial crosstalk regulation of cancer and it was shown that stromal fibroblast-induced TGF- β signaling can be important for the suppression of tumorigenesis in adjacent epithelium (46). These results indicated that distinct TGF- β responses mediated by stromal fibroblasts can regulate tumor initiation and progression of adjacent epithelium. Systemic delivery of compounds used to inhibit TGF- β usually inhibit all host-tumor interactions that are regulated by TGF- β including those involving immune evasion, angiogenesis, stromal-epithelial crosstalk and tumor cell autonomous signaling (45,47,48).

Downregulation of E-cadherin and subsequent release of β -catenin is a central regulatory event in epithelial to mesenchymal transition (EMT), E-cadherin and other adherens junction proteins are major targets of signaling pathways downstream from the TGF- β receptor. TGF- β has a role in cell attachment, migration, invasion, wound healing and fibrosis (3). EMT, like TGF- β , is a well-coordinated process during embryonic development and a pathological feature in neoplasia and fibrosis (49). Epithelium in mature or adult tissues can also undergo EMT following epithelial stress, such as inflammation or wounding that leads to fibroblast production and fibrogenesis. Epithelia forming tumors involves EMT when carcinoma become metastatic (50). One such example is TGF- β 's triggering of EMT in hepatocellular carcinoma (HCC) (51) and, TGF- β mediated hepatocyte EMT contribution to liver fibrogenesis (52). TGF- β promotes EMT in late stages of tumorigenesis by a combination of Smad-dependent transcriptional events and Smad-independent effects on cell junction complexes (2,3). *In*

vitro experiments provided the first clues about the role of TGF- β superfamily members in modulating EMT. In fact, all three TGF- β isoforms, TGF- β 1, - β 2 and - β 3 are capable of eliciting EMT *in vitro* (53). TGF- β also induces EMT *in vivo* during tumor progression. In mouse tumors and cell lines, TGF- β -induced EMT is Smad-dependent and is enhanced by Ras signaling (2). TGF- β -induced EMT is observed in transformed epithelial progenitor cells with tumor-propagating ability (54). Smads act as regulatory factors of EMT and directly or indirectly activate transcription of mesenchymal markers (53). A number of transcriptional repressors of E-cadherin (Snail, Slug, Lef-1, Sip1) have been recently recognized in the transcriptomic analyses of epithelial cells undergoing TGF- β induced EMT. However, recent evidence suggests that EMT inducing factors such as Twist, Snail and TGF- β may also promote the expression of cell surface markers in presumptive tumor-propagating cells, also referred to as 'cancer stem cells' (54). The recent finding that transcriptional repression of Id (Id2, Id3) gene expression by TGF- β is linked to the process of EMT, has provided new mechanistic insight into the signaling pathways mediating this process (55). One characteristic event in TGF- β mediated EMT is the formation of focal adhesions where various tyrosine kinases and their substrates reside. It has been shown that c-Src, a non-receptor tyrosine kinase is involved in the turnover of focal adhesions and also in disruption of adherens junctions (56). Since c-Src has been reported to be activated by TGF- β in various types of cells (40,57,58), it has been hypothesized that c-Src activity is likely one driving force behind TGF- β mediated EMT.

Direct evidence to support TGF- β 's role in driving tumor metastasis comes from genetically engineered mouse models and preclinical studies of TGF- β antagonists (3,59). Recent studies have shown that TGF β RII knockout animals developed significantly more pulmonary metastases compared with control mice (60–62), suggesting that the role of TGF- β in metastasis depends on multiple factors, including the tumor-initiating mutation, process of TGF- β inactivation, and the timing of TGF- β signaling (13). To conclude, the studies in model systems have described a broad range of potential and sometimes contradictory TGF- β effects on metastasis.

5. Novel therapeutic inhibitors of TGF- β signaling pathway

The genetic and preclinical studies support targeting TGF- β signaling as therapeutic strategy for combating cancer. To date there have been three approaches to inhibit the TGF- β signaling pathway that have been investigated. They are: (1) the inhibition at the translational level using antisense oligonucleotides that can be engineered into immune cells or delivered directly into tumors, (2) inhibition of the ligand-receptor interaction using monoclonal antibodies, and (3) inhibition of the receptor-mediated signaling cascade using inhibitors of TGF- β receptor kinases (3). For each of these approaches, several drugs have been developed and are either in non-clinical or in early stages of clinical investigation. Some of these have already been shown to be efficacious in limiting tumor invasion and metastasis *in vivo*. One of the challenges of anti-TGF- β therapy will be in targeting the tumor promoting arm of TGF- β signaling while maintaining the tumor suppressive arm. (63) It has already been found that some conventional drugs appear to selectively block the EMT versus growth-inhibitory activities of TGF- β . For example, Rapamycin can reinstate the growth-inhibitory activity of TGF- β in cells that have lost this response and can also, potentiate TGF- β induced growth inhibition in responsive cells (64). This same drug inhibits EMT in mesothelial cells by blocking TGF- β induced Snail transcription (65).

The inhibition of EMT may have unexpected therapeutic advantages in targeting adenovirus-based therapies to cancer (66). Recent studies have shown that small-molecule inhibitors of the TGF- β receptors enhance expression of the adenoviral receptor protein, CAR, with resultant increases in adenoviral infectivity of carcinoma cells *in vitro*. This potentiates opportunities for combinational approaches with TGF- β inhibitors to enhance efficacy of viral drug delivery

(67). Therapeutic intervention can involve blocking the generation of TGF- β and its isoforms using DNA or RNA based inhibition and can also involve large molecules such as monoclonal antibodies. Some of the monoclonal antibodies are currently in advanced clinical investigation. Finally, there is significant focus on the development of small molecule inhibitors designed to block the TGF- β signaling downstream of TGF- β receptor. Companies such as GlaxoSmithKline, Scios, Pfizer, Biogen and Eli Lilly have active programs to develop small molecule kinase inhibitors targeting TGF β RI and TGF β RII kinases (Table 1).

(a) Large Molecule Inhibitors

The advances in antisense oligonucleotides (ASOs) technology have allowed several ASOs to enter clinical investigation (Table 1). However, recent clinical failures of the phosphorothioate ASOs, oblimersen and aprinocarsen have shown specific pharmacokinetic behavior that limits their ability to reach tumor tissue (68). Antisense Pharma has two TGF- β specific phosphorothioate antisense oligonucleotides in development (69) but have recognized the above limitations for the clinical development of AP-12009, a specific phosphorothioate ASO directed against the mRNA of TGF- β 2 (70) (Figure 2). TGF- β 2 plays a pivotal role as a multimodally acting cytokine by regulating key mechanisms of tumor progression. Immunosuppression, invasion, migration, proliferation and angiogenesis are simultaneously promoted in a variety of malignant tumors. This multiple impact on cancer cells is inhibited by AP-12009. *In vitro* experiments have been performed to prove specificity and efficacy of AP-12009 employing patient-derived malignant glioma cells as well as peripheral blood mononuclear cells (PBMCs) from patients. Currently, phase I/II studies in advanced pancreatic carcinoma, metastatic melanoma, and metastatic colorectal carcinoma and a phase IIb study in recurrent or refractory high-grade glioma are ongoing. The high efficacy of AP 12009 in high-grade glioma patients is underscored in the active-controlled Phase IIb study AP12009-G004. This study showed a strong survival benefit in patients treated with AP 12009 compared to standard chemotherapy treatment. The survival data observed in this Phase I/II study looks promising given the poor prognosis associated with pancreatic cancer and melanoma. These results support targeted TGF- β 2-suppression using AP-12009 as a promising novel approach for malignant gliomas and other highly aggressive, TGF- β 2-overexpressing tumors (70). Additionally, a TGF- β 1 specific ASO, AP-11014, designed by the same company, is under preclinical development. AP-11014 is being developed for the treatment of non-small cell lung carcinoma (NSCLC), colorectal and prostate carcinomas. Based on data from a clinical trial with AP-11014 in TGF- β 1 over-expressing tumors was planned (71). This drug is currently in advanced stage of pre-clinical development. The antisense drug AP-15012 for the treatment of cancer and other molecules for cancer and non-cancer indications is in the stage of discovery. Lucanix from NovaRx is a non-viral gene based allogeneic tumor cell vaccine which demonstrates enhancement of tumor antigen recognition as a result of TGF- β 2 inhibition. Recently, randomized dose variable phase II trial was performed with stage IIIB/IV NSCLC patients. Lucanix was demonstrated to be safe and well tolerated. A survival advantage is suggested in patients who receive $\geq 2.5 \times 10^7$ cells/injection, thereby supporting the justification for further phase III evaluation (72).

Although strategies using ASO technology or vaccines have not yet produced an approved drug, monoclonal antibodies (e.g., trastuzumab and rituximab) have been successfully developed for a number of cancer indications (73,74). The antibodies must overcome significant physical barriers to penetrate a solid tumor mass including the vascular endothelium, stromal barriers, high interstitial pressure and epithelial barriers (75). Monoclonal antibodies are frequently the therapeutic method of choice for inhibiting TGF- β family signaling since they are particularly effective; they bind directly to the ligand and prevent binding to its receptor, effectively shutting down ligand signaling. Some of the monoclonal antibodies are currently in advanced clinical investigation, including the monoclonal antibodies

lerdelimumab and metelimumab. Lerdelimumab (CAT-152, Trabio™) is a recombinant human IgG4 directed against TGF-β2. It is currently being investigated for the treatment of post-surgical fibrosis. Results of Phase II clinical trials of lerdelimumab in patients undergoing glaucoma surgery justified proceeding with further clinical trials. It is currently being investigated in Phase III trials for the prevention of post-operative scarring following primary trabeculectomy (76), and two large Phase II/III trials are currently ongoing at Cambridge Antibody Technology (London, United Kingdom). Metelimumab (CAT-192), a human IgG4 monoclonal antibody directed against TGF-β1 is being developed by Cambridge Antibody Technology in collaboration with Genzyme (Framingham, MA). It is currently undergoing Phase I/II trials in patients with diffuse scleroderma, but no studies in cancer have been conducted. Cambridge Antibody Technology is also developing a series of human pan-TGF-β monoclonal antibodies (GC-1000 series) in collaboration with Genzyme. Although non-clinical investigations in cancer are ongoing with this series, GC-1008 is currently the only monoclonal antibody from this series selected for clinical investigation (Cambridge Antibody Technology, 2004 press release). GC-1008 is a pan-neutralizing IgG4 human antibody directed against all three isoforms of TGF-β. Metastatic melanoma and renal cell carcinoma (RCC) are being investigated as possible targets for anti-TGF-β therapy as both are known to be immunogenic cancers. GC-1008 is currently being studied in a Phase I/II dose-escalation study in patients with advanced metastatic melanoma or renal cell carcinoma (RCC) (Genzyme, Press Release). In mice, a TGF-β neutralizing antibody suppressed radiation-induced acceleration of metastatic breast cancer progression (59). Other companies such as Genentech have announced that they are also pursuing monoclonal antibodies against TGF-β (March 28-April 2, 2005, Keystone Meeting).

Soluble receptors (sRs) coupled to Fc portions are an alternative to monoclonal antibodies. They also abrogate signaling at the ligand level by binding ligand and preventing it from binding to cell surface receptors. Soluble TGFβRII has anti-cancer effects in mice, it suppresses the growth and metastasis of pancreatic cancer cells (77) and inhibits breast cancer cell growth, migration, invasion and metastasis (78). Studies have been performed using transfected construct for soluble TGFβRII (sTGFβRII) in the pancreatic cancer cell line PANC-1 in a xenograft model and demonstrated advantages including suppression of intrapancreatic tumor growth and local as well as distant metastases, suppression of angiogenesis, inhibition of PAI-1 and uPA overexpression, and potentially, suppression of uPA-mediated TGF-β activation. These results suggest that sTGFβRII targets many of the deleterious aspects that occur as a consequence of TGF-β overexpression in pancreatic ductal adenocarcinoma (PDAC) (77). Soluble TGFβRIII has demonstrated efficacy of inhibiting the growth and angiogenesis of human colon and breast cancer cells *in vivo* (79).

However, since tumors are likely to be under the influence of multiple TGF-β isoforms, pan-TGF-β antibodies might be more effective than isoform-specific ones. Two pan-TGF-β monoclonal antibodies, 1D11 and 2G7 have been described (80). Interestingly, although the 2G7 antibody had no effect against MDA-231 cells *in vitro*, it suppressed the establishment of MDA-231 tumors and lung metastases, possibly via inhibition of TGF-β mediated immunosuppressive mechanisms (80). Ananth et al (81) showed that the treatment of human renal cell cancer xenografts in nude mice with anti-TGF-β neutralizing antibody resulted in tumor regression in association with a marked decrease in microvascular density. Further strategies aimed at blocking ligand access to TGF-β receptors resulted in the development of soluble TGF-β receptors consisting of recombinant proteins containing the extracellular domain of TGFβRII fused with the Fc portion of the heavy chain of murine IgG1 (Fc:TGFβRII). This study showed that systemic administration of Fc:TGFβRII protein to MMTV-polyomavirus middle T-antigen transgenic mice increased apoptosis in primary tumors and inhibited lung metastases. Fc:TGFβRII also inhibited metastases from transplanted 4T1 and EMT-6 mammary tumors in syngeneic BALB/c mice (78).

Molecular targeting of the TGF- β pathway in tumors is based on the rationale that TGF- β exerts strong immunosuppressive effects in these tumors and blocking TGF- β function might enable the immune system against the tumor. Overall, these non-clinical studies show that TGF- β inhibition results in antitumor effect and is well-tolerated in mice over a long period of treatment. Whether this strategy will work in humans warrants further investigation.

(b) Small Molecule Inhibitors

Small molecule inhibitors have been developed to block TGF- β superfamily signaling at the receptor level. Biochemical mechanism of action and crystallography studies confirmed that several different classes of highly selective and potent chemical inhibitors competitively bind to the ATP-binding pocket of the target enzyme (82–84). Initial drug discovery efforts were focused on TGF β RI kinase as a therapeutic target (83,84). Most TGF- β signaling small molecule inhibitors are targeted to the kinase domain of TGF β RI (Figure 2), which differs considerably from that of TGF β RII, thus giving specificity for inhibition of TGF β RI versus TGF β RII signaling. However, to date there are no reports of signals mediated directly via TGF β RII independently of a TGF β RI. The main strategies for inhibition of TGF- β signaling pathway is to include compounds that interfere with the binding of TGF- β to its receptors, drugs that block intracellular signaling, and antisense oligonucleotides. The strategies, aimed at directly blocking the catalytic activity of TGF β RI, include small molecules such as SB-431542 and SB-505124 (GlaxoSmithKline), SD-093 and SD-208 (Scios), and LY580276 (Lilly Research Laboratories) (references in Table 1), which act as competitive inhibitors for the ATP-binding site of TGF β RI kinase and whose structural properties have been reviewed (83) (Figure 2). SD-093 and LY580276 have been shown to block EMT and tumor cell migration in pancreatic cancer and mouse mammary epithelial cells, respectively (82,85). Furthermore, SD-093 inhibits the basal migratory and invasive phenotype that is generated by autocrine TGF- β signaling in Smad4-deficient BxPC-3 pancreatic cancer cells. SD-208 was recently tested in non-clinical cancer models. Recent study with administration of the TGF β RI kinase inhibitor SD-208 to mice bearing intracranial SMA-560 gliomas resulted in increased infiltration of the tumor with immune effector cells and prolonged survival (86). Similar results in the SMA-560 model have been obtained using SX-007, an orally bioavailable pyridopyrimidine TGF β RI kinase inhibitor (87). Indeed, SB-431542, SB-505124, and LY580276 also inhibit activin receptor-like kinases ALK-4 and ALK-7 besides TGF β RI (ALK-5), which indicates that they can inhibit activin-mediated activation of Smad2 and Smad3 (82,88). A small molecule inhibitor of TGF- β /ALK5 kinase activity, LY573636, is currently being assessed in patients with malignant melanoma, soft tissue sarcoma, NSCLC, and ovarian cancer (89,90). Similar observations have been made for A-83-01 (91). IN-1130, a TGF β RI kinase inhibitor was recently developed by In2Gen Co., which suppresses renal fibrosis in obstructive nephropathy (92). The involvement of these biological processes in tumorigenesis has not yet been fully addressed, although they could also play a significant role in cancer.

Recently, Akhurst group (93) used LY2109761, which has a K_i of 38 nM in blocking TGF β RI kinase and an IC_{50} of 300 nM in blocking TGF β RII, and stated that the advantage of this drug over more TGF β RI-specific ones is that it is relatively metabolically stable and may be used for *in vivo* studies. In our recent study with *in vivo* mouse colon metastasis model, LY2109761 decreased liver metastases and prolonged survival (94), and another study demonstrated a decrease in metastasis *in vivo* in pancreatic cancer mouse model (95). HTS-466284 (also referred as LY364947) is an inhibitor of TGF β RI and showed TGF- β signaling reporter gene activation when compared to the query compound, SB203580 (96). The relevance of HTS-466284 to inhibiting TGF- β signaling has been underscored with the independent identification of this molecule by Eli Lilly and Company in a conventional high-throughput screen (96). Moreover, Jinnin et al (97) recently described a specific inhibitor of

Smad3 (SIS3), by selectively inhibiting Smad3 activation and Smad3-mediated DNA binding and gene expression with as yet unknown molecular mechanism of action. Further evidence for activity of these small molecule TGF β RI kinase inhibitors is their ability to modulate TGF- β induced EMT which leads to tumor cell invasion and metastasis. One compound, LY2157299, that can be orally administered, has entered phase I trials for advanced/metastatic cancer (83). Daily oral administration of LY2157299 was safe and well tolerated at the two dose levels and the pharmacokinetic profile was consistent with the prediction derived from preclinical pharmacokinetic/pharmacodynamic (PK/PD) model (98). Other compounds with similar characteristics are still in preclinical stages.

TGF- β has been shown to protect transformed cells from apoptosis. This protection from cell death is blocked by the PI3 kinase inhibitor LY294002 (99) or TGF β RI kinase inhibitors (100). Activated Ras can inhibit TGF- β induced nuclear accumulation of Smad2 and Smad3, Smad-dependent transcription, and the growth-inhibitory effect of TGF- β (101). TGF- β can activate Ras-MAP kinase signaling in transformed cells (36) and inhibition of endogenous TGF- β function with dominant-negative truncated TGF β RII blocks the growth of Ras-transformed Jnk^{-/-} fibroblast as tumors (102). Oncogenes that activate Ras-MAP kinase signaling may also engage and require TGF- β for tumor progression and tumors with activating Ras mutations or with an activated Ras-MAP kinase pathway are attractive targets for TGF- β inhibitors. It has been shown that seemingly independent pathways such as the Ras-MEK-ERK and TGF- β signaling pathways can synergize to stimulate EMT, tumor invasion and metastasis. Thus, inhibitors of the Ras-MEK-ERK pathway, together with TGF- β inhibitors, may reduce EMT and tumor spread. Current experience with some of the above mentioned agents suggests that they are surprisingly nontoxic (83). These data suggest a basis for the combinations of TGF- β inhibitors with anticancer therapy. Deciding which of these molecules to advance into clinical investigation remains challenging. In summary, selective TGF- β /Smad inhibitors have been identified via target based drug discovery. TGF- β /Smad inhibitors should be investigated in patients with enriched expression of the respective target or where the target plays a critical role in tumor growth. The novel TGF- β signaling inhibitors should be developed using pharmacokinetic prediction models as well as biomarkers that allow rapid determination whether the TGF β RI kinase inhibitors achieve biologically effective doses at the predicted pharmacokinetic exposure.

6. Conclusions

TGF- β is the most potent and naturally occurring inhibitor of cell growth as well as one of the most potent naturally occurring tumor suppressors. Among several other signal transduction pathways, the TGF- β pathway is being extensively evaluated as a potential therapeutic target (83). However, the dual role of TGF- β in oncogenesis requires the detailed understanding of the TGF- β biology in order to design successful therapeutic approaches and prevent undesired side effects. Understanding the detailed nature of the interactions between TGF- β and its receptors represents a critical new step forward and provides an opportunity to discover new drugs that mimic the interactions between TGF- β and its receptors. Mechanistically, they should block assembly of the TGF- β signaling complex and in turn eliminate the tumor-promoting activities of TGF- β s. TGF- β is very selective in its interactions with its receptors. The complexity of Smad and non-Smad proteins, that orchestrate the EMT and metastatic responses, promises several additional important players to be discovered. Recent discovery that TGF- β /BMP/Smad signaling also regulates microRNA expression has pointed out a new path toward yet another unexplored territory of TGF- β research (103). The TGF- β inhibitors in preclinical and clinical development suggest that the therapy in combination with other conventional and molecular targeted anticancer agents will be addressed in the near future (69). Many research groups are developing anti-TGF- β therapies to address disease situations in which the tumor-promoting activities are fueled by TGF- β over-expression. Design of EMT,

tumor metastasis and invasion specific drugs await the discovery of novel mediators of the processes that are amenable to pharmaceutical intervention. Preclinical studies have provided convincing evidence that targeting the TGF- β pathway is able to inhibit tumor growth and metastasis *in vivo*.

7. Expert opinion

TGF- β signaling certainly has an important role in the development of most human solid tumors, and as such it has been considered as a very viable therapeutic target. The current evidence suggests that it is possible to differentiate between the tumor suppressor and promoting effects of TGF- β . Among the cellular mechanisms that explain the tumor promoting action of TGF- β , EMT still represents an active research area that requires deeper investigation. In TGF- β signaling, Smads transduce the signals from ligand-receptor complexes at the cell surface to gene transcription in the nucleus. Specific Smad-protein interactions determine signaling specificity. The inhibitory Smads, coreceptors at the surface, and intracellular kinases can modify the signaling strength of Smads and represent another strategic area to be considered in targeting TGF- β pathway. For example, although Smad2/3/4 signaling plays a tumor suppressor role, it also exhibits a pro-metastatic function in breast cancer (104) and melanoma metastasis to bone (105). In contrast, mutation or reduced level of Smad4 in colorectal cancer is directly correlated to increased metastasis and poor survival (106,107).

Several strategies are under development and include natural and synthetic inhibitors of TGF- β , TGF- β neutralizing antibodies, soluble forms of TGF β RII and TGF β RIII, and TGF β RI and TGF β RII kinase inhibitors. All these approaches have promising therapeutic potential given the magnitude of all the tumor promoting activities of TGF- β signaling. If the new TGF- β inhibitors prove to have adverse side effects due to the multifunctionality of TGF- β , the more targeted approaches towards Smad function or towards some of the regulators of the pathway will be warranted. In order to design effective cancer therapeutics that targets the TGF- β signaling pathway, it is crucial to identify the factors that determine the balance between the opposing properties of TGF- β signals. Also, in order to formulate personalized therapies based on the manipulation of TGF- β signaling in tumors, it is necessary to establish the influence of other signaling cascades on TGF- β signals. Therapeutic approaches designed with such underlying principles should potentially have better efficacy and less toxicity than the ones that are in use currently. Some of the key issues to be solved are: (1) to identify the mechanisms through which Smad-independent signaling pathways are activated by the receptors and how these pathways contribute to the cellular response, (2) determine the relative importance of the pro-apoptotic and anti-migration/invasion effects of TGF- β are compared with other potential tumor suppressor activities and (3) to consider therapeutic approaches based on the regulation of the specific signal transduction pathways that result aberrantly up- or down-regulated as consequence of TGF- β signaling inactivation.

Progress in delineating the pro-tumorigenic effects and mechanisms of TGF β in specific tumor types and in different stages of cancer progression is essential for determining when and how anti-TGF β targeted therapy might be feasible. Although TGF- β is recognized as important carcinogenesis associated factor, it is still not clear whether it is a general tumor related factor or if this is its role is etiological. For example, TGF- β is well recognized as most important pro-fibrogenetic cytokine in liver fibrogenesis (108) and a recent study demonstrated that there are major etiology dependent differences, that is TGF- β tightly correlated with fibrotic stage in chronic HBV, but not in chronic HCV. Thus, when designing a therapeutic strategy based on inhibiting TGF- β signaling, it maybe required to first indicate the TGF- β role before performing a clinical trial.

Currently, there are emerging developments with various TGF- β inhibitors and significant developments will include the introduction of oral agents, such as the small-molecule TGF β RI kinase inhibitors. In contrast to the monoclonal antibodies blocking TGF- β ligands, small molecules are likely to have a higher penetration in solid tumors and maybe active in broader range of tumor types. TGF- β pathway inhibitors, including small and large molecules, have now entered clinical trials and large companies such as GlaxoSmithKline, Eli Lilly, Johnson and Johnson and Scios are pursuing this approach with considerable effort.

The combination of *in vivo* imaging of TGF- β receptors, their regulators and their effectors, loss of function analysis using RNAi and knockout mouse models, and 'omics' technologies will be powerful tools in answering some of these questions. Discovering new molecular markers that can be used either prognostically or diagnostically to define EMT is an important area of study in the field of cancer targeting therapeutics. Identification of signaling effectors of TGF- β that contribute to tumor progression and EMT may allow the development of even more sophisticated pharmacological approaches in the battle against cancer.

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Figure 1. The TGF- β signaling through Smad-dependent and Smad-independent pathways
 TGF- β receptor signals through multiple signaling cascades. TGF- β activates Smad and non-Smad pathways. The ligand-activated TGF β RI and TGF β RII receptor complex recruits and phosphorylates the receptor specific R-Smads. The R-Smads are presented to the TGF β RI by a membrane-bound protein, SARA. R-Smad proteins are cytoplasmic molecules which, after receptor activation, associate with Co-Smad. Hetero-oligomeric complex of R-Smad-Co-Smad translocates to the nucleus and binds to specific DNA sequence and interacts with transcription factors (TF) and Co-factors (CF) to regulate transcription. R-Smads and Co-Smad shuttle between nucleus and cytoplasm. The pathway is negatively regulated by the I-Smads which binds activated TGF β RI, thereby preventing phosphorylation of R-Smads, or recruits the E3 ubiquitin ligases, Smurf1 and Smurf2 to induce proteasomal degradation of the R-Smads.

STRAP interacts with TGF β RI and TGF β RII and with I-Smad, thus stabilizing the interaction of I-Smad with the receptor complex. Activation of the epidermal growth factor (EGF) receptor and other tyrosine kinase receptors, interferon (IFN)- γ signaling, and activation of NF- κ B by tumor-necrosis factor (TNF)- α , also induce I-Smad expression, leading to inhibition of TGF- β signaling. Although the mechanism of activation of non-Smad pathways by the receptor complex remains largely unknown, these pathways are known to be involved in the pro-oncogenic responses to TGF- β in cooperation with or without Smad pathway. The activated receptor complex activates non-Smad signaling pathways, such as PI3K/Akt, Rho/Rac GTPases and Ras/MAPK and TAK1/MEKK1. TAK1 (TGF- β activated kinase 1) may mediate TGF- β effects with respect to the activation of distinct MAP kinase pathways (JNK, p38). TGF- β also induces activation of the Cdc42 GTPase which stimulates p21 activated kinase (PAK) downstream from Rac and Cdc42. TGF- β activation of PP2A, that inhibits S6K activity through the mTOR/FRAPPP2A-S6K pathway, mediates Smad-independent growth suppression. SBE: Smad binding element and TBE: Transcription factor binding element.

Figure 2. TGF-β signaling pathway inhibitors that are currently under development for potential cancer therapy

The TGF-β signaling pathway offers many different avenues for therapeutic intervention, each of which offers advantages and drawbacks. Current therapeutic approaches for modulating TGF-β signaling involve antagonism of TGF-β ligand binding to the heteromeric receptor complex with isoform-selective antibodies as large molecule inhibitors, such as metelimumab (for TGF-β2) and lerdelimumab (for TGF-β2/3) from Genzyme, and pan-TGF-β antibodies such as GC-1008 (Genzyme), ID-11 (Genzyme), SR-2F (NCI/NIH) and 2G7 (Genentech) antibodies, or soluble TGF-βRII receptor fusion proteins (Biogen). Alternatively, the intracellular inhibition of the TGF-βRI kinase can be achieved with small molecule inhibitors,

such as SB431542 and SB505124 from GlaxoSmithKline; LY580276, LY550410, LY364947, LY573636 and LY2157299 from Eli Lilly; SD093 and SD208 from Scios. Other inhibitors for TGF- β receptor kinase are NPC30345 (Scios), Ki-26894 (Kirin brewery), SM16, A-83-01 (Kyoto Pharma), SX-007 (Scios) and IN-1130 (In2Gen), and dual inhibitors of both the TGF β RI and TGF β RII kinases, such as LY2109761 (Eli Lilly). Alternatively, expression of TGF- β isoforms can be affected by antisense technology targeting mRNA sequence-specific degradation, such as AP-12009 and AP-11014 which were developed by Antisense Pharma and targeted to TGF β II and TGF β I, respectively. SIS3 is a selective chemical inhibitor of Smad3 activation and Smad3-mediated DNA binding and gene expression.

Table 1Development of TGF- β signaling pathway inhibitors and their current status.

Agent	Drug target	Clinical studies	Company/sponsor	Reference
<i>Antisense oligonucleotides</i>				
AP-12009	mRNA TGF- β 2	Phase III	Antisense Pharma	(109)
AP-11014	mRNA TGF- β 1	Preclinical	Antisense Pharma	(71)
NovaRx	TGF- β 1 & TGF- β 2	Phase I/II	NovaRx	(110)
<i>Large molecule inhibitors</i>				
Lerdelimumab (CAT-152)	TGF- β 2/-3	Phase III	CAT	(111)
Metelimumab (CAT-192)	TGF- β 2	Phase II	CAT/Genzyme	(112)
GC-1008	Pan TGF- β	Phase I	CAT/Genzyme	(110)
ID11	Pan TGF- β	Preclinical	Genzyme	(113)
SR-2F	Pan TGF- β		NCI/NIH	(110)
2G7	Pan TGF- β	Phase III	Genentech	(80)
<i>Small molecule inhibitors</i>				
LY-550410	TGF β RI kinase	Preclinical	Eli Lilly Research	(83)
LY-580276	TGF β RI kinase	Preclinical	Eli Lilly Research	(84)
LY-364947	TGF β RI & RII kinase	Preclinical	Eli Lilly Research	(114)
LY-2109761	TGF β RI & RII kinase	Preclinical	Eli Lilly Research	(84)
LY-2157299	TGF β RI kinase	Preclinical	Eli Lilly Research	(83)
LY-573636	TGF β RI kinase	Phase II	Eli Lilly Research	[§]
SB-505124	TGF β RI kinase	Preclinical	GlaxoSmithKline	(71)
SB-431542	TGF β RI kinase	Preclinical	GlaxoSmithKline	(115)
SD-208	TGF β RI kinase	Preclinical	Scios	(86)
SD-093	TGF β RI kinase	Preclinical	Scios	(85)
Ki-26894	TGF β RI kinase	Preclinical	Kirin Brewery Co.	(116)
Sm16	TGF β RI kinase	Preclinical		(117)
NPC-30345	TGF β RI kinase	Preclinical	Scios	(118)
A-83-01	TGF β RI kinase		Kyoto Pharma	(91)
SX-007	TGF β RI kinase		Scios	(87)
IN-1130	TGF β RI kinase		In2Gen Co.,	(92)
<i>Other inhibitors</i>				
Trx-xFoxH1b (Interacting peptide aptamers)	Smads	Preclinical		(119)
Trx-Lef1 Antisense-transfected tumor cells (Vaccine)	TGF- β 2	Phase I and II		(72)
Soluble TGF β RII:Fc (Stabilized soluble protein)	TGF- β Rs	Preclinical	Biogen	(78)
Lucanix (Vaccine)	TGF- β 2	Phase II	NovaRx	(72)
Betaglycan / TGF β RIII (Recombinant soluble)	TGF β RIII			(120)

CAT: Cambridge Antibody Technology; NCI: National Cancer Institute; NIH: National Institutes of Health; TGF: Transforming growth factor

[§]<http://clinicaltrials.gov/show/NCT00383292>