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Targeting the Transforming Growth Factor-β Signaling in Cancer Therapy

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Abstract

TGF-β pathway is being extensively evaluated as a potential therapeutic target. The transforming growth factor-β (TGF-β) signaling pathway has the dual role in both tumor suppression and tumor promotion. To design cancer therapeutics successfully, it is important to understand TGF-β related functional contexts. This review discusses the molecular mechanism of the TGF-β pathway and describes the different ways of tumor suppression and promotion by TGF-β. In the last part of the review, the data on targeting TGF-β pathway for cancer treatment is assessed. The TGF-β inhibitors in pre-clinical studies, and Phase I and II clinical trials are updated.

Key Words: Transforming growth factor-β (TGF-β), EW-7197, ALK5, Breast cancer, Metastasis

INTRODUCTION

Transforming growth factor-β (TGF-β) is a multifunctional cytokine that regulates proliferation, differentiation, development, angiogenesis, wound healing and other functions in many cell types (Massagué, 2008). Regarding pathological disorders, such as cancer, TGF-β plays two conflicting roles of a tumor suppressor and a tumor promoter. TGF-β acts as a tumor suppressor in the early stage of cancer development, whereas in late stage it can take on role of tumor promoter, favoring of invasion and metastasis (Mishra *et al*., 2005; Padua and Massagué, 2009). TGF-β is a one of members of a large superfamily of secreted proteins that include three TGF-β isoforms (TGF-β1, -β2 and -β3), activins, bone morphogenetic proteins (BMPs), inhibins, nodal, and others. Cancer cells, in general, secrete larger amounts of TGF-β than their normal counterparts (Kingsley, 1994; Massagué, 2000). TGF-β is secreted from the cell as an inactive latent homodimeric polypeptide bound to other extracellular proteins (Roberts and Wakefield, 2003; Muraoka-Cook *et al*., 2005). The mature, bioactive TGF-β is produced on proteolytic cleavage of the latent complex. TGF-β interacts with its four receptor subunits that interact not only with TGF-β, but also with each other. Understanding the detailed mechanism of the interactions between TGF-β and its receptors presents potential opportunities to find new drugs. Such drugs or blockades could be designed to inhibit the assembly of the TGF-β signaling complex and in turn eliminate its tumor-promoting activities.

THE MECHANISM OF TGF-β SIGNALING

TGF-β1 binds with high affinity and selectivity to the transmembrane TGF-β type II receptor (TβRII), and this recruits the TGF-β type I receptor (TβRI or ALK5) and activates TβR1 kinase (Fig. 1). Activated TβR1 then initiates canonical Smad signaling by phosphorylation of receptor-associated Smads (R-Smads), Smad2, and Smad3. The phosphorylated Smad2/3 forms a complex with Smad4, the common mediator Smad, and translocate into the nucleus. In the nucleus, Smad complexes accomplish high affinity binding to Smad-binding elements within the promoter region of TGF-β target genes, showing TGF-β dependent transcription (Shi and Massagué, 2003). TβRI and TβRII are transmembrane serine/threonine kinases, and there are seven TβRIs and five TβRIIs are identified in humans (Padua and Massagué, 2009). TβRI and TβRII are paired in different combinations for different ligands, for example, TGF-β1 signaling needs ALK5/ TβRII combination in most cells. Smads are intracellular proteins characterized by Mad homologous domain at N-terminus (MH-1) and at C-

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terminus (MH-2), and linker connecting between these two MH-1 and MH-2 domains. The linker recruits ubiquitin ligases (E3 ligase, and smurfs) and is phosphorylated by other kinases such as MAPK and cyclin dependent kinases (Massagué, 2008). TGF-β can also signal through Smad-independent signaling, including the PI3 kinase, MAPK, TRAF6-TAK1 and RhoA-Rock pathways (Derynck and Zhang, 2003) (Fig. 1). The antagonistic Smads, Smad-6 and Smad-7, are thought to function by blocking ligand-dependent signaling (Heldin *et al*., 1997; Zhang and Derynck, 1999). The canonical TGF-β/Smad signaling pathway is essential to the growth inhibitory action of TGF-β, however, the relative contribution of canonical and non-canonical pathways to other TGF-β regulated biological processes, such as EMT and apoptosis, is still under investigation.

TGF-β AS A TUMOR SUPPRESSOR

Mutations in genes of the components of TGF-β signaling have been identified and mutations in TβRII, TβRI, Smad-2 and Smad-4 are most common. These mutations are observed in GI tract cancers such as colorectal cancer (CRC), in gastric, pancreatic, biliary tract, lung and brain (glioma) tumors (Grady *et al*., 1998; Levy and Hill, 2006). The TβRII gene mutations are abundant, because TβRII is a mutational hotspot due to its 10 base poly-A repeat within its coding sequence (Markowitz *et al*., 1995; Parsons *et al*., 1995; Akiyama *et al*., 1997; Takenoshita *et al*., 1997; Grady *et al*., 1999; Connolly *et al*., 2012). Mutations in TβRI are not as frequent as Tβ RII, although they have been described in pancreatic, colorectal, ovarian and head and neck cancers (Goggins *et al*., 1998; Wang *et al*., 2000; Chen *et al*., 2001). Mutations of TβRII and TβRI are relatively rare in breast, skin and hematological cancers (Dong and Blobe, 2006; Levy and Hill, 2006; Connolly *et al*., 2012). A number of mutations in Smad-2 and Smad-4 have been identified in pancreas, ovarian, cervical, liver, CRC, lung, and other cancers (Eppert *et al*., 1996; Yakicier *et al*., 1999; Wang *et al*., 2000; Maliekal *et al*., 2003). The genetic studies from human tumors argue that the Smad-dependent TGF-β pathway acts as a tumor suppressor in many types of human cancers, particularly those of the GI tract. The action of TGF-β as a tumor suppressor is shown by functional inactivation of receptors and Smads, and elevated expression of TGF-β sig-

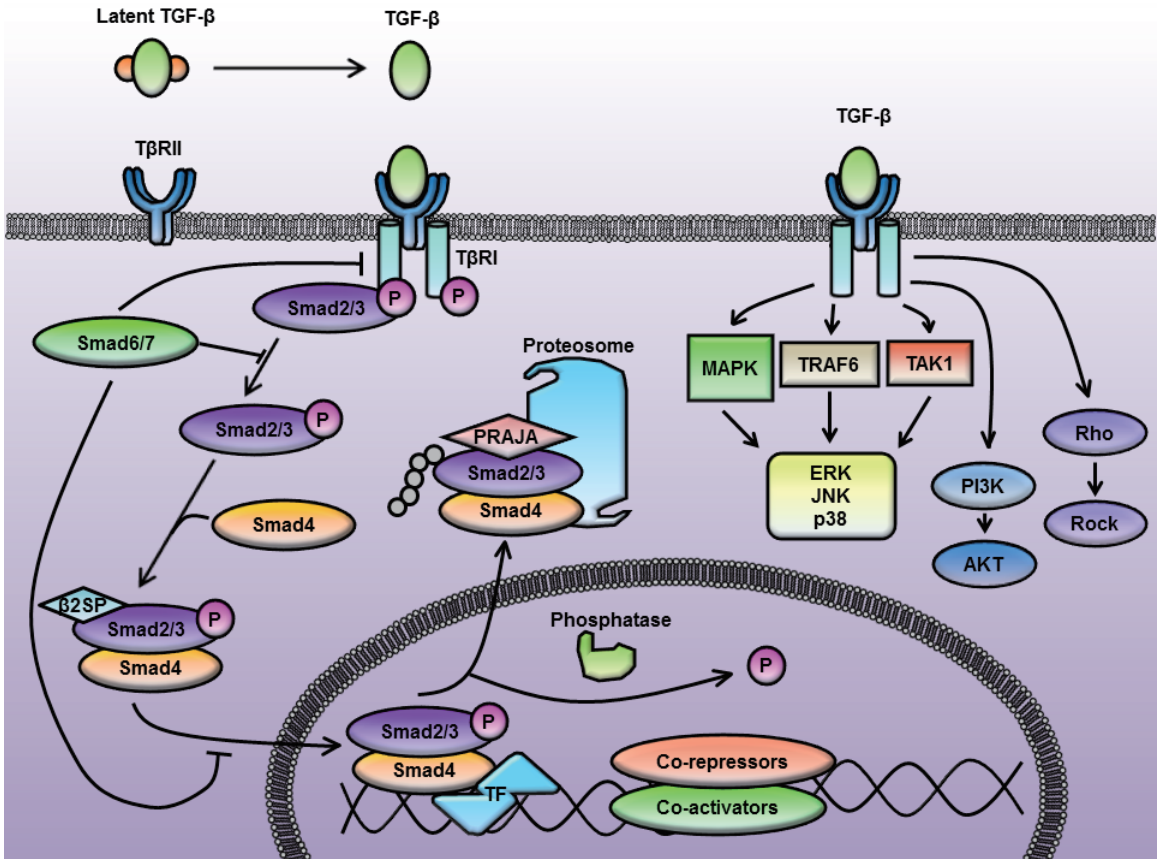

Fig. 1. TGF-β signaling through Smad-dependent and Smad-independent pathways. (A) Canonical pathway. TGF-β ligands activated TGF-βRI and TGF-βRII receptor complex recruits and phosphorylates the receptor specific Smad2/3. Hetero-oligomeric complex of Smad2/3-Smad4 translocates to the nucleus and binds to specific DNA sequence and interacts with transcription factors and cofactors to regulate transcription. The pathway is negatively regulated by the Smad6/7, which bind activated TGF-βRI, thereby preventing phosphorylation of Smad2/3, or recruit the E3 ubiquitine ligases to induce proteasomal degradation of the Smad2/3 (B) Noncanonical pathway. Smad-independent signaling. TGF-β can promote the activity of several signaling pathways other than Smad, including MAPKs, PI3K kinases, TRAF6-TAK1-p38/JNK, Rho-Rock, among others.

naling in human carcinoma and in mouse tumor models (Datta and Mann, 2008). TGF-β controls cell proliferation mainly by inhibiting cell cycle progression through G1-arrest (Fig. 2). In most epithelial, endothelial and hematopoietic cells, TGF-β arrest cell cycle at G1 by inducing or activating cdk inhibitors such as, p16INK4A, p15INK4B, p21CIP1 and/or p27Kip1 (Massagué and Gomis, 2006). TGF-β inhibited transcription factors, such as Myc, Id1 and Id2, involved in proliferation and differentiation. In most tumor cells where TGF-β signaling is disrupted by somatic mutation, TGF-β is incapable of inducing p15Ink4b andp21Cip1 and inhibiting Myc and Id proteins. This tumor-suppressive function of TGF-β has raised concerns about the use of TGF-β antagonists to treat cancer, despite the increasingly strong evidence that TGF-β1 can promote tumor metastasis.

TGF-β AS A TUMOR PROMOTER

It is widely accepted that during the late stage of tumor progress, inhibitory effects of TGF-β on cell proliferation are lost (Porter, 2009; Travis *et al*., 2010; Sánchez-Zamorano *et al*., 2011) (Fig. 3). The prominent mechanisms of TGF-β on tumor progression are epithelial-to-mesenchymal transition (EMT), tumor stroma interaction and microenvironment, and circumvention of the immune system (Wakefield and Roberts, 2002; Derynck and Akhurst, 2007). During EMT, the cells lose their polarity and cell-cell contact by decreasing the expression of E-cadherin and other components of the cell junction and increasing the expression of N-cadherin and other components of extracellular matrix (Thiery *et al*., 2009). EMT is a highly coordinated process in response to stress, such as inflammation or wounding during embryonic development, neoplasia and fibrosis (Chen *et al*., 2001). TGF-β induces EMT by Smad-dependent transcriptional events and enhanced by Smad-independent Ras signaling late stages of tumorigenesis (Mishra *et al*., 2005). Smads act as transcriptional regulatory factors of EMT regulators such as Snail/Slug/Twist, Cripto-1 and Six1 (Thuault *et al*., 2006; Micalizzi *et al*., 2009; Viloria-Petit *et al*., 2009; Lindley and Briegel, 2010; Micalizzi *et al*., 2010; Wendt *et al*., 2010; Shirakihara *et al*., 2011). Recent study suggests that EMT-inducing factors such as Twist, Snail and TGF-β may induce the expression of cell surface markers associated with cancer stem cells and these cells share high homology to bone marrow-derived mesenchymal stem cells (Mani *et al*., 2008). When the immunosuppressive effects of TGF-β become dominant, the net effect favors tumor progression, even though TGF-β has anti-inflammatory effects which result in tumor suppression (Massagué, 2008). In CTLs, TGF-β suppresses transcription of factors, such as

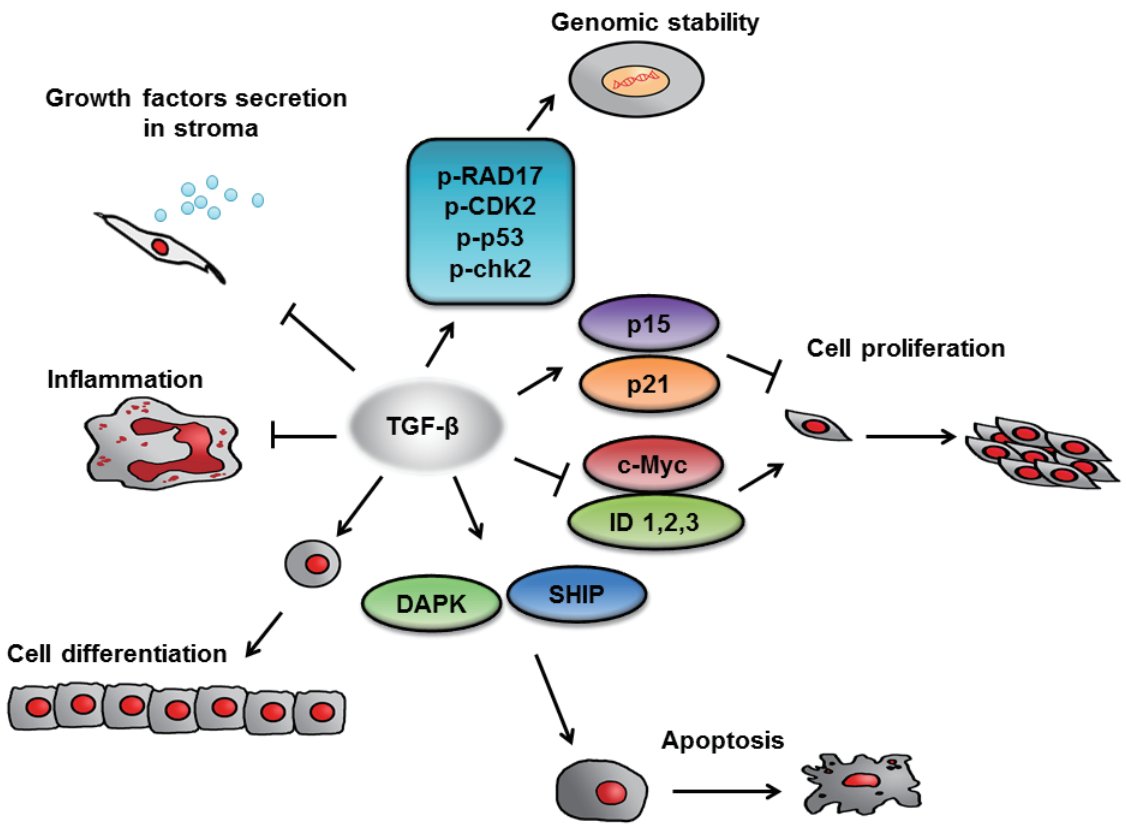

Fig. 2. TGF-β signaling in tumor suppression. TGF-β inhibits cell proliferation by increases in expression of cyclin-dependent kinase (CDK) inhibitors p21 and p15 and decrease in expression of proliferative drivers such as c-Myc and ID. TGF-β also effects on apoptosis and cell differentiation, genomic stability, tumor stroma, and inflammation.

granzyme, perforin, interferon-g and FAS ligand (Thomas and Massagué, 2005; Meulmeester and ten Dijke, 2011). TGF-β can inhibit both $CD4^+$ and $CD8^+$ T cells as well as on natural killer (NK) cells by inhibiting the function of antigen presenting cells (Arteaga *et al*., 1993). TGF-β shifts the immune response from differentiated anti-tumor cells into the immature cells that can release TGF-β and IL-11 into the tumor environment (Flavell *et al*., 2010; Connolly *et al*., 2012).TGF-β increases angiogenesis, activities of metalloproteases MMP-2 and MMP-9, and inhibits protease inhibitor TIMP (Derynck *et al*., 2001; Hagedorn *et al*., 2001; Sánchez-Elsner *et al*., 2001; Kang *et al*., 2003; Padua and Massagué, 2009). Role of TGF-β in tumor metastasis was observed in numerous models of cancer, such as the collagen-embedded spheroid system (Wiercinska *et al*., 2011) and other three-dimensional co-culture assays (Lee *et al*., 2007). TGF-β is a major contributor to the bone metastases, and TGF-β is released from bone matrix by the activated osteoclasts that degrade the bone matrix. Secreted TGF-β stimulates releasing of other osteolytic cytokines, such as parathyroid hormone related protein (PTH-rP), IL-11 and CTGF from the metastatic cells to maintain the metastatic process (Kingsley *et al*., 2007). Smad-3 and -4 for bone, and Smad-2 for lung, liver and brain are necessary for the metastatic expansion, respectively (Kang *et al*., 2005; Massagué, 2008; Meulmeester and ten Dijke, 2011). The induction of the angiopoietin-like 4 (ANGPTL4) gene by TGF-β in the primary tumor is the key for cancer cells to leave the breast in order to disrupt the lung capillary walls, results in the TGF-βinduced lung metastasis (Padua *et al*., 2008). Because ANGPTL4 do not work on the fenestrated capillaries of the bone marrow TGF-β can direct breast cancer cell to lung and not to bone metastasis (Massagué, 2008).

THERAPEUTIC INHIBITORS OF TGF-β SIGNALING

Studies support targeting TGF-β signaling as an anti-cancer therapeutic strategy (Fig. 4). Currently, therapeutic strategies against TGF-β can be divided into three approaches: i) prevention of TGF-β synthesis by using antisense molecules; ii) inhibition of the ligand-receptor interaction by ligand traps (monoclonal antibodies and soluble receptors) and anti-receptor monoclonal antibodies; and iii) inhibition of the receptor-mediated signaling cascade, using TGF-β receptor kinases inhibitors and aptamers (Padua and Massagué, 2009). For each of these approaches, several drugs have been developed and are either in non-clinical or in early stages of clinical investigation.

Since TGF-β level is often elevated during tumor progression, inhibiting its synthesis has the potential to decrease TGF-β levels within the tumor microenvironment. AP12009 (Trabedersen, Antisense, Pharma) is an antisense molecule against TGF-β 2, whose expression is associated with poor prognosis in glioblastoma and pancreatic cancer. Trabedersen (AP12009) showed the efficacy in a mouse model of pancreatic cancer and was successfully tested in Phase I/II study in patients with refractory high grade glioma (Hau *et al*., 2007; Schlingensiepen *et al*., 2011). AP11014 and AP15012 are oth-

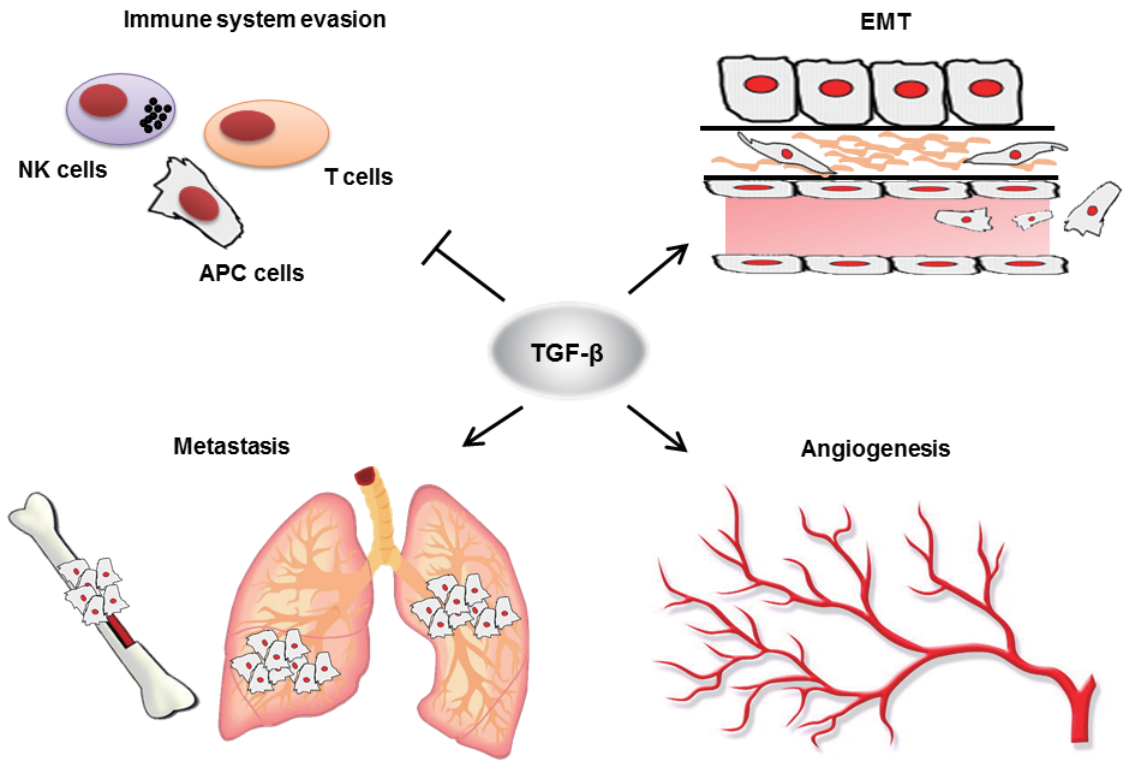

Fig. 3. TGF-β signaling in tumor promotion. TGF-β promotes tumor progression by mechanisms, such as EMT, metastasis, angiogenesis, immune modulation, and tumor microenvironment. APC: antigen presenting cells; EMT: epithelial-to-mesenchymal transition; NK: natural killer.

er two antisense molecules in pre-clinical trials for treatment of non-small cell lung cancer, prostate carcinoma and CRC, and malignant melanoma (MM), respectively (Lampropoulos *et al*., 2012).

A monoclonal antibody (mAB), 1D11 (Genzyme Corp., Sanofi), that binds TGF-β 1, 2 and 3, resulted in suppression of lung metastasis in metastatic breast cancer mouse model, by increase in the antitumor response of $CD8^+$ T-cells (Nam *et al*., 2008). It also decreased bone loss by reduced expression of PTHrP and its regulatorGli2 (Connolly *et al*., 2012). 2G7, another mAB in pre-clinical trials, showed efficacy in inhibiting breast cancer metastasis by increasing NK cells activity (Arteaga *et al*., 1993; Biswas *et al*., 2007; Ganapathy *et al*., 2010). Three humanized mABs, GC-1008 (Fresolimumab), CAT-152 (Lerdelimimab) and CAT-192 (Metelimumab) were developed by Genzyme and tried in clinical trial. GC-1008 was tested in Phase I/II clinical trial and two trials of GC-1008 are in recruitment phase: Fresolimumab and radiotherapy in metastatic breast cancer (NCT01401062) and safety and imaging study of GC1008 in glioma (NCT01472731). Soluble TβRII and TβRIII (betaglycan) have been tested in pre-clinical studies in breast and pancreatic cancer metastasis (Muraoka *et al*., 2002; Rowland-Goldsmith *et al*., 2002; Yang *et al*., 2002; Meulmeester and ten Dijke, 2011), but no clinical trials have been undertaken with these soluble receptors. PF-03446962 is an anti-TβRI mAB which inhibits binding to ligands BMP9 and TGF-β to TβRI and can serve as an anti-angiogenesis agent by inhibiting endothelial cell sprouting (van Meeteren *et al*., 2012). PF-03446962 is in recruitment phase: malignant pleural mesothelioma (NCT01486368).

Small molecules targeting receptor kinases have been extensively investigated and these are more efficient TGF-β signaling blockers than mAB and antisense molecules (Connolly *et al*., 2012). SB-431542 and SB-505124 (GlaxoSmithKline), small molecule inhibitors of TβRI, inhibit phosphorylation of Smad-2/3, TGF-β -induced proliferation, motility and angiogenesis of glioma cells, and transcription of collagen and fibronectin in renal carcinoma cells (Matsuyama *et al*., 2003; DaCosta Byfield *et al*., 2004; Hjelmeland *et al*., 2004; Calone and Souchelnytskyi, 2012). SB-431542 also increases dendritic cells maturation, $CD8^+$ T cell activity (Takeuchi *et al*., 2010; Tanaka *et al*., 2010; Connolly *et al*., 2012). However, these two inhibitors are not stable and specific so that they can draw to unpredictable outcomes and side effects.Ki26894 and LY364937 (Eli-Lilly & Co) are other TβRI inhibitors which appeared to be promising in terms of inhibiting metastasis to bone and experiments using breast and gastric cell line in in vitro (Ehata *et al*., 2007; Shinto *et al*., 2010) and xenografts mouse model *in vivo* (Bandyopadhyay *et al*., 2006; Ehata *et al*., 2007). LY2109761 (Eli-Lilly & Co) is a small molecule

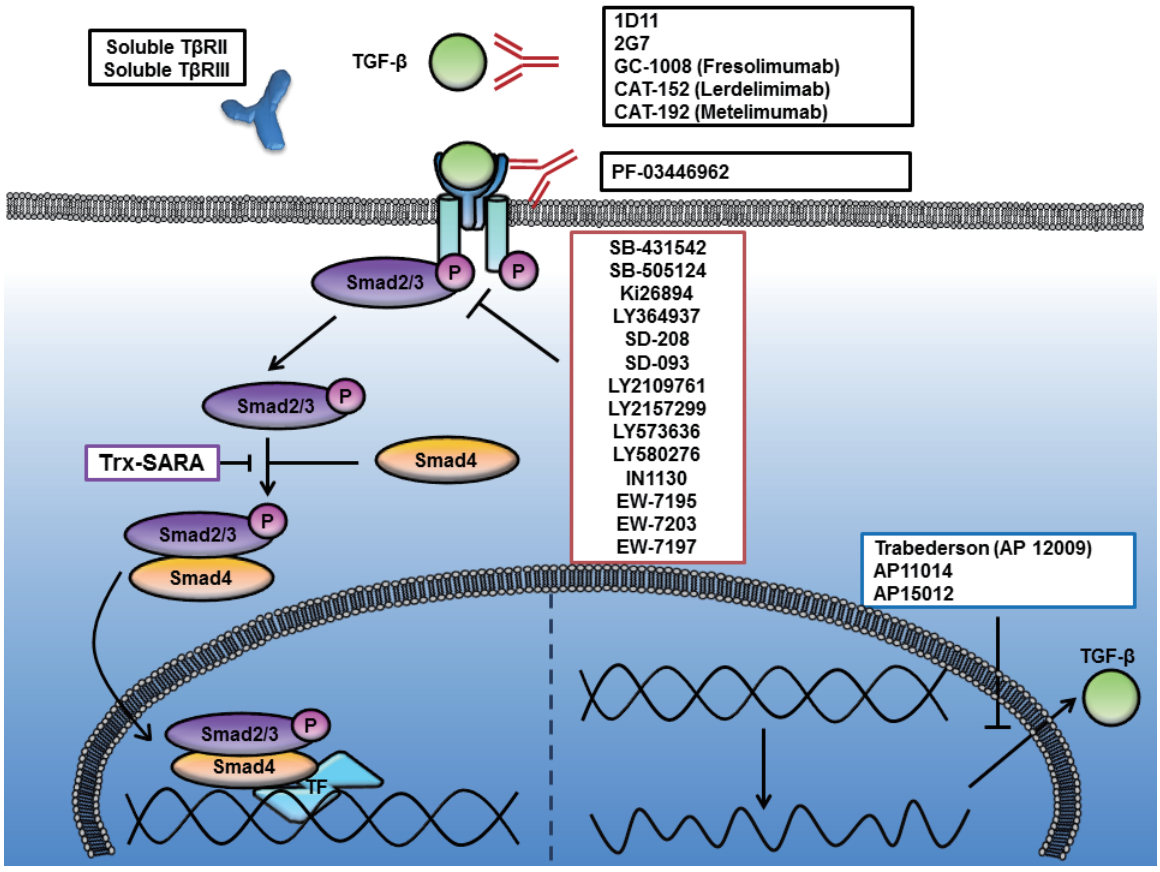

Fig. 4. TGF-β signaling pathway inhibitors under development for potential cancer therapy. (blue line box) Antisense molecules inhibit TGF-β synthesis; (black line box) monoclonal antibodies, soluble receptors and anti-receptor monoclonal antibodies inhibit ligand-receptor interaction; (red line box) receptor kinase inhibitors and peptide aptamers (purple line box) inhibit signal transduction.

which is TβRI-specific (Ki= 38 nM for TβRI kinase vs IC50 of 300 nM for TβRII) inhibitor, and is relatively metabolically stable and suitable for *in vivo* studies. *In vivo* mouse pancreas and colon metastasis model, LY-2109761 decreased liver metastases and prolonged survival (Capocasale *et al*., 1995; Fernandez *et al*., 2002; Melisi *et al*., 2008; Korpal *et al*., 2009; Zhang *et al*., 2009). However, long-term use of this drug in a skin cancer mouse model resulted in resistance and cancer progression (Connolly *et al*., 2011), suggesting that more than one drug may be needed for long-term inhibition of one signaling pathway (Connolly *et al*., 2012). SD-093 and SD-208, LY-580276 (Sawyer, 2004), which act as competitive inhibitors for the ATP-binding site of TβRI kinase showed anti-metastasis effect in glioma (Uhl *et al*., 2004) and metastatic mouse models (Subramanian *et al*., 2004; Uhl *et al*., 2004; Yingling *et al*., 2004; Mohammad *et al*., 2011). SD-093 and LY-580276 have been shown to block EMT and tumor cell migration in pancreatic cancer and mouse mammary epithelial cells, respectively (Subramanian *et al*., 2004; Peng *et al*., 2005).TGF-β/ALK5 kinase inhibitor, LY-573636, is tested in patients with malignant melanoma, soft-tissue sarcoma, NSCLC, and ovarian cancer (Gordon *et al*., 2013). IN-1130, a TβRI kinase inhibitor suppresses renal fibrosis in obstructive nephropathy and metastasis from breast to lung (Moon *et al*., 2006). Recently, potent and highly specific TGF-β/ALK5 inhibitors, EW-7203 (Park *et al*., 2011b), EW-7195 (Park *et al*., 2011a), and EW-7197 (Kim *et al*., 2011) were developed as orally available drugs. EW-7203, EW-7195, and EW-7197 inhibited Smad/TGF-β signaling, cell migration, invasion, and lung metastasis of breast cancer cells in 4T1 and MDA-MB-231 orthotropic xenograft mice and MMTV/cNeu transgenic mice. They inhibited epithelial to mesenchymal transition (EMT) in both TGF-β treated breast cancer cells and 4T1 orthotropic xenograft mice. 1.25 mg/Kg EW-7197 increased the survival time of 4T1-Luc and 4T1 breast tumor bearing mice (Kim *et al*., 2011). Pre-clinical study with EW-7197 was completed and ready for the clinical trial. LY2157299 (Eli-Lilly & Co) is the only TGF-β receptor kinase inhibitor currently in clinical trial and a TβRI kinase inhibitor that reduces growth of lung and breast cell lines (Bueno *et al*., 2008). LY2157299 was well tolerated at all doses from patient with Grade IV glioma. A pulmonary embolism and thrombocytopenia were two drug-related dose limiting toxicities and currently, LY2157299 is tested in four clinical trials, all of them are still recruiting patients: Phase Ib/II in stage II-IV pancreatic cancer of LY2157299 combined with gemcitabine versus gemcitabine plus placebo (NCT01373164); Phase II in HCC patients who have had disease progression on Sorafenib or are not eligible to receive sorafenib (NCT01246986); Phase Ib/IIa study combining LY2157299 with standard Temozolomide based radiochemotherapy in patients with newly diagnosed malignant glioma (NCT01220271); and Phase II Study ofLY2157299 mono therapy or LY2157299 plus Lomustine therapy compared to Lomustine monotherapy in patients with recurrent glioblastoma (NCT01582269).

CONCLUSIONS

TGF-β pathway is being extensively evaluated as a potential therapeutic target (Yingling *et al*., 2004). Because of the dual role of TGF-β in tumorigenesis, a comprehensive understanding of TGF-β biology is required for the design successful therapeutics. It is important to discover new drugs that mimic the interactions between TGF-β and its receptors and mechanistically inhibit transduction of the TGF-β signaling and in turn eliminate the tumor-promoting activities of TGF-βs. The TGF-β inhibitors are in pre-clinical studies, and Phase I and II clinical trials. Preclinical studies have provided convincing evidence that targeting the TGF-β pathway is able to inhibit tumor growth and metastasis *in vivo*. And the results from clinical trial are encouraging for further new drug development.

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