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## TGF- $\beta$ Signaling in Cancer

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### ABSTRACT

The transforming growth factor- $\beta$  (TGF- $\beta$ ) is a family of structurally related proteins that comprises of TGF- $\beta$ , activins/inhibins, and bone morphogenic proteins (BMPs). Members of the TGF- $\beta$  family control numerous cellular functions including proliferation, apoptosis, differentiation, epithelial-mesenchymal transition (EMT), and migration. The first identified member, TGF- $\beta$  is implicated in several human diseases, such as vascular diseases, autoimmune disorders, and carcinogenesis. Activation of the TGF- $\beta$  receptor by its ligands induces the phosphorylation of serine/threonine residues and triggers phosphorylation of the intracellular effectors, SMADs. Upon activation, SMAD proteins translocate to the nucleus and induce transcription of their target genes, regulating several cellular functions. TGF- $\beta$  dysregulation has been implicated in carcinogenesis. In early stages of cancer, TGF- $\beta$  exhibits tumor suppressive effects by inhibiting cell cycle progression and promoting apoptosis. However, in late stages TGF- $\beta$  exerts tumor promoting effects, increasing tumor invasiveness, and metastasis. Furthermore, the TGF- $\beta$  signaling pathway communicates with other signaling pathways in a synergistic or antagonistic manner and regulates cellular functions. Elevated TGF- $\beta$  activity has been associated with poor clinical outcome. Given the pivotal role of TGF- $\beta$  in tumor progression, this pathway is an attractive target for cancer therapy. Several therapeutic tools such as TGF- $\beta$  antibodies, antisense oligonucleotides, and small molecules inhibitors of TGF- $\beta$  receptor-1 (TGF- $\beta$ R1) have shown immense potential to inhibit TGF- $\beta$  signaling. Finally, in the interest of developing future therapies, further studies are warranted to identify novel points of convergence of TGF- $\beta$  with other signaling pathways and oncogenic factors in the tumor microenvironment. *J. Cell. Biochem.* 117: 1279–1287, 2016. © 2016 Wiley Periodicals, Inc.

**KEY WORDS:** CELL SIGNALING; PROLIFERATION; EMT; METASTASIS; INVASION

**T**GF- $\beta$  is a sizeable superfamily of cytokines that include the TGF- $\beta$ s (TGF- $\beta$ 1, - $\beta$ 2, and - $\beta$ 3), activins, inhibins, Anti-Mullerian Hormone (AMH), Bone Morphogenic Protein (BMPs), Growth Differentiation Factors (GDFs), Glial-derived Neurotrophic Factors (GDNFs), and nodals [Massagué, 2012]. The TGF- $\beta$  signaling pathway is instrumental in regulating crucial cellular activities such as cell growth, differentiation, apoptosis, motility, invasion, extracellular matrix production, angiogenesis, and immune response. In cancer, TGF- $\beta$  has a dual role. In the early stages of cancer development, TGF- $\beta$  operates as a tumor suppressor, whereas the opposite is true in late stages, supporting invasion and metastasis by modulating the

immune system and tumor microenvironment. All three isoforms of TGF- $\beta$  (TGF- $\beta$ 1, TGF- $\beta$ 2, and TGF- $\beta$ 3) are synthesized in cells as precursor molecules containing a propeptide region in addition to the TGF- $\beta$  homodimer. The TGF- $\beta$  homodimer interacts with a Latency Associated Peptide (LAP) forming the Small Latent Complex (SLC). This complex remains in the cell until it binds to the Latent TGF- $\beta$ -Binding Protein (LTBP), forming a larger complex termed as Large Latent Complex (LLC). The LLC is secreted into the extracellular matrix in an inactive form, which prevents its binding to its receptors. The release of the LLC from the matrix, followed by proteolysis of the LAP releases the active TGF- $\beta$  to its receptors [Hyytiainen et al., 2004].

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There are three TGF- $\beta$  receptors (TGF $\beta$ R1, TGF $\beta$ R2, and TGF $\beta$ R3). Both TGF $\beta$ R1 and TGF $\beta$ R2 contain serine/threonine protein kinases in their cytoplasmic domains while TGF $\beta$ R3 has no kinase activity. Binding of TGF- $\beta$  to TGF $\beta$ R2, and hetero-tetramerization with TGF $\beta$ R1 initiates the intracellular signaling via SMADs. TGF $\beta$ R3 functions as a coreceptor to increase the binding of ligands to TGF $\beta$ R2 [Massagué, 2012].

### TGF- $\beta$ SIGNALING PATHWAY

The active TGF- $\beta$  mediates its effect by binding to TGF $\beta$ R2 and modifying its conformation, then the type II receptor phosphorylates TGF $\beta$ R1 on specific serine and threonine residues (Fig. 1). The internalization of the heteromeric receptor complex materializes via the clathrin- and lipid raft-caveolae endocytic routes. The two discrete internalization paths determine whether the receptor complex will induce a signaling response or receptor degradation [Itoh and ten, 2007; Neuzillet et al., 2015; Papageorgis, 2015].

In the canonical pathway, the activated receptor complex phosphorylates receptor-SMADs (R-SMADs), which are cytosolic effectors responsible for activating multiple downstream signaling

pathways. The clathrin-dependent internalization of TGF- $\beta$  receptor complex initiates signaling by steering the receptor complex to early endosomes. The SMAD anchor for receptor activation (SARA) and cytoplasmic form of promyelocytic leukemia protein (cPML) on early endosomes recruit R-SMADs (SMAD-1, 2, and 3) to the activated receptor complex. Eventually R-SMADs are phosphorylated by activated type I receptor permitting R-SMADs association with SMAD4. The activated SMAD complex is transported to the nucleus, where it directly or indirectly regulates gene expression by controlling epigenetic processes, such as chromatin remodeling or by upholding promoter DNA methylation, which is vital in silencing epithelial gene expression in cells that have undergone epithelial-to-mesenchymal transition [Neuzillet et al., 2015; Papageorgis, 2015].

Trafficking of TGF- $\beta$  receptor complex controls the activity and termination of signaling events. The lipid rafts-caveolae microdomains are enriched with cholesterol and caveolin. The receptor complex that is endocytosed into lipid rafts-caveolae is bound to degrade. The activity of TGF- $\beta$  signaling is regulated by the inhibitory activity of SMAD7. Under basal conditions, SMAD7 is localized within the nucleus of the cell. However, upon formation of

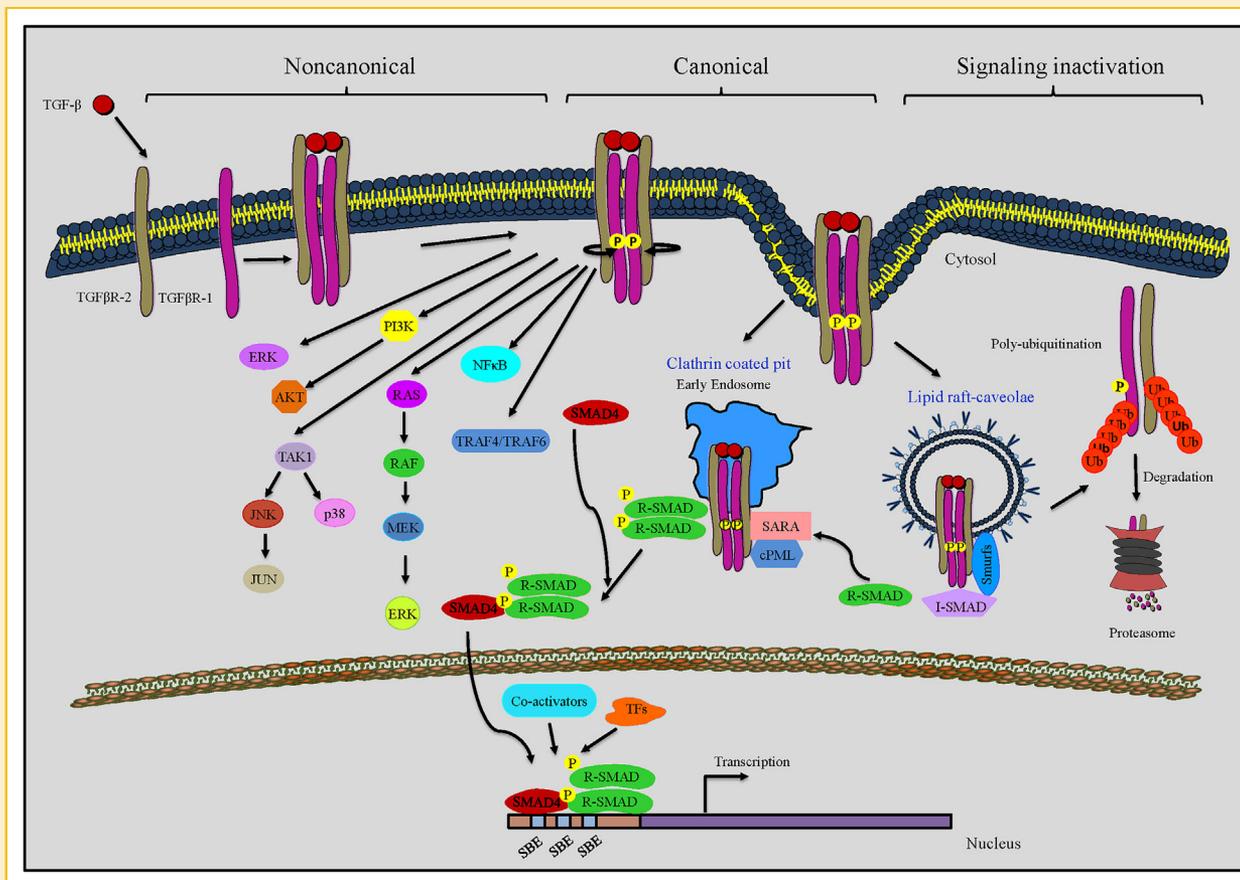


Fig. 1. TGF- $\beta$  ligand binds to TGF $\beta$ R2 prompting the formation of a hetero-tetrameric receptor complex composed of TGF $\beta$ R1 and TGF $\beta$ R2. The binding of ligand and subsequent oligomerization triggers TGF $\beta$ R2 to phosphorylate TGF $\beta$ R1 on serine or threonine residues. In the canonical pathway the activated receptor complex phosphorylates R-SMAD (SMAD2 or SMAD3) which then forms a heteromeric complex with SMAD4 and translocates to the nucleus where it interacts with various transcription factors, coactivators, or corepressors to regulate target gene expression. In the non-canonical pathway, TGF- $\beta$  can activate the MAPKs, NF $\kappa$ B, Ras, TRAF6, TAK1-p38/JNK, and PI3K kinases, leading to various biological effects. Receptor complexes endocytosed into lipid rafts-caveolae are degraded by I-SMADs recruitment of Smurfs.

TGF $\beta$ -induced receptor complex, it moves to the cell membrane and binds to a receptor complex, blocking interaction between R-SMADs and receptor complex and hence the inhibition of downstream TGF- $\beta$  signaling. In addition, SMAD7 can antagonize the formation of a functional SMAD-DNA complex by binding to DNA and consequently blocking TGF- $\beta$ -mediated transcriptional events. In some instances, SMAD7 represses TGF- $\beta$  signaling by interacting with E3-ubiquitin ligases. In the nucleus, SMAD7 forms a complex with E3-ubiquitin ligases Smurf1 or Smurf2, and upon TGF- $\beta$  activation, the complex translocates to the plasma membrane where Smurf induces ubiquitination for proteasomal degradation of the TGF- $\beta$  receptors [Itoh and ten, 2007; Neuzillet et al., 2015; Papageorgis, 2015].

Although the SMADs are fundamental for the TGF- $\beta$  modulated diverse cellular responses, there are numerous signaling responses stimulated by TGF- $\beta$  that occur independently of the SMAD proteins. Mounting evidence has revealed that in the non-canonical pathway, the activated TGF- $\beta$  receptor complex conveys signals through a number of factors such as tumor necrosis factor (TNF), receptor-associated factor 4 (TRAF4), TRAF6, TGF $\beta$ -activated kinase 1 (TAK1), p38 mitogen-activated protein kinase (p38 MAPK), p42/p44 MAPK, RHO, phosphoinositide 3-kinase PI3K/AKT, extracellular signal-regulated kinase (ERK), JUN N-terminal kinase (JNK), or nuclear factor- $\kappa$ B (NF- $\kappa$ B) to reinforce or attenuate downstream cellular responses [Itoh and ten, 2007; Mu et al., 2012; Papageorgis, 2015].

### CONTRASTING ROLES OF TGF- $\beta$

It is widely recognized that TGF- $\beta$  plays central roles in carcinogenesis. Several studies have clearly revealed dual roles of TGF- $\beta$  in this process. TGF- $\beta$  is reported to suppress growth of normal and premalignant epithelial cells. However, accumulation of genetic and epigenetic alterations in cancer cells switches the TGF- $\beta$  function. Cancer cells produce and secrete TGF- $\beta$  which promotes their invasive and metastatic potential [Nagaraj and Datta, 2010; Massagué, 2012]. In normal epithelial cells, TGF- $\beta$  acts as a tumor suppressor and regulates cell proliferation, apoptosis, differentiation, senescence, adhesion, and motility. TGF- $\beta$  restrains the proliferation of many human cell lines and tissues including thyroid, hepatocytes, colon and mammary epithelial cells [Massagué, 2012]. Studies have shown tissue specific inactivation of TGF $\beta$ RII results in spontaneous tumor formation with no pathology in many organs implying that TGF- $\beta$  elicits its antiproliferative effects in precise settings [Nagaraj and Datta, 2010]. Loss of TGF- $\beta$  induced growth arrest can be due to either the abnormal expression of positive regulators of cell cycle, for example the cyclins and cyclin-dependent kinases (cdks), or the negative regulators, for instance the cdk inhibitors. The loss of c-myc in epithelial cells and in cancer cells coincides with a loss of c-myc promoter responsiveness to TGF- $\beta$  [Massagué, 2012; Principe et al., 2014]. TGF- $\beta$  inhibits epithelial cell proliferation by causing cycle arrest at the G1 phase, and inducing or activating cyclin-dependent kinase inhibitors (p16INK4A, p15INK4B, p21CIP1, and/or p27Kip1), and attenuating myc, DNA-binding protein inhibitor ID-1 (Id1) and Id2. In cancer cells TGF- $\beta$  is unable to elicit changes in myc, ID-1, and ID-2 due to somatic mutations in components of the TGF- $\beta$  signaling pathway [Massagué, 2012; Neuzillet et al., 2015]. The mechanism by which

TGF- $\beta$  acts as a tumor suppressor is through inactivation of TGF- $\beta$  receptors and SMADs. The downregulation of receptors, and increased expression of TGF- $\beta$  signaling inhibitors has been reported in human cancers [Nagaraj and Datta, 2010; Principe et al., 2014]. It is well established that TGF- $\beta$  produced by tumor cells acts on itself in an autocrine manner to stimulate tumor suppressive biological responses. A number of studies suggest that TGF $\beta$ R1 and TGF $\beta$ R2 are tumor suppressor genes. Mutations in TGF $\beta$ R1 and TGF $\beta$ R2 are linked with ovarian, cervical, gastric, head, and neck carcinomas. Furthermore, inactivating mutations in TGF $\beta$ R1 and TGF $\beta$ R2 have also been described in human lymphoma [Nagaraj and Datta, 2010]. TGF $\beta$ R3 is expressed in epithelial cells and considered as a suppressor of cancer progression. However, loss of TGF $\beta$ R3 expression has been correlated with disease progression and poor patient prognosis. Furthermore, restoration of TGF $\beta$ R3 in cancer cells demonstrated a direct role for TGF $\beta$ R3 in regulating cell migration, invasion, angiogenesis, and metastasis in vitro and in vivo [Massagué, 2012; Principe et al., 2014; Neuzillet et al., 2015].

Most types of cancers exhibit insensitivity to TGF $\beta$ -mediated growth inhibition. In colorectal, pancreatic, gastric, and prostate tumors insensitivity to TGF $\beta$ -mediated growth inhibition is attributed to the loss of function or truncating mutations in TGF $\beta$ R1, TGF $\beta$ R2, SMAD2, and SMAD4 genes. In pancreatic and colorectal cancers, loss of 18q21 chromosome, which harbors the SMAD4 gene is reported. Restitution of SMAD4 expression in pancreatic cancer cell lines decreases VEGF levels resulting in suppression of tumor growth and angiogenesis [Principe et al., 2014; Papageorgis, 2015]. In some cancers, genetic alterations or gene mutations are not detected, suggesting existence of other methods for acquiring resistance to TGF- $\beta$ -mediated growth inhibition. In mammary and lung epithelial cells, activation of the Ras oncogene and its downstream target ERK leads to phosphorylation of SMAD2/3 in the linker region, as a consequence SMADs are retained in the cytoplasm and degraded by ubiquitin [Schwarte-Waldhoff et al., 2000; Papageorgis, 2015].

### TGF- $\beta$ AND EPITHELIAL TO MESENCHYMAL TRANSITION

One of the key mechanisms by which TGF- $\beta$  promotes cell migration, invasion, and metastasis is through induction of epithelial-mesenchymal transition (EMT). It is a multi-step process in which cells lose apical-basal cell polarity, and gain spindle-shaped, fibroblast-like morphology distinctive for mesenchymal cells. The EMT process can be categorized into three well-defined subtypes based the biological settings and circumstances in which the EMT event is activated. Type 1 EMT, also known as developmental EMT, occurs during embryogenesis to assist development of organs and tissues. Commencement of type 1 EMT stimulates gastrulation and formation of ectodermal, mesodermal, and endodermal tissues from the primitive streak. It also instigates neural tube formation during spinal and cerebral development. Disruption of TGF- $\beta$  isoforms and their receptors has been associated with defects in the initiation of type 1 EMT during organogenesis and tissue morphogenesis [Principe et al., 2014; Neuzillet et al., 2015]. Type 2 EMT is induced in response to inflammation, particularly during wound healing and tissue regeneration, but ceases once inflammation is diminished [López-Novoa and Nieto, 2009]. TGF- $\beta$  plays an instrumental role in mediating the type 2

EMT process by promoting myofibroblast activation and differentiation in conjunction with extracellular matrix (ECM) remodeling, a process that is essential for tissue repair [Taylor et al., 2010]. Type 3 EMT is most prevalent for oncogenically transformed cells that have the capacity to metastasize. This EMT program is a requisite for the acquisition of malignant phenotypes by epithelial cancer cells. In vitro studies suggest a major role of TGF- $\beta$ /SMAD signaling in the induction of EMT in cancer cells. The adhesion molecule E-cadherin located at cell-cell adhesion junctions is an essential molecule for the formation and maintenance of the epithelial phenotype. The hallmark of EMT is loss of E-cadherin and up regulation of vimentin, slug, snail, and fibronectin which are critical for attainment of motility and invasive properties, and permits the cells to migrate through the extracellular matrix and form metastases at distant sites [Thiery et al., 2009; Principe et al., 2014].

The entire range of signaling mediators that contribute to EMT of carcinoma cells is far from complete. It is suggested that the genetic and epigenetic changes experienced by cancer cells during the process of primary tumor formation make them particularly responsive to EMT-inducing heterotypic signals initiating in the tumor-associated stroma. EMT-inducing signals stemming from the tumor-associated stroma, mainly hepatocyte growth factor (HGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and TGF- $\beta$ , seem to be accountable for the induction or functional activation of a series of EMT-inducing transcription factors, especially slug, snail, twist, zinc finger E-box binding homeobox 1 (ZEB1), Gooseoid, and FOXC2 in cancer cells. Upon expression and activation, the transcription factors choreograph the EMT in conjunction with the other transcription factors. The execution of EMT in cancer cells depends on additional signal transducers including SMADs, ERK, MAPK, PI3K, AKT, RhoB,  $\beta$ -catenin, lymphoid enhancer binding factor, Ras, c-Fos, and cell surface ( $\beta$ 4 integrins,  $\alpha$ 5 $\beta$ 1 integrin, and  $\alpha$ V $\beta$ 6 integrin) proteins [Lindsey and Langhans, 2014; Barriere et al., 2015].

TGF- $\beta$  has been shown to induce EMT in certain types of cancer cells. TGF- $\beta$  induces EMT either through canonical or non-canonical pathways. The TGF $\beta$ R1 and its effector SMAD proteins mediate the epithelial to mesenchymal transition in NMuMG breast epithelial cells [Piek et al., 1999]. TGF- $\beta$  produced by an autocrine mechanism support and strengthen the EMT program. Evidence of collaboration of  $\beta$ -catenin and LEF with SMADs in inducing an EMT has been reported. Studies have demonstrated interaction of LEF and  $\beta$ -catenin in PDGF-induced EMT. Together these studies exhibit the TGF- $\beta$ /SMAD/LEF/PDGF axis as a key player of an EMT phenotype in cancer [Eger et al., 2000; Principe et al., 2014; Neuzillet et al., 2015].

In addition to SMADs mediated EMT process, TGF- $\beta$  induces EMT via the non-canonical pathway. In NMuMG mouse mammary epithelial cells, integrin  $\beta$ 1 mediates the activation of latent TGF- $\beta$  by  $\alpha$ V $\beta$ 6 integrin, which in association with p38 MAPK and RhoA, mediates an autocrine TGF- $\beta$ -induced EMT. Blocking RhoA or its downstream target p160<sup>ROCK</sup>, by the expression of dominant-negative mutants, inhibits TGF- $\beta$ -mediated EMT. Fibulin-5 (FBLN5), a TGF-beta target gene is shown not only to stimulate matrix metalloproteinase expression and activity, but also initiates EMT by elevating twist expression and reducing E-cadherin expression in breast cancer. In H-Ras transformed EpH4 mammary epithelial cells

(EpRas), TGF- $\beta$  induces EMT by decreasing the expression of glutaredoxin 1 (Grx1), MEK/MAP kinase and phosphatidylinositol-3 kinase (PI3K) signaling. Pre-treatment of H-Ras transformed EpH4 cells with ROS scavenger N-acetylcysteine (NAC) inhibited TGF- $\beta$ 1-induced EMT [Lee et al., 2010; Lindsey and Langhans, 2014].

### BLOCKING TGF- $\beta$ SIGNALING

TGF- $\beta$  has a wide array of effects on tumorigenesis. Cancer initiation and progression is attributed to elevated TGF- $\beta$  levels. Thus blockade of TGF- $\beta$  signaling may provide therapeutic opportunities to inhibit tumor growth and metastasis. A number of strategies such as the use of ligand traps, small molecule inhibitors, antisense oligonucleotides, and small molecule receptor kinase inhibitors are employed to disrupt the TGF- $\beta$  signaling pathway [Korpai and Kang, 2010; Lampropoulos et al., 2012]. Ligand traps include neutralizing antibodies developed against each TGF- $\beta$  ligands and soluble decoy receptor proteins that incorporate the ectodomains from either TGF $\beta$ R2 or TGF $\beta$ R3. Ligand traps effectively block the excess TGF- $\beta$  produced by tumor cells and fibroblasts during cancer progression that contribute to tumor aggressiveness. Studies employing a pan-neutralizing anti-mouse TGF- $\beta$  monoclonal antibody, 1D11 (Genzyme Corp., Sanofi), which binds to all three TGF- $\beta$  isoforms or recombinant Fc-fusion proteins containing the soluble ectodomain of either TGF $\beta$ R2 (TGF $\beta$ R2-Fc) or TGF $\beta$ R3 have shown to decrease metastasis in breast cancer models [Connolly et al., 2012]. Another way of inhibiting TGF- $\beta$  signaling is to use antisense oligonucleotides. Targeting human TGF- $\beta$ 2 RNA with specific antisense oligonucleotide demonstrated inhibition of TGF- $\beta$ 2 expression and reduced proliferation and migration in pancreatic cancer in vitro and in vivo [Schwarte-Waldhoff et al., 2000]. Preclinical data suggest that small molecule inhibitors, SB-431542 or Ki26894 (GlaxoSmithKline) developed for blocking TGF $\beta$ R1 when used in orthotopic xenograft model, showed decreased metastasis and prolonged mouse survival [Ehata et al., 2007]. An alternative strategy to block TGF- $\beta$  signaling is to interfere with the SMADs. Peptide aptamers have a target-binding domain and a scaffolding domain which stabilizes the resulting molecular complex. A peptide aptamer Trx-SARA binds to SMAD2 and SMAD3 and disrupts their interactions with SMAD4. Treatment of murine mammary epithelial cells with Trx-SARA resulted in reduction of SMAD2/3 levels and inhibition of TGF- $\beta$ -induced EMT [Zhao and Hoffmann, 2006].

Treatment of tumor cells with a combination of vaccines and antisense TGF- $\beta$  therapy showed a reduction of tumor size and increased survival benefit [Principe et al., 2014; Neuzillet et al., 2015]. Furthermore, the results of preclinical studies have shown that TGF- $\beta$  inhibition can enhance therapeutic efficacy of cytotoxic agents [Neuzillet et al., 2015]. The anti-TGF- $\beta$  treatments deemed safe as daily administration of a high dose of neutralizing TGF- $\beta$  antibody in adult mice showed no adverse effects on their health [Kulkarni et al., 1993].

### CROSSTALK OF TGF- $\beta$ SIGNALING WITH OTHER PATHWAYS

#### INTERFERONS

The TGF- $\beta$  pathway crosstalks with several pathways associated with tumor progression (Fig. 2). Interferons (IFNs) are a group of

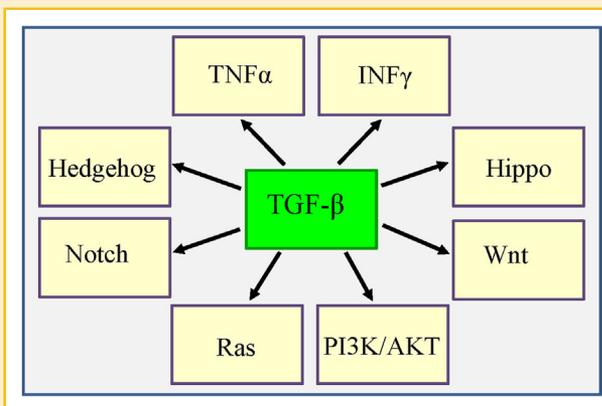


Fig. 2. TGF- $\beta$  pathway communicates with other signaling pathways and regulates numerous key tumorigenic processes.

pleiotropic cytokines and have a pivotal role in host defense and immune surveillance. In mammals, there are two types of IFNs, type I and type II. Type I IFN, includes IFN- $\alpha$  and IFN- $\beta$ . Type II IFN has a solo member IFN- $\gamma$ . Dual role of IFN- $\gamma$  has been reported in carcinogenesis. Interaction between TGF- $\beta$  and IFN- $\gamma$  has been alluded by several investigators. In poorly differentiated human gastric carcinoma cell line (GCTM-1), TGF- $\beta$  enhances invasive potential of cells whereas IFN- $\gamma$  inhibits TGF- $\beta$  dependent invasion by inhibiting the phosphorylation of SMAD2/3 and therefore the translocation of SMAD complex to the nucleus. In addition to this mechanism, IFN- $\gamma$  via JAK1 and STAT1 induces the expression of SMAD7, an antagonistic SMAD which excludes the interaction of SMAD3 with the TGF- $\beta$  receptor [Kuga et al., 2003]. Crosstalk of two pathways was further substantiated during wound healing. Although platelets are the main source of TGF- $\beta$ , fibroblasts also produce TGF- $\beta$  mRNA and protein. TGF- $\beta$  has been shown to stimulate proliferation of normal fibroblasts and healing of wounds. Collagen plays a crucial role in healing of wounds of skin and other tissues while TGF- $\beta$  is the major growth factor involved in collagen synthesis. Compared to normal fibroblasts, collagen synthesis is shown to be greater in wounds and can be induced by TGF- $\beta$ . IFN- $\gamma$  has been shown to negatively modulate the wound healing process by suppressing the production and functional activity of TGF- $\beta$ 1. Thus, blocking the IFN- $\gamma$  signal transduction pathway may accelerate wound healing by augmenting TGF- $\beta$ 1 production [Tredget et al., 2000].

#### TNF- $\alpha$ PATHWAY

Tumor necrosis factor alpha (TNF- $\alpha$ ) is a multifunctional cytokine implicated in apoptosis, cell survival, inflammation, and immunity. It acts via TNF receptor 1 and TNF receptor 2. The latter is expressed only on immune cells, while the former is expressed in every cell. TNF receptor 1 has the death domain (DD), while it is absent in TNF receptor 2. TNF- $\alpha$  is a modulator of tumor growth in vitro and in vivo. TNF- $\alpha$  has been shown to increase the secretion of TGF- $\beta$  in MCF-7 and ZR-75-1 breast cancer cell lines. Both cytokines were able to induce apoptosis synergistically in Schwann, endothelial and in SNU620 human gastric cancer cells. The TGF- $\beta$  and TNF- $\alpha$ -induced

apoptosis is shown to be mediated by crosstalk between SMAD3 and JNK signaling, regulating the expression and stability of Bim (a pro-apoptotic member of the BCL-2 protein family). Furthermore, knockdown of Bim has been shown to block both TGF- $\beta$  and TNF- $\alpha$ -induced apoptosis. A recent study has suggested crosstalk between TNF- $\alpha$  and TGF- $\beta$  during induction of EMT in A549 lung adenocarcinoma cells. In these cells TNF- $\alpha$  induces phosphorylation of SMAD2 in the linker region, and modulates SMAD regulated gene transcription which results in EMT [Saito et al., 2013].

#### NOTCH PATHWAY

Notch pathway comprises of four Notch receptors (Notch1-4) and five canonical ligands of the Delta-Serrate-Lag (DSL) type [Jag1, Jag2, delta-like 1 (Dll1), Dll3, and Dll4]. These receptors and ligands can make a large number of receptor-ligand combinations, with the ability to produce multiple responses. Notch signaling is initiated by ligand Delta-Serrate-LAG2 (or DSL) binding to transmembrane Notch receptor, which undergoes a two-step proteolytic cleavage by ADAM family proteases and  $\gamma$ -secretase, releasing the Notch Intracellular Domain (NICD). The NICD translocates to the nucleus where it binds to the C protein binding factor 1 (CSL) and changes the complex from a repressor to an activator of Notch target genes. Notch pathway is activated in a number of cancers. Crosstalk between Notch and TGF- $\beta$  pathway has been well demonstrated. In mammary epithelial cells at the onset of EMT, TGF- $\beta$  induces expression of the hairy/enhancer-of-split-related transcriptional repressor, Hey1 and the Notch-ligand, Jagged1 (Jag1). If either Hey1, Jagged1, Notch, or SMAD3 is inactivated, TGF- $\beta$  fails to induce EMT in mammary epithelial cells [Zavadil et al., 2004]. Notch1 abrogates the anti-proliferative property of TGF- $\beta$  by sequestering the p300 co-factor from SMADs, while silencing Notch1 expression rescues the responsiveness to TGF- $\beta$  in human cervical carcinoma cell line, CaSki [Masuda et al., 2005].

#### HEDGEHOG PATHWAY

In mammals, three hedgehog (HH) ligand proteins (Sonic HH-sHH, Indian HH-IHH, and Desert HH-DHH) are reported. The two transmembrane protein receptors, Patched 1 and Patched 2, function as tumor suppressors inhibiting downstream signaling proteins Smoothed (Smo) and Gli from activating downstream signaling components and transcription of target genes. The HH pathway is activated upon binding of any of the three ligands to Patched receptor, which eases Patched-mediated suppression of Smo and triggers the translocation of the active form of Gli to the nucleus to activate target genes expression [Amakye et al., 2013]. Activation of the HH pathway has been found in the majority of human cancers. Crosstalk between HH and TGF- $\beta$  signaling is evident as TGF- $\beta$  induces Gli2 transcription and upregulation of Gli1 in normal fibroblasts, keratinocytes, and MDA-MB-231 breast carcinoma cells. In vivo studies have also shown the modulation of Gli expression in mouse models overexpressing TGF- $\beta$ 1 in the epidermis. Similarly, HH signaling has been shown to induce the expression of TGF- $\beta$  signaling proteins that are involved in SMO-dependent tumorigenesis affecting motility and invasiveness of gastric cancer cells. Blocking TGF- $\beta$  expression and function diminished Gli-mediated HH signaling and reduced cell growth [Denkler et al., 2007]. During

the development of the cerebellum, it is demonstrated that SHH accelerates the growth of granule cell precursors (GCPs). These cells also express bone morphogenetic proteins (BMPs) that belong to the transforming growth factor beta (TGF- $\beta$ ) family. It is reported that in granule cell progenitors (GCPs) the BMP-2 and -4 antagonize the proliferative function of sHH through SMAD5. In addition, BMP downregulates sHH signaling proteins Smo and Gli1. One such mechanism by which BMP-2 inhibits proliferation and promotes cell differentiation of GCPs is downregulation of oncogene *n-myc* [Alvarez-Rodriguez et al., 2007].

#### WNT PATHWAY

There are three types of Wnt signaling pathways. The canonical Wnt pathway, the non-canonical planar cell polarity pathway, and the non-canonical Wnt/calcium pathway. All of these pathways direct signals from outside of a cell through cell surface receptors to the inside of the cell. They are activated by the binding of a Wnt-protein ligand to a frizzled family receptor, which delivers the biological signal to the protein dishevelled inside the cell. The canonical Wnt pathway manages to regulate gene transcription, the non-canonical planar cell polarity pathway controls the cytoskeleton that is accountable for the shape of the cell, and the non-canonical Wnt/calcium pathway regulates calcium inside the cell [Blagodatski et al., 2014; Tai et al., 2015]. The Wnt signaling pathway is pro-oncogenic, and the TGF- $\beta$  signaling pathway exhibits both inhibitory and proliferative effects. However, both pathways participate in cancer formation and progression. The Wnt and TGF- $\beta$  pathways crosstalk at multiple levels. The two pathways mutually regulate their ligand production. A functional interaction between SMAD7 and  $\beta$ -catenin was demonstrated in prostate cancer cells. Treatment of PC-3 cells with TGF- $\beta$  increased the levels of  $\beta$ -catenin and LEF1 in a SMAD7-dependent manner. This interaction was critical for TGF- $\beta$ -induced  $\beta$ -catenin-regulated apoptosis. Transfection of PC-3 cells with  $\beta$ -catenin siRNA protected cells from TGF- $\beta$  induced apoptosis, further demonstrating the cooperation of SMAD7 and  $\beta$ -catenin in apoptosis of cells [Edlund et al., 2005]. In human mesenchymal stem cells, a new form of crosstalk between the TGF- $\beta$  and Wnt signaling which is independent of alterations in  $\beta$ -catenin stability and phosphorylation status has been reported. In these cells, the signaling was initiated by the TGF- $\beta$  receptor-mediated phosphorylation of SMAD3, which disrupted the protein complex as revealed by the diminished interactions between SMAD3 and glycogen synthase kinase 3 $\beta$  (GSK-3 $\beta$ ). Dissociation of protein complex conceded cotranslocation of  $\beta$ -catenin and SMAD3 into the nucleus [Jian et al., 2006]. In mammary gland epithelial cells, the interdependence of Wnt and TGF- $\beta$  was shown by identifying Wnt and TGF- $\beta$  target genes. Cells treated with a combination of Wnt and TGF- $\beta$  expressed *Ankrd1*, *Ccnd1*, *Ctgf*, *Gpc1*, *Hs6st2*, *IL11*, *Inhba*, *Mmp14*, and *Robo1*, genes that were not expressed in single ligand treated cells. In breast and colon cancer transgenic mouse models, many of the genes identified in *in vitro* studies were found also overexpressed *in vivo*. TGF- $\beta$  pathway inactivation in animals reduced the expression of some of the identified genes and impeded tumor growth, emphasizing that TGF- $\beta$  and Wnt synergistically promote tumorigenesis [Labbe et al., 2007]. In colon cancer cells, decreased expression of SMAD4 was associated with increased

expression of  $\beta$ -catenin mRNA and Wnt target genes, *c-myc* and *Axin2*. In human embryonic kidney (HEK293T) cells, inhibition of SMAD4 expression and BMP receptor signaling results in augmentation of  $\beta$ -catenin expression and Wnt signaling [Freeman et al., 2012; Peng et al., 2015].

#### HIPPO PATHWAY

The Hippo pathway is a key regulator of cell growth, proliferation, apoptosis, and cell-fate decisions. The main components of this pathway are the STE20 kinases (MST1 and MST2) and the NDR kinases (LATS1 and LATS2), which controls the phosphorylation of TAZ and YAP. One of the functions of TAZ/YAP pathway is to assign active SMAD complex a cytoplasmic or nuclear localization in the cells. Following TGF- $\beta$  stimulation, TAZ binds to SMAD2 and translocates to the nucleus to activate target genes. TAZ knockdown in HepG2 cells inhibited nuclear accumulation of SMAD2 confirming that TAZ is essential for TGF $\beta$  signaling. TAZ is overexpressed in cancer cells and in the absence of TGF- $\beta$ , TAZ showed no effects of SMAD2 distribution in the cytoplasm and nucleus of the cells [Varelas et al., 2008; Boggiano and Fehon, 2012]. In high-density cell cultures, crumbs polarity complex is formed, which interacts with and relays cell density information to TAZ/YAP by promoting its phosphorylation, cytoplasmic retention, and suppression of TGF- $\beta$  signaling. Studies have shown that disruption of the crumbs complex results in increased SMAD, TAZ, and YAP nuclear accumulation, intensify sensitivity to the TGF- $\beta$  ligand, and promotes TGF- $\beta$ -mediated epithelial-to-mesenchymal transition [Varelas et al., 2010].

#### RAS PATHWAY

RAS, also called small GTPase, is a family of related proteins that are expressed in cells and implicated in transmitting signals within cells. Once RAS is "switched on" via incoming signals, it then switches on other proteins, which eventually turn on genes associated with cell growth, differentiation, and survival. Consequently, mutations in RAS genes result in production of activated RAS proteins, which cause unintentional and overactive signaling inside the cell, leading to cancer development. TGF- $\beta$  is a potent inhibitor of epithelial cell proliferation, and the mechanism by which it inhibits is through activation of the cyclin-dependent kinase inhibitors (CDKIs) p15<sup>Ink4B</sup> and p21<sup>Cip1</sup> and by suppression of *c-myc* and inhibitors of differentiation (IDs). The tumor-suppressive functions of TGF- $\beta$  signaling are detected in epithelial tissues under physiological conditions and during early stages of tumorigenesis. RAS signaling is suggested to be involved in the conversion of anti- to pro-oncogenic TGF- $\beta$  signaling effects in late stages of cancer [Grusch et al., 2010]. There is clear evidence that TGF- $\beta$  and RAS play a significant role in advancing benign mammary, prostate and lung tumors to undifferentiated invasive tumors [Grusch et al., 2010]. In the intestinal epithelium, the crosstalk between TGF- $\beta$  and RAS is revealed by overexpression of K-RAS and deletion of TGF $\beta$ RII, resulting in the generation of metastatic adenocarcinomas in conjunction with stimulated epidermal growth factor (EGF) signaling independent of Wnt/ $\beta$ -catenin. In breast and pancreatic cancer cells, interaction of K-RAS and TGF- $\beta$  signaling is delineated by induction of Snail, a transcriptional repressor of RIN1, a RAB5

guanine nucleotide exchange factor (GEF). RAB5 is known to downregulate receptor tyrosine kinases and promote TGF- $\beta$  signaling by augmenting endocytosis of TGF $\beta$ R complex, causing an increased cell migration and invasion [Hu et al., 2008; Grusch et al., 2010].

### PI3K/AKT PATHWAY

Phosphatidylinositol 3-kinase, (PI3K)/AKT signaling pathway is activated in cancers and contributes to proliferation, survival, motility, and angiogenesis that are important for the growth and/or maintenance of tumors. The PI3K/AKT activity is known to abate TGF- $\beta$ -induced apoptosis and/or cell cycle arrest in cells by blocking nuclear translocation of SMAD3. An alternative pathway by which PI3K/ AKT obstruct SMAD3 is via inactivation of nuclear factors essential for SMAD3 function.

TGF- $\beta$  treatment of HER2-expressing breast cancer cells increases AKT activity which is essential for cell migration. Crosstalk of TGF- $\beta$  and PI3K/AKT is revealed in oncogene-overexpressing mammary epithelial cells as PI3K inhibitors have shown inhibition of TGF- $\beta$ -induced motility. Further studies confirmed the interaction by showing blockade of TGF- $\beta$ -induced epithelial to mesenchymal transition using PI3K inhibitor LY294002 or with expression of dn-AKT. Upon treatment of epithelial pancreatic carcinoma cells with TGF- $\beta$ 1, the TGF $\beta$ RII associates with the E-cadherin/catenin complex and facilitates the disassembly of the E-cadherin adhesion complex which decreases E-cadherin and increases phosphorylation of  $\beta$ -catenin and affects cell-cell adhesion. Crosstalk between the two pathways is further substantiated in mammary epithelial cell lines where TGF- $\beta$ 1 induced EMT activates PI3-kinase and AKT, which results in alteration of E-cadherin at adherens junctions and changes in SMAD4 regulated gene expression [Guo and Wang, 2009; Papageorgis, 2015].

### LONG NON-CODING RNAs

Apart from interaction of TGF- $\beta$  with other signaling pathways, emerging studies suggest interactions of Long non-coding RNAs (lncRNAs) and TGF- $\beta$ . The lncRNAs are transcribed RNA molecules of more than 200 nucleotides that either do not encode proteins or lack >100 amino acid open reading frame. They play a crucial role in various biological processes. Increasing evidence suggests their association with carcinogenesis. Crosstalks between TGF- $\beta$  and a number of lncRNAs such as Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), Maternally expressed gene 3 (MEG3), plasmacytoma variant translocation 1 (PVT1), Linc00974, urothelial carcinoma antigen 1 (UCA1), and BRAF-activated non-coding RNA (BANCR) are reported to regulate the TGF- $\beta$  signaling in cancer. Interaction of TGF- $\beta$  and MALAT1 was shown in multiple myeloma cells where knockdown of MALAT1 inhibited production of TGF- $\beta$  and subsequently suppressed cell growth. Furthermore, MALAT1 has been shown to regulate the bioavailability of TGF- $\beta$  in mesenchymal stem cells by promoting the activation of latent TGF- $\beta$  binding protein 3 (LTBP3) promoter via recruitment of Sp1 to the LTBP3 gene. Interaction between lncRNAs and TGF- $\beta$  is further supported by investigations revealing inhibition of TGF- $\beta$  induced EMT in bladder cancer cells by silencing MALAT1 [Wang et al., 2016]. In addition, many TGF- $\beta$  pathway genes such as TGF- $\beta$ 2, TGF- $\beta$ 2RI,

and SMAD2 have binding sites for MEG3 RNA. He et al. [2014] found downregulation of MEG3 in TGF- $\beta$  treated human hepatic stellate cell line, LX-2. Ectopic expression of MEG3 in LX-2 cells inhibited TGF- $\beta$ -induced cell proliferation and stimulated apoptosis. Although new knowledge of lncRNAs and TGF- $\beta$  crosstalk is yet to emerge, lncRNAs appear to be potential targets to adjust TGF- $\beta$  signaling pathway.

### PROGESTINS

Progestins emerge to offer protection against gynecological cancers. They are routinely used to treat endometrial and ovarian cancer patients. The mechanisms by which progestins act as anti-cancer agents is not known. Endometrial cancer cells produce and secrete high levels of TGF- $\beta$ , which is associated with tumor aggressiveness. Progesterone significantly reduced TGF- $\beta$  ligands, receptors and SMAD2/3 expression in endometrial cancer cells. The mechanism by which progesterone illustrates its inhibitory effects on TGF- $\beta$  signaling is by preventing translocation of activated SMAD2/3 to the nucleus. This inhibitory effect was not seen when cells were exposed to progesterone receptor antagonist Mifepristone. In addition, progesterone inhibited TGF- $\beta$ 1 induced cell proliferation and invasive potential of endometrial cancer cells while a TGF- $\beta$ R1 blocker abolished inhibitory effects of progesterone. Furthermore, we have demonstrated inhibition of snail, slug, vimentin, N-cadherin and enhancement of E-cadherin in progesterone exposed cells [Bokhari et al., 2014]. Another study has also reported EMT inhibition in response to progesterone [van der Horst et al., 2012]. All these results point to crosstalk between TGF- $\beta$  signaling and progesterone.

### NESTIN

Nestin is an intermediate filament protein and is a marker of stem cells. Nestin by interacting with vimentin or desmin forms polymers which provide structural support to cells and maintain cellular membranes. There is limited literature on nestin and TGF- $\beta$  interaction. A study indicated a positive relationship between nestin and TGF $\beta$  signaling pathway in pancreatic cancer [Su et al., 2013]. We compared expression of nestin and TGF- $\beta$  in immortalized epithelial endometrial cells and in endometrial cancer cells and showed higher expression of both in cancer cells. A direct relationship between the two have been shown by shRNA mediated knockdown or by ectopic expression of nestin in endometrial cancer cells. Knockdown of nestin diminished TGF- $\beta$ 1, TGF $\beta$ Rs, and phosphorylated SMAD2/3 but not SMAD4 and inhibited endometrial cancer cell growth in vitro and in vivo. Furthermore, nestin knockdown inhibited TGF- $\beta$ 1 induced EMT as revealed by down-regulation of EMT markers and upregulation of E-cadherin. In contrast, nestin overexpression showed an inverse expression pattern of TGF $\beta$  signaling components, EMT proteins expression and enhanced invasive potential of cancer cells (our unpublished data). These results strongly support interdependence of nestin and TGF- $\beta$  signaling on regulating tumor progression.

## CONCLUSIONS AND FUTURE PERSPECTIVES

TGF- $\beta$  has a major role in a variety of cancer types during progression and metastasis. Increased levels of TGF- $\beta$  in the

tumor and its microenvironment promote tumorigenesis by inducing EMT and facilitating tumor cell proliferation, thus making it a druggable target. At present, diverse therapeutic approaches are being tested in clinical trials and several preclinical investigations are evaluating the efficacy of synthetic and natural anticancer/antimetastatic molecules. Emphasis is placed on blockers of TGF- $\beta$  activation, neutralizing antibodies against TGF- $\beta$ -receptor interaction, inhibitors of TGF- $\beta$  receptor kinase activity, ligand traps, antisense oligonucleotides, peptide aptamer, and natural products.

TGF- $\beta$  signaling is altered in tumor cells, thus TGF- $\beta$  inhibitors may have restricted effects on these cells but may affect TGF- $\beta$  responsive cells in the tumor microenvironment. Studies have linked TGF- $\beta$  signaling with tumor microenvironment demonstrating the promotion of angiogenesis and metastasis. TGF- $\beta$  inhibition therapy may be tailored to normalize tumor microenvironment by inhibiting stromal stimulation, which results from excess production of TGF- $\beta$  and other factors from tumors. Knowledge gained from tumor and stromal cells may be instrumental in identifying new molecular targets for developing novel TGF- $\beta$  inhibitors. Nonetheless, there are still many hurdles in developing and employing TGF- $\beta$  inhibitors in patients. Timing of treatment and selection of patients who would benefit from TGF- $\beta$  inhibition treatment are critical. TGF- $\beta$  inhibition may be a better preventive strategy for early stage cancers, as tumors resulting from chronic inflammation have elevated levels of TGF- $\beta$  prior to tumor formation and form an attractive microenvironment for cancer cells. TGF- $\beta$  inhibitor treatment can be beneficial as adjuvant therapy after complete resection of tumor. TGF- $\beta$  inhibitors target the tumor microenvironment and have minimal effect on cell proliferation. To circumvent this issue, a combination of cytotoxic agents along with TGF- $\beta$  inhibitors would enhance antitumorogenic effects compared to TGF- $\beta$  inhibitors alone. Thus, targeting the TGF- $\beta$  signaling pathway alone may not be sufficient to abrogate tumor growth. A synergistic response may be attained by concurrently targeting other aberrant signaling pathways such as EGFR, PI3K/AKT, NF- $\kappa$ B, JAK/STAT. Preclinical studies in pancreatic tumors targeting the EGFR and TGF- $\beta$  signaling pathways simultaneously exhibited better efficacy than targeting either signaling pathway alone. Clinical studies assessing the effects of the TGF $\beta$ R1 kinase inhibitor LY-2157299 in combination with Sorafenib (a small molecular inhibitor of multiple tyrosine protein kinases and Raf kinases) in hepatocellular carcinoma support the notion that combined treatment is more effective than a single treatment in inhibiting tumor growth. While several antagonists are being assessed in clinical trials, their long-term efficiency and potential adverse side effects are not known. It is challenging to predict whether complete blocking of TGF- $\beta$  with inhibitors would be sufficient to block the tumor promoting effects of TGF- $\beta$  without affecting its tumor suppressive functions. Thus, it is important to develop novel strategies to target or manipulate the downstream signaling components of the TGF- $\beta$  pathway. To avoid the side effects and drug resistance associated with long-term use of existing TGF- $\beta$  blockers, future studies should explore whether combinatorial or sequential treatments of anti-TGF- $\beta$  drugs with irradiation or chemotherapy will be beneficial. Furthermore, concomitantly

aiming multiple targets may prove to be an effective therapy as TGF- $\beta$  interacts with many signaling pathways. Profound understanding of TGF- $\beta$  and its networking pathways that regulate its outcome in tumors cells will assist in selecting drug combinations for tumors.

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