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Commentary

TGF-β in cancer and as a therapeutic target

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ABSTRACT

Cancer develops through a series of genetic changes leading to malignant transformation. Numerous gene and pathways involved in stages of progression to frank malignancy have been elucidated. These genetic changes result in aberrations in fundamental cellular processes controlling proliferation, apoptosis, differentiation and genomic stability. Metastasis is the hallmark of malignancy. The process of metastasis is extremely complex and involves steps including dissemination of tumor cells from the primary tumor through the vascular and lymphatic system and growth selectively in distant tissues and organs. Transforming growth factor-β which is a growth suppressive cytokine in many normal situations becomes an active and important participant in malignant disease including angiogenesis, extracellular matrix deposition, immuno-suppression and metastasis growth promotion. Transforming growth factor-β and its receptors are targets for antibody therapeutics and small molecule kinase inhibitors.

The development of cancer has been shown to occur through a process of malignant transformation which involves a series of genetic changes. Research over the past few decades has identified numerous genes and pathways involved in various stages of tumor progression [1]. These genetic changes invariably disrupt fundamental cellular processes controlling proliferation, apoptosis, differentiation and genome stability. It is the combinatorial effect of these genetic changes that result in malignant transformation [1]. Proliferating hematopoietic and epithelial cell populations are particularly susceptible to the accumulation of genetic changes leading to malignancy. Nearly 90% of human solid tumors arise from epithelial cells. The majority of patients who succumb to cancer die as a result of metastatic disease progression rather then from the primary tumor. The process of metastasis is extremely complex and involves many steps including dissemination of tumor cells from the primary tumor through the vascular and lymphatic system coupled with the ability to selectively colonize distant tissues and organs [2]. The pleiotropic cytokine transforming growth factor-β (TGF-β) and its signaling effectors have been shown to be involved in malignancy, thus making TGF-β and its signaling pathway a potentially important target for anticancer therapeutics.

1. TGF-β in cancer

TGF-β is a growth inhibitor of many normal tissues and early stage lesions. However, TGF-β activity facilitates growth and metastasis in late stage cancer. During normal development and tissue homeostasis TGF-β functions to restrain proliferation through induction of cytostatic and apoptotic gene programs [3]. The tumor suppressive ability of TGF-β has been demonstrated in model systems and in studies of human disease. The TGF-β-induced cytostatic gene expression programs elucidated in studies with epithelial and lymphoid cells include activation of cyclin-dependent kinase inhibitors $p15^{INK4B}$ and $p21^{WAF1}$ and repression growth-enhancing

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transcription factors c-Myc and ID1-3 [4]. In animals, transgenic expression of TGF-β1 in mammary gland, skin, pancreas and liver promoted growth arrest and in some settings enhanced apoptosis [5]. Furthermore, TGF-β1 over-expression suppressed mammary tumor development by transgenic expression of TGF-α or carcinogen exposure. The growth inhibitory effects of TGF-β result in tumor suppression in these models.

Attenuation of TGF-β signaling correlates with tumor progression and enhanced metastatic potential in both animal models and human patient samples. Transgenic expression of dominant negative TGF-β type II receptor enhanced carcinogen-induced lung and mammary tumorigenesis [6]. Mice with ablation of *Tgf-β1* or *Smad3* genes develop colon adenomas that progress to carcinomas. Mice heterozygous for the *Tgf-β1* gene which expresses 10–25% of normal protein levels demonstrate enhanced carcinogen-induced tumor development [7].

Germline or sporadic mutation of TGF-β signal transduction pathways components occurs in human cancers. Inactivating mutations in TGF-β type II receptor are found in sporadic and hereditary colon cancers characterized by microsatellite instability (MSI). Inactivating mutations in the *Smad2* and *Smad4* genes are found in colorectal and pancreatic carcinomas [8]. Sporadic mutations in the TGF-β type I receptor are found at low frequency in breast, ovarian and pancreatic carcinomas [9]. The frequency of germline or sporadic mutations in components of TGF-β signal transduction pathways occur at low frequency except in pancreatic carcinoma and MSI colorectal carcinoma. Recently, *TGF-β1* gene polymorphisms were associated with decreased 5-year survival in breast cancer patients, and multiple polymorphisms in the *TGFBRI* gene were correlated with an increased risk of breast, ovarian cancer and hematologic malignancies but not prostate cancer [10]. The functional consequences of polymorphisms on TGF-β signaling pathways remain unclear.

Although the majority of cancers retain TGF-β type I/II receptors, malignant cells are not growth inhibited by TGF-β possibly due to activation of the PI3K and Ras signal transduction pathways. Loss of TGF-β growth control allows tumor cells increased motility, elevated matrix protease activity, increased TGF-β production and fibroblast-mediated extracellular matrix (ECM) expression and angiogenesis [11]. Elevated TGF induces epithelial to mesenchymal transition (EMT) of normal and transformed epithelial cells and thus an enhanced migratory ability. Increased migration by epithelial cells that have undergone EMT is mediated by repression of cell–cell adhesion protein, E-cadherin, and induction of fibroblast specific markers. Smad-dependent and independent signal transduction pathways are implicated in TGF-β-induced EMT. Both TGF-β and Ras-MAPK pathways are required for EMT and enhancement of invasive and metastatic properties of malignant cells [12].

Strong evidence exists from animal models that TGF-β can promote late stage tumor growth and metastasis. Mice heterozygous for the *Tgf-β1* gene have enhanced carcinogen-induced tumorigenesis. Tumors that developed retained the remaining wild-type *Tgf-β1* allele indicating that there was no selective pressure to abrogate TGF-β signaling [7]. In addition, transgenic mice expressing TGF-β1 in keratinocytes developed fewer carcinogen-induced skin papillomas; however, conversion of benign lesions into invasive spindle cell carcinomas increased in TGF-β1 transgenic mice.

TGF-β is a potent suppressive cytokine with effects on normal hematopoietic homeostasis and immune responses [11,13]. Genetically engineered mice allowed identification of a critical role for TGF-β1 in homeostasis of T lymphocytes. Genetic deletion of the *Tgf-β1* gene, abrogation of signaling through transgenic expression of dominant negative TGF-β type II receptor in T cells or reconstitution of lethally irradiated mice with dominant negative TGF-β type II receptor-transduced bone marrow resulted in development of lethal multifocal inflammatory disease [14]. Thus, TGF-β1 has an important role in limiting T cell proliferation in normal tissue homeostasis. TGF-β1 expression by malignant epithelial cells and/or associated stromal cells in the tumor microenvironment mediates tumor progression through suppressive effects on immune effector cell populations.

The ability of the immune system to induce tumor-specific T cells in patients with advanced cancer is well documented but eradication of malignant disease by endogenous immune response is rare [15]. Tumors can tolerize host immune responses mounted against the malignant lesion. Recent data implicates regulatory T (T_{reg}) cells as key in tumor-mediated immunosuppression. Elevated T_{reg} cell levels were identified in the blood and tumor tissue of cancer patients [16]. There is a strong predictive correlation between increasing intratumoral T_{reg} cell numbers and reduced survival in patients with ovarian carcinoma [99]. Numerous studies show that T_{reg} cells potently suppress activation of $CD4^+$, $CD8^+$ T cells and natural killer (NK) cells [16,17]. TGF-β1 directly regulates development of T_{reg} cells in vitro and in vivo [18]. This may be a major mechanism by which TGF-β1 promotes tumor progression. Experiments with mouse lymphoma, melanoma and prostate carcinoma tumors support the hypothesis that inhibition of TGF-β1 mediated immunosuppression can have strong therapeutic potential in cancer. Transgenic mice expressing dominant negative TGF-β type II receptor in T cells mounted anti-tumor immune responses after inoculation of EL-4 lymphoma or B16-F10 melanoma cells. In contrast control mice were unable to activate $CD4^+$ and $CD8^+$ T cell anti-tumor immune responses and rapidly succumbed to tumor growth [19]. Mice reconstituted with dominant negative TGF-β type II receptor-transduced bone marrow were insensitive to TGF-β mediated suppression of T cell responses and survived challenge with B16-F10 melanoma or TRAMP-C2 metastatic prostate carcinoma cells better than control mice [20]. Studies with a mouse fibrosarcoma tumor model identified a novel mechanism that can suppress $CD8^+$ T cell anti-tumor immune responses independent of T_{reg} cell function. A nonlymphoid splenic cell of myeloid lineage has been described that can be induced to secrete TGF-β1 and directly suppress anti-tumor activity of cytolytic T cells [21]. Elevated TGF-β in tumor-bearing animals can suppress host T cell responses.

In addition to suppression of cytolytic T cell responses, TGF-β inhibits activation of NK cells in cancer patients. Plasma TGF-β1 was elevated in lung and colorectal carcinoma patients and cell surface expression of the NK cell activation marker NKG2D inversely correlated with plasma TGF-β1 [22]. Incubation of NK cells with plasma from cancer patients or

exogenous TGF-β1 specifically reduced cell surface NKG2D without modulating expression of other receptors [22]. TGF-β1 down regulates the expression of NKp30 a receptor involved in NK mediated cell killing of immature dendritic cells (DC). TGF-β1 impairs multiple NK cell functions through down regulation of specific cell surface receptors. Immunosuppression in cancer patients can also be mediated through direct effects on DC which are specialized antigen presenting cells that initiate primary immune responses [23]. TGF-β1 can interfere with DC function through down regulation of cell surface classes I and II MHC antigens, co-stimulatory molecules and chemokine receptors. Tumor-derived TGF-β1 reduces the efficacy of DC vaccines in mouse tumors [24]. TGF-β is a potent immunosuppressive cytokine acting on cells from multiple lineages. In summary, alterations in the TGFβ signaling pathway in malignancy can be epigenetic or genetic deletions or mutations, amplifications or downregulation of components that result in enhancement of tumor growth or increase in host tolerance for the malignancy [4,8].

2. Breast cancer

The role of TGF-β in the development and progression of breast cancer has been studied extensively. TGF-β acts as a growth inhibitor in early stage disease and as a pro-oncogenic factor in late stage disease. The majority of breast cancers secrete elevated TGF-β1 in tumor microenvironment associated with either malignant epithelial cells, stromal cells or both [25]. In a large panel of breast cancers (456 cases) more then 90% of the tumors had phosphorylated Smad2 tending to indicate that TGF-β signaling was intact; however, receptors other than TGF-β receptors can modulate phosphorylation of Smad2 [26]. Numerous studies show a strong correlation between elevated plasma TGF-β1 and breast cancer disease progression [25,27]. Pre- and post-operative plasma TGF-β1 associated tightly with clinical outcome in a small study of newly diagnosed breast cancer patients [25]. Patients whose plasma TGF-β1 normalized after tumor resection had a favorable prognosis, and patients with persistently elevated plasma TGF-β1 had an increased risk of lymph node metastases and disease progression [25]. Plasma TGF-β1 may be a useful prognostic factor in breast cancer.

Increased immuno-reactivity for TGF-β protein correlated with poor prognosis and increased lymph node involvement [28]. Elevated TGF-β was associated with tamoxifen resistance. Studies with human MCF-7 breast carcinoma cells demonstrated induction of TGF-β signal transduction pathways after culture in the absence of estrogen [29]. Clonal MCF-7 cell lines expressing elevated TGF-β1 promoted tumor development in the absence of estrogen. This tumor development was attenuated by treatment with tamoxifen and an antibody that neutralizes the three active TGF-β isoforms but not after treatment with either agent alone [30]. There is a highly significant association between TGF-β type II receptor expression and reduced survival of patients with estrogen receptor negative breast cancer [31].

Recent studies have elucidated the dual tumor suppressive and pro-metastatic functions of TGF-β signaling using bi-transgenic mouse models of breast cancer. Transgenic expression of Neu oncogene driven by the mouse mammary tumor virus (MMTV) long-terminal repeat resulted in multifocal mammary adenocarcinomas that metastasized to lung. Amplification of the *Her2/Neu* epidermal growth factor receptor gene occurs frequently in human breast cancer [32]. Transgenic mice expressing constitutively activated and dominant negative TGF-β receptors were developed to examine TGF-β signaling effects on Neu-induced mammary tumorigenesis and metastasis [33]. Co-expression of constitutively activated TGF-β type I receptor increased the latency of Neu-induced primary mammary tumors, while transgenic expression of dominant negative TGF-β type II receptor decreased the latency of tumor development [33]. The pro-metastatic effect of TGF-β was observed in mice co-expressing activated TGF-β type I receptor or activated TGF-β1 and Neu by increased incidence of extra-vascular lung metastases [33].

Studies utilizing a doxycycline (DOX)-inducible transgenic promoter provide evidence that TGF-β1 is pro-metastatic in a mouse breast cancer model [34]. Earlier studies showed that transgenic expression of polyoma virus middle T oncogene (PyMT) driven by MMTV long-terminal repeat induced multifocal mammary tumors with lung metastases [35]. The role of TGF-β1 in tumor progression and metastasis was elucidated by generating triple transgenic mice where DOX-inducible active TGF-β1 could be expressed in PyMT-transformed mammary epithelial cells in a temporal and spatial fashion [34]. When mice with palpable mammary tumors were treated with DOX to induce expression of active TGF-β1, there was no effect on the growth of primary mammary tumors, but there was a more then 10-fold increase in lung metastases [34]. Active TGF-β1 mediated this effect by acting on the mammary tumor cells rather then by a paracrine effect on tumor stromal, endothelial or immune cells. Elevated active TGF-β1 and phosphorylated Smad2 were found in PyMT expressing mammary epithelium, and a three-fold elevation in serum TGF-β1 occurred after administration of DOX suggesting that systemic changes could contribute to increased lung metastasis [35]. The challenge is to distinguish TGF-β1-mediated autocrine versus paracrine/systemic effects in this model.

Attenuation of TGF-β signaling can modulate tumor progression and metastasis in a cell autonomous fashion. Expression of dominant negative TGF-β type II receptor in genetically related cell lines derived from non-transformed MCF-10A human mammary epithelial cells could cooperate with Ras enhance to tumorigenesis by these cells [36]. Conversely, dominant negative TGF-β type II receptor expression in a high-grade metastatic cell line had no effect on primary tumor development but markedly inhibited metastasis [36]. TGF-β pathways also play a critical role in breast cancer metastasis to bone. Human breast cancers frequently metastasize to bone resulting in pathologic fractures and pain due to osteolytic bone destruction [37]. Studies with human breast carcinoma cell lines in a mouse bone tumor model identified a molecular basis for bone tropism. Inoculation of MDA-MB-231 human breast carcinoma cells into the left ventricle of nude mice seeds cells into the arterial circulation and consistently produces osteolytic bone lesions and visceral metastases with short latency. Tumor cells growing in the bone microenvironment express parathyroid hormone-related peptide (PTHrP) which stimulates osteoclasts and

bone resorption [42]. Constitutively activated TGF-β type I receptor enhanced expression of PTHrP in MDA-MB-231 cells in culture and increased osteolytic bone destruction in vivo [38]. Expression of dominant negative TGF-β type II receptor inhibited PTHrP expression and osteolytic bone tumor development. Recently, a multi-gene program activated by TGF-β which regulates bone and adrenal gland specific metastasis of MDA-MB-231 human breast carcinoma was defined linking dysregulated TGF-β signaling with osteolytic breast cancer metastases and suggesting a point for therapeutic intervention in advanced breast cancer [39]. Thus, microenvironmental signaling from stromal cells can regulate adjacent epithelial cell tumorigeneic behavior [4].

3. Prostate cancer

Elevated TGF-β1 is consistently found in prostate cancer compared with normal prostate tissue from patients [40]. Expression of TGF-β1 appears early in prostate cancer and increases during tumor progression and metastasis [40]. Hormone refractory and recurrent prostate cancers frequently produce osteoblastic bone lesions stimulated by TGF-β released from tumor microenvironment or bone matrix [41]. No roles for TGF-β2 or 3 in prostate cancer have been identified; however, prostate-specific antigen can activate latent TGF-β2 suggesting a mechanism for autocrine regulation of TGF-β activation in prostate cancer [42]. In addition, a strong correlation was found between elevated preoperative plasma TGF-β1 and prostate cancer progression and metastasis in locally advanced disease patients [43]. Elevated postoperative plasma TGF-β1 was predictive for disease recurrence and metastasis indicating that micrometastases were established at the time of primary tumor surgical resection [43]. Microarray analyses of a panel of prostate carcinomas identified gene expression signatures that correlated with tumor Gleason score [44]. TGF-β type II receptor was down-regulated during disease progression. However, expression of several TGF-β-induced genes involved in ECM deposition (*Collagen 1A*) and bone-specific metastasis (*Osteopontin*) were elevated and correlated with increasing Gleason grade [44].

In prostate epithelial cell lines loss of growth inhibition by TGF-β by decreased expression of the types I and II receptors and increased expression of TGF-β1 correlated with malignant transformation. Prostate carcinoma cell lines in culture no longer respond to TGF-β1-mediated growth inhibition compared with non-tumorigenic prostate epithelial cells [45]. Prostate carcinoma sublines, DU145, PC-3 and LNCaP, resistant to chemotherapy secreted more TGF-β1 into culture medium, and animals bearing xenografts of these tumor lines had increased plasma TGF-β1 [46]. Treatment of animals bearing prostate carcinoma xenografts with cytotoxic chemotherapy resulted in increased plasma TGF-β1 that correlated with the drug resistance of the tumor cell lines [46]. These data support the hypothesis that TGF-β1 contributes to drug resistance.

TGF-β1 over-expression in the Dunning R3327 MATLyLu rat prostate carcinoma line resulted in enhanced primary tumor growth and lung and lymph node metastasis after subcutaneous implantation. Reduced TGF-β1 expression by stably expressing an antisense oligonucleotide in MATLyLu prostate carcinoma line inhibited primary tumor growth and metastasis [47]. Thus, TGF-β1 expression can enhance metastasis of rat prostate carcinoma cells. It is unclear whether this pro-metastatic activity is mediated through autocrine effects on the prostate carcinoma cells or through paracrine effects on associated stromal, endothelial and immune cells.

The stroma plays a significant role in prostate tumorigenesis. Carcinoma-associated fibroblasts (CAF) from prostate carcinomas can stimulate tumor progression of non-transformed prostate epithelial cells in an in vivo tissue recombination model. TGF-β1 expression was higher in CAF than in fibroblasts from normal human prostate and increased TGF-β1 expression correlated with the capacity of CAF to promote transformation of normal prostate epithelial cells [48]. Normal prostate fibroblasts were converted to CAF in culture by exposure to TGF-β1 suggesting that TGF-β1 can support development of reactive stroma. It is unclear whether CAF influence tumor development by secretion of factors that promote a reactive ECM and enhance angiogenesis or by effects on tumor cells. The role of stromal cells in prostate tumorigenesis was addressed using LNCaP prostate carcinoma cells that formed tumors very inefficiently when implanted into immune compromised mice. Mixing LNCaP cells with mouse tumor derived ECM components (Matrigel) increased tumor takes [49]. Tumor incidence was dramatically increased by co-transplanting LNCaP cells with CAF and Matrigel. The resulting tumors had a more then 10-fold increase in micro-vessel density [49]. Inclusion of latency-associated protein (LAP) to neutralize TGF-β during the generation of LNCaP differential reactive stroma tumors reduced microvessel density 3.5-fold and tumor weight by nearly 50% [50]. It is unclear whether TGF-β1 is being secreted by the prostate carcinoma cells, the CAF or both. TGF-β produced in the tumor micro-environment has direct effects on prostate epithelial cells as well as paracrine effects on tumor associated stromal and endothelial cells. In summary, in prostate cancer, up-regulation of TGF-β1 has been associated with angiogenesis, metastasis and poor patient prognosis [4].

4. Therapeutic interventions

Small molecule, antibody and antisense TGF-β antagonists are in development for the treatment of cancer and fibrotic disorders [51,52]. These approaches include large molecule inhibitors which include monoclonal antibodies, soluble receptors and antisense oligonucleotides directed against the ligands and small molecules directed against the receptor kinase activities. Multiple human and mouse monoclonal and rabbit polyclonal antibodies that neutralize active TGF-β isoforms by blocking ligand access to the receptors have been described. Phage display technology was utilized to develop human antibodies that selectively neutralize specific TGF-β isoforms. These antibodies are being evaluated in clinical trials for treatment of fibrotic disorders. A human monoclonal antibody, CAT-152, that neutralizes TGF-β2 is currently in Phase III trials for prevention of scarring induced by glaucoma

surgery, and a second human monoclonal antibody, CAT-192, that selectively targets TGF-β1 was examined in Phase I/II trial for the treatment of scleroderma. Two pan-neutralizing mouse monoclonal antibodies, 1D11 and 2G7, have been used extensively to demonstrate the therapeutic potential of TGF-β antagonism in tumor models. Mouse and human tumor cell lines frequently secrete elevated TGF-βs in vitro and in vivo and have been used to examine the therapeutic potential of TGF-β antagonists. Growth of MDA-MB-231 human breast carcinoma implanted intra-abdominally was reduced after treatment with 2G7, and anti-tumor activity correlated with enhanced cell killing mediated by splenic NK cell.

Tumor resistance to chemotherapy in vitro and in vivo can be reversed by TGF-β antagonist treatment. Tamoxifen resistant LCC2 breast carcinoma cells express elevated TGF-β2 compared with tamoxifen sensitive cell line LCC1. Combination treatment with tamoxifen and the pan-neutralizing TGF-β antibody 2G7 inhibited growth of LCC2 human breast carcinoma xenografts compared with either agent alone [33]. A critical role for NK cells in anti-tumor activity was identified supporting a role for tumor-derived TGF-β in host immune suppression [33]. The killing of MDA-MB-231 breast cancer spheroids was enhanced by exposure to 2G7 in combination with the anticancer agent cisplatin (CDDP) suggesting that tumor-derived TGF-β may contribute to drug resistance [52,53]. EMT6 mouse mammary carcinoma drug resistant sublines were generated in vivo by treatment of tumor bearing animals with anti-tumor agents cyclophosphamide (CTX) and CDDP [54]. Treatment of animals bearing drug resistant tumors with TGF-β neutralizing antibodies or the leucine-rich proteoglycan decorin increased the sensitivity of EMT6/CTX tumors to CTX and EMT6/CDDP tumors to CDDP [55,56]. Thus, TGF-β in the tumor microenvironment may contribute to drug resistance in vivo and TGF-β antagonists may be an effective addition to treatment of drug resistant tumors.

Several protein therapeutics that neutralize active TGF-β isoforms are being developed [51]. The extracellular ligand-binding domain of the TGF-β type II receptor was fused to the Fc domain of human IgG1 (abbreviated Fc: TβRII or SR2F) to produce a high-affinity, stable antagonist capable of neutralizing TGF-β1 and -β3 isoforms. Transgenic expression of systemic SR2F did not result in pathology as occurred in TGF-$\beta^{-/-}$ gene deletion mice, but did decrease metastasis from primary mammary tumors [57]. Purified Fc:TβRII protein inhibited spontaneous lung metastasis from orthotopically implanted 4T1 and EMT6 mammary carcinomas [58]. CD8+ T cells were identified as key mediators of Fc:TβRII anti-tumor activity in a syngeneic mouse malignant mesothelioma [59]. Antibodies that neutralize active TGF-β isoforms have potent anti-tumor activities in rodent models of melanoma, multiple myeloma and prostate carcinoma.

Small molecule inhibitors of the serine/threonine kinase activity of TGF-β type I receptor, thus blocking phosphorylation of Smad2/3, are under development [60,61]. These compounds block TGF-β-induced EMT of normal mammary epithelial cells and migration and invasion of pancreatic carcinoma cells [62]. SD-208 inhibited the growth of intracranial gliomas in a syngeneic mouse model in a manner that correlated with increased tumor infiltration of NK cells, CD8+ T cells and macrophage [63]. Several of the small molecules also inhibit other TGF-β type I receptors such as ALK4 resulting in the modulation of activin-dependent Smad activation. The significance of this broad spectrum activity is unclear.

5. Conclusion

The concepts described in this review will soon have the opportunity to be tested in the clinic. Both a fully human pan-TGF-β neutralizing antibody and a small molecule TGF-β type I receptor kinase inhibitor are likely to enter Phase I clinical trial in cancer patients soon. An antisense to TGF-β2 is currently in Phase II clinical trial. LY215799, a dihydropyrrolopyrazole derivative, is a selective TGF-β RI kinase inhibitor that abrogates TGF-β-dependent biology in a variety of cell types and in vivo inhibits phospho-Smad2 formation in multiple tumors and normal tissues. The entry of this molecule into Phase I clinical trial in metastatic cancer patients will help elucidate the potential clinical benefit as well as potential side effects of modulating TGF-β signaling in cancer patients with LY215799.

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