
Review

Clinically Translatable Approaches of Inhibiting TGF- β to Target Cancer Stem Cells in TNBC

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Abstract: Triple-negative breast cancer (TNBC) is a subtype of breast cancer that disproportionately accounts for the majority of breast cancer-related deaths due to the lack of specific targets for effective treatments. In this review, we highlight the complexity of the transforming growth factor-beta family (TGF- β) pathway and discuss how the dysregulation of the TGF- β pathway promotes oncogenic attributes in TNBC which negatively affects patient prognosis. Moreover, we discuss recent findings highlighting TGF- β inhibition as a potent method to target mesenchymal (CD44⁺/CD24⁻) and epithelial (ALDH^{high}) cancer stem cell (CSC) populations. CSCs are associated with tumorigenesis, metastasis, relapse, resistance, and diminished patient prognosis; however, due to differential signal pathway enrichment and plasticity, these populations remain difficult to target and persist as a major barrier barring successful therapy. This review highlights the importance of TGF- β as a driver of chemoresistance, radioresistance and reduced patient prognosis in breast cancer as well highlights novel treatment strategies which modulate TGF- β , impede cancer progression and reduce the rate of resistance generation via targeting the CSC populations in TNBC and thus reducing tumorigenicity. Potential TGF- β inhibitors targeting based on clinical trials are summarized for further investigation which may lead to the development of novel therapies to improve TNBC patient prognosis.

Keywords: Triple Negative Breast Cancer, Cancer Stem Cell, TGF- β

1.0 Introduction

A GLOBOCAN study in 2020 demonstrated that there were approximately 19.31 million new cancer cases and 9.96 million cancer-related deaths worldwide[1-2]. In line with these shocking numbers, a recent report by Dagenais et al demonstrated that while cardiovascular disease is still the number one cause of mortality (40%) worldwide, in high-income countries, deaths attributed to cancer (55%) exceeded deaths due to cardiovascular disease (23%) among adults aged 35-70 [3]. Together this data suggests that in the developed world and probably in the future for other nations; cancer will overtake cardiovascular disease as the leading cause of mortality making the treatment and research of this disease a major medical priority [3].

Further breakdown of the GLOBOCAN 2018 study revealed over 2 million breast cancer diagnoses and over 600,000 breast cancer-related mortalities that year. Thus breast cancer is the most frequent cancer affecting women, accounting for 1 in 4 cancer cases amongst the female population throughout the world and this disease remains the leading cause of cancer-related deaths amongst women [1]. Triple negative breast cancer only accounts for 15-20% of breast cancer incidences; however, this subtype is disproportionately associated with decreased patient prognosis and relapse in comparison [4,5]. Additionally, in comparison with other breast cancer subtypes, TNBC due to lack of expression of the estrogen receptor, progesterone receptor, and HER-2 is primarily treated with surgery and non-specific chemotherapy and radiotherapy regimens. As such, the combination of a highly aggressive breast cancer subtype paired with inadequate treatment options contributes towards the dismal prognosis of TNBC compared to other breast cancer subtypes.

Treatment for TNBC remains an unmet medical need and the development of novel approaches/therapeutics are required to overcome this hurdle.

1.1 Overview of TGF- β Signaling

In brief, TGF- β signaling is mediated primarily through SMAD or non-SMAD mechanisms [6]. There are three main isoforms of TGF- β (TGF- β 1, TGF- β 2, and TGF- β 3); however, in mammals, TGF- β 1 is the predominant isoform and its inactivated form is secreted by cells and is bound to extracellular proteins [7]. Various proteins/conditions have been found to activate TGF- β such as pH, ROS, plasminogens, metalloproteinases, and thrombospondin [6,8,9]. Activated TGF- β then binds to the TGF- β type II serine/threonine kinase receptor which recruits, dimerizes, and phosphorylates the TGF- β type I receptor promoting its activation. Activated TGF- β type I receptor then phosphorylates and activates SMAD2 and SMAD3. Following their activation, SMAD2 and SMAD3 trimerizes with co-SMAD4. The activated SMAD transcription complex then translocates to the nucleus and induces transcription of numerous target genes regulating extracellular matrix production, inflammation, proliferation, immunoregulation and survival [6,8,9].

TGF- β signaling can also be mediated through non-SMAD dependent mechanisms through direct phosphorylation of various proteins by the activated TGF- β type 1 receptor. It has been found that TGF- β signaling can then promote MAPK/ERK, PI3K/Akt/mTOR/S6K, RhoA/Rac signaling [10-12]. As these pathways are deregulated in TNBC, due to its position as a key branchpoint regulator of these downstream pathways, the modulation of TGF- β may demonstrate to have potent therapeutic effects in the treatment of TNBC[13,14].

1.2 The Complicated Regulation of TGF- β in Cancer

TGF- β has been demonstrated to play a biphasic role during tumorigenesis [15]. During the early stage of tumor development, TGF- β 1 has been demonstrated to act as a tumor suppressor through induction of p21^{CIP1} which prevents cell cycle progression and through the suppression of c-Myc[15]. TGF- β 1 is capable of stimulating apoptosis through SMAD dependant activation of GADD45b which then binds and activates p38 triggering programmed cell death [16]. TGF- β 1 can also modulate apoptosis through the regulation of pro-apoptotic (BIM and BIK) and anti-apoptotic factors (Bcl-xL) [17-20]. Tang et al demonstrated using xenograft models of early-stage breast cancer that TGF- β induced differentiation through the downregulation of Id1 (Inhibitor of differentiation/DNA binding, a member of the helix-loop-helix protein family which binds to basic helix-loop-helix transcription factors to inhibit differentiation and promote self-renewal) [21,22]. Using an in vivo serial dilution assay, the gold standard to assess tumorigenicity, Tang et al observed that compared to the control MCF10A-Ca1h xenografts, TGF- β unresponsive tumors through transfection of a dominant negative type II TGF- β receptor were 10-20 times more effective at tumor formation, supporting the tumor suppressor role of TGF- β in early carcinoma development [21].

While TGF- β takes on tumor-suppressive roles during early carcinoma development, it has been found that in various late-stage models of cancer (carcinomas including breast, prostate, lung, and colorectal cancers) TGF- β signaling is associated with angiogenic, proliferative, and pro-metastatic phenotypes [15,23-26]. The exact mechanism behind this process remains convoluted; however, it has been found that as cancer progresses, mutations within the TGF- β ligands, receptors and downstream/upstream mediators affecting signaling are widespread and promote dysregulation [27-29]. One such example is p53, upon p53 mutation (one of the most frequently mutations in cancer), TGF- β signaling switched from a tumor suppressor to instead promoting migration and proliferation in ovarian cancer cell line models [27]. A report by Ji et al sheds light on the complicated crosstalk between p53 and TGF- β where using non-small-cell lung carcinoma (H1299) and mouse oral cancer-derived (J4708) cells (both p53^{-/-}) it was demonstrated that transfection of mutant p53 (R175H) binds to the MH2 domain in SMAD3 which led to the disruption of the formation of the SMAD3-4 complex[30]. This correlated with increased migration and proliferation with reduced responsiveness upon TGF- β administration, whereas TGF- β addition to control cells induced the expression of p21^{WAF1} and suppressed growth and migration[30]. Compared to the controls, gene analysis demonstrated that mutant p53 cell lines decreased the expression of p21 and p15 tumor suppressors upon TGF- β stimulation; however, the gene expression of MMPs and Slug were increased compared to the control which was correlated with enhanced cellular migration[30]. Treatment with SB431542 (a TGF- β /ALK4/5 inhibitor) restored TGF- β -induced gene expression in both the control and p53 mutant cell lines [28]. Furthermore, siRNA knockdown of SMAD3 demonstrated similar results upon TGF- β stimulation revealing that it was through p53 antagonism of SMAD3 that TGF- β dysregulation was mediated[30]. Furthermore, mechanistic analysis revealed that it was through ERK signaling that mutant p53 was associating with SMAD3 and upon inhibition

of MEK and ERK, the interaction between mutant p53 and SMAD 3 alongside aberrant signaling, was abolished[30]. Together this research highlights the complicated network facilitating proper TGF- β tumor suppression, how this pathway may be deregulated, the antagonistic role of SMAD3 towards Slug and MMP expression, and how deregulation of this pathway may affect cellular proliferation, migration, and even malignancy.

Other pathways have also been found to modulate TGF- β signaling; it was found that the Akt protein physically interacts with SMAD3 translocating it outside the nucleus and preventing signaling thus halting TGF- β mediated apoptosis, highlighting that dysregulated P13K/Akt signaling can also alter TGF- β signaling [28]. A more recent study by David et al shed further light on the complicated TGF- β switch in pancreatic ductal adenocarcinoma models. They demonstrated that TGF- β through SMAD4 stimulates EMT (epithelial to mesenchymal transition) and migration; however, TGF- β signaling simultaneously promoted apoptosis through upregulation of SNAI1 (an EMT associated factor) which in turn inhibited KLF5 allowing for SOX4 levels to increase and trigger apoptosis[29]. This was interesting as SOX4 is traditionally associated with tumorigenicity; however, it was found that in pancreatic ductal adenocarcinoma model, SOX4 induced apoptosis and it was only upon SOX4 complexing with KLF5 (upon downregulation of SNAI1) was there increased tumorigenesis[29]. This serves to highlight the complicated, contextual balance of TGF- β signaling. As signal modifications are common in cancer, there are a plethora of potential mechanisms that can dysregulate TGF- β signaling switching it from a tumor suppressor to an oncogene in carcinoma cells. Pro-oncogenic signal pathways such as MAPK, PI3K/Akt/mTOR and c-Myc are also frequently altered in TNBC which may oppose/antagonize the tumor suppressive signalling of TGF- β and mechanistically alter the TGF- β pathway [31-33].

1.3 The Clinical Correlation of Dysregulated TGF- β Signaling.

TGF- β has been found to be negatively correlated with patient prognosis in TNBC. Jiang et al demonstrated that highly metastatic TNBC is associated with RAB1B (of the RAS oncogene family) suppression. This resulted in elevated TGF- β R1 expression which led to increased SMAD3 levels and metastasis. When correlated with TNBC patients it was found that patients with decreased RAB1B expression demonstrated reduced prognosis[34].

Additionally, it was found that patients with increased cytoplasmic TGF β 1 demonstrated a positive correlation with increased tumor grade, lymph infiltration, and diminished disease-free survival [35-37]. Using cBioportal and the TCGA PanCancer Atlas in our own analysis, we assessed 1082 breast cancer patients and grouped them into two categories based on TGF- β pathway gene expression (TGF- β high vs. low)[38-41]. We found that high TGF- β signaling was associated with diminished overall survival (Figure 1A, 16.8% mortality with a 122.83 median month survival in TGF- β high vs. 12.7% with a 140.28 median month survival in TGF- β low groups, * p <0.05). This database analysis supports other studies which demonstrate that TNBC is associated with increased TGF- β signaling contributing to a reduced patient prognosis; thereby, supporting the need for the advancement of therapeutic modulation of TGF- β [35,36,42].

Figure 1

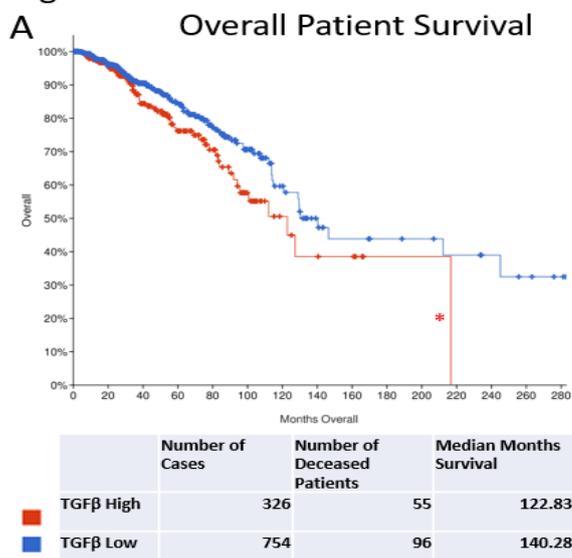


Figure 1: Database Analysis of TGF- β Gene Expression and Survival in Breast Cancer Patients (A) Kaplan–Meier curves for overall survival of the patients with high expression of TGF- β signaling in cancer samples (red curve) in comparison with patients with unaltered expression (TGF- β low, blue curve). n = 1082, *P = 0.0303, log-rank test. The

1.4 Clinical Importance of CSCs in TNBC

Breast cancer stem cells (CSCs) represent a small percentage of cells within tumors that exhibit stem cell-like properties, such as self-renewal, differentiation, and quiescence[43]. CSCs are at the apex of the cellular hierarchy within tumors, capable of maintaining CSC pools and giving rise to non-CSC bulk tumor cells to promote disease progression, resistance generation, and facilitate tumor metastasis[44-46].

In breast cancer, there are two major CSC populations which are characterized by CD44+/CD24- and ALDHhigh markers[47,48]. Al Hajj et al fractionated breast cancer cells using flow cytometry and then through serial dilution assays demonstrated that the CD44+/CD24- CSC population possesses an impressive 100-fold increased tumorigenicity upon mammary fat pad transplantation compared to unfractionated cells[49]. The CD44+/CD24- CSC population in breast cancer is associated with a mesenchymal phenotype, increased N-cadherin expression, decreased E-cadherin, and increased YAP, Twist, Snail, and Slug gene expression[47,50-52]. This population also demonstrates increased migration, resistance to conventional chemotherapeutics, increased reliance on glycolysis and quiescence[47,50].

The ALDH high CSC population is characterized by being able to form a tumor with as little as 1500 breast cancer cells [53,54]. In contrast to the mesenchymal CD44+/CD24-, ALDHhigh CSCs demonstrate an epithelial phenotype with high E-cadherin expression, low N-cadherin, vimentin, Slug, Wnt, Twist, and Snail expression[47,51,55]. ALDHhigh CSCs were found to be highly enriched for HIF-1 α signaling, angiogenic promotion and were highly proliferative[47]. Importantly, both epithelial and mesenchymal CSCs possess differential signaling enrichment/repression, can interconvert, exist on a gradient and work together to facilitate metastasis and secondary tumor formation[47,51,56].

Conventional therapy using anthocyanins, taxols, and other antimetabolite or antineoplastic agents, while effective against the bulk population are ineffective at targeting CSCs and even lead to the enrichment of CSCs in breast cancer patients and cell line models [51,57,58]. Thus methods to increase therapeutic efficacy and in the development of assessing CSC populations before and following treatment may present a useful clinical indicator of therapeutic efficacy.

Yu *et al* illustrated this using a dual-colorimetric RNA-in situ hybridization approach to assess cells for epithelial/mesenchymal gene expression that breast CSCs possessed epithelial, mesenchymal, and epithelial/mesenchymal hybrid signatures [59]. Pre- and post-chemotherapy analysis was performed (post-treatment with cisplatin, taxol, and adriamycin) on circulating tumor population numbers and CSC plasticity[59]. It was found that chemotherapy-responsive patients demonstrated decreased CSCs and a proportional decrease in mesenchymal in comparison to epithelial CSC populations. In patients with progressive disease, there were increased mesenchymal CSCs and increased multicellular CSC clusters which were also highly positive for mesenchymal markers thus demonstrating how non-specific chemotherapy can influence CSC plasticity and promote increased tumor cell dissemination[59].

Another report by Papadaki *et al* used ALDH1 (an epithelial marker) and Twist (a mesenchymal marker) to determine epithelial, mesenchymal, or epithelial/mesenchymal populations in the CSCs of 130 breast cancer patients[60]. It was found that hybrid epithelial/mesenchymal CSCs were associated with increased lung metastasis, increase patient relapse, and decreased progression-free survival (10.2 months vs. 13.5 months)[60]. Chemotherapy treatment increased hybrid epithelial/mesenchymal CSCs whereas the epithelial and mesenchymal CSCs was reduced [60]. These findings in combination with other reports advocate that chemotherapy treatment alters the plasticity and population dynamics of epithelial, mesenchymal, and epithelial/mesenchymal CSCs, decreases patient prognosis and increases the rates of metastasis/relapse[47,48,51,57,61].

Such findings highlight the magnitude of CSCs in patient outcome, the need for novel therapeutic treatment, and support further studies in investigating CSC enrichment as indicators for patient prognosis.

1.5 TGF- β as a Therapeutic Target to Inhibit TNBC and its CSC population

TGF- β has been demonstrated to be enriched alongside ALDH^{high} and CD44⁺/CD24⁻ (epithelial, and mesenchymal CSC markers) in chemotherapy-treated TNBC patients[62]. Upon direct administration of paclitaxel to TNBC cell lines, similar results were observed with an increase in tumorigenesis and mammosphere formation[62]. Importantly, it was found that the CSC enriching effects of paclitaxel chemotherapy were due to TGF- β mediated SMAD4 dependant expression of IL-8 and upon siRNA to inhibit SMAD4 or exposure to LY2157299 (a TGF- β type I receptor kinase inhibitor), tumorigenesis was rescued and epithelial, and mesenchymal CSC populations were inhibited. These findings were verified in vivo using mouse TNBC tumor models and it was found using serial dilution tumorigenesis assays that compared to the control (3/5 tumors formed at an injection concentration of 1×10^3 cells) paclitaxel treatment increased tumorigenesis (4/5 tumors formed at an injection concentration of 1×10^3 cells) while the combination of paclitaxel and LY2157299 was able to reduce tumorigenicity (2/5 tumors formed at an injection concentration of 1×10^3 cells)[62].

These results correlate with recent findings from Yadav et al where it was demonstrated in breast cancer cell lines that after treatment with radiotherapy, the surviving cells possessed increased proliferation and TGF- β 1, TGF- β 2 and TGF- β 3. Interestingly, these cells also demonstrated increased CSC markers (CD44⁺/CD24⁻/ALDH^{high}) and enhanced migration. Further treatment was met with resistance; however, treatment with TGF- β 1 inhibitors was able to rescue and re-sensitize cells to radiotherapy [63].

Epirubicin is another widely used anthracycline to treat TNBC. It has been shown to cause enrich CD44⁺/CD24⁻ CSCs and tumorigenicity of breast cancer following treatment[64]. A study by Xu et al transformed MDA-MB-231 TNBC cells (epirubicin-sensitive) into an epirubicin-resistant cell line (MB-231/Epi) through chronic exposure to epirubicin. Resistance was correlated with higher levels TGF- β expression, chemotherapy resistance and CD44⁺/CD24⁻ CSC enrichment. In addition to this, MB-231/Epi cells showed increased migration and invasion which indicated potentially enhanced metastatic potential. Thus this paper highlights the potential associated between TGF- β , chemoresistance and CSC enrichment leading to enhanced tumor progression and metastasis, highlighting the importance of targeting TGF- β in TNBC[65].

A study by Sun *et al* further looked into the associated between TGF- β , CSC enrichment and radioresistance. Sun et al demonstrated that following initial radiotherapy, breast cancer patients who demonstrated radioresistance and recurrence within 5 years of their initial therapy were found to have increased expression of ALG3 (Alpha-1,3- Mannosyltransferase)[66]. These findings were correlated with breast cancer cell lines where basal-like and HER-2+ breast cancer lines demonstrated increased levels of radioresistance and ALG3 expression. Moreover, upon the creation of an ALG3-overexpression model, previously radiosensitive breast cancer cell lines demonstrated radioresistance, and ALG3-overexpressing breast cancer cell lines, when injected subcutaneously into mice, displayed an increased tumor growth rate and OCT4 gene expression (a commonly used marker to assess CSC enrichment). Conversely, it was also demonstrated in the basal-like TNBC cell lines that upon ALG3 knockout models, previously radioresistance cell lines were sensitized, tumor growth in vivo was delayed and OCT4 expression was decreased. Further assessment of ALG3 modulation of CSCs in breast cancer demonstrated that ALG3 overexpressing cell lines also demonstrated increased NANOG, OCT4, and SOX2 expression (CSC associated genes) and increased tumorsphere formation capabilities. FACs analysis demonstrated increased CD44⁺/CD24⁻ CSCs in wild type ALG3 overexpressing breast cancer cell lines; however, this population was severely diminished upon ALG3 knockdown (control MDA MB-231 TNBC cells were 75.3% CD44⁺/CD24⁻ while ALG3 knockdown MDA MB-231 cells were only 42.1% CD44⁺/CD24⁻) highlighting that ALG3 may serve as a potential target to decrease radioresistance in breast cancer.[67] Mechanistic analysis through luciferase assay determined that ALG3 downregulation reduced the luciferase signal of SMAD-luc demonstrating TGF- β signal modulation via ALG3. Further assessment demonstrated that ALG3 expression promoted the glycosylation of TGF β R2 which mediated TGF β signaling. It has previously been demonstrated that glycosylation of TGF β R2 affects its ligand-binding sensitivity and reduced glycosylation of TGF β R2 leads to disrupted binding capacity with TGF β R1 which in turn reduced phosphorylation of smad2 and ultimately TGF β signaling [67,68].

Usage of Tunicamycin (a N-linked glycosylation inhibitor) demonstrated similar effects on TGF β R2 as the ALG3 knockdown cell lines. Finally, co-immunoprecipitation demonstrated an interaction between TGF β R1 and TGF β R2, as well as TGF β R1 and p-smad2 in ALG3 expressing breast cancer cell lines. This co-immunoprecipitation was not observed in ALG3 knockout cell lines. A TGF β R2 inhibitor (LY2109761) was then used to inhibit ALG2 overexpressing breast cancer

cell lines which induced apoptosis post-radiotherapy and diminished tumorsphere formation as well as CD44+/CD24-CSCs[66].

As indicated through the above studies, CSC enrichment and resistance post-chemotherapy and radiotherapy may be targeted through TGF- β inhibition. Thus, TGF- β signaling may provide a promising target for CSC inhibition in TNBC to be used in conjunction with conventional therapy. Other studies have produced similar findings using TGF- β inhibitors on breast cancer models in vitro and in vivo. Schech et al demonstrated the efficacy of entinostat (a class I HDAC inhibitor with TGF- β modulating properties) in inhibiting CD44+/CD24- CSCs in TNBC cell lines (from 63.1% to 3.66% in MDA MB-231 cells)[69,70]. Additionally immortalized non-cancerous breast cancer lines (MCF-10a and 184B5) cells were induced to form mammospheres and enrich their CSC population through TGF- β exposure. This effect was inhibited upon treatment with entinostat or LY2109761. Moreover, TNBC cells were inoculated into the fat pads of mice and lung metastasis was assessed after 3 weeks. Mice treated with entinostat demonstrated reduced tumor growth in vivo as well as reduced rates of lung metastasis.

For future investigations, active interventional clinical trials listed in Clinicaltrials.gov database for the treatment of patients with various cancers through TGF- β inhibition are summarized in Table 1. These potential TGF- β modulators/inhibitors seem to be safe for usage in the clinic and have been demonstrated to suppress the TGF- β signaling pathway in preclinical studies though their efficacy in the treatment for TNBC remains to be determined. We have also listed completed clinical trials for the treatment of breast cancer with TGF- β inhibitors for further investigation (Table 2). Future translational research to determine the clinical efficacy of TGF- β inhibitors in targeting TNBC CSCs and impeding tumorigenicity in combination with other inhibitors and chemotherapeutic drugs may lead to the development of a tangible therapy to improve patient prognosis.

Table 1: TGF- β Inhibitors in Active Cancer Clinical Trials. The Clinicaltrials.gov database was used to assess active, interventional clinical trials for cancer treatment within all phases of development. Clinical Trial Search link (accessed on August 1, 2021): https://clinicaltrials.gov/ct2/results?term=tgf&cond=Cancer&flds=abky&Search=Apply&recrs=f&recrs=d&age_v=&gndr=&type=&rslt=

Inhibitor	Clinical Trial Number	Mechanism of Action	Tumor Type
AVID200	NCT03834662	A receptor ectodomain trap that inhibits TGF-beta1 and TGF-beta3	Malignant Solid Tumor
Bintrafusp alfa (M7824)	NCT04246489 NCT04551950 NCT03833661	Bifunctional fusion protein with a ectodomain of TGFB-RII fused to human IgG1 blocking PD-L1	Uterine Cervical Neoplasms, biliary tract cancer, cholangiocarcinoma, gallbladder cancer
Fresolimumab	NCT02581787	A recombinant anti-TGB growth factor antibody against TGFB-1,2,3.	Stage IA-B NSCLC
Vactosertib (TEW-7197)	NCT03732274	Potent TGFB receptor ALK4/ALK5 inhibitor	Metastatic NSCLC
GT90001	NCT03893695	Fully human anti-ALK1 monoclonal antibody that inhibits TGFB and ALK-1	Metastatic HCC
Nimotuzumab	NCT00957086	Recombinant humanized murine immune antibody that blocks TGF and EGF	Squamous cell carcinoma of the head and neck
LY3200882	NCT02937272	An ATP competitive inhibitor of the serine-threonine kinase domain of TGFβRI	Solid Tumors
Galunisertib (LY2157299)	NCT02423343 NCT02906397 NCT02178358 NCT03206177	A small molecule inhibitor of the TGFβ receptor I kinase	Solid Tumor, NSCLC, HCC Recurrent, Lung Neoplasms, Esophageal Neoplasms, Stomach Neoplasms, Hepatocellular Carcinoma
Losartan	NCT01821729	angiotensin II receptor antagonist	Pancreatic Cancer
Vactosertib	NCT04103645	Selective ALK5 Inhibitor	Myeloproliferative Neoplasm
M7824	NCT03833661	A fusion protein inhibitor comprised of human TGFβRII fused to the extracellular domain of human linked to the C-terminus of human anti-PD-L1 heavy chain	Biliary Tract Cancer, Cholangiocarcinoma, Gallbladder Cancer

Table 2: TGF- β Inhibitors in Completed Breast Cancer Clinical Trials. The Clinicaltrials.gov database was used to assess completed, interventional clinical trials for breast cancer/neoplasm treatment within all phases of development. Clinical Trial Search link (accessed on August 1, 2021):https://clinicaltrials.gov/ct2/results?cond=Breast+Cancer&term=tgf&type=Intr&rslt=&recrs=e&age_v=&gndr=&intr=&titles=&outc=&spons=&lead=&id=&cntry=&state=&city=&dist=&locn=&rsub=&strd_s=&strd_e=&prcd_s=&prcd_e=&sfpd_s=&sfpd_e=&rfpd_s=&rfpd_e=&lupd_s=&lupd_e=&sort=

Inhibitor	Clinical Trial Number	Mechanism of Action	Tumor type
Imiquimod[71]	NCT00821964	Inducer of interferon-gamma, known to inhibit TGF- β	Male breast cancer, Recurrent breast cancer, skin metastases, stage IV breast cancer
Fenretinide	NCT00001378	Retinoid inducing dimerization of retinoid acid receptors which has been shown to regulate affect multiple signal transduction pathways, including IGF, TGF- β , and AP-1[72]	Breast cancer and neoplasms
Fresolimumab[52]	NCT01401062	a human anti- (TGF- β) monoclonal antibody	Metastatic breast cancer
Bevacizumab	NCT01959490	humanized monoclonal antibody that targets VEGF-A and has demonstrated inhibited TGF- β following treatment [73]	Breast cancer stages II-III C
Galunisertib (LY2157299)	NCT02423343 NCT02178358 NCT02734160 NCT01246986	A small molecule inhibitor of the TGF β receptor I kinase	Solid Tumor, NSCLC, HCC Recurrent
Vactosertib (TEW-7197)	NCT02160106	Potent TGFB receptor ALK4/ALK5 inhibitor	Advanced stage solid tumors
NIS793	NCT02947165	mAb that binds to human TGFB and prevents of activation of downstream signaling	Breast, lung, hepatocellular, colorectal, pancreatic and renal cancer

1.6 Conclusion

For the development of effective therapeutic approaches, future preclinical research must consider targeting both epithelial and mesenchymal CSCs and assess how experimental treatments affect these populations using clinically translatable models. While tumor shrinkage models demonstrate time point efficacy of therapy, CSC composition assessment must be preformed to determine whether the investigated therapy reduces or enriches for CSC populations within the tumor to determine long-term clinical efficacy. To that end we advocate for serial dilution assessments and FACS assessment post therapy to determine tumor population assessment and functional tumorigenicity post therapy. Furthermore, we endorse multiple rounds of serial dilutions/ treatment and CSC assessment may be preformed to mimic long term survival and effects on tumorigenicity with multiple rounds of therapy which would provide substantial evidence into the long term clinical efficacy and patient prognosis.

In regards to TNBC treatment, there currently exists no specific therapy. Given the preclinical and clinical evidence of TGF- β inhibitors, future studies using known and novel regulators of the TGF- β pathway may lead to a clinically translatable breakthrough therapy.

2.0 Materials and Methods

Breast cancer datasets from the Cancer Genome Atlas PanCancer Atlas (TCGA, <https://www.cell.com/pb-assets/consortium/pancanceratlas/pancani3/index.html>)[41] were used and analyzed with cBioportal (<http://www.cbioportal.org/index.do>). High TGF- β gene expression was defined based on the following gene set available at cBioportal consisting of 30 genes associated with the TGF- β superfamily with the following genes each having an mRNA expression greater than 3 standard deviations above the mean: *TGFB1 TGFB2 TGFB3 TGFB1 TGFB2 TGFB3 BMP2 BMP3 BMP4 BMP5 BMP6 BMP10 BMP15 BMP2 ACVR1 ACVR1B ACVR1C ACVR2A ACVR2B ACVRL1 SMAD2 SMAD3 SMAD1 SMAD5 SMAD4 SMAD9 SMAD6 SMAD7 BMPR1A and BMPR1B*. Expression data, correlation data, mutational frequency, breast cancer subtype analysis and Kaplan–Meier survival curves were generated using the datasets compiled by June 2020 from the following database IDs: <https://bit.ly/2MVN0KN3>.

Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used “Conceptualization, A.S. and S.M.; methodology, A.S.; writing—original draft preparation, A.S, S.M, R.K, S.C, V.V.; writing—review and editing, A.S, S.M, R.K, S.C, V.V.; visualization, A.S and S.M.; supervision, A.S. All authors have read and agreed to the published version of the manuscript.” Please turn to the CRediT taxonomy for the term explanation. Authorship must be limited to those who have contributed substantially to the work reported.

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