Blocking Fibrotic Signaling In Fibroblasts from Patients with Carpal Tunnel Syndrome

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Abstract

Fibrosis of the subsynovial connective tissue (SSCT) in carpal tunnel syndrome (CTS) patients is increasingly recognized as an important aspect of CTS pathophysiology. In this study, we evaluated the effect of blocking profibrotic pathways in fibroblasts from the SSCT in CTS patients. Fibroblasts were stimulated with transforming growth factor β1 (TGF-β1), and then treated either with a specific fibrosis pathway inhibitor targeting TGF-β receptor type 1 (TβRI), platelet-derived growth factor receptor (PDGFR), epidermal growth factor receptor (EGFR) or vascular endothelial growth factor receptor (VEGFR). Fibrosis array and quantitative real-time polymerase chain reaction of fibrotic genes were evaluated. Array gene expression analysis revealed significant down-regulation of multiple fibrotic genes after treatment with TβRI, PDGFR and VEGFR inhibitors. No array fibrotic genes were significantly down-regulated with EGFR inhibition. Further gene expression analysis of known CTS fibrosis markers collagen type I A2 (Col1), collagen type III A1 (Col3), connective tissue growth factor (CTGF) and SERPINE1 showed significantly down-regulation after TβRI inhibition. In contrast, VEGFR inhibition significantly down-regulated CTGF and SERPINE1, whereas PDGFR and EGFR inhibition significantly down-regulated Col3. Taken together the inhibition of TβRI appears to be the primary mediator of fibrotic gene expression in fibroblasts from CTS patients. TGF-β/Smad activity was further evaluated, and as expected inhibition of Smad activity was significantly down-regulated after inhibition of TβRI, but not with PDGFR, VEGFR or EGFR inhibition. These results indicate that local therapies specifically targeting TGF-β signaling alone or in combination offer the potential of a novel local antifibrosis therapy for patients with CTS.

Keywords

Carpal tunnel syndrome; Subsynovial connective tissue; transforming growth factor β; Fibrosis

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Introduction

Carpal tunnel syndrome (CTS) is the most common compression neuropathy (de Krom et al., 1992; Stevens et al., 1988). Non-inflammatory fibrosis of the subsynovial connective tissue (SSCT) is a characteristic of idiopathic CTS, with some studies suggesting that the fibrosis may be the cause, rather than a consequence of the nerve compression (Ettema et al., 2004; Freeland et al., 2002). Several studies have reported that the up-regulation of transforming growth factor β1 (TGF-β1) signaling and other fibrosis-related cytokines plays an important role in the SSCT fibrosis associated with idiopathic CTS (Chikenji et al., 2014; Gingery et al., 2014).

TGF-β1 regulates the expression of many extracellular matrix proteins, such as collagen, fibronectin, and elastin, as well as down-regulating matrix metalloproteases (Branton and Kopp, 1999; Mogyorosi and Ziyadeh, 1998). Thus, TGF-β1 stimulation leads to a vicious cycle of fibrosis which may compromise normal organ function (Kagami et al., 1996; Leask and Abraham, 2004). Previous reports have also noted that TGF-β1 affects the expression of other profibrotic cytokine receptors, such as the platelet-derived growth factor receptor (PDGFR), the epidermal growth factor receptor (EGFR), and the vascular endothelial growth factor receptor (VEGFR) (Deharvengt et al., 2012; Ferrari et al., 2006; Trojanowska, 2008). However, the effect of inhibition of these cytokines on the metabolism of fibroblasts derived from the SSCT of patients with CTS is unknown.

This study tested the hypothesis that inhibition of profibrotic cytokine receptors would reduce the expression of profibrotic genes in fibroblasts derived from the SSCT of CTS patients, using target specific inhibitors of TGF-β receptor type1 (TβRI) (SD208, an ATP-competitive TβRI inhibitor), PDGFR (AG1296, a protein kinase inhibitor of PDGFR), EGFR (Lapatinib, a small molecule kinase inhibitor of EGFR1 and 2 (Her2)) and VEGFR (Axitinib, a small molecule tyrosine kinase inhibitor of VEGFR). In addition, since canonical TGF-β/Smad activation is an important mediator of fibrosis in CTS we also evaluated the effect of inhibition on Smad reporter activity.

Materials and Methods

This study was approved by our Institutional Review Board. SSCT was harvested from patients with CTS at the time of carpal tunnel release. The inclusion criteria for patients with CTS included a clinical diagnosis of idiopathic CTS, confirmatory electrodiagnostic studies performed in conformance with the American Association of Neuromuscular and Electrodiagnostic Medicine guidelines (Stevens, 1997) and a lack of response to nonsurgical treatment. Patients with a history of previous volar wrist surgery, hand/wrist tumor or deformity, cervical radiculopathy, inflammatory arthritis, wrist osteoarthritis, flexor tendinitis, hemodialysis, morbid obesity (body mass index > 40 kg/m^2), sarcoidosis, peripheral nerve disease, metabolic disorders such as diabetes mellitus or thyroid disease, amyloidosis, or major trauma to the ipsilateral wrist were excluded. This study included 5 patients (2 males, 3 females; mean age 63 years; range 59 to 65 years) who had undergone carpal tunnel release surgery.

Cell culture

Primary SSCT fibroblasts were derived from harvested CTS patient SSCT tissue. SSCT was minced and cultured at 37°C in minimal essential medium with Earle's salts supplemented with 10 % fetal bovine serum and 1 % antibiotic-antimycotic. Media were changed every 3 days and adherent cells were passaged after reaching 70% confluence. We previously reported that cultured SSCT fibroblasts maintained phenotypic gene expression at least until passage 5 (Gingery et al., 2014). Therefore, we used passage 4 or 5 cells in this study. Cells were seeded at 5×10^5 fibroblast cells per well in 6 well-plates and cultured overnight. In order to determine the role of TGF-β, PDGF, EGF and VEGF signaling plays a role in CTS fibrosis, we used chemical inhibitors that targeted receptor signaling in primary fibroblasts from CTS patients. SD208 (IC50: 49 nM) (Bio-Techne, Devens, MA, 3269) is an ATP-competitive TβRI inhibitor (Uhl et al., 2004). AG1296 (IC50: 0.3–0.5 μM) (Selleck Chemical, Houston, TX, S8024) is a protein kinase inhibitor that is specific for PDGFR (Kovalenko et al., 1997). Lapatinib (IC50: 10.8 nM) (Selleck Chemical, Houston, TX, S1028) is a small molecule kinase inhibitor that targets both EGFR1 and 2 (Her2) (Moy et al., 2007). Axitinib (IC50: 0.1–0.3 nM) (Selleck Chemical, Houston, TX, S1005) is a small molecule tyrosine kinase inhibitor that primarily targets VEGFR and with some to a lesser extent PDGFR and c-kit (Keating, 2015). Given that TGF-β1 is a known mediator of CTS (Chikenji et al., 2014; Gingery et al., 2014) inhibitor treatments were performed in the presence of 5 ng/mL TGF-β1 (R&D Systems, Inc., Minneapolis, MN, 240-B-010). Six experimental groups were used to evaluate the effect on fibrotic gene signaling; vehicle control (Veh), Veh with TGF-β1 (TGF-β1), SD208 with TGF-β1 (SD208), AG1296 with TGF-β1 (AG1296), Lapatinib with TGF-β1 (Lapatinib) and Axitinib with TGF-β1 (Axitinib). All cell cultures other than the Veh only groups were pretreated with an inhibitor (SD208, 1 μM; AG1296, 20 μM; Lapatinib, 2 μM; or Axitinib, 1 μM) for 60 minutes prior to experiments, and then cultured with TGF-β1 for 24 hours.

Fibrosis Array and Quantitative real-time polymerase chain reaction

Total RNA was isolated using Trizol reagent (Invitrogen, Grand Island, NY, 15596026). RNA was quantitated using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Equal amounts of total RNA were used to synthesize cDNA, using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, 170-8891) as previously reported (Gingery et al., 2014).

Gene expression analysis was completed using Human Fibrosis polymerase chain reaction (PCR) arrays (SA Biosciences, Frederick, MD, PAHS-120Z) according to the manufacturer's protocol. The geometric mean of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and ribosomal protein lateral stalk subunit P0 (RPLP0) served as an internal control. Fold changes were calculated on a log 2 scale, comparing treatment groups.

Additionally, genes whose expression were up-regulated in CTS patient SSCT fibroblasts compared to control normal human SSCT fibroblasts; collagen type I A2 (Col1), collagen type III A1 (Col3) and connective tissue growth factor (CTGF) (Gingery et al., 2014), were analyzed using quantitative real-time PCR (qRT-PCR). SERPINE1, a TGF-β responsive gene and a genes highly regulated in our arrays were also analyzed (Kutz et al., 2001).

GAPDH served as a housekeeping gene for the qRT-PCR studies. C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) was used for the quantification and analysis. All PCR primers were designed using Primer3 software (http://frodo.wi.mit.edu/primer3/) and were purchased from Integrated DNA Technologies (Coralville, IA). The probe sequences are listed in Table 1.

Smad Luciferase Reporter Assay

SSCT fibroblasts were stably transfected with a reporter plasmid containing the Smad binding element. The Smad binding element comprises a CAGACA motif that serves as a direct binding site for Smad proteins using the Cignal Reporter Assay (Qiagen, Valencia, CA, CCS-017L) as the Smad binding element. Cells were plated at 1.3×10^5 fibroblast cells per well and the following day cells were transfected with pathway reporters using FuGENE 6 transfection reagent (Promega Corporation, Madison, WI, E2691) according to the manufacturer's protocols. After 24 hours culture, the transfected cells were pretreated with each inhibitor for 60 minutes and then treated with ± TGF-β (5ng/mL) as noted. After 24 hours cells were lysed with passive lysis buffer (Promega Corporation, Madison, WI, E1941) and cell lysates luciferase activity was measured upon addition of luciferase assay reagent (Promega Corporation, Madison, WI, E1483) using the Dual-Luciferase® Reporter Assay System (Promega Corporation, Madison, WI, E1910) as previously reported (Gingery et al., 2014). Relative units of luciferase activity were reported after subtracting the basal expression levels observed in the Veh group.

Statistical analysis

The gene expression of the fibrosis array result was normalized to housekeeping genes as determined by SA Biosciences RT^2 Profiler™ PCR Array Data Analysis software (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php). Statistical analysis for qRT-PCR gene expression experiments and Smad luciferase reporter assay was analyzed by an unpaired Student's t-test. All measurements were expressed as mean values and standard error. The level of statistical significance was set at $p < 0.05$ or $p < 0.01$ (where noted). The statistical analysis was performed using Statistical Package for the Social Sciences 21.0 for Windows (SPSS Inc., Chicago, IL).

Results

Fibrosis array - regulation of fibrotic genes upon cytokine receptor inhibition

Multiple fibrotic genes were significantly ($p < 0.05$) up-regulated in SSCT cells treated with TGF-β1 as compared to Veh, including SERPINS, CTGF, VEGFA, PDGFA, TGF-β1 and TβRI. In addition, multiple genes were significantly ($p < 0.05$) down-regulated greater than 2-fold including integrins, TGF-β2, Smad6 and Smad3 (Fig. 1A, Suppl. Table 1). Upon inhibition of TβRI kinase activity with SD208 there were significant ($p < 0.05$) changes including down-regulation of SERPINE1, CTGF, VEGFA, PDGFA, TGF-β1, TβRI (Fig. 1B, Suppl. Table 2). Given that both PDGFA and VEGFA were significantly ($p < 0.05$) regulated by TGF-β1 and by inhibition of TβRI, and that EGFR has been shown to be an important fibrotic signal mediator (Liu et al., 2015; Peng et al., 2016; Wang et al., 2016) we explored whether inhibiting these cytokines would down-regulate pro-fibrotic genes. In both

cases, we found that the number of fibrotic genes that were regulated by blocking PDGFR and VEGFR tyrosine kinase activity impacted fewer fibrotic target genes than TβRI inhibition. PDGFR inhibition resulted in down-regulation of TGF-β3, PLAU and CCL2 expression and increased IL1A expression (Fig. 2A, Suppl. Table 3). VEGFR inhibition resulted in down-regulation of multiple profibrotic genes including CTGF, SERPINE1, ACTB, STAT1, and TIMP3 (Fig. 2B, Suppl. Table 4). However, EGFR inhibitor had no significant effect on gene expression (Suppl. Table 5).

Fibrotic expression of known SSCT CTS fibrotic markers

Inhibition with the TβRI inhibitor SD208, significantly down-regulated Col1 expression ($p < 0.01$), whereas all other receptor inhibitors did not regulate this gene (Fig. 3A). Col3 regulation was significantly decreased with TβRI ($p < 0.01$), PDGFR and EGFR ($p < 0.05$) inhibition; however VEGFR inhibition did not significantly regulate this gene expression (Fig. 3B). CTGF, another important marker of CTS fibrosis, was significantly down-regulated by TβRI ($p < 0.01$) and VEGFR ($p < 0.05$) inhibition; however inhibition of PDGFR and EGFR had no impact on CTGF expression (Fig. 3C). Finally, we evaluated SERPINE1 expression with inhibition and found, just as in the fibrosis arrays, that only TβRI and VEGFR significantly ($p < 0.01$) inhibited SERPINE1 expression (Fig. 3D).

Smad Luciferase Reporter Assay

Smad reporter activity was significantly ($p < 0.01$) up-regulated in TGF-β1 treated cells; however inhibition with TβRI inhibitor significantly ($p < 0.01$) decreased reporter activity. Inhibitors to PDGFR, EGFR and VEGFR did not result in changes of Smad reporter activity (Fig. 4).

Discussion

TGF-β is a central mediator in fibrosis in many different organs, such as lung, liver and kidney (Kato et al., 2004; Krein and Winston, 2002; Meng et al., 2015; Pohlers et al., 2009). The effect of TGF-β on the cell is mediated by canonical TGF-β/Smad signaling as well as non-canonical pathways including mitogen-activated protein kinase (MAPK) pathways, Rho-like GTPase signaling pathways, and phosphatidylinositol- 3-kinase (PI3K) /Akt pathways (Rockey et al., 2015; Zhang, 2009). Compared to normal SSCT fibroblasts, iSSCT fibroblasts from CTS patients have significantly increased TGF-β1 and CTGF protein levels and show significantly up-regulated fibrosis related genes (Chikenji et al., 2014; Gingery et al., 2014). Further the patients have increased collagen deposition, hypervascularity, decreased permeability and increased stiffness (Ettema et al., 2004; Werthel et al., 2014). In this study, we sought to assess the effect of inhibiting various components of fibrotic signaling on the expression of profibrotic genes. The chemical inhibitors targeted TβRI (SD208), PDGFR (AG1296), EGFR (Lapatinib), and VEGFR (Axitinib) were used. Inhibitor concentrations were selected based on effective doses in cell culture experiments from previous reports (Andrianifahanana et al., 2013; Baroni et al., 2006; Gingery et al., 2014; Hu-Lowe et al., 2008; Nahta et al., 2007; Uhl et al., 2004).

The fibrosis arrays showed that TGF-β1 activated the expression of several genes, including SERPINE1 and growth factors such as CTGF, VEGFA and PDGFA. SD208 acts by inhibiting the TβRI kinase (ALK5) (Nasim et al., 2012). We and others have reported that the inhibition of TβRI by SD208 can down-regulate the expression of fibrotic markers in fibrotic diseases (Akhurst and Hata, 2012; Chen et al., 2006; Gingery et al., 2014). SSCT markers of fibrosis include Col1, 3 and CTGF (Chikenji et al., 2014; Gingery et al., 2014). Our gene expression results confirm this finding, showing significant up-regulation of Col1, 3 and CTGF and SERPINE1 expression. This up-regulation of genes associated with fibrosis is significantly down-regulated by SD208 treatment, indicating that TGF-β signaling is an important regulator of CTS fibrosis. Consistent with this was the significant down-regulation of many profibrotic genes by treatment with SD208 in the fibrosis array.

PDGFA and B chain dimeric isoforms (PDGF-AA, -AB, and -BB) also play important roles in the pathogenesis of fibrosis. These isoforms bind PDGFR α and β, respectively, and stimulate the expression of collagen (Bonner, 2004). PDGF signaling involves multiple pathways including the MAPK pathway, protein kinase C and calcium, c-jun n-terminal kinase, PI3K and the signal transducers and activators of transcription (STAT) pathway (Demoulin and Essaghir, 2014). AG1296 can reduce pulmonary fibrosis in rats by acting as a selective inhibitor of autophosphorylation of PDGFR α and β (Baroni et al., 2006; Kovalenko et al., 1997; Rice et al., 1999). In this study, the PDGFR inhibitor, AG1296, had limited effects on regulating fibrotic genes down-regulated the expression of Col3, however had limited effects on regulating other fibrotic genes. This data suggests that PDGFR may not be a significant target for the treatment of fibrosis that is seen in patients with CTS.

EGF/EGFR signaling has been associated with several types of human organ fibrosis including renal fibrosis, pulmonary fibrosis, and liver fibrosis (Fuchs et al., 2014; Vallath et al., 2014; Zhuang and Liu, 2014). EGF is a protein with 53 amino acid polypeptide and three intramolecular disulfide bonds, with a molecular weight of 6045-Da (Harris et al., 2003). EGFR (also known as human epidermal growth factor receptor (HER) 1 or erbB-1) is a tyrosine kinase receptor that is frequently expressed on the cell surface and is activated by binding of its specific ligands, including EGF and TGF-α (Scaltriti and Baselga, 2006). Depending on the ligand-receptor combination, multiple downstream signaling pathways such as MAPK/Extracellular signal regulated kinase (ERK), PI3K/Akt and Janus kinase / STAT can be activated (Holbro and Hynes, 2004). Lapatinib (originally known as GW572016) is a small molecule that inhibits the intracellular tyrosine kinase domains of EGFR and HER2 (Moy et al., 2007), and an effect of Lapatinib on fibrotic disease has been demonstrated (Andrianifahanana et al., 2013; Beyer et al., 2010; Cho et al., 2009). In this study, Lapatinib was found to significantly suppress one gene Col3. Taken together EGFR inhibition appears to have a limited response blocking profibrotic gene expression in CTS fibroblasts.

VEGF, a crucial regulator of blood-vessel formation in adults is also involved in fibrotic signaling (Olsson et al., 2006). VEGF signaling stimulates cellular responses through activation of multiple signaling pathways, such as the MAPK/ ERK and PI3K/Akt signaling pathways (Ferrara et al., 2003). Some studies suggested a fibrogenic effect of VEGF through other mechanisms as well, including promotion of inflammation, release of fibrosis-

enhancing molecules from VEGF-activated endothelial cells, and direct effects of VEGF on hematopoietic stem cells (Sahin et al., 2012; Yoshiji et al., 2003). These studies suggest that VEGF inhibition might also have beneficial effects on fibrosis resolution. Axitinib is a potent, selective inhibitor of VEGFR1, 2, 3, PDGFR-β, and c-kit (Keating, 2015). One report describes a beneficial effect of Axitinib on pulmonary fibrosis (Hillman et al., 2014). In our study Axitinib suppressed the expression of CTGF and SERPINE1, which are downstream of TGF-β1 signaling. However, Smad signaling was not suppressed by Axitinib. This result suggests that the suppression of CTGF and SERPINE1 by Axitinib involves a different mechanism, separate from canonical TGF-β/Smad signaling, and that therefore VEGFR targeted treatment may provide an additional effect beyond that of a TGF-β1 inhibitor such as SD208.

Smad reporter activity was significantly down-regulated by inhibition of TβRI signaling via SD208, no other receptor inhibitors blocked Smad activity. These results suggest that SD208 targeting of TβRI blocks TGF-β/Smad signaling, and suppresses not only the expression of growth factors but also several profibrotic genes.

The strength of this study is that we evaluated receptor level fibrosis inhibition in cells derived from patients with CTS on expression candidate fibrotic cytokine/receptor genes. As such, this study may be useful in helping to identify candidate therapeutic targets both alone and in combination for the treatment of fibrosis in CTS patients. The principal weakness of this study is *in vitro* analysis and not an exploration at the tissue or organism level. Additionally, this study was limited to gene expression changes and future work will need to confirm these effects in vivo. Finally, patients with CTS tend to be aged 50 and above (Bland and Rudolfer, 2003), and so the role of cellular senescence in these results should be investigated. Indeed, we plan this in future studies.

In conclusion, we believe that these results confirm the key role of TGF-β signaling in the fibrosis associated with CTS, and that further studies into the effect of TGF-β inhibition, alone or in combination with each other targeted inhibitors, are warranted. Such studies might offer, for the first time, a mechanism-specific targeted drug therapy for CTS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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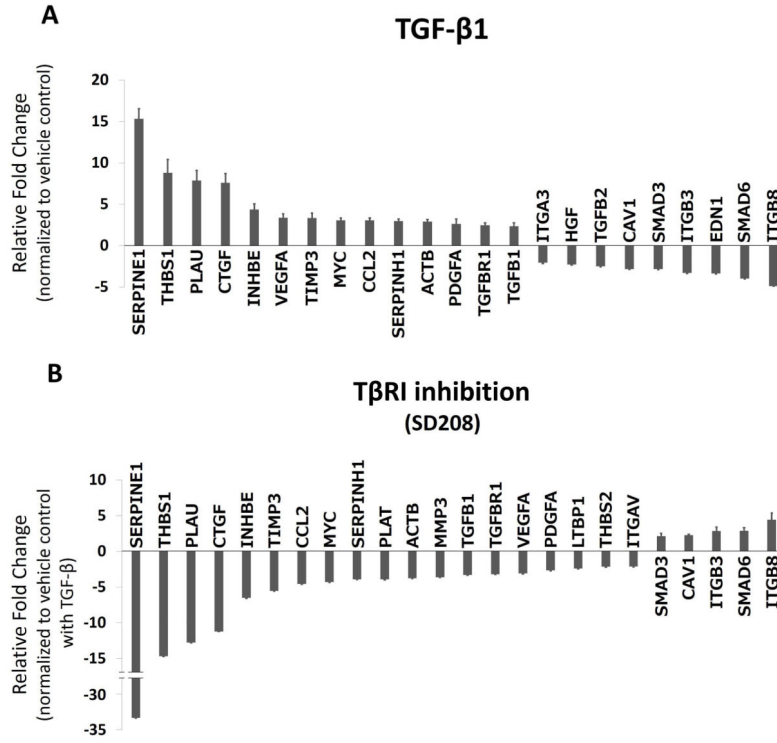
Figure 1.
Fibrotic array gene expression in fibroblasts derived from the SSCT in CTS patients treated with TGF-β1 and TβRI inhibitor (SD208). Significant (p < 0.05) 2-fold changes of fibrotic gene expression of TGF-β1 compared to vehicle control (Fig. A) and TGF-β1 + SD208 compared to vehicle control with TGF-β1 (Fig. B) in CTS fibroblast after 24 hours of treatment. All data are significant (p < 0.05) and reported as regulated 2-fold relative change mean ± SE. n=5.
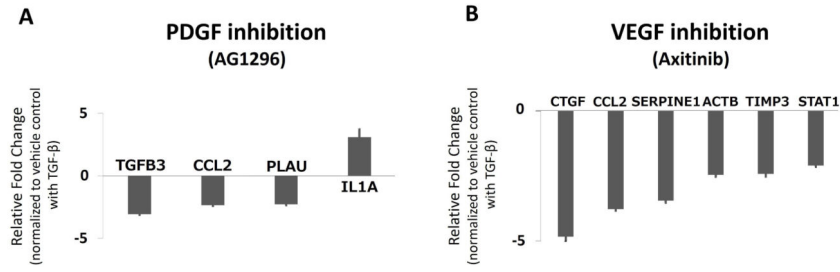
Figure 2.
Fibrotic array gene expression in fibroblasts derived from the SSCT in CTS patients treated with PDGFR inhibitor (AG1296) and VEGRF inhibitor (Axitinib). Significant (p < 0.05) 2-fold changes of fibrotic gene expression of TGF-β1 + AG1296 (Fig. A) and TGF-β1 + Axitinib (Fig. B) in CTS fibroblast compared to vehicle control with TGF-β1. All data are reported as up-regulated more than 2-fold or down-regulated less than 0.5-fold significant regulation, mean ± SE. n=5.
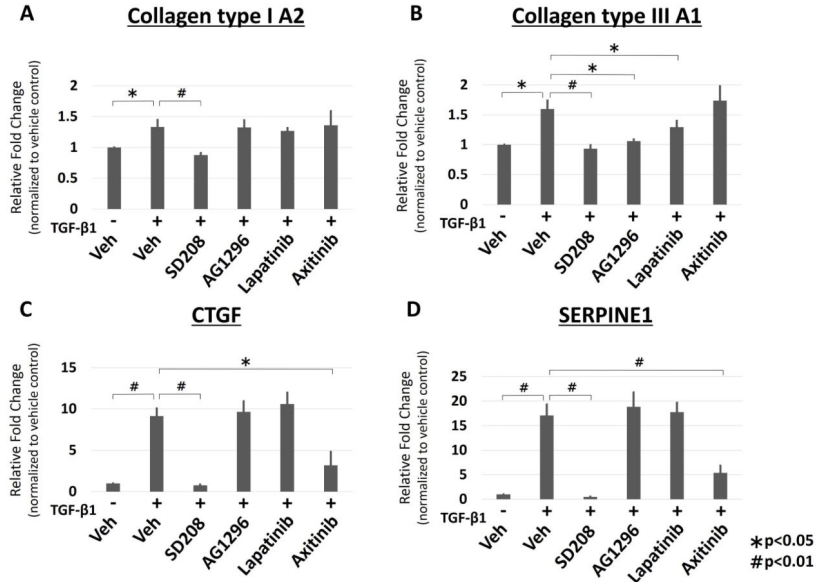
Figure 3.
Gene expression analysis using qRT-PCR of known CTS fibrosis marker genes Col1, 3, CTGF, SERPINE1 upon cytokine receptor level inhibition. CTS fibroblasts were treated with TGF-β1 and TβRI (SD208), PDGFR (AG1296), EGFR (Lapatinib) and VEGFR (Axitinib) inhibitor for 24 hours. Gene expression is normalized to vehicle control. All data are expressed as mean ± SE. n=5. (* indicates p < 0.05, # indicates p < 0.01).
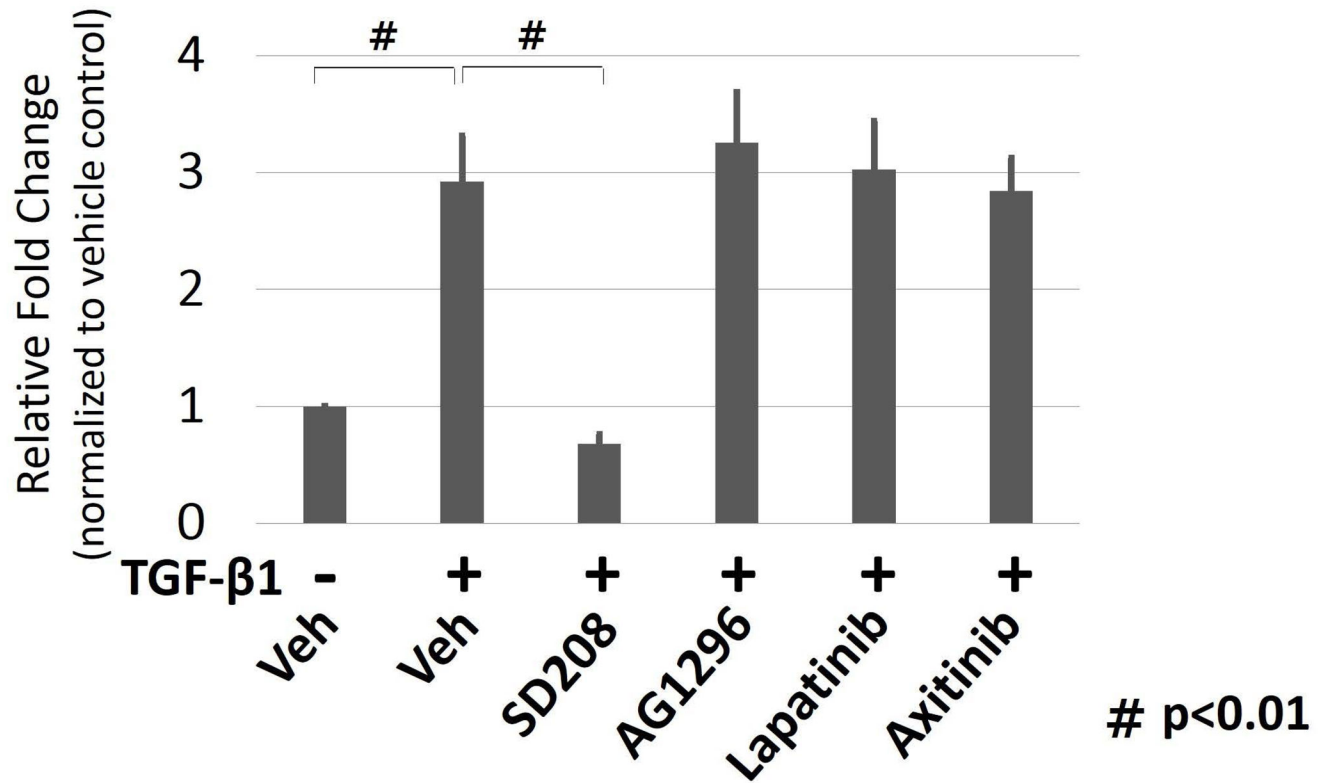
Smad Reporter Activity

Figure 4.
Smad reporter activity comparing cytokine receptor inhibition. Comparison of CTS fibroblasts treated with TGF-β1 and TβRI (SD208), PDGFR (AG1296), EGFR (Lapatinib) and VEGFR (Axitinib) inhibitor for 24 hours. Data are expressed as relative fold change from vehicle control. All data are expressed as mean ± SE. n=3. (# indicates $p < 0.01$).

Table 1

Primer sequences used for qRT-PCR

Gene	Forward	Reverse	Accession Number
Col1	ttgaccctaaccaaggatgc	cagttcttggctgggatgtt	NM_000089
Col3	gatcaggccagtggaaatgt	gtgtgtttcgtgcaaccatc	NM_000090
CTGF	tcccacccaattcaaaacat	tgctcctaaagccacacctt	NM_001901
SERPINE1	ctctctctgccctcaccaac	gtggagaggctcttggtctg	NM_000602
GAPDH	cagcctcaagatcatcagca	tgtggtcatgagtccttcca	NM_001256799