Inactivation of cancer-associated-fibroblasts (CAF) disrupts oncogenic signaling in pancreatic cancer cells and promotes its regression

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Conflict of Interest
University of Minnesota has a patent for Minnelide (WO/2010/129918/Triptolide Prodrugs), which has been licensed to Minneamrita Therapeutics, LLC. AKS and SMV are inventors on this patent. AKS is the co-founder and the Chief Scientific Officer of this company. Dr. Banerjee is a compensated consultant with Minneamrita Therapeutics LLC and this relationship is managed by University of Miami.

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Abstract (250 words)

Resident fibroblasts that contact tumor epithelial cells (TEC) can become irreversibly activated as cancer-associated-fibroblasts (CAF) which stimulate oncogenic signaling in TEC. In this study, we evaluated the crosstalk between CAF and TEC isolated from tumors generated in a mouse model of KRAS/mutp53-induced pancreatic cancer (KPC mice). Transcriptomic profiling conducted after treatment with the anticancer compound Minnelide revealed deregulation of the TGF-β signaling pathway in CAF, resulting in an apparent reversal of their activated state to a quiescent, non-proliferative state. TEC exposed to media conditioned by drug-treated CAF exhibited a decrease in oncogenic signaling as manifested by downregulation of the transcription factor Sp1. This inhibition was rescued by treating TEC with TGF-β. Given promising early clinical studies with Minnelide, our findings suggest that approaches to inactivate CAF and prevent tumor-stroma crosstalk may offer a viable strategy to treat pancreatic cancer.

Precis

In an established mouse model of pancreatic cancer, administration of the promising experimental drug Minnelide was found to actively deplete reactive stromal fibroblasts and trigger tumor regression, with implications for stromal-based strategies to attack this disease.
Introduction

Pancreatic cancer is one of the most devastating cancers with a dismal 5-year survival rate of less than 6% (www.cancer.gov)\(^1\). Recent studies suggest that the dense desmoplastic stroma, consisting of intense fibrosis, increased production of extracellular matrix (ECM), and proliferation of myofibroblast-like cells\(^2,3\), contributes to the aggressiveness and chemotherapeutic resistance, thereby leading to poor survival. This fibro-inflammatory stroma, besides demonstrating multiple pro-cancerous features, contributes to an increase in tumor interstitial fluid pressure thus inhibiting delivery of anticancer therapies to the tumor cells\(^4-6\). However, stroma-targeted therapies have not been beneficial in terms of survival or prognosis\(^7\). An ideal therapeutic regimen for PDAC would thus be a combination of anti-tumorigenic drugs that are minimally toxic, and anti-stromal to deplete the dense stroma and/or disrupt its crosstalk with the tumor cells\(^8,9\).

The PDAC stroma consists of activated fibroblasts, also known as cancer associated fibroblasts (CAFs), immune cells, vasculature, and an abundance of ECM proteins\(^10\). This reactive milieu of cells modulates both tumor epithelial cells (TECs) and its microenvironment to promote tumor progression. CAFs secrete chemokines and cytokines that are pro-tumorigenic and help the tumor proliferate and metastasize. Whilst stromal cells do not exhibit the genetic transformations seen in malignant pancreatic cancer cells, they are altered by cytokines, and growth factors secreted by inflammatory cells and tumor cells\(^11,12\). In the initial phases of tumor development, stroma production is stimulated by cancer-cell derived growth factors including transforming growth factor-\(\beta\) (TGF-\(\beta\)), hepatocyte growth factor (HGF), fibroblast growth factor (FGF), and epidermal growth factor (EGF)\(^13\). These tumor-derived factors and immune cell derived factors activate the quiescent stellate cells in the pancreas and make them myofibroblast-like. During these transformations under the influence of the tumor, the activated stellate cells further transform to cancer-associated fibroblasts. In their activated state, CAFs
secrete ECM components, primarily type I and III collagen, fibronectin, and proteoglycans. Till date it has not been elucidated if CAFs can revert to normal quiescent fibroblasts(14,15).

Studies in our laboratory show that triptolide, a diterpene triepoxide from the Chinese plant *Tripterygium wilfordii*, induces cell death in pancreatic cancer cells, and is effective in reducing tumor growth and locoregional spread in several complementary models of pancreatic cancer(16). For ease of clinical application, we have developed a water-soluble prodrug, Minnelide for this compound (17-22). Mechanistically, we have demonstrated that Minnelide downregulates HSP70 via inhibition of the activity of the transcription factor Sp1, thereby leading to pancreatic cancer cell death(23,24). Our recent publications showed that in addition to being effective against the epithelial pancreatic cancer cells, Minnelide also depletes the stroma by preventing the synthesis of hyaluronan and collagen stabilization. Furthermore, treatment with Minnelide reduces the viability of CAFs isolated from the pancreatic tumors(6).

Our pre-clinical studies show that at a dose of 0.4 mg/kg, Minnelide is an effective cytotoxic compound that targets multiple pathways in a tumor cell. At this dose, Minnelide eliminates stromal cells and decreases ECM components like collagen and HA, thereby relieving the pressure on blood vessels allowing them to be functional, which results in better drug delivery(6). Minnelide has just completed the Phase I clinical trial against advanced gastrointestinal malignancies and is currently awaiting Phase 2 trials. The Phase 1 has yielded very encouraging results with significant tumor responses observed in terms of reduced tumor activity on PET-CT and many patients with partial response or stable disease(25) (Manuscript under preparation). This Phase I trial revealed that the maximum tolerated dose for Minnelide is 0.67 mg/m². This roughly translates to 0.2 mg/kg in mice. At this dose, Minnelide depletes the stromal ECM, resulting in relieving the interstitial pressure on the blood vessels and leading to better drug delivery(6).
In the current study, we performed an exhaustive transcriptome analysis on CAFs and determined that the profound effect of Minnelide on the pancreatic tumor stroma is due to inactivation of CAFs in the tumor. This further results in a low ECM production via suppression of the TGF-β signaling pathway in CAFs. Inactivation of CAFs lead to a decreased “cross-talk” between the tumor and the stroma, leading to decreased oncogenic signaling, suppressed tumor growth, and invasion.

Materials and Methods

Cell lines and cell culture

Four primary cell lines were isolated from Kras^{G12D}; Trp53^{R172H}; Pdx-1-Cre (KPC) mice. The TECs were isolated according to our previous study(22). Isolation of CAFs (CAF-1, CAF-5 and CAF-7) from three KPC mice was performed following the protocol described by Sharon et al(26). The purity of fibroblasts was checked by flow-cytometry after staining isolated fibroblasts with fibroblast surface protein (FSP) antibody and CK19 antibody. Population with FSP+CK19-staining was used for downstream experiments. All the established cell lines were used between passages 5 and 18. We also used three pre-established cell lines, the mouse PDAC cell line Panc02 and human PDAC cell line S2-VP10 (gift from Dr. Masato Yamamoto’s lab, University of Minnesota) and the human pancreas fibroblasts SC00A5 (Vitro Biopharma, CO, USA). KPC-1 and CAFs were maintained in DMEM (Gibco, ThermoFisher Scientific, NY, USA) containing 10% fetal bovine serum (FBS) and 1% Pen Strep (Gibco). Panc02 and S2-VP10 were cultured in RPMI 1640 (Gibco) containing 10% FBS and 1% Pen Strep (Gibco). SC00A5 was maintained in MSC-GRO™ Low serum, Complete Media (Vitro Biopharma). All cell lines were routinely tested for mycoplasma and STR profiles (ATCC).
Fluorescence activated cell sorter analysis

Single-cell suspensions were prepared from fresh cell culture. Cell fixation and permeabilization was performed with the BD Bioscience Cytofix/Cytoperm kit (BD Biosciences, CA, USA), according to the manufacturer’s instructions. Apoptosis and BrDU incorporation for proliferation was done using Apoptosis and Cell Proliferation Kit following manufacturer’s instructions (BD Biosciences). Analysis of α-SMA, TGF-Beta receptor type 1, and TGFBR2 (Abcam) were conducted by FACS. All samples were analyzed on BD FACSCANTO II flow cytometers (BD Biosciences). Data was acquired and analyzed with FACSDiVa software (BD Biosciences) and FlowJo Software.

Invasion assay

Tumor cell invasion was measured by counting the number of tumor cells that invaded through matrigel pre-coated transwell chambers with 8-mm pores (BD Biosciences). On the top of inserts: 24 hour FBS starved; untreated; or 100 nM 24 hour triptolide treated tumor cells (KPC-1, Panc02 and S2-VP10, 1.5×10⁵ each) were placed. On the bottom chamber: untreated or 100 nM 24 hour triptolide treated CAFs (CAF-1, CAF-5 and SC00A5, 1.5×10⁵ each) in 1% FBS were added. The negative control group was added with 1% FBS in the bottom chamber. After incubation for 24 hours (KPC-1 and S2-VP10) to 48 hours (Panc02) for invasion, the invaded cells were fixed with 70% ethanol, stained with crystal violet, and 5 random fields were counted under a light microscope. Each experiment was repeated thrice.

Production of conditioned media
Conditioned media (CM) of tumor cells and CAFs was produced using FBS-free basal media to exclude the effects of growth factors in serum for downstream experiments. In normal conditions, 70% confluent cells were cultured in FBS-free basal media for 48 hours. In experiments designed to analyze the effects of triptolide, 100% confluent cells were cultured in FBS-free basal media containing 25 nM triptolide for 24 hours and then changed to no drug, FBS-free, basal media for 48 hours. The resulting CM were centrifuged for 10 minutes at 1,000 rpm after collection and stored at -80°C for no more than two months before use.

**Measurement of ECM and TGF-β secretion**

To evaluate the effects of triptolide on ECM secretion of CAFs, we measured concentration of total collagens, fibronectin (FN), periostin, hyaluronic acid (HA), matrix metallopeptidase 2 (MMP2), and MMP9 in CM derived from CAFs. Enzyme-linked immuno sorbent assay (ELISA) was used to quantify FN (BioVision, CA, USA), periostin (Thermo Scientific), HA (TSZELISA, MA, USA), MMP2 (Abcam), and MMP9 (Abcam). Total collagen was quantified by Sircol collagen assay kit (Biocolor Life Science Assays, County Antrim, UK). Meanwhile, autocrine signaling of TGF-Beta 1 (Abcam) and TGF-Beta 2 (R&D Systems, MN, USA) were determined by ELISA in CM. All the experiments were performed according to the manufacturer’s protocol.

**Animal model**

All animal experiments were performed according to the University of Miami Animal Care Committee guidelines. Both transgenic mouse model of spontaneous PDAC and orthotopic PDAC mouse model were included. KPC animals were generated by crossing Lox-Stop-Lox (LSL) KRas\textsuperscript{G12D}, Trp53\textsuperscript{R172H} animals with Pdx-1 Cre animals. Minnelide treatment with 0.2 mg/kg body weight was started when animals were 8 weeks of age. Animals in saline and treatment...
groups were age-matched. After 30 days of Minnelide treatment, animals were sacrificed and tumor tissues were collected. For one arm of orthotopic PDAC mouse model, 1,000 KPC-1 cells were suspended in matrigel (BD Biosciences) and injected in the pancreas of 20 C57BL/6 mice, for another arm, 1,000 KPC-1 cells plus 9,000 CAF-1 cells were suspended in matrigel (BD Biosciences) and injected in the pancreas of 20 C57BL/6 mice. Ten mice from each arm were treated with Minnelide (0.2 mg/kg body weight) for 30 days before sacrifice.

**Immunofluorescence and immunohistochemistry**

For immunofluorescence, 4 μm paraffin tissue sections were deparaffinized in xylene and rehydrated through graded ethanol. Hematoxylin and Eosin (H&E) staining were conducted to confirm histological features. Primary antibodies for αSMA (Abcam), cleaved-PARP (Abcam), FN (Santa Cruz Biotechnologies) and vimentin (Cell Signaling Technology) were diluted according to vendor’s instruction and incubated overnight at 4°C. The primary antibody was omitted for the negative controls. Alexa Fluor® conjugated secondary antibody (Invitrogen) were used for visualizing. Slides were counterstained with DAPI and observed in a Leica fluorescent microscope. Immunohistochemistry was used to detect BrdU in tissue sections according to manufacturer’s protocol (Abcam).

**Sirius red staining and measurements**

Tissue sections were stained using picrosirius red staining solution (Chondrex Inc, WA, USA) according to manufacturer’s instructions. The Sirius red–stained area was quantified using ImageJ software by selecting stained fibers in five fields at a magnification of ×100 under a light microscope.

**Quantitative real-time polymerase chain reaction assay**
Messenger RNA (mRNA) expression was analyzed through quantitative real-time polymerase chain reaction (qRT-PCR) using LightCycler 480 System (Roche, Basel, Switzerland) and SYBR Green (Qiagen). The 18s ribosomal RNA expression was used to normalize the results obtained in different conditions. Primers used in this article were listed in Supplementary Table 1.

**Transcriptome deep sequencing and analysis**

Aliquots of RNA were derived from the qRT-PCR samples. CAF-1, CAF-5 and CAF-7 control group, triptolide short-term treatment group, and long-term treatment group were analyzed. The RNA was quality tested using a Bioanalyzer 2100 (Agilent Technologies, CA, USA). cDNA was created by reverse transcription of oligo-dT purified polyadenylated RNA and fragmented, blunt-ended, and then ligated to barcoded adaptors. Then, the library was size selected, and the selection process was validated and quantified by capillary electrophoresis and qPCR, respectively. Samples were load on the HiSeq 2500 (Illumina Inc., CA, USA) to generate around 25 million paired-end 50bp reads for each sample.

Quality control was conducted by FastQC 0.11.2 according to [http://www.bioinformatics.babraham.ac.uk/projects/fastqc](http://www.bioinformatics.babraham.ac.uk/projects/fastqc) (27). TopHat 2.0.5, was used to map the paired reads to the UCSC mm10 assembly of the mouse genome(28). The mean inner distance was established using the insertion size metrics feature of Picard-tools. The resulting TopHat data served as input to other Cufflinks tools 2.2.0(29). Transcripts were also assembled using Cufflinks, with stipulating the reference transcriptome Mus_musculus. GRCm38.74. All cufflinks assemblies were merged with Cuffmerge. Differentially expressed genes (DEGs) were calculated with Cuffdiff and presented the data in terms of fragments per kilobase of transcript per million mapped reads (FPKMs). Visualization of data from Cuffdiff outputs was used CummeRbund v2.0.0(30). clusterProfiler(31) and Ingenuity Pathway Analysis (Qiagen) were

Estimation of active and total TGF-β in the cells and media

Conditioned media from TECs (MIA-PaCa2 and S2VP10) and activated pancreatic stellate cells (PSC) were collected after 48h of plating. Cell lysates from the TECs and PSCs were prepared by lysing the cells in RIPA buffer. Total and active TGF-b was estimated in the media and cell lysate using LegendMax TGF-b1 ELISA lit and LegendMax Free active TGF-b1 ELISA kit (Biolegends).

Vitamin A Accumulation Assay

5,000 CAF-7 cells were plated in each well of a black 96-well plate with clear bottom. The following day, cells were either left untreated, or treated with 25 nM triptolide for 48 hours. After treatment, 10 ng/mL TGF-β was added for 30 minutes to two untreated rows (12 wells), and two rows treated with triptolide (12 wells). After 30 minutes, cells were washed gently with PBS, and phenol-free media was replaced. Fluorescence was measured with excitation of 300 nm and emission of 338 nm on half of the plate. The other half of the plate was used for determining viability, as previously described.

Oil red staining

20,000 CAF-7 cells were plated in each well of a four-well chamber slide. The following day, two wells were treated with 25 nM triptolide for 48 hours. After treatment, 10 ng/mL TGF-β was added for 30 minutes to one untreated well, and one well treated with triptolide. Cells were then
washed gently with PBS, and fixed with 2% paraformaldehyde for 30 minutes. Cells were washed with water, and incubated with 60% isopropanol/40% water for 5 minutes. 60% isopropanol was removed, and replaced with Oil Red O working solution (3 parts Oil Red O:2 parts water) for 20 minutes. Cells were again washed with water, and counterstained with hematoxylin for 1 minute. Cells were washed, mounted with permount, and imaged with a light microscope.

**Sp1 activity assay**

Human pancreatic stellate cells (hPSC) were seeded in four, 10 cm plates, and cultured until 70-80% confluent, and were then treated with 25 nM triptolide for 48 hours. Meanwhile, 300,000 MIA PaCa-2 and S2-VP10 cells were seeded in 6 cm plates. After 48 hours, 10 ng/mL TGF-β was added to one untreated hPSC plate and one triptolide treated hPSC plate for 30 minutes. The conditioned media from the hPSCs was collected. Any DMEM on the MIA PaCa-2 cells was removed and the cells were rinsed with PBS. Conditioned media from the hPSCs was added to the MIA PaCa-2 cells for an additional 24 hours as follows: untreated hPSC, hPSC + TGF-β, hPSC + triptolide, and hPSC + triptolide + TGF-β. Cell lysates were collected for each sample and added in duplicate to the Sp1 TransAM ELISA plate (Active Motif). The ELISA was performed per the manufacturer’s instructions, and the Sp1 binding was normalized to protein concentration.

**Dual luciferase reporter assay**

MIA PaCa-2 and human Pancreatic stellate cells (hPSC’s) were plated at a density of 80,000 cells per well in a 24 well plate. Cells were treated with Triptolide (25 nM for 48 hours) and TGF-β (10ng/ml for 30 minutes). The supernatant from hPSC cells treated with triptolide was plated on top of the MIA PaCa-2 cells. After 48 hours of the hPSC conditioned media on MIA PaCa-2 cells, the cells were lysed with 1X Passive lysis buffer. The plate was then kept at -80°C.
overnight and the luciferase activity was measured the next day using the stop and glow kit from Promega according to the manufacturer's protocol.

**ECIS**

CAF-7 proliferation was measured by Electric Cell-substrate Impedance Sensing (ECIS). CAF-7 cells were seeded in 8W10E+ PET arrays with or without TGF-β (10ng/ml) and or triptolide (25 nM). Impedance (Z), capacitance (C) and resistance (R) were monitored for 60 hours by an ECIS Model ZΘ instrument (Applied BioPhysics Inc., Troy, NY) and normalized to $Z_0$.

**Statistical analysis**

Values are expressed as the mean ± SEM. Two-group data were analyzed using a t-test. Multigroup data were first analyzed using one-way ANOVA, if there are positive findings; Bonferroni's multiple comparisons test was performed to finish pairwise comparisons. A p-value < 0.05 was considered statistically significant.
Results

Low dose Minnelide decreased CAF-induced tumor growth in pancreatic cancer animal model

It is well established that CAFs promote tumor development and invasion in number of cancers. To evaluate the effects of triptolide (the active compound of Minnelide) on TEC invasion in the presence and absence of CAFs, a Boyden chamber assay was conducted. Three cell line pairs, CAF-1/Panc02, CAF-5/KPC-1, and SC00A5/S2-VP10 were set up (Figure 1A-C). CAFs increased invasion rate in untreated-tumor cells from two to four fold. Interestingly, when CAFs were treated with triptolide before co-culture with tumor cells, their invasion inducing roles decreased dramatically compared to untreated CAFs (Figure 1A-C). Inhibition of invasion of CAFs on TECs was similar to that observed when TECs were treated with same dose of triptolide. In addition to the promoting invasion, the untreated CAFs also induced tumor cell growth (Supplementary Figure 1 A, B). These results demonstrated that tumor growth and invasion-promoting functions of CAFs were impaired by triptolide.

To see if Minnelide affected CAF-induced tumor burden at a low dose (within the maximum tolerated dose of 0.67 mg/m² that is equivalent to 0.2 mg/kg in mice), in vivo we co-injected CAF:TEC (9:1) orthotopically in the pancreas of C57/Bl6 mice (n=8-9). Animals were treated with 0.2 mg/kg Minnelide for 30 days. Tumor weight was measured at the completion of the experiment. Our results showed that the presence of CAF cells generated larger tumors compared to those that did not have CAF cells injected (Figure 1D). When treated with Minnelide, tumors having CAF cells showed greater decrease in tumor weight compared to those that did not have CAFs (p=<0.05). Further, Minnelide treatment resulted in decreased collagen in the tissue and decreased α-SMA expression (Figure 1E, Supplementary Figure 2A,B). TUNEL staining
indicated increased apoptosis in the group with CAF/TEC treated with Minnelide compared to the TEC alone treated with Minnelide. Further, decreased BrdU staining indicated decreased proliferation was observed in the CAF/TEC group compared to the TEC group alone (Figure 1E, Supplementary Figure 2C). Upon scoring the metastatic lesions, we observed that the group having TEC had a 37.5% metastatic index which, when treated with Minnelide was reduced to 22.2%. However, in the TEC/CAF group had a higher metastatic index (75%) which was decreased to 44.4% upon treatment with Minnelide (Figure 1F). This indicated that presence of CAFs in a tumor increased proliferation of the TECs, production of collagen, and expression of α-SMA. Upon treatment with 0.2 mg/kg Minnelide, both group responded equally to the compound and decreased the collagen secretion, α-SMA secretion, proliferation, and increased the apoptosis in these cells.

In the KPC mouse model that fully recapitulates disease progression of human PDAC, we measured the amount of desmoplastic markers, such as collagen, FN, and the cellular component fibroblasts, in response to Minnelide treatment. Heavy desmoplasia was seen in KPC mice without Minnelide treatment, while in the Minnelide treatment group, less collagen and FN were expressed (Supplementary Figure 3A-I).

**Transcriptomic analysis of Cancer Associated Fibroblast after triptolide treatment**

To study which pathways in CAFs were being downregulated by triptolide, we next conducted a transcriptome analysis based on RNA-Seq. Cell lines CAF-1, CAF-5, and CAF-7 were either untreated or treated with triptolide (100 nM) for 6 hours or 24 hours. All samples had around 25 million paired reads with over 90% mapped uniquely mapped (Supplementary Table 2). A total of 3859 DEGs were identified between control group and triptolide 24h group with a FDR of 0.05
To further analyze the pathways that are deregulated by triptolide, we conducted pathway enrichment analysis and found that most significantly downregulated pathways were the RAR/RXR signaling pathway and TGF-β signaling (Figure 2C). Interestingly, RAR/RXR pathway is a retinoic acid (RA) stimulated pathway. In the presence of RA, which is a morphogen derived from vitamin A, RA translocates to the nucleus and stimulates the RAR/RXR to bind to their response elements (RARE), resulting in transcription of genes that regulate differentiation, survival, and TGF-β secretion. Our analysis showed that several genes in this pathway were being differentially regulated (Supplementary Figure 4 A,B).

IPA analysis showed that TGF-β signaling pathway was significantly downregulated in CAFs (Figure 2D). Furthermore, the downstream genes of TGF-β signaling pathway were also downregulated in triptolide treated group (Figure 2E and F). The top ten up-and downregulated genes are tabulated in Supplementary Figure 4C. Interestingly, in the TECs, these pathways were not among the top significant pathways that were deregulated (Data not shown).

**Triptolide downregulated the TGF-β pathway in CAFs at a sub-lethal dose**

It is well known that upon activation, the stromal cells, specifically fibroblasts, secrete TGF-β. Our results confirmed this (Figure 3A,B). Since our transcriptomics analysis was done at a dose of 100 nM triptolide, we next studied the effect of low dose of triptolide on the TGF-β signaling. We treated the CAFs with a very low dose of 25 nM triptolide and analyzed all the components of the TGF-β pathway. Our study showed that the autocrine TGF-β signaling pathway in the CAFs was downregulated even at 25 nM triptolide. The expression of TGF-β was significantly decreased both at the RNA level (Figure 3C) as well as secretion level in both cells and supernatant (Figure 3D, E). Additionally, effectors of TGF-β pathway (SMAD2, 3, 4) were downregulated both at the RNA (Figure 3F) and protein level (Figure 3G). In addition, several other genes that were directly being regulated by the TGF-β signaling were also downregulated (Figure 3F). The expression and secretion of the TGF-β, as well as all its components that were
decreased by triptolide treatment were rescued when treated with recombinant TGF-β (Figure 3 F,G).

**Downregulation of TGF-β pathway in CAFs reverted them from activated to inactivated state.**

Activation of pancreatic stellate cells (PSCs) is characterized by loss of Vitamin A droplets in these cells and a subsequent increase in α-SMA expression. Thus, an increase in Vitamin A and lipid droplet accumulation in the fibroblast as well as a loss of α-SMA would indicate an inactivation of CAFs. We observed that treatment with triptolide decreased proliferation of CAFs without affecting their viability (Figure 4A). To study if this was due to “inactivation” of CAFs, we next analyzed the vitamin A droplets in these cells after treatment with triptolide. Our results showed that treatment with triptolide increased vitamin A accumulation (seen by monitoring fluorescence at 338 nM) in CAFs. Interestingly, upon treatment with TGF-β in the presence of triptolide, the vitamin A fluorescence decreased to that observed in untreated cells (Figure 4B). This indicated that TGF-β was capable of reverting the triptolide-induced inactivation. This was further supplemented by our IPA analysis of the RAR/RXR pathway. We observed an increased expression of RBP (Retinol Binding Protein) in our triptolide treated CAFs (Supplementary Figure 4A). RBP is required for stabilization of retinol in the cells, thus, this further conformed to our hypothesis that triptolide was indeed inactivating the CAFs, resulting in accumulation of vitamin A. To confirm this observation further, we next performed an Oil Red staining on the CAF cells. We observed an increased Oil Red stain in the triptolide treated CAFs, which decreased upon treatment with TGF-β (Figure 4C).

α-SMA is a classic marker for activation of pancreatic stellate cells (PSC). When in their quiescent state, PSCs do not express α-SMA. However, upon activation, this protein is
upregulated. Treatment of CAF cells with triptolide decreased the α-SMA+ cells as seen by flow cytometry (Figure 4D).

To study if treatment with triptolide is affecting the other functions of CAFs as well, we next analyzed their ECM secretion. Our results showed that treatment with 25 nM triptolide decreased the secretion of fibronectin (Figure 5A), periostin (Figure 5B), collagen (Figure 5C), hyaluronic acid (Figure 5D), MMP2 (Figure 5E), and MMP9 (Figure 5F). Further, treatment with TGF-β reverted this decreased ECM production (Figure 5G, H).

**Decreased TGF-β signaling in CAFs affected oncogenic signaling in tumor epithelial cells**

It is well established that the tumor-stroma crosstalk is involved in tumor progression and metastasis. Since our data indicated that triptolide decreased the TGF-β signaling in the CAF cells and also induced inactivation, we next studied the effect of this inactivation on the TEC. Our previously published data showed that Sp1 was one of the transcription factors that was overexpressed in pancreatic cancer, and this in turn upregulated the anti-apoptotic pathways and proteins like NF-κB pathway and HSP70. To study if the decreased TGF-β from the “inactivated” CAFs decreased these pathways, we next set used conditioned media from activated PSC cells (+/- triptolide) and added it to MIA PaCa-2 cells. In a parallel set, we added 10 ng/mL TGF-β to the MIA PaCa-2 cells with conditioned media from 25 nM triptolide treated PSC cells. A dual luciferase reporter assay for SMAD showed that the conditioned media from triptolide treated PSC cells downregulated the SMAD transcriptional activity, which was rescued in the presence of TGF-β (Figure 6A). Since SMAD transcriptional activity leads to transcription of Sp1 (33-35), we next tested the Sp1 gene expression and activity. Our results showed that Sp1 mRNA expression was downregulated in the set with conditioned media from 25 nM triptolide. This expression was subsequently rescued upon adding TGF-β to the MIA PaCa-2 cells (Figure 6B). We next studied the effect of CAF inactivation on Sp1 DNA binding and
transcriptional activity. Our results showed that in the presence of conditioned media from the 25 nM triptolide treated PSC, Sp1 binding was significantly decreased (Figure 6C), indicating that CAF inactivation led to inhibition of transcriptional activity as well. Treatment with TGF-β reverted the Sp1 DNA binding, confirming that this inhibition of DNA binding was mediated via downregulation of TGF-β secretion by the PSCs. Our previously published data show that in the TECs, Sp1 downregulation inhibits anti-apoptotic proteins like NF-κB and HSP70. To study if inactivation of PSCs also led to this phenotype we studied the HSP70 and NF-κB gene expression after treating cancer cells with conditioned media from 25 nM triptolide. Our results showed that MIA PaCa-2 cells treated with conditioned media from PSCs plus triptolide downregulated p50 and p65 expression, and adding TGF-β upregulated expression, when compared to untreated PSC conditioned media (Figure 6D).

Discussion

Pancreatic tumors are characterized by a robust desmoplastic stroma; a significant part of which is comprised of myo-fibroblast like cells. The origin of these cells remains unclear but a substantial part of these cells are derived from pancreatic stellate cells (PSC). These cells are very similar to those found in the liver (hepatic stellate cells, HSC), which have been well studied in context of liver injury. In a normal pancreas, the PSCs are a quiescent population typically characterized by the presence of a large amount of vitamin A droplets or retinol (36-38). Exposure of PSCs to UV light at 300-338 nm elicits a transient blue-green fluorescence that can be quantitated(11). Upon inflammatory stimulation from the environment, these cells become activated, “losing” their retinoid containing lipid droplets and become myo-fibroblastic, secreting α-SMA. Even though a number of studies have been focused on the biology of the pancreatic stellate cells and their activation, the mechanism of loss of retinoids has not been studied in PSCs as they have been studied in HSCs. In HSCs, activation leads to the
metabolism of retinol to retinoic acid, which acts as a ligand for the RAR/RXR signaling pathway. This pathway is one of the key pathways involved in differentiation and is very tightly regulated. Upon activation, the RAR/RXR bind to their response elements (RARE) in the promoter of specific genes and regulate transcription of genes involved in differentiation, proliferation and survival. One such pathway stimulated by the RAR/RXR is the TGF-β signaling pathway.

It is classically believed that resident fibroblasts in any tissue become activated during wound healing and revert to their inactive state upon resolution (14). However, when associated with TECs, this activation often become irreversible, leading to formation of CAFs. In the pancreas, an injury or insult leads to activation of the PSCs. During the course of pancreatic tumor progression, the PSCs become activated by the TECs and reach a stage where they presumably become irreversibly activated and drive tumor growth. At this point, they express Fibroblast Activation Protein and Fibroblast Secretory Protein and become cancer associated fibroblasts or CAFs that cannot go back to the quiescent stage. Thus, targeting the CAFs at this stage with molecules that can revert the back to “quiescent” state can be considered an attractive therapeutic strategy, as this will disrupt the tumor-stroma crosstalk and inhibit the tumor growth and progression.

Our transcriptomics data showed that treatment of CAFs (isolated from a mature KPC mouse tumor) with triptolide deregulated the TGF-β pathway and the RAR/RXR signaling pathway (Figure 2, Supplementary Figure 4). Interestingly, the expression of RBP was increased by this analysis and the expression of RDH was downregulated. This indicated that triptolide was affecting the retinol-metabolizing pathway. Instead of the retinol being metabolized to RA (by RDH), there was more retinol in the CAFs that needed stabilization by RBPs. This was confirmed by both our Oil Red staining and vitamin A accumulation assay (Figure 4). We further
observed, this could be that the RAR/RXR pathway is a key developmental and differentiation pathway in the cells, it is very tightly regulated. While retinoic acid (ATRA and 9cisRA) can stimulate the RAR/RXR transcriptional activity, an excess of it acts as an inhibitor of RDH and shuts down the conversion of retinol to RA. A number of studies have shown that ATRA can prevent activation of PSCs and this only observed at high doses (39,40). Our studies showed that treatment with ATRA at a low dose increased proliferation of CAFs, while at an increased dose of 1uM, it inhibited proliferation presumably by causing a feedback inhibition of the RAR/RXR pathway (Supplementary Figure 5).

Previous work from our laboratory has shown that Minnelide, the water-soluble prodrug of triptolide, is an effective anti-tumor and anti-stromal compound(6). At a dose of 0.4 mg/kg, Minnelide depletes the stroma, induces stromal and tumor cell death, and prevents metastasis. However, our recently completed Phase 1 clinical trial shows that the maximum tolerated dose of Minnelide in patients in 0.67 mg/m$^2$, which translates to 0.2 mg/kg in mice. At this dose, Minnelide still decreases the ECM in the stroma, but does not have any profound effect on viability of TECs or CAFs. In the current work, we show that while CAFs promoted tumor growth and invasion, treatment with Minnelide did not significantly decrease metastasis in vivo (Figure 1). However, treatment with Minnelide did result in less number of proliferating cells in the tumor as was seen by BrdU uptake (Figure 1, Supplementary Figure 2). This effect was confirmed in vitro as well, where treatment with conditioned media from triptolide treated CAFs decreased proliferation of TECs as well as CAFs (Figure 4A).

It is well known that CAFs produce TGF-β, which in turn activates oncogenic signaling in the tumor epithelial cells via the SMAD group of transcription factors$^{23,24}$. It is also known that TGF-β signaling leads to induction of Sp1 transcriptional activity in tumors$^{25}$. Though Sp1 is not
present is terminally differentiated tissues, we and others have seen that pancreatic cancer has an increased Sp1 activity\textsuperscript{26} and its inhibition leads to cancer cell death. Our data suggest that triptolide treatment of CAFs leads to inhibition of TGF-β mRNA levels as well as its secretion (Figure 3). Upon treating MIA PaCa-2 cells with conditioned media from 25 nM triptolide treated PSCs, we saw a distinct decrease in the SMAD transcriptional activity (Figure 6), Sp1 expression, as well as Sp1 activity (Figure 6). In addition, other anti-apoptotic pathways that are known to be upregulated in pancreatic cancer (and regulated by Sp1 activity) were also found to be downregulated.

In addition to decreased proliferation, treatment with triptolide also decreased ECM secretion by CAFs. Interestingly, all the “inhibition” effect of triptolide was recovered upon treatment with TGF-β, which indicated that triptolide induced inactivation of CAFs led to a decreased TGF-β secretion by these cells, which deregulated both the autocrine signaling in the CAFs as well as the paracrine signaling in the TECs. This has been demonstrated in the schematic figure (Figure 7).

**Conclusion**

The role of CAFs in promoting tumor growth and invasion has been established in a number of cancers including pancreatic cancer. Several studies in pancreatic cancer have also been focused on depletion of stromal components specifically the ECM. In the current study, we show for the first time that Minnelide, and its active compound triptolide, is able to revert the presumably “irreversible” CAFs to an inactive state, where they show decreased TGF-β secretion and less ECM production. This in turn, affects the TECs and lowers their proliferation, decreases their oncogenic signaling, leading to a tumor regression. This mechanistic insight on the effect of triptolide on CAF inactivation will pave the way for developing viable and attractive therapy for pancreatic cancer, a disease that still lacks efficient chemotherapeutic options.
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Figure legends

**Figure 1. Triptolide/Minnelide inhibited CAF mediated invasion, tumor development and desmoplasia of TECs in pancreatic cancer cells and animal model.** Co-culture of TEC (mouse derived KPC, Panc-1 or human derived S2VP10) with CAF cells (mouse derived CAF-1, CAF-5 or human derived SC00A5) resulted in increased invasion in a boyden chamber, this was decreased upon treatment with 25nM triptolide of CAF cells, TECs or both. (A-C). Co-injection of CAFs with tumor cells increased tumor growth (D) in orthotopic PDAC mouse model, but Minnelide treatment suppressed tumor-promoting roles of CAFs in vivo as can be seen by the end of study tumor weight. Analysis of tumors by HE staining, sirius red staining and BrdU staining showed that CAFs induced increased collagen synthesis, proliferation in TECs, however, this was decreased upon treatment with Minnelide. Minnelide also induced apoptosis as seen by cleaved PARP staining and TUNEL staining (E). Though CAFs promoted metastasis, treatment with Minnelide resulted in decreased metastatic lesions as can be observed by the metastatic index (F). In summary, Minnelide suppressed CAFs mediated collagen deposition, cell proliferation, metastasis and αSMA and induced apoptosis in orthotopic PDAC model. (*, P<0.05; **, P<0.01).

**Figure 2. Transcriptome analysis of triptolide treated CAFs.** Differentially expressed genes in CAFs before and after triptolide treatment were shown in heatmap (A). Principal component analysis showed three distinct groups of all samples that correlate to different treatments (B). Pathways that are significantly deregulated in 24h triptolide treatment samples were shown as bar plot (C). Visualization of TGF-β pathway in response to triptolide treatment in CAFs. Green label means down-regulated genes and red-label mean up-regulated genes (D). GSEA indicated obvious suppressive role of triptolide on TGF-β pathway down-stream genes’ expression (E). Heatmap of TGF-β pathway down-stream genes’ expression before and after triptolide treatment. Red means high-expression and blue means low-expression (F).
Figure 3. Triptolide downregulated TGFβ pathway in the CAFs. Activated pancreatic stellate cells (PSCs) produced more active as well as total TGF-β compared to the TECs in culture supernatant (A) as well as in the cell lysate (B) as seen by the ratio of the active: total TGF-β. Treatment with 25nM triptolide (sub-lethal) decreased mRNA expression (C) as well as active: total TGF-β in hPSC supernatant (D) as well as cell lysate (E). mRNA expression of several genes in the TGF-β pathway were downregulated by triptolide treatment of activated PSC. This was rescued by addition of recombinant TGF-β (F). Triptolide treatment also downregulated the activity of SMAD2 and SMAD3 proteins as seen by decreased phosphorylation in western blot. This was rescued by addition of recombinant TGF-β (G). * signifies P<0.05 in control vs. triptolide treatment ; # signifies P<0.05 in triptolide treated vs. TGF–β + triptolide–treated samples.

Figure 4. Downregulation of TGF-β pathway in CAFs by triptolide reverts them from activated to inactivated state. Treatment of TEC (KPC001) with conditioned media from CAF7 treated with 25nM triptolide decreased proliferation rate of KPC001 as measured by ECIS (A), resulted in Vitamin A accumulation as observed by change of fluorescence at 338nM (B) and lipid droplet accumulation by Oil Red staining (C). Further, analysis α-SMA expression by flow cytometry showed a decrease in the intensity of staining indicating an inactivation of CAF cells in the presence of triptolide (D). * signifies P<0.05 in control vs. triptolide treatment ; # signifies P<0.05 in triptolide treated vs. TGF-β + triptolide–treated samples.

Figure 5. Treatment with triptolide decreased ECM secretion by CAFs. Treatment with 25 nM triptolide decreased the secretion of fibronectin (Figure 5A), periostin (Figure 5B), collagen (Figure 5C), hyaluronic acid (Figure 5D), MMP2 (Figure 5E), and MMP9 (Figure 5F). Further, treatment with TGF-β reverted this decreased ECM production (Figure 5G, H). * signifies
P<0.05 in control vs. triptolide treatment ; # signifies P<0.05 in triptolide treated vs. TGF-β + triptolide–treated samples.

Figure 6. Downregulation of TGFβ in CAFs inhibits oncogenic signaling in TECs. Treatment of TEC (MIA-PACA2 or S2VP10) with conditioned media from activated PSC treated with 25nM triptolide decreased SMAD transcriptional activity as seen by dual luciferase assay (A). This resulted in decreased Sp1 expression in the TECs (B). Further, DNA binding ability of Sp1 was inhibited (C). Expression of genes downstream of Sp1 like HSP70, HSF1 and components of NF-kB pathway (RelA and NFKB1) were also downregulated. These were rescued upon addition of recombinant TGF-β (D). * signifies P<0.05 in control vs. triptolide treatment; # signifies P<0.05 in triptolide treated vs. TGF-β + triptolide–treated samples.

Figure 7. Schematic Figure illustration of how Minnelide mediated inactivation of CAFs affects oncogenic signaling in TECs. Minnelide inactivates the CAF cells leading to decreased production of TGFβ, which in turn downregulates pro-oncogenic signaling in the tumor epithelial cells.
Figure 2

A

B

C

Canonical pathways affected

D

E

F

Enrichment of TGF-Beta pathway regulated genes
Figure 4

A

- Control
- Triptolide
- TGFbeta
- Triptolide + TGFbeta

B

Vitamin A RFU normalized to cell viability

C

CAF7
CAF7 + triptolide

CAF7 + TGF-beta

CAF7 + triptolide + TGF-beta

D

Oil Red O staining normalized to cell area

CAF7
CAF7 + triptolide
CAF7 + TGF-beta
CAF7 + triptolide + TGF-beta

Control CAF αSMA staining
25nM TPL treated CAF:αSMA staining

α-SMA low
α-SMA High

No. of events

α SMA intensity
Figure 7.
Inactivation of cancer-associated-fibroblasts (CAF) disrupts oncogenic signaling in pancreatic cancer cells and promotes its regression

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