TNFα and TGFβ-1 synergistically increase the cancer stem cell properties of MiaPaCa-2 cells

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Abstract. Increased serum concentrations of tumor necrosis factor α (TNFα) and transforming growth factor β-1 (TGFβ-1) in the blood of patients with pancreatic cancer (PC) have previously been demonstrated. In addition, exogenous exposure to these cytokines promotes various cancer cell invasive and cancer stem cell (CSC) phenotypes. However, their importance in pancreatic CSCs remains elusive. In the present study, the effects of TNFα and TGFβ-1 on the human PC cell line MiaPaCa-2 were examined. Using flow cytometry, it was revealed that TNFα and TGFβ-1 synergistically increase cluster of differentiation (CD) 44v6, CD133 and ATP-binding cassette transporter G2 (ABCG2) expressing populations in adherent tumor cell culture conditions. Furthermore, a similar trend was observed in cells pretreated with these cytokines grown in sphere forming culture conditions. Similar to previous studies, TNFα treatment increased the proportion of epidermal growth factor receptor (EGFR) expressing cells in adherent culture, and this data was further supported by the results of the sphere formation assay, in which the subculture with a high proportion of EGFR expressing cells exhibited the most efficient sphere forming ability. However, the proportion of vascular endothelial growth factor receptor 1 (VEGFR1) expressing cells did not increase upon treatment with these cytokines individually or in combination. This data was subsequently supported by the results of the wound healing assay in which cytokine treatment did not increase the migration of cells. The MTT cell proliferation and cytotoxicity assay revealed that TNFα + TGFβ-1 treatment significantly increased cell proliferation and daunorubicin resistance, but not gemcitabine resistance. In conclusion, the data of the current study provide a mechanistic association between TNFα, TGFβ-1 and the CSC properties of MiaPaCa-2 cells. In addition, it suggests that targeting TNFα and TGFβ-1 is beneficial for improving the therapeutic efficacy of treatments for patients with PC.

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Abbreviations: PC, pancreatic cancer; TNFα, tumor necrosis factor α; TGFβ-1, transforming growth factor β-1; CSC, cancer stem cell; ABCG2, ATP-binding cassette transporter G2

Key words: MiaPaCa-2, TNFα, TGFβ-1, cancer stem cell, sphere forming ability, CD44v6, CD133, ABCG2

Introduction

Deregulated expression of cytokines by tumor cells and their surrounding stromal cells including fibroblasts and immune cells have been found to be essential for cancer cells to acquire aggressive phenotype (1). These resulting highly tumorigenic cells are now referred to as cancer stem cells (CSCs) or tumor initiating cells, often associated with stem cell (SC) properties including resistance to chemotherapy, increased capacity of anchorage independent growth, expression of SC antigens (2). CSCs have been found and characterized in various cancers on the basis of their SC markers and functional properties such as sphere forming ability or *in vivo* tumorigenicity. There were several SC markers have been identified as universal markers for most cancer types. CD44, CD133 and ATP-binding cassette transporter G2 (ABCG2), among many SC markers, have been used individually or in combination with other markers to identify and isolate CSC from cancers of breast (3), colon (4), skin (5), ovary (6) and pancreas (7). Although initially CD44 was broadly considered as a CSC marker in various cancers (8), more detailed recent reports revealed that the variant 6 isoform (CD44v6) is found to specifically expresses in CSCs of brain (9) and colon cancers (10), and in an earlier clinical study (11) CD44v6 was found in metastatic lesions of PC suggesting this isoform may be associated with metastasis.

Another prospective cell surface antigen is CD133, which is now established as a putative CSC marker for most prevalent solid human cancers including brain (12), colon (4), head and neck cancers (13). In the case of PC, CD133 has been defined not only as a CSC marker, *in vitro* and *in vivo* functional studies also established the CD133 positive cancer cells (sometimes in combination with other markers) as a core population responsible for drug resistance, invasion, tumorigenicity and metastasis (14). In their cohort study Maeda *et al* examined clinical relevance of CD133 in PC via immunohistochemistry, in which CD133 expression in PC tumor samples

correlated with lymph node metastasis and poor prognosis (15). Overexpression of ABCG2 in various cancer cells has been associated with multi-drug resistance due to its ability to efflux the drugs outside the cell, and reports also demonstrated that ABCG2 can be used as a CSC marker independently (16). Although essential roles of CSC in PC progression have been proved beyond doubt, however little is known about the cytokines that increase CSC properties in this cancer.

TNFα and TGFβ-1, among others, have been found to be most abundant cytokines that play crucial roles not only in augmenting cancer cells invasion and migration capacities, but also promote their 'stemness' as demonstrated by mechanistically overexpression or suppression and exogenously stimulating approaches (17,18). For example, targeting TNFα by monoclonal antibody (mAB) attenuated tumor growth and made the tumor cells sensible to drug treatment in a mouse model of PC (19). Clinical observation also support those cellular and animal studies, since overexpression of these cytokines have been found in many different human tumor samples and patient blood and correlated with poor prognosis (20). For example Lin *et al* reported that high level of TGFβ-1 in serum of PC patients was associated with increased risk of death (21). Elevated serum concentrations of TNFα and TGFβ-1 have been observed in blood from PC patients (22). Moreover, recent reports further expanded our understanding of these cytokines in the CSC biology (17). For example treatment with TGFβ for 7 days resulted in increased self-renewal capacity of patient-derived glioma-initiating cells (GICs) via inducing leukemia inhibitory factor, and prevented GICs differentiation and promoted *in vivo* oncogenesis (23). In their blood cancer study, Kagoya *et al* revealed a potential role of TNFα in leukemia initiating cells' (LICs) maintenance, in which constitutive NF-κB activity is maintained through autocrine TNFα secretion by LICs (24). However, the possible effects of TNFα and TGFβ-1 on CSC populations of PC have not yet been studied.

In this report, we examined the effects of TNFα and TGFβ-1 on PC cell line MiaPaCa-2 cells, and our phenotypic and functional data showed that these cytokines substantially increase CSC populations in this cell line when worked together and significantly increase self-renewal and proliferation and probably ABCG2 dependent drug resistance.

Materials and methods

Reagents. Culture media and reagents for maintaining parental MiaPaCa-2 cells and tumor spheres are as following: DMEM, DMEM-F12, recombinant epidermal growth factor (rEGF), recombinant basic fibroblast growth factor (rbFGF), B27 and N2 supplements (Life Technologies, Grand Island, NY, USA), insulin, fetal bovine serum (FBS), L-glutamine, TrypLEX, Poly-HEMA (all from Sigma, St. Louis, MO, USA, unless otherwise specified). Antibodies for flow cytometry: CD133/1 (AC133)-APC (Miltenyi Biotech, Cambridge, MA, USA), EGF-R-PE, purified anti-CD32 anti-CD16 (BD Biosciences, San Diego, CA,), CD44v6-AlexaFluor 488, VEGF-R1/Flt1-PE, ABCG2-PE (R&D Systems, Minneapolis, MN, USA) (BD Biosciences). For MTT assay: 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), daunorubicin and gemcitabine (Eli Lilly).

Cell culture. MiaPaCa-2 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), and maintained in DMEM supplemented with 10% FBS, glutamine, and antibiotics (penicillin and streptomycin). Cells were incubated in a humidified incubator containing 5% CO_2 at 37˚C. To obtain tumor spheres from MiaPaCa-2 cells and their cytokine-treated subcultures, the cells growing in adherent culture condition were detached using TrypLEX when cell confluence reached to 80-90%, and were washed with ice-cold PBS. Single cells were seeded at a density of 2,000 cells per well in Poly-HEMA-coated or ultralow attachment 24-well plates. DMEM/F12 was used as the basic sphere-culture medium, which supplemented with 50 ng/ml rEGF, 20 ng/ml rbFGF, 5 μg/ml insulin, 1X B27 supplement without vitamin A, 1X N2 supplement and 1% FBS. Sphere cells were incubated in an incubator containing no CO_2 at 37˚C. After 10-12 days, the sphere cells were harvested by gentle centrifugation and dissociated with TrypLEX.

Sphere formation assay. The culture condition was similar to the above description, except seeding density. To evaluate sphere forming ability, cells were seeded at a density of 200 cells per well in Poly-HEMA-coated 96-well plates. 5 days later, fresh sphere-culture medium was added to each well. On day 11-12, the numbers of spheres were counted under an inverted microscope (DMIL; Leica, Mannheim, Germany), and sphere forming efficiency was calculated by dividing number of spheres to initially seeded cells, and by multiplying with 100%.

Flow cytometry. Prior to the labeling, cells were incubated with anti-CD32/anti-CD16 cocktail to block the Fc receptors. Then, for surface and intracellular staining, cells were subsequently incubated with mAbs specific for surface markers according to the manufacturer's protocols, then fixed and permeabilized with Fixation/Permeabilization solution (BD Pharmingen; BD Biosciences) and incubated for 20 min in the dark at room temperature. Cells were then washed with Perm/Wash Buffer (BD Biosciences) and stained with mAbs specific for intracellular cytokines. Afterwards, cells were washed with PBS, re-suspended in flow solution, and immediately analyzed by flow cytometry on FACSCalibur (BD Biosciences) using CellQuest Pro software (BD Biosciences). All cells were gated based on FSC and SSC properties, from this gate, cells were analyzed for expression of CSC markers. Anti-Mouse Ig, κ/Negative Control Compensation Particles Set (BD Biosciences), unstained cells, single fluorochrome stained cells, and cells stained as fluorescence-minus-one (FMO) controls were used to set up the machine.

MTT cell proliferation and cytotoxicity assay. The sensitivities of the four subcultures to daunorubicin and gemcitabine were assessed by MTT assay. Each subculture divided in two groups, one is control group where no drug will be added, and second group is experimental group where a drug will be added. Cells were seeded in 96-well plates at a density of 1,000 cells/100 μl in culture media and plus their own corresponding cytokines. After 24 h incubation, 100 μl culture media were added to control groups and equal volume of culture media containing daunorubicin or gemcitabine, with

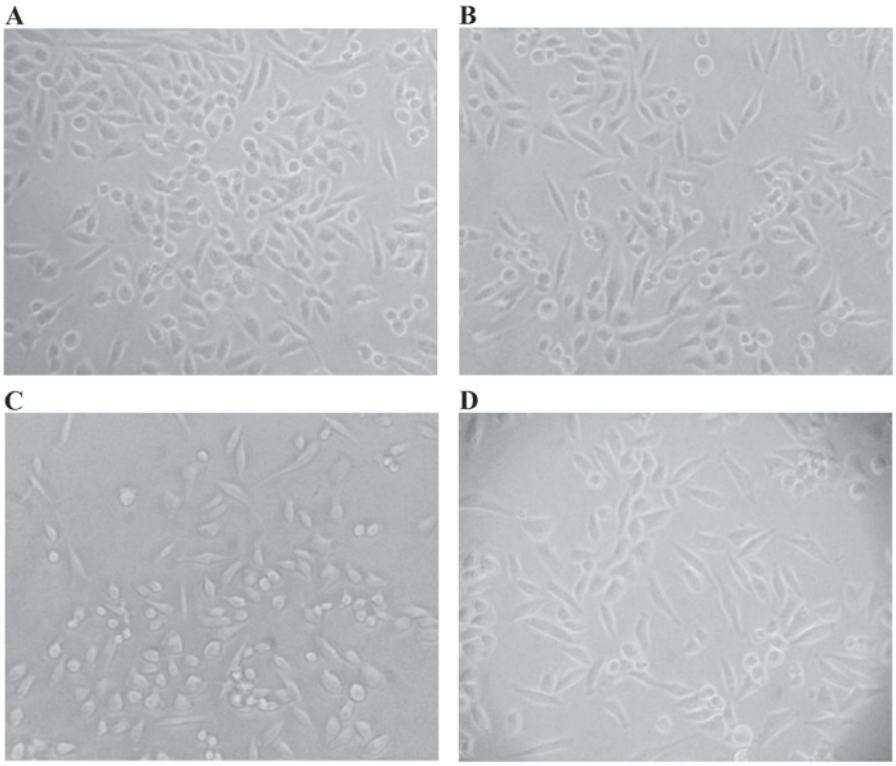

Figure 1. TNFα and TGFβ-1 induce morphological changes of MiaPaCa-2 cells. Representative images of three independent experiments. (A) pMia cells, (B) pMiaTN cells, (C) pMiaTB cells and (D) pMiaTT cells. Original magnification, x200. TNFα, tumor necrosis factor α; TGFβ-1, transforming growth factor β-1 pMia, untreated MiaPaCa-2 cells; pMiaTN, TNFα + TGFβ-1-treated MiaPaCa-2 cells; pMiaTB, TGFβ-1-treated MiaPaCa-2 cells; pMiaTT, TNFα + TGFβ-1-treated. MiaPaCa-2 cells.

a final concentration of 200 ng/ml or 6.8 μg/ml respectively, were added to experimental groups. After another 24 h incubation, 20 μl MTT solution (5 mg/ml) was added to each well, and incubated for 4 h at 37˚C, then supernatant was carefully removed and let the plates dried out at room temperature. Then 100 μl of DMSO was added to each well. The absorbance of each well was measured at 492/630 nm using a microplate reader.

Wound healing assay. Appropriate number of cells from four subcultures were seeded in adherent culture dishes and allowed them to grow until reached to confluency. A straight stretch was made using a pipette tip in the central area of the confluent culture. The cells were rinsed with fresh culture media to remove detached cells. New media with low serum concentration were added to each subculture. Phase contrast images were recorded at indicated time points.

Statistics. The Wilcoxon signed-rank test was used to obtain P-values when compared the proportions of CSC populations between untreated and cytokine treated cells. For the rest of the data Student's t-test was used to determine statistically significant difference and the results were represented as the mean ± SD. $P<0.05$ was considered as significant.

Results

Treatment of MiaPaCa-2 with TNFα and TGFβ-1 increase CSC populations in adherent culture condition. TGFβ-1 has long been used as a master stimulator of epithelial-mesenchymal transition (EMT), in which epithelial cells lose their coble-stone like morphology and convert to spindle-shaped, fibroblastoid cells (25). Although less frequently used than TGFβ-1, TNFα has also been accepted as an EMT stimulator (26). In some reports combination of these two cytokines were used and exhibited more profound effects on cells than used alone (27), and the cells undergone EMT often share CSC characteristics (25). Therefore, we cultured MiaPaCa-2 cells with low concentrations of TNFα (2 ng/ml) and TGFβ-1 (2 ng/ml) and combination of both in adherent culture dishes. So we had four subcultures including untreated parental cells designated as follows: pMia (untreated parental MiaPaCa-2), pMiaTN (TNFα-treated), pMiaTB (TGFβ-1-treated), and pMiaTT (TNFα + TGFβ-1-treated). Two days later, the proportion of spindle-shaped cells moderately increased among TNFα alone (Fig. 1B) and in combination with TGFβ-1 (TNFα + TGFβ-1) treated cells (Fig. 1D). Interestingly, the proportion of small and round-shaped cells was increased after treatment with TGFβ-1 alone (Fig. 1C). After in total three days of incubation, the cells were subjected to flow cytometry analyses to assess a series of markers and a single case of flow cytometry data obtained at the same day and comparison of the proportions of the phenotypic markers between untreated (pMia or Mia) and double treated (pMiaTT or MiaTT) cells were shown by flow dot plots and graphs respectively. As shown in Fig. 2A combinatorial treatment resulted in the most substantial increase of CD44v6 positive cells (20.7%), CD133 positive cells (3.5%) and double positive (CD44v6/CD133) cells (2.4%) among four subcultures, while individual treatment also considerably increased when compared to untreated parental pMia cells.

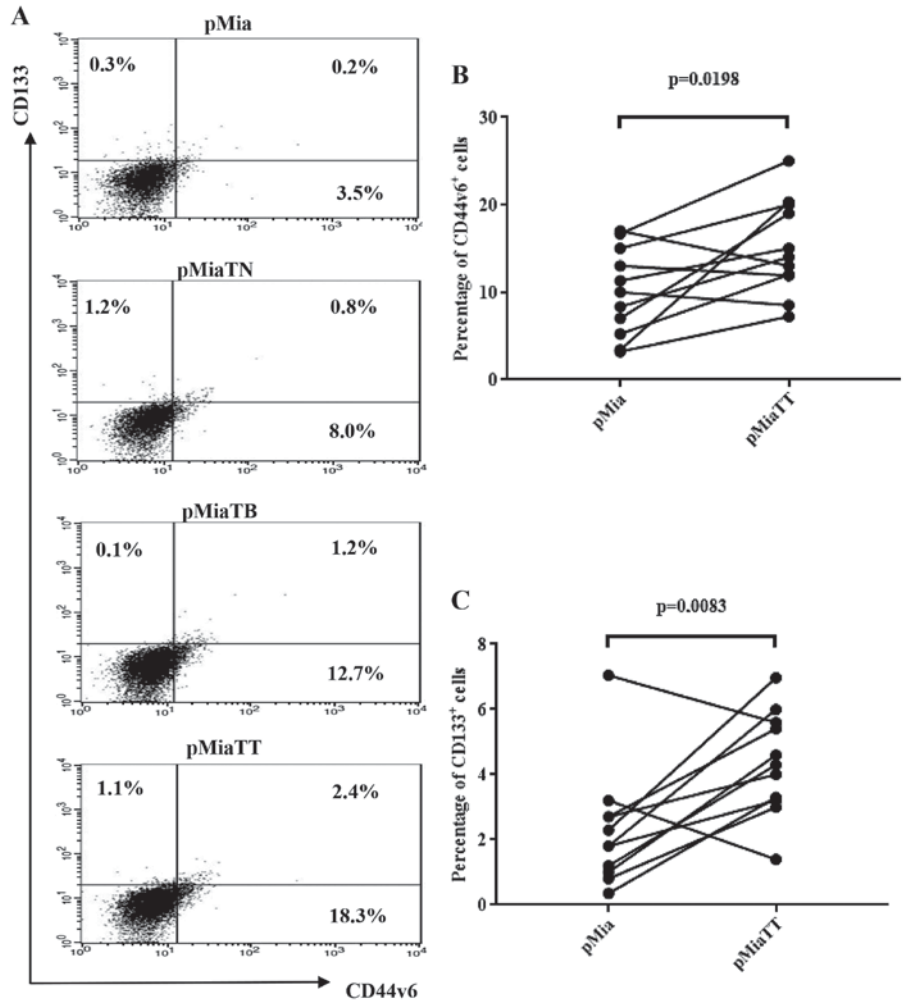

Figure 2. TNFα and TGFβ-1 treatment increase CD44v6 and CD133 and CD44v6/CD133 expressing populations among MiaPaCa-2 cells in adherent culture. (A) Flow cytometry dot plots demonstrating a single case of CD44v6 and CD133 and CD44v6/CD133 expressing populations among untreated and cytokine treated MiaPaCa-2 cells. Comparison of the proportions of CD44v6 (B) and CD133 (C) expressing cells between pMia and pMiaTT cells were shown in the graphs. The experiments were repeated eleven times. TNFα, tumor necrosis factor α; TGFβ-1, transforming growth factor β-1; pMia, untreated parental MiaPaCa-2 cells; pMiaTT, TNFα + TGFβ-1-treated MiaPaCa-2 cells.

Specifically, pMiaTN cells included 8.8% of CD44v6 positive cells, 2.0% of CD133 positive cells, and 0.8% of CD44v6/CD133 positive cells, while pMiaTB cells included 13.9% of CD44v6 positive cells, 1.3% of CD133 positive cells, and 1.2% of CD44v6/CD133 positive cells. Parental pMia cells included 3.7% of CD44v6 positive cells, 0.5% of CD133 positive cells, and 0.2% of CD44v6/CD133 positive cells. The significant changes in the proportions of CD44v6 and CD133 expressing cells between pMia and pMiaTT cells were shown in Fig. 2B and C. In the case of ABCG2 (Fig. 3), pMiaTT cells still showed the greatest proportion of ABCG2 positive cells (21.3%) when compared to control pMia cells (5.3%) and individually treated pMiaTN cells (12.7%) and pMiaTB cells (10.1%). Fig. 3B shows a significant change in the proportion of ABCG2 expressing cells between pMia and pMiaTT cells. Taken together, these data suggest that TNFα and TGFβ-1 could synergistically increase CSC populations among MiaPaCa-2 cells.

Pretreatment of MiaPaCa-2 cells with TNFα and TGFβ-1 increase CSC populations in sphere forming culture condition. Meanwhile, we succeeded culturing of tumorspheres from MiaPaCa-2 cells, and furthered the assessment of CSC marker expression on sphere cells derived from the four individual subcultures designated as follows: Mia (derived from untreated parental MiaPaCa-2), MiaTN (TNFα-pretreated), MiaTB (TGFβ-1-pretreated), and MiaTT (TNFα + TGFβ-1-pretreated). Procedures of obtaining of tumor spheres described in the materials and methods. After 12-14 days of culture, the tumor spheres were harvested and dissociated with TrypLEX, and flow cytometry analyses were performed. In consistent with the data obtained from adherent culture, pretreatment with TNFα + TGFβ-1 gave rise to the highest number of CSCs (Fig. 4A). Specifically, 24.9% of CD446v positive cells and 20% of CD133 positive cells and 8.7% of double positive cells, while MiaTN cells possessed 14.7% of CD44v6 positive cells, 8% of CD133 positive cells, and 4.3% of double positive cells. MiaTB cells possessed 20.4, 10.2 and 5.9% of respective CSC populations. Control Mia cells included 11.4, 4.4 and 2.6% of respective CSC populations. The significant change (Fig. 4B) in the proportion of CD44v6 expressing cells between Mia and MiaTT cells was obtained,

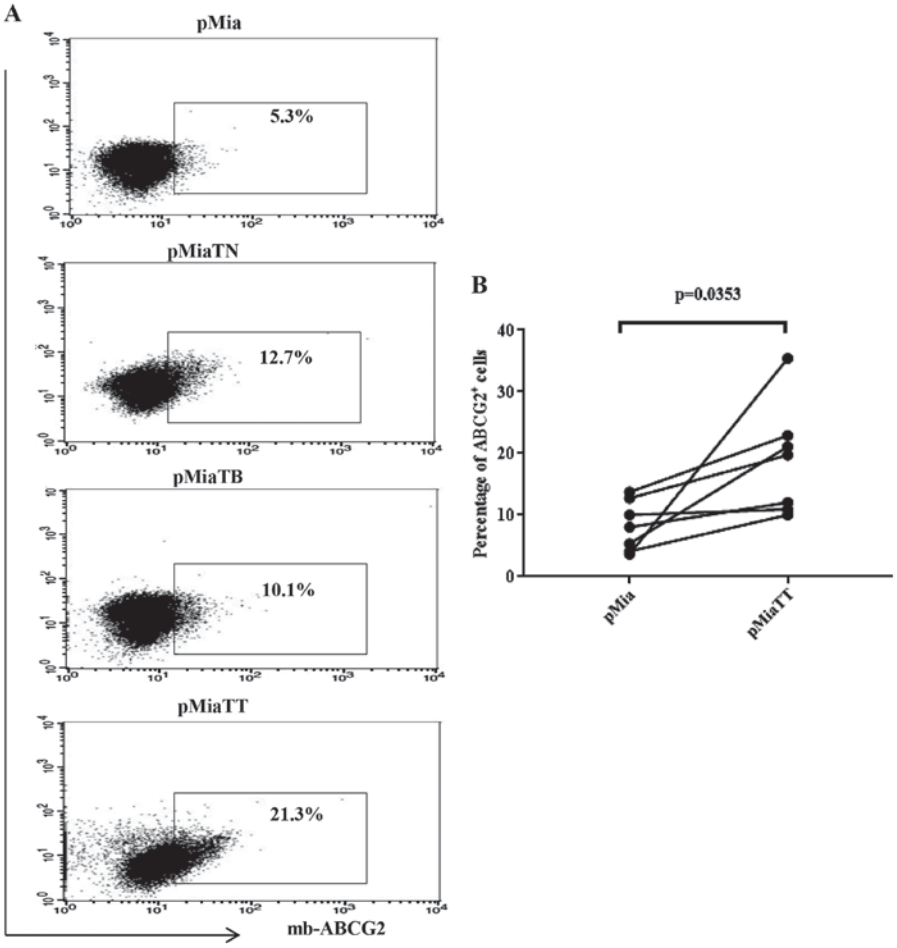

Figure 3. Treatment with TNFα and TGFβ-1increase ABCG2 expressing population among MiaPaCa-2 cells in adherent culture. (A) Flow cytometry dot plots demonstrating a single case of ABCG2 expressing cells among untreated and cytokine treated MiaPaCa-2 cells. (B) Comparison of the proportion of ABCG2 expressing cells between pMia and pMiaTT cells was shown in the graph. The experiments were repeated seven times. TNFα, tumor necrosis factor α; TGFβ-1, transforming growth factor β-1; pMia, untreated parental MiaPaCa-2 cells; pMiaTT, TNFα + TGFβ-1-treated MiaPaCa-2 cells; ABCG2, ATP-binding cassette transporter G2.

however, there was no significant change in terms of CD133 expressing cells between Mia and MiaTT cells as shown in Fig. 4C. In general, cells pretreated with cytokines showed higher proportion of ABCG2 positive cells compared to the control (Fig. 5A), however, in this case MiaTB cells possessed the highest proportion of ABCG2 positive cells which reached 25.2%, while MiaTT and MiaTN cells had similar percentages of ABCG2 positive cells 18.3% and 17.3% respectively. Control Mia cells possessed 13.3% of ABCG2 positive cells. Accordingly, comparison of the proportion of ABCG2 positive cells between Mia and MiaTB cells was reached to statistical significance (Fig. 5B). Of note, the proportions of CSCs in sphere-forming cultures were substantially increased compared to adherent cultures as sphere-forming culture media have the ability to enrich SC populations (Figs. 2, 3, 4, and 5).

Treatment with TNFα increases the proportion of EGFR positive cells among adherent MiaPaCa-2 cells. Previously, induction of EGFR expression by TNFα at mRNA and protein level in six PC cell lines has been reported (28). However, in that study MiaPaCa-2 was not included. So we tested using flow cytometry whether TNFα and TGFβ-1 have any effect on EGFR expressing population in MiaPaCa-2 cells. As indicated in Fig. 6A, TNF-α treatment, in concert with previous works, increased EGFR positive cells (7.7%) compared to untreated (2.4%) and TGFβ-1 treated cells (3.1%), while treatment with combination of cytokines increased EGFR expressing cells nearly by two-fold (4.3%) when compared to untreated cells. Fig. 6B and C show that the comparisons of the proportion of EGFR positive cells between pMia and pMiaTT cells and between pMia and pMiaTN cells were reached to statistical significance.

TNFα and TGFβ-1 treatment do not increase the proportion of vascular endothelial growth factor receptor 1 (VEGFR1) expressing cells among MiaPaCa-2 cells in adherent culture. Because presence of VEGFR1 on PC cell lines including MiaPaCa-2 have been demonstrated by RT-PCR and western blot analysis, and upon stimulation with VEGF-A or VEGF-B Panc-1 cells exhibited increased migration and invasion abilities (29), therefore we tested whether TNFα and TGFβ-1 could increase the proportion of VEGFR1 expressing cells among MiaPaCa-2. As indicated in Fig. 7A, the flow cytometry data revealed that neither individual nor combinatorial treatment led to substantial increase of the proportion of VEGFR1

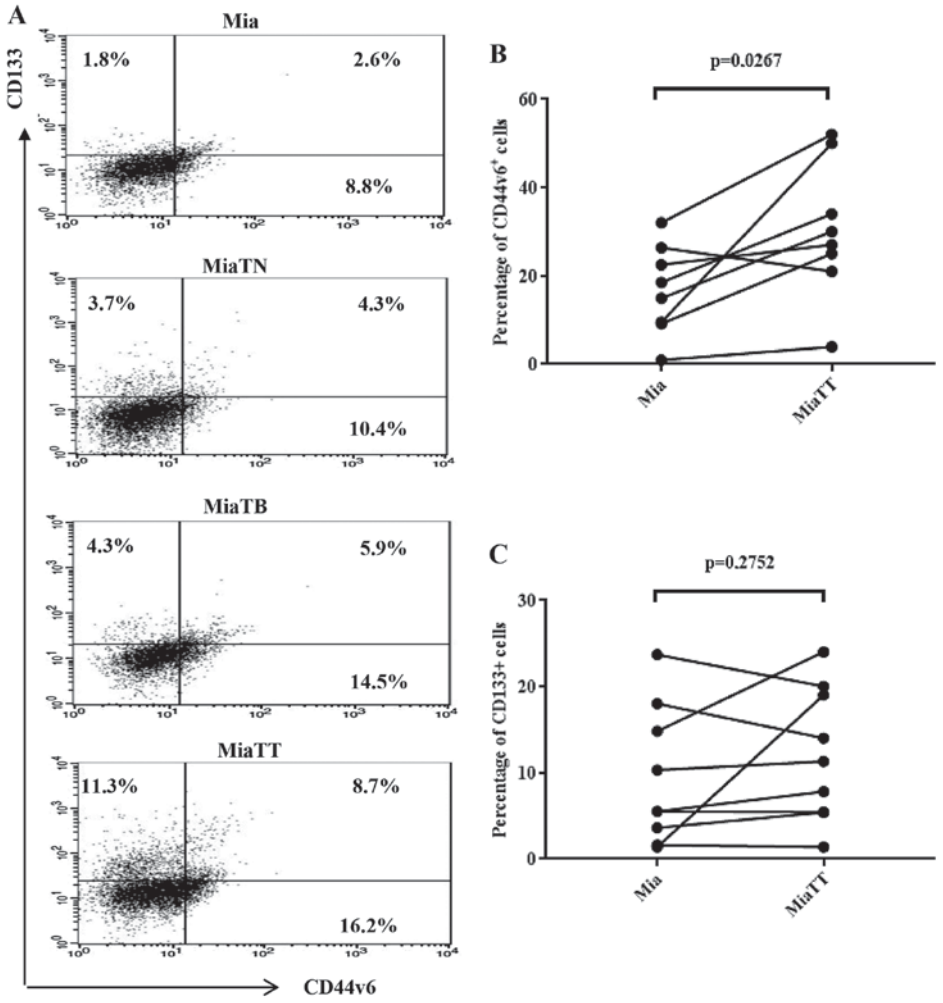

Figure 4. TNFα and TGFβ-1 treatment increase CD44v6 and CD133 and CD44v6/CD133 expressing populations among MiaPaCa-2 cells in sphere forming culture. (A) Flow cytometry dot plots demonstrating a single case of CD44v6 and CD133 and CD44v6/CD133 expressing populations among untreated and cytokine pretreated MiaPaCa-2 cells. Comparison of the proportions of CD44v6 (B) and CD133 (C) expressing cells between Mia and MiaTT cells were shown in the graphs. The experiments were repeated nine times. TNFα, tumor necrosis factor α; TGFβ-1, transforming growth factor β-1.

expressing cells. Likewise, there was no significant change in the proportion of VEGFR1 expressing cells between pMia and pMiaTT cells (Fig. 7B).

TNFα and TGFβ-1 treatment do not promote migration of MiaPaCa-2 cells. In order to confirm our phenotypic data on VEGFR1 at functional level, we performed wound healing assay. Indeed, the migration assay revealed that treatment of MiaPaCa-2 cells with TNF-α and TGFβ-1 neither individually nor combinatorially promoted migration capacity of the cells (data not shown) suggesting TNFα and TGFβ-1 may not bestow a migratory capacity on MiaPaCa-2 cells as shown in other types of cancer cells.

TNFα + TGFβ treatment lead to increased proliferation and daunorubicine resistance, but not gemcitabine. In our study ABCG2 expressing cells were increased upon exposure to TNFα + TGFβ, therefore we carried out cytotoxicity assay in order to examine the phenotypic data. We first chose daunorubicin, because it is one of the substrates of ABCG2 (16). As indicated in Fig. 8B combinatorial treatment significantly (P=0.00407) increased daunorubicin resistance compared to untreated cells. At the same time we also tested the proliferation ability of the cells upon treatment with these cytokines. As we expected combinatorial treatment significantly (P=0.039416) increased the proliferation ability of the cells (Fig. 8A). Then we further tested if this cytokine treatment will also give the cells more resistance to gemcitabine which is a one of the most frequently used chemodrugs in basic research and clinical setting in particular for PC. Surprisingly, the cytokine treatments with both individually and combinatorially did not increase drug resistance to gemcitabine (data not shown).

Pretreatment of MiaPaCa-2 cells with TNFα alone and TNFα + TGFβ promote sphere forming ability. To assess self-renewal capacity of these four subcultures, we performed sphere formation assay. The four subcultures were plated in sphere forming condition, and after 11 to 12 days of incubation the numbers of spheres were counted under an inverted microscope (Fig. 9). Interestingly, the cells pre-treated with TNFα generated spheres with the highest efficiency (P=0.0475578), while the cells pretreated with combination of cytokines (TNFα + TGFβ-1) still had a significant (P=0.0385558) increase in sphere forming ability when compared to untreated

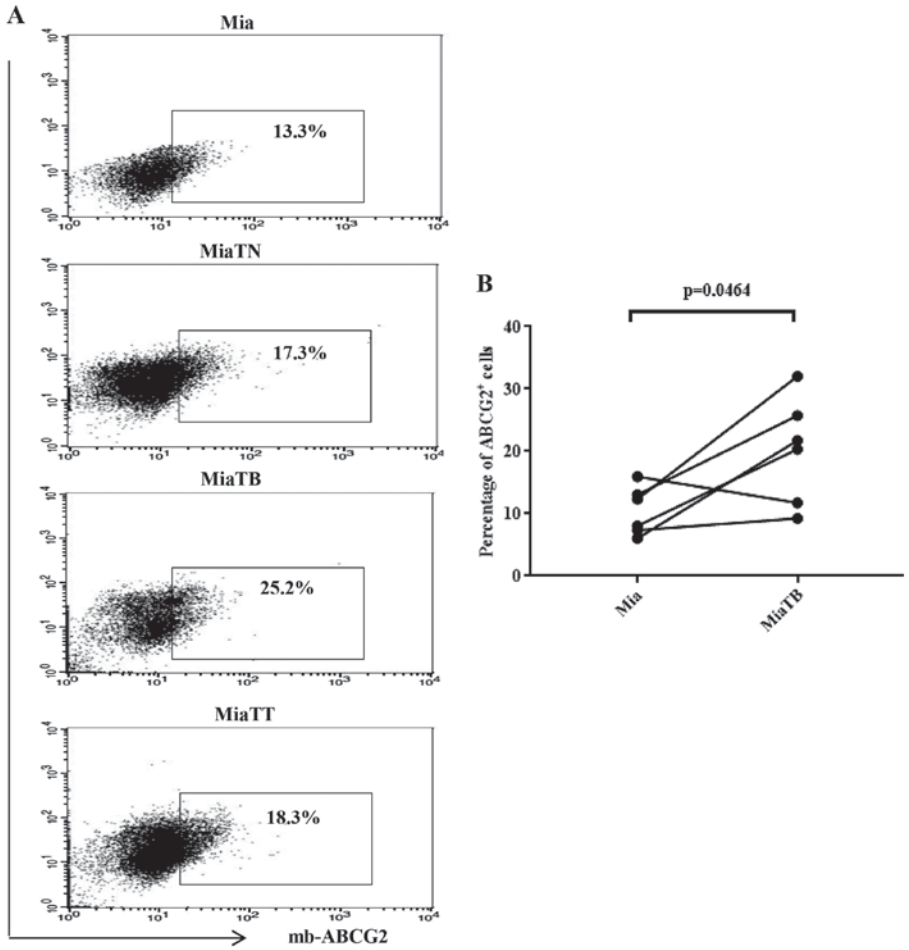

Figure 5. Treatment with TNFα and TGFβ-1 increase ABCG2 expressing population among MiaPaCa-2 cells in sphere forming culture. (A) Flow cytometry dot plots demonstrating a single case of ABCG2 expressing cells among untreated and cytokine pretreated MiaPaCa-2 cells. (B) Comparison of the proportion of ABCG2 expressing cells between Mia and MiaTB cells was shown in the graph. The experiments were repeated six times. TNFα, tumor necrosis factor α; TGFβ-1, transforming growth factor β-1; ABCG2, ATP-binding cassette transporter G2.

cells. TGFβ-1 pretreated cells generated slightly higher number of spheres than untreated parental cells (Fig. 10).

Discussion

Pancreatic CSCs have been defined based on the expression of putative CSCs markers including CD133, CD44, CD24, CXCR4, EpCAM, and ABCG2, among others (7,14,30-32). These distinct cell populations often associated with increased resistance to chemodrugs, anchorage independent growth potential, *in vivo* tumorigenesity and metastatic activity. Although there are still lacks of evidence about the stromal factors that increase the CSC populations in PC, however, the preclinical and clinical studies can provide important clues about the potential candidate factors responsible for augmenting CSC populations in PC.

Elevated levels of TNFα and TGFβ-1 in PC patients' blood and tumor tissues have been found in a number of clinical studies (20). Preclinical studies also constantly demonstrate the involvement of these cytokines in various aspects of cancer cell progression including invasion, migration, and metastasis (17,18). Thus, we aimed to examine whether TNFα and TGFβ-1 have effects on CSC populations expressing above mentioned CSC markers in PC.

Here, we found that TNFα and TGFβ-1 can increase the proportion of CSC populations, which defined by expression of putative CSC markers: CD44v6, CD133, and ABCG2. These increases in CSC populations were seen both in adherent culture and sphere forming culture conditions, in the latter case the cells pretreated with these cytokines.

Tumor-promoting roles of TNFα and TGFβ-1have been shown in numerous works, and recent reports even linked these two cytokines with regulation of CSC properties of breast cancer (33), gilaoblastoma (23), leukemia (24) and PC (34). For example, Wang *et al* revealed that TGFβ-1-induced EMT could increase CD44/CD24 expressing CSC population in PANC-1 cells, another well known PC cell line (34). A quite recent report revealed that TNFα can maintain leukemia initiating cells via autocrine fashion by forming a positive feedback loop with NF-κB (24). The importance of TNFα in PC progression has been well exemplified in a study of Egberts *et al*, in which several PC cell lines (MiaPaCa-2 was not included) acquired invasive properties *in vitro* upon treatment with TNFα, and they further injected these cells into mice and subsequently treated with TNFα, and observed strong enhancement of tumor growth and metastasis. They also showed a reduction of tumor growth and metastasis after inhibition of TNFα with its inhibitors (19). There were also

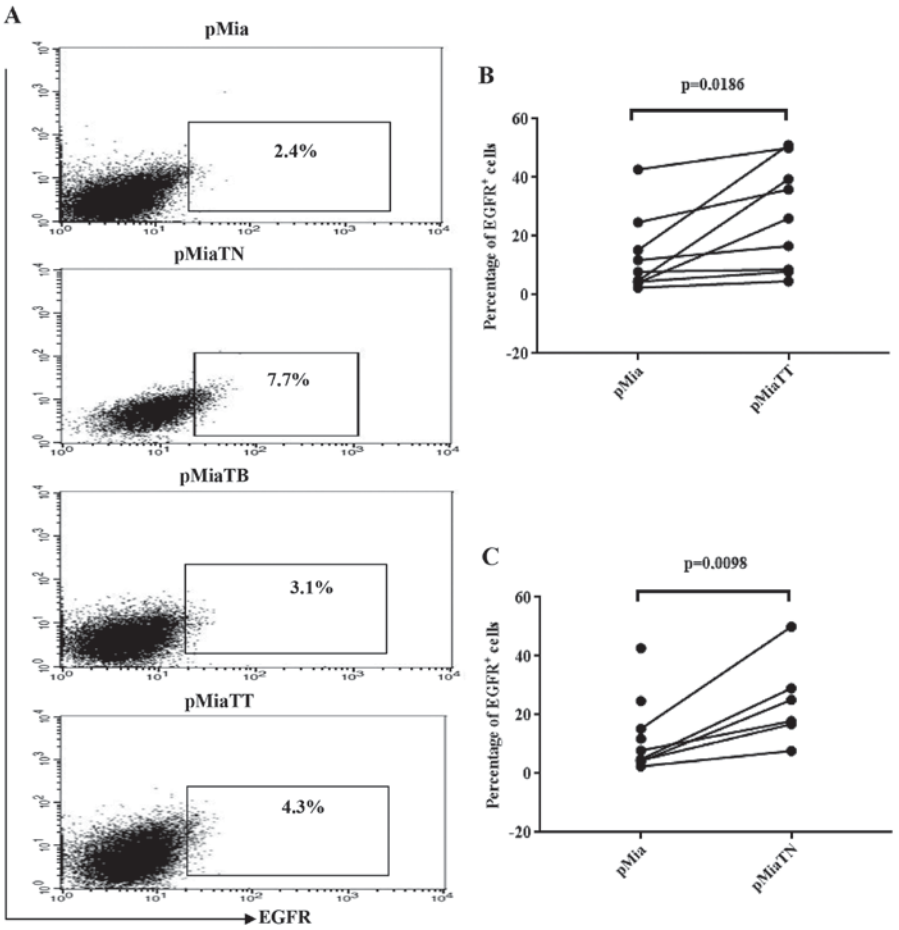

Figure 6. Treatment with TNFα and TGFβ-1 increase EGFR positive cells among MiaPaCa-2 cells in adherent culture. (A) Flow cytometry dot plots demonstrating a single case of EGFR expressing cells among untreated and cytokine treated MiaPaCa-2 cells. (B) Comparison of the proportion of EGFR expressing cells among pMia and pMiaTT cells was shown in the graph. The experiments were repeated nine times. (C) Comparison of the proportion of EGFR expressing cells between pMia and pMiaTN cells was shown in the graph. The experiments were repeated six times. TNFα, tumor necrosis factor α; TGFβ-1, transforming growth factor β-1; EGFR, epidermal growth factor receptor; pMia, untreated parental MiaPaCa-2 cells; pMiaTT, TNFα + TGFβ-1-treated MiaPaCa-2 cells; pMiaTN, TNFα-treated MiaPaCa-2 cells.

few reports showed synergistic effects of TNFα and TGFβ-1 on cancer cell progression (27,35).

To our knowledge this is the first study to show increased properties of CSCs in MiaPaCa-2 cells upon exposure to TNFα and TGFβ-1. These data suggest that cytokines secreted by tumor associated stromal and immune cells have an important role in PC progression through propagating CSC populations within pancreatic tumor mass, it can also explain, at least in part, why there were elevated concentrations of these cytokines in advanced PC patients. However, it remains unknown, in our study, as to why the proportion of smaller and round-shaped cells was increased upon TGFβ-1 treatment. Because it is contrary to most studies in which TGFβ-1 treated cells often exhibit mesenchymal like morphology. For example, in their study Wang *et al* showed that PANC-1 cells acquired mesenchymal morphology upon exposure to TGFβ-1 (34). This may due to the different PC cell lines respond distinctly to TGFβ-1 signaling.

A recent report highlighted the importance of EGFR signaling, in corporation with HH/GLI signaling, for the oncogenic phenotype of basal cell carcinoma and tumor initiating PC cells (36), and in an earlier study overexpression of EGFR at both mRNA and protein level in response to TNFα stimulation has been found (28) suggesting there should be a close relationship between EGFR and CSC properties of PC. Thus, we examined EGFR expressing population using flow cytometry. In consistent with the previous reports, treatment with TNFα substantially increased the proportion of EGFR expressing population among MiaPaCa-2 cells compared to untreated and TGFβ-1 alone and combinatorial treatment.

Because activation of VEGFR1 upon stimulation with VEGF-A and VEGF-B was associated with increased migration and invasiveness of PC cells (29), we examined the effect of TNFα and TGFβ-1 on VEGFR1 expressing MiaPaCa-2 cells. Surprisingly, exposure to TNFα and TGFβ-1 individually and combination of both cytokines only marginally increased the number of VEGFR1 expressing cells, suggesting TNFα and TGFβ-1 are may not implicate in migration and invasion of MiaPaCa-2 cells. To test this assumption we further performed wound healing assay. Indeed, the wound healing assay supported our phenotypic data, as neither cytokine individually or combinatorially promoted migration of MiaPaCa-2 cells. Overall, these findings suggest that TNFα and TGFβ-1 may not contribute directly to invasion and metastasis of MiaPaCa-2 cells. At this point, we should admit that there was a limitation in our experiments regarding migration of

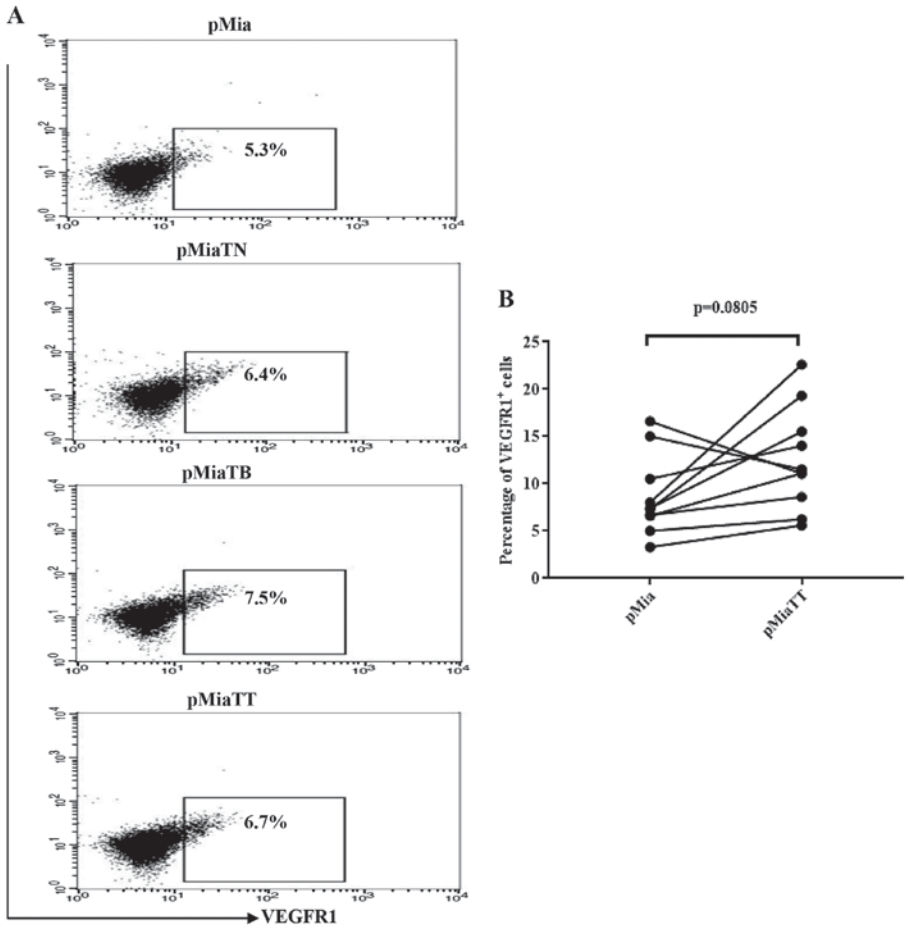

Figure 7. Treatment with TNFα and TGFβ-1 slightly increase the number of VEGFR1 expressing cells among MiaPaCa-2 cells in adherent culture. (A) Flow cytometry dot plots demonstrating a single case of VEGFR1 expressing cells among untreated and cytokine treated MiaPaCa-2 cells. (B) Comparison of the proportion of EGFR expressing cells between pMia and pMiaTT cells was shown in the graph. The experiments were repeated ten times. TNFα, tumor necrosis factor α; TGFβ-1, transforming growth factor β-1; VEGFR1, vascular endothelial growth factor receptor 1; pMia, untreated parental MiaPaCa-2 cells; pMiaTT, TNFα + TGFβ-1-treated MiaPaCa-2 cells.

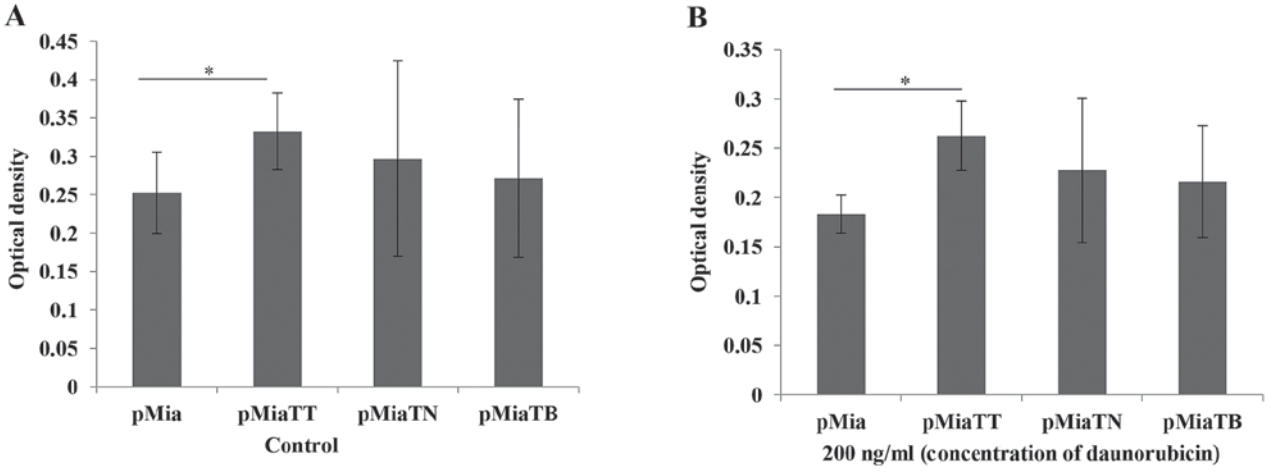

Figure 8. TNFα + TGFβ-1 treatment increases (A) proliferation (P=0.039416) and (B) daunorubicin (P=0.00407) resistance of MiaPaCa-2 cells. The experiments were repeated five times. All data were expressed as mean ± SD. TNFα, tumor necrosis factor α; TGFβ-1, transforming growth factor β-1; SD, standard deviation.

cytokine-treated cells. That is we used very low concentration of TNFα. In many previous cancer works the concentration of TNFα were ranged from 10 to 50 ng/ml. The reason we chose this particular concentration (2 ng/ml) for TNFα was the morphological changes have already occurred in MiaPaCa-2 cells at this concentration in the beginning of the experiments.

The substantially increased proportion of ABCG2 expressing cells among MiaPaCa-2 upon treatment with

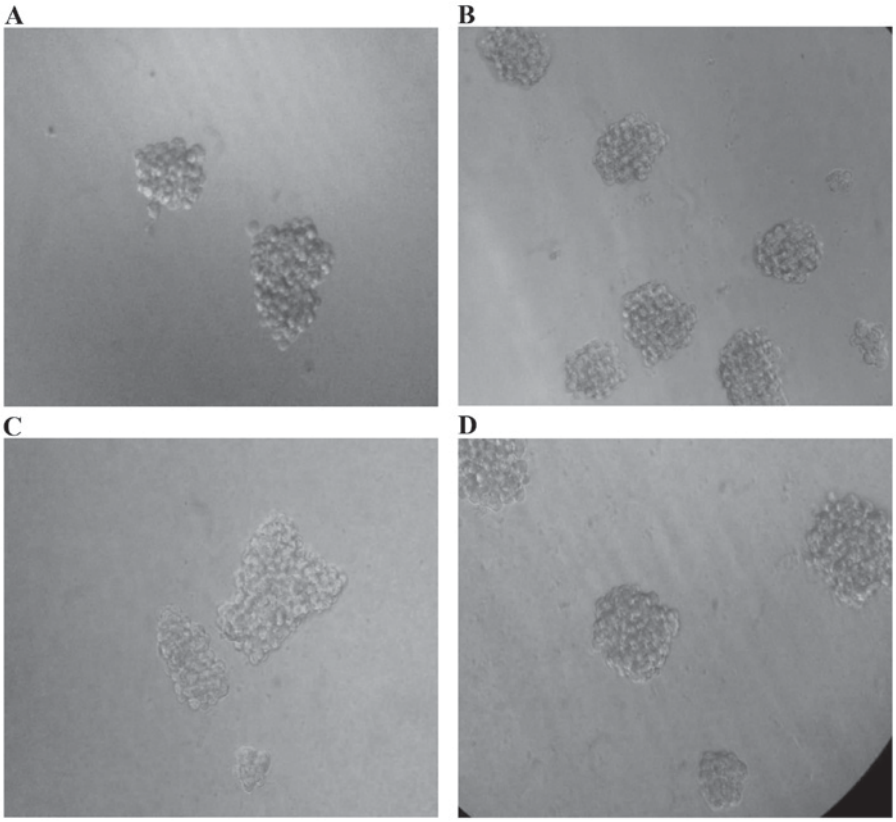

Figure 9. Representative images of tumor spheres derived from MiaPaCa-2 cells and the cells pretreated with TNFα and TGFβ-1 individually and combinatorially. (A) Mia cells, (B) MiaTN cells, (C) MiaTB cells, (D) MiaTT cells. Original magnification, x200. TNFα, tumor necrosis factor α; TGFβ-1, transforming growth factor β-1.

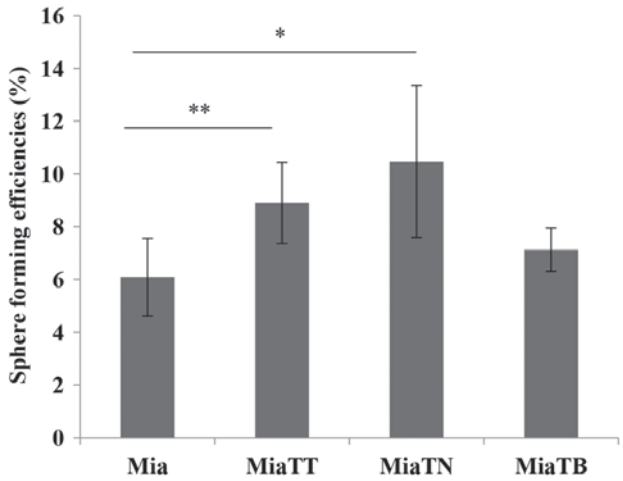

Figure 10. Pretreatment of MiaPaCa-2 cells with TNFα alone and in combination with TGFβ-1 promote sphere forming ability. The experiments were repeated four times. All data were expressed as mean ± SD. *P=0.0475578; **P=0.0385558. SD, standard deviation.

combination of both cytokines gave us an implication that TNFα and TGFβ-1may play a role in drug resistance of the cells. Therefore, we treated the four subcultures with daunorubicin, as it is one of the main substrates of ABCG2 (16). As expected treatment with combination of the cytokines significantly increased daunorubicin resistance. We subsequently tested if the cytokine treated cells also acquired a resistance to gemcitabine, a widely used chemodrug to treat PC and other solid cancers. However, quite unexpectedly, TNFα and TGFβ-1 treatment did not improve gemcitabine resistance of the cells. There are two possible explanations may account for this failure. Firstly, the proportion of the CSC populations in MiaPaCa-2 cells after treatment with these cytokines is still not adequate to show a dramatic change in drug (against gemcitabine) resistance, and MTT assay may not sensible enough to detect the small changes that occurred in the cells. Another explanation is MiaPaCa-2 itself is intrinsically resistant to gemcitabine (37) and there is little room for further increasing drug resistance (against gemcitabine). Our microscopic observations agree with the second explanation, while not denying the first one, because unlike treatment with daunorubicin, which dramatically inhibited proliferation of MiaPaCa-2 cells and led to apoptotic death, gemcitabine treatment exhibited little effect on cell proliferation and led to unremarkable apoptotic death (unpublished observation). At the same time we also showed that the proliferation rate of the cells was significantly increased upon treatment with TNFα + TGFβ-1.

In order to assess our phenotypic data about CSC markers at the functional level, we performed sphere forming assay, and interestingly, the highest sphere forming ability was seen in TNFα pretreated cells, while pretreated with both cytokines still yielded significant number of spheres. The increased number of EGFR expressing cells may explain the reason as to why pretreatment with TNFα increased self-renewal of the cells, despite lower proportion of CD44v6 and CD133 expressing

cells compared to TGFβ-1 and TNFα + TGFβ-1treated cells. It is noteworthy that the self-renewal assay was conducted *in vitro* condition, in which included recombinant EGF, a direct ligand for EGFR, therefore it is not surprising that the cells pretreated with TNFα exhibited the highest sphere forming capacity. Therefore, this finding should be validated through *in vivo* tumorigenesis study in the future.

Since these two cytokines, TNFα and TGFβ-1, have been considered as pleiotropic molecules which are enable to initiate multiple signaling cascades in a cell, so we can assume multiple signaling pathways as candidate mechanisms for these increases of CSC populations in MiaPaCa-2 cells. Among them aberrant NF-κB and STAT3 signalings can be considered as most promising candidate mechanisms, since constitutive activations of NF-κB and STAT3 and their positive feedback relationships with CSC markers such as CD44 and CD133 have been shown in some cancer types including PC (38-40). Furthermore, TNFα is a well known activator of both NF-kB and STAT3, and a recent study showed that TGFβ-1is also enable to activate NF-kB in PC cells (41) suggesting simultaneous presence of TNFα and TGFβ-1 in the tumor microenvironment may further augment the CSC populations of PC through activating those master transcription factors and our data support this hypothesis to some degree as combination of TNFα and TGFβ-1 has increased CSC populations of MiaPaCa-2 cells greater than when used them individually.

In summary, our data provide a mechanistic link between TNFα, TGFβ-1 and CSC properties of PC. This link was demonstrated by synergistically increased CSC populations among MiaPaCa-2 cells upon treatment with TNFα and TGFβ-1 and increased sphere forming ability, and probably ABCG2 dependent drug resistance. These findings highlight the importance of stromal factors during tumor progression and imply a better understanding of the more functional roles and downstream signaling pathways of TNFα and TGFβ-1 in PC will give further insight into underlying mechanisms of PC progression.

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