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Original Article

Therapeutic anti-CD147 antibody sensitizes cells to chemoradiotherapy *via* targeting pancreatic cancer stem cells

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Abstract: We have previously demonstrated that anti-CD44s H4C4 or liposomal-delivered STAT3 inhibitor FLLL32 sensitized pancreatic cancer cells to radiotherapy through the elimination or inhibition of cancer stem cells (CSCs) and that HAb18G/CD147 promoted STAT3-mediated pancreatic tumor development by forming a signaling complex with CD44s. In this paper, we therefore explored whether anti-CD147 HAb18IgG sensitized pancreatic cancer cells to chemoradiotherapy *via* the targeting of CSCs. We tested the influence of HAb18IgG on the sensitivity of pancreatic cancer cells to chemoradiotherapy by clonogenic and MTT assays and on pancreatic CSCs by colony and sphere formation assays, flow cytometry, quantitative real-time RT-PCR (qRT-PCR) and stem cell transcription factors PCR array analysis. Changes in CD147 signaling were examined by immunoblot and reporter assays. We found that HAb18IgG sensitized pancreatic cancer cells to chemoradiotherapy by dose-dependently decreasing colony and sphere formation. Furthermore, HAb18IgG reduced the pancreatic CSC subpopulation and the expression of stem cell transcription factors OCT4, SOX2 and NANOG. Mechanistically, HAb18IgG inhibited CSCs by blocking CD44s-pSTAT3 signaling. The present findings indicated the promising therapeutic role of anti-CD147 HAb18IgG in suppressing pancreatic tumor initiation and overcoming post-chemoradiotherapy recurrence through the direct targeting of CSCs.

Keywords: CD147, antibody, HAb18IgG, pancreatic cancer, CSCs

Introduction

Emerging evidence suggests that cancer stem cells (CSCs), a minor population of cancer cells that display profound chemoradiotherapy resistance, play a critical role in tumor initiation, malignant progression, disease relapse and distant metastasis [1]. CSCs possess the capacity for self-renewal and multi-differentiation similar to stem cells. CSCs are also characterized by slow cell cycle kinetics, efficient disposal of chemotherapeutic agents by drug efflux transporters, increased aldehyde dehydrogenase 1 activity and altered mitochondrial metabolism [2, 3]. Moreover, the microenvironment signals that mediate CSC plasticity accelerate the therapeutic inefficiency and onset of metastasis [4]. In various blood and solid tumors, including pancreatic cancer, the existence of CSCs has been verified [5, 6]. Therefore, the current new therapeutic strategy has shifted towards targeting CSCs.

Compared to regularly proliferating cancer cells, CSCs have unique gene profiles and intracellular constitutions and express specific membrane markers. Therefore, therapeutic strategies targeting CSCs include targeting CSC surface/intrinsic markers or signaling pathways and targeting CSC metabolism or the microenvironment by means of antibodies, aptamers, peptide ligands, small molecules or RNA-based therapeutics [1, 2, 7]. Among these strategies, antibody therapeutics targeting CSC surface markers (CD44, CD47, EpCAM, EGFR, *etc.*) and small molecule inhibitors targeting CSC signaling pathways (STAT3, Notch, Wnt, *etc.*) are the most promising and have already been investigated in clinical trials [1, 2, 7].

CD44 is the most common CSC marker, and three antibody therapeutics against CD44 are currently in phase I clinical trials: anti-CD44v6 labeled with 186Re or conjugated with mertansine [8] and humanized anti-CD44 designed to inhibit CD44-HA interactions [9] or to target a glycosylated epitope [1]. In pancreatic cancer, we have reported that anti-CD44s H4C4 blocked tumor initiation and postradiotherapy recurrence *via* affecting both TICs and bulk tumor cells [10]. Unfortunately, CD44 is also present on normal stem cells and cancer cells, and CD44 has several alternative splicing and post-translational modifications. Moreover, resistance to anti-CD44 therapy was reported in the AML [11].

Recently, the targeting of signaling pathways shared by CSCs and non-CSCs, such as the STAT3 signaling pathway that is particularly hyperactivated in CSCs, has been shown to be effective in killing CSCs and non-CSCs and disrupting the interconversion between the two subpopulations. In our previous study, targeting pancreatic CSCs with STAT3 inhibitor FLLL32 blocked pancreatic tumor formation and overcame radioresistance [12]. In addition, the combination of a STAT3 inhibitor, napabucasin (BBI608), with paclitaxel or the FOLFIRI regimen is under investigation in a phase 3 clinical trial for treating non-small cell lung cancer (NSCLC) or metastatic colorectal carcinoma [1]. As STAT3 is activated by multiple factors, the most effective way to abrogate STAT3 activation could be the blockage of the STAT3 upstream signal. We have identified HAb18G/CD147 as a novel upstream activator of STAT3 signaling *via* interaction with CD44s and thus as a surrogate marker for STAT3-targeted therapies in pancreatic cancer [13].

CD147, also named EMMPRIN or HAb18G/CD147, has been reported to be linked with CSC characteristics, such as epithelial-mesenchymal transition (EMT) [14], anoikis resistance [15] and chemoradiotherapy resistance [16, 17]. Anti-CD147 drug, metuximab (Licartin), has been successfully applied to prevent tumor recurrence of post liver transplantation or radiofrequency ablation in patients with advanced hepatocellular carcinoma [18, 19]. However, the effect of anti-CD147 against pancreatic CSCs remains unclear.

In this paper, we demonstrated that anti-CD147 HAb18IgG sensitized pancreatic cancer cells to chemoradiotherapy by inhibiting the potential of CSCs and suppressing CSC CD44s-pSTAT3 signaling. Our data revealed a potential therapeutic application of the anti-CD147 drug metuximab for fatal and incurable pancreatic cancers.

Materials and methods

Antibodies and drugs

Anti-STAT3 and anti-phospho-STAT3 (Tyr705) were purchased from Cell Signaling Technology (Danvers, MA), and the anti-CD44s clone MEM-263 was purchased from Abnova (Walnut, CA). Goat anti-rabbit horseradish peroxidase (HRP), goat anti-mouse HRP and mouse IgG were purchased from Invitrogen (Carlsbad, CA). WP1066 was obtained from Calbiochem (Billerica, MA), and gemcitabine was obtained from Sigma (St. Louis, MO), genfitinib was obtained from MedChemExpress (Monmouth Junction, NJ). Anti-CD147 HAb18IgG was prepared as reported [13].

Cell culture and treatment

Human pancreatic cancer cell lines (MIA PaCa-2, CFPAC-1, PANC-1 and BxPC3) were purchased from American Type Culture Collection and cultured in DMEM containing 10% fetal bovine serum (HyClone). Cells were treated with 0-20 μg/ml HAb18IgG or mouse IgG (nIgG) and then used for the following experiments: MTT and clonogenic assays, cell growth and colony/sphere formation assays, ALDEFLUOR assay, stem cell transcription factors PCR array and quantitative real-time RT-PCR, immunoblotting assays and STAT3 reporter assay.

MTT assay

A total of 8×10^3 cells were seeded into 96-well plates and cultured for 24 hours. The next day, varying concentrations of gemcitabine and genfitinib with nIgG or HAb18IgG were added to the cells and incubated for 72 hours. The cell viability was determined by measuring the WST-8 dye absorbance at 450 nm and was presented as relative cell viability normalized to the individual nIgG controls. Chemo-sensitivity was expressed as IC_{50} values [12].

Colony formation and clonogenic assays

For the colony formation assay, cells were cultured in DMEM containing 10% FBS with 250

Table 1. The sequences of the oligonucleotide primers used for qRT-PCR

Genes	Primer Sequence (5'-3')
OCT4	Forward: GGGCTCTCCCATGCATTCAAAC
	Reverse: ACCTTCCCTCCAACCAGTTGC
SOX2	Forward: TGGACAGTTACGCGCACAT
	Reverse: CGAGTAGGACATGCTGTAGGT
NANOG	Forward: CCCCAGCCTTTACTCTTCCTA
	Reverse: CCAGGTTGAATTGTTCCAGGTC
GAPDH	Forward: TGATGACATCAAGAAGGTGGTGAAG
	Reverse: TCCTTGGAGGCCATGTGGGCCAT

cells in each well of a 6-well plate for 7-10 days. For the clonogenic assay, different numbers of cells for various doses (200~10,000 cells/well) were subjected to X-ray radiation (0, 2, 4, 6, or 8 Gy) and then incubated for 2-3 weeks in 10% FBS supplemented DMEM. For both assays, the colonies were stained by 0.1% crystal violet and counted (> 50 cells) manually with the aid of an Olympus INT-2 inverted microscope. Data from radiation-treated cells were normalized to the nIgG treated cells. Plating efficiencies and survival fractions were calculated to obtain survival parameters and plot cell survival curves as described [10].

Cell growth assay

Cells were plated at a density of 1×10^5/ml with 2 ml per well in 6-well plates in complete media, collected and counted every 24 hours for 3 days using a haemocytometer. A cell growth curve was drawn according to the cell numbers at the specified incubation time.

Apoptosis assay

Cells were labeled with Annexin V-FITC and propidium iodide (PI) double staining kit (Trevigen, Gaithersburg, MD) and analyzed by a FACSCalibur flow cytometer, and the data were analyzed with CellQuest software (BD Biosciences, San Jose, CA).

Cell cycle analysis

A total of 1×10^6 cells were fixed with 70% cold ethanol at 4°C overnight and incubated in 200 µl of PBS containing 0.5 mg/ml RNase, 0.05% Triton X-100 and 10 µg/ml PI at 37°C for 1 hour, and the data were analyzed using a BD FACSCalibur flow cytometer.

Tumorsphere culture

The tumorsphere culture method and medium were described previously [10]. Briefly, cells were plated in 24-well ultralow attachment plates (Corning) at a concentration of 2,000 cells per well. Ten to 14 days later, spheres ≥50 µm in diameter were counted under a phase-contrast microscope. To quantify sphere size, spheres were collected with a 40 µm sieve (BD Biosciences) and disassociated into a single cell suspension with TrypLE™. Sphere size was then calculated as the number of cells in the total tumorsphere divided by the number of spheres.

ALDEFLUOR assay

The ALDEFLUOR kit (Stem Cell Technologies) was applied to analyze the population with ALDH enzymatic activity [12]. Briefly, cells were incubated in the ALDEFLUOR assay buffer containing ALDH substrate BAAA (1 µM per 1×10^6 cells) at 37°C for 45 min, whereas negative control cells were incubated with 50 mM of ALDH inhibitor diethylamino-benzaldehyde (DEAB) under the same conditions. BAAA-stained cells were analyzed using a FACSCalibur flow cytometer and CellQuest software.

Human stem cell transcription factors PCR array

Total RNA isolated from MIA PaCa-2 cells treated with 10 µg/mL HAb18IgG or nIgG was reverse transcribed into complementary DNA and then analyzed by the real-time RT² Profiler PCR Array (QIAGEN, Cat. no. PAHS-501Z) in combination with RT² SYBR® Green qPCR MasterMix (Cat. no. 330529).

Quantitative real-time RT-PCR (qRT-PCR)

Total RNA extraction and cDNA synthesis were carried out using a method described previously [20]. qRT-PCR was carried out in an ABI 7700® real-time PCR system (Applied Biosystems). The primers used for OCT4, SOX2, NANOG, and GAPDH are listed in **Table 1**. Individual genes of interest (GOIs) were normalized to the housekeeping gene GAPDH. Relative mRNA levels are calculated as $2^{-\Delta Ct} = 2^{-(Ct\ (HKG) - Ct\ (GOI))}$.

Immunoblotting assay

Immunoblotting assay were performed as reported [13]. Proteins in the cellular lysates

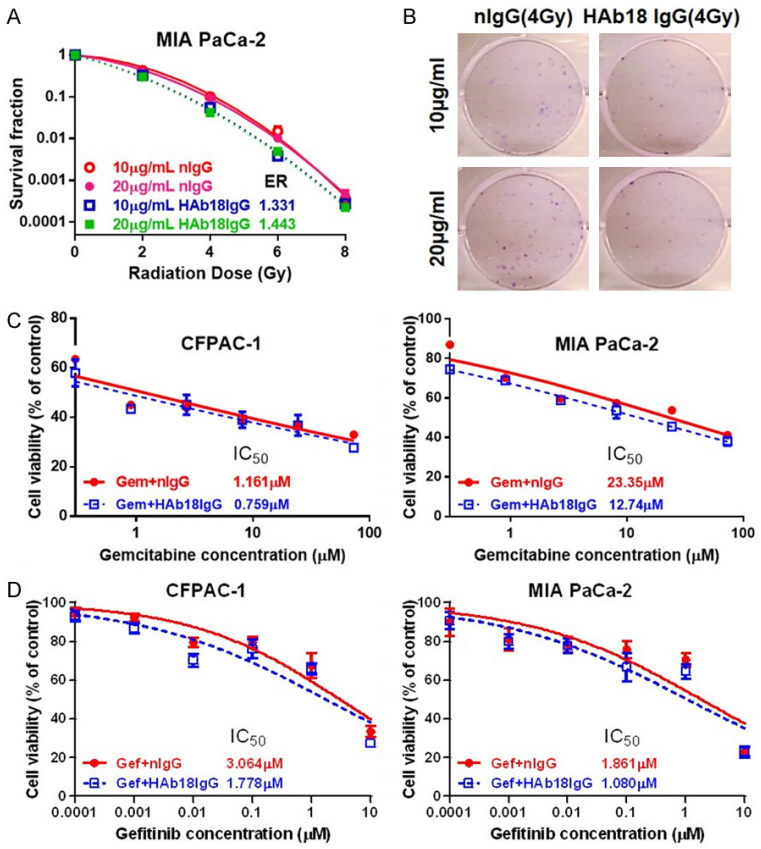

Figure 1. HAb18IgG sensitized pancreatic cancer cells to chemo-/radiotherapy. (A) Clonogenic survival assay of MIA PaCa-2 cells treated with 10 μg/ml or 20 μg/ml HAb18IgG or nIgG. Survival fractions were plotted, and the enhancement ratios (ER) were calculated by HAb18IgG *vs.* nIgG at the corresponding dose. (B) Respective images of MIA PaCa-2 cells treated with 4 Gy radiation combined with or without 10 μg/ml or 20 μg/ml HAb18IgG or nIgG. (C, D) Cytotoxicity of gemcitabine (C, Gem) or EGFR inhibitor genfitinib (D, Gef) in CFPAC-1 and MIA PaCa-2 cells combined with or without 10 μg/ml HAb18IgG or nIgG.

were equalized, and then separated and analyzed by probing with individual antibodies.

STAT3 reporter assay

STAT3 transcriptional activity was detected using the pSTAT3-TA-luc reporter plasmid (Beyotime). Briefly, cells were transfected with 0.5 μg pSTAT3-TA-luc reporter constructs or 0.5 μg of a pGL6-TA vector control using lipofectamine 2000. Sixteen hours after transfection, the cells were treated with 10 μg/mL HAb18IgG or nIgG for 1 hour. Luciferase activity was measured in the cell lysates by an Epoch™ microvolume spectrophotometer (BioTek) using the Firefly Luciferase Reporter Gene Assay Kit (Beyotime).

Statistical analysis

All data are shown as the mean ± SD of triplicate values from three independent experiments. The significance of the differences between groups was determined with *t*-tests and one-way ANOVA using GraphPad Prism 6.0 (GraphPad Software, http://www.graphpad.com). $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ were considered to be statistically significant as indicated.

Results

HAb18IgG sensitized pancreatic cancer cells to chemoradiotherapy

As CD147 has been linked with CSC features such as chemoradiotherapy resistance [13, 17], we first tested whether HAb18IgG, an anti-CD147 antibody, enhanced the growth inhibitory effect of radiation or gemcitabine/genfitinib on pancreatic cancer cells. As shown in clonogenic survival assay, compared to nIgG, HAb18IgG enhanced radiation-induced clonogenic cell death in a dose-dependent manner with a radiation dose enhancement ratio (ER) of 1.331 and 1.443 for 10 μg/mL and 20 μg/mL HAb18IgG, respectively (**Figure 1A**, **1B**). Herein, the ER values are larger than 1.2 [12]; thus, HAb18IgG is considered to be able to sensitize pancreatic cancer cells to radiation. In MTT assay, with the HAb18IgG combinations, the IC_{50} value of gemcitabine greatly decreased by 34.6% and 45.4% in CFPAC-1 and MIA PaCa-2 cells, respectively, suggesting that HAb18IgG sensitized pancreatic cancer cells to gemcitabine (**Figure 1C**). Likewise, EGFR inhibitor genfitinib exhibited a similar extent reduction of IC_{50} values when combined with HAb18IgG and decreased the IC_{50} values both by 41.9% in CFPAC-1 and MIA PaCa-2 (**Figure 1D**). Together, these results demonstrate that HAb18IgG potentially sensi-

tized pancreatic cancer cells to chemoradiotherapy.

HAb18IgG suppressed cellular growth and colony formation

Our previous paper showed that CD147 promotes cellular and clonogenic growth in pancreatic cancer cells [13]. We then explored whether the enhancement effect of HAb18IgG on chemoradiotherapy was due to its inhibition of cellular and clonogenic growth (or colony formation). We found that HAb18IgG significantly decreased the growth of pancreatic cancer cells (**Figure 2A**). In addition, HAb18IgG suppressed the ability for colony formation by dose-dependently reducing the number of clones in MIA PaCa-2 and PANC-1 cells (**Figure 2B**). Thus, HAb18IgG sensitized pancreatic cancer cells to chemoradiotherapy by suppressing cellular growth and colony formation.

Colony formation assay is designed to measure the cellular proliferative capacity and indirectly indicated cell death. To investigate whether the inhibition of HAb18IgG on cellular and clonogenic survival was the result of direct cell death, we examined cell apoptosis in HAb18IgG-treated cells. Unexpectedly, the apoptosis ratio in HAb18IgG-treated cells was almost the same as that in nIgG-treated cells (**Figure 2C**). Furthermore, the unchanged apoptosis level between HAb18IgG- and nIgG-treated cells coincided with a similar percentage of cells in the G_0/G_1 phase or in the S/G_2 phase as determined by cell cycle analysis (**Figure 2D**). Therefore, the ability of HAb18IgG to inhibit colony formation may not derive from the decrease of cell death or cell proliferation in the total population.

HAb18IgG decreased the ratio and potential of CSCs

CSCs are the root of cancers and resisting chemoradiotherapy. Thus, we investigated whether the colony formation inhibition of HAb18IgG originated from its inhibition of CSCs using tumorsphere formation assay. **Figure 3A** and **3B** showed that the number of spheres of HAb18IgG-treated cells was significantly lower than those of nIgG-treated cells and that the average sphere size of HAb18IgG-treated cells was smaller than that of nIgG-treated cells. Moreover, HAb18IgG decreased sphere number and size in a dose-dependent manner, with a maximal decrease at the concentration of 20 μg/mL. The abilities of chemoradiotherapy resistance and of colony and sphere formation are CSC features; thus, HAb18IgG affected the potentials of CSCs.

We further determined the alteration of CSC numbers upon HAb18IgG treatment using the ALDEFLUOR assay. As shown in **Figure 3C** and **3D**, incubation with HAb18IgG significantly reduced the ratio of $ALDH^+$ subpopulations in MIA PaCa-2 (average 54.9% reduction by 10 μg/mL HAb18IgG) and PANC-1 (average 77.6% reduction by 10 μg/mL HAb18IgG) compared to that of nIgG. As HAb18IgG reduced the number and potentials of CSCs, HAb18IgG could be a potential therapeutic agent for eliminating pancreatic CSCs.

To explore potential stem cell transcription factors that would be affected by HAb18IgG, we carried out the PCR array. In total, 27 of the 84 stem cell transcription factors screened (32.1%), including NANOG and STAT1, had altered expression (> 2-fold) after HAb18IgG treatment (**Figure 4A**; **Table 2**). As seen in qRT-PCR validation analysis, HAb18IgG reduced the mRNA levels of NANOG and the level of SOX2/OCT4 in pancreatic cancer cells (**Figure 4B**). As HAb18IgG reduced the level of stem cell transcription factors involving in pluripotent cell maintenance and differentiation, these data indicated HAb18IgG affected the CSCs differentiation.

HAb18IgG eliminated CSCs via CD44s-pSTAT3 signaling

We previously reported that CD147 promoted cellular and clonogenic growth in pancreatic cancer cells *via* CD44s-STAT3 signaling [13]. Additionally, targeting pancreatic CSCs with anti-CD44s H4C4 [10] or STAT3 inhibitor FLLL32 [12] blocked pancreatic tumor formation and overcame radioresistance. To explore whether HAb18IgG eliminated CSCs by inhibiting CD44s-STAT3 signaling, we performed western blotting. We observed that HAb18IgG greatly decreased the protein levels of CD44s, pSTAT3 and STAT3 in pancreatic cancer cells (**Figure 5A**). Furthermore, a functional reporter assay showed that HAb18IgG significantly decreased STAT3 transcriptional activity compared to nIgG, with more inhibition in MIA PaCa-

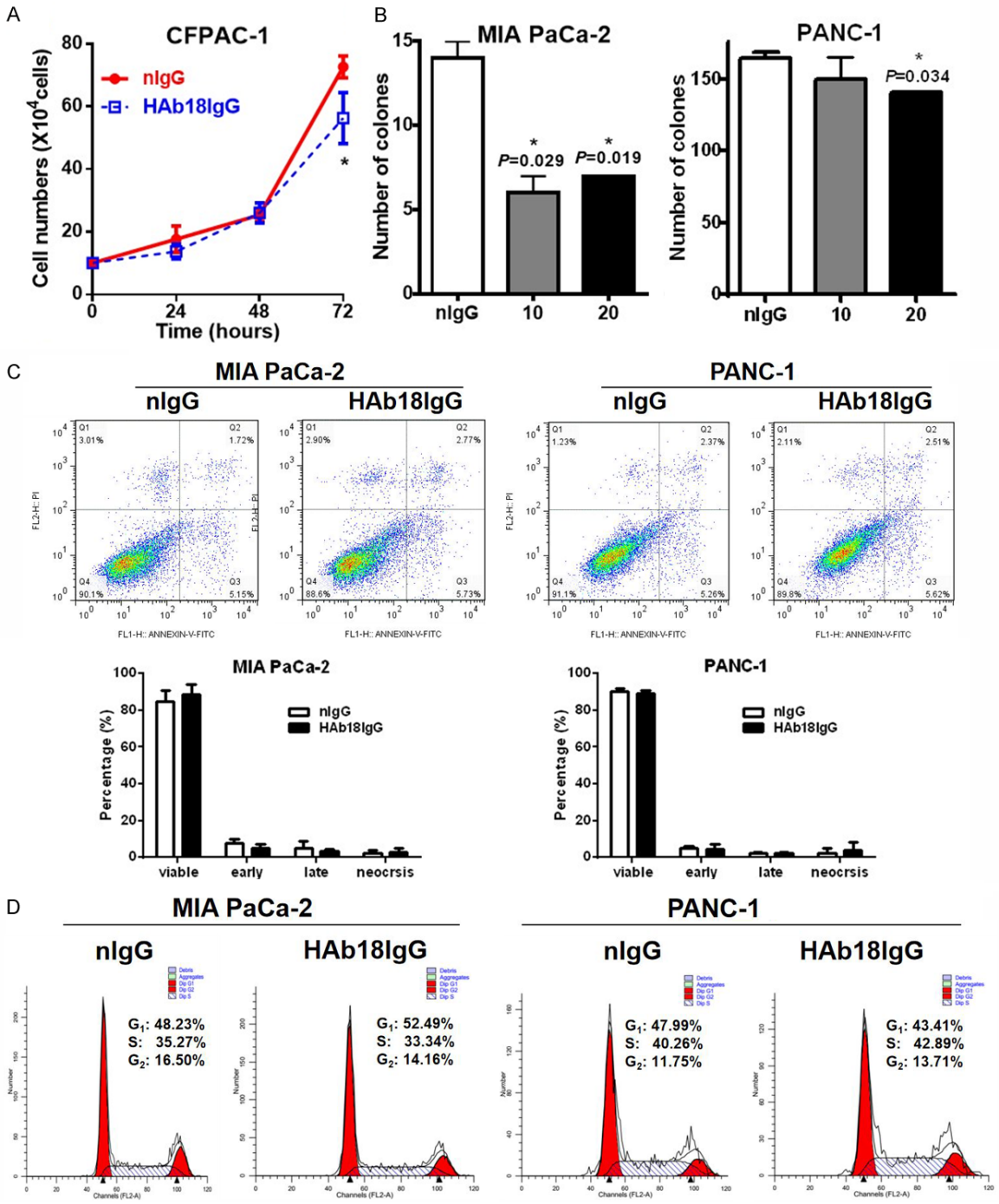

Figure 2. HAb18IgG suppressed cellular growth and colony formation. A. Cellular growth assay in CFPAC-1 cells treated with 10 μg/ml HAb18IgG or nIgG for 3 days. B. Colony formation assay in MIA PaCa-2 and PANC-1 cells treated with 10 μg/ml or 20 μg/ml HAb18IgG or 20 μg/ml nIgG for 14 days. C. Apoptosis analysis in MIA PaCa-2 and PANC-1 cells treated with 10 μg/ml HAb18IgG or nIgG for 2 days. D. Cell cycle analysis in MIA PaCa-2 and PANC-1 cells treated with 10 μg/ml HAb18IgG or nIgG for 2 days.

2 cells (27.3%) with relatively high pSTAT3 expression and less inhibition in PANC-1 (17.9%) with relatively low pSTAT3 signaling (**Figure 5B**).

Finally, we tested whether inhibiting STAT3 signaling could reinforce the inhibitory effect of HAb18IgG on the ratio of pancreatic CSCs. As

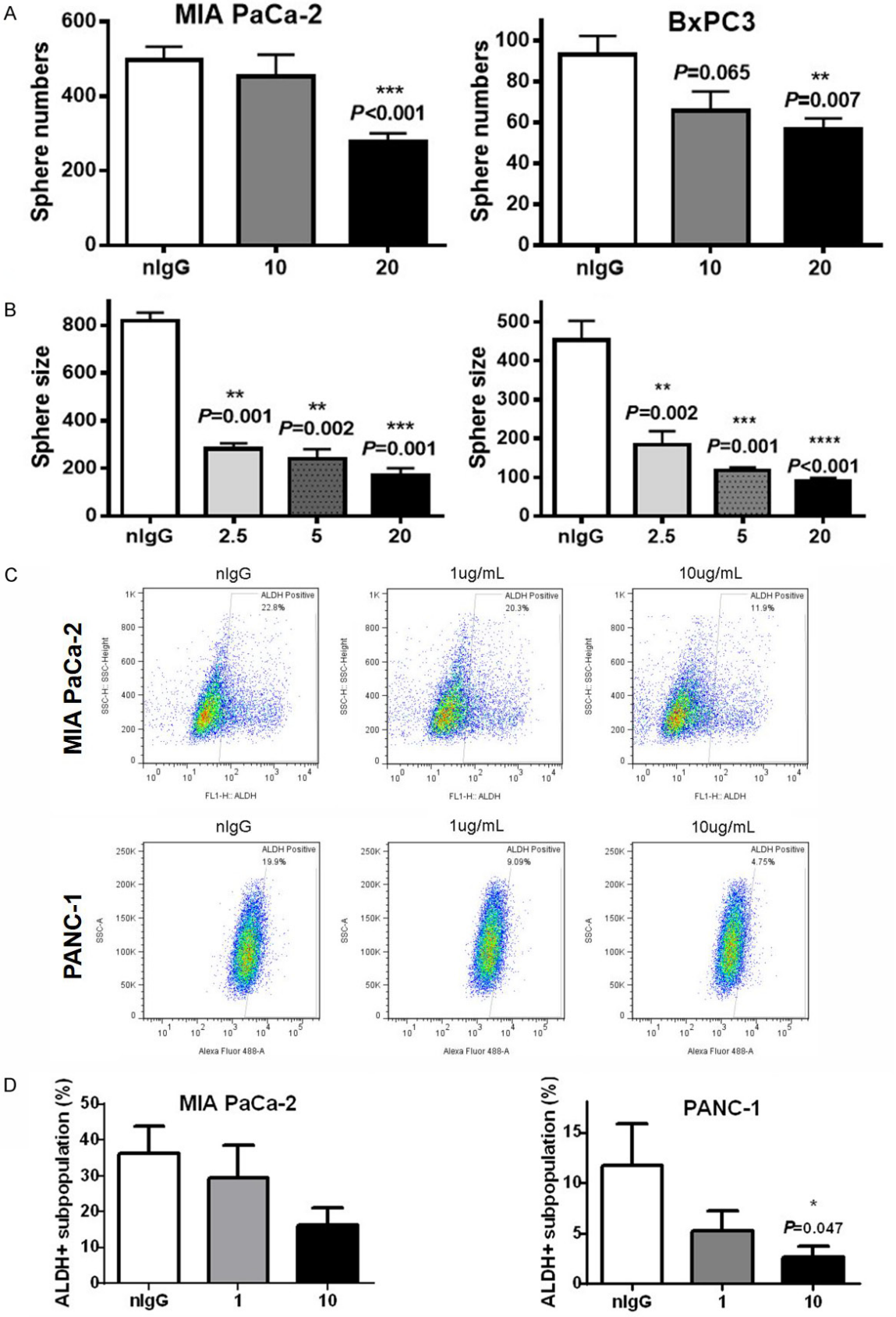
A
MIA PaCa-2
Sphere numbers
600
400
200
0
***
P<0.001
nIgG
10
20
BxPC3
Sphere numbers
100
80
60
40
20
0
P=0.065
**
P=0.007
nIgG
10
20
B
Sphere size
800
600
400
200
0
**
P=0.001
**
P=0.002
***
P=0.001
nIgG
2.5
5
20
Sphere size
500
400
300
200
100
0
**
P=0.002
***
P=0.001
****
P<0.001
nIgG
2.5
5
20
C
nIgG
1ug/mL
10ug/mL
MIA PaCa-2
ALDH Positive
22.8%
20.3%
11.9%
SSC-H: SSC-Height
FL1-H: ALDH
PANC-1
ALDH Positive
19.9%
9.09%
4.75%
SSC-A
Alexa Fluor 488-A
D
MIA PaCa-2
ALDH+ subpopulation (%)
50
40
30
20
10
0
nIgG
1
10
PANC-1
ALDH+ subpopulation (%)
15
10
5
0
*
P=0.047
nIgG
1
10

Figure 3. HAb18IgG decreased the ratio and potential of CSCs. (A, B) Sphere numbers (A) and sphere size (B) in 2.5, 5, 10, or 20 μg/ml HAb18IgG- or 20 μg/ml nIgG-treated cells. (C, D) ALDH$^+$ subpopulation ratios in MIA PaCa-2 and PANC-1 cells treated with 1 μg/ml or 10 μg/ml HAb18IgG or 10 μg/ml nIgG for 2 days.

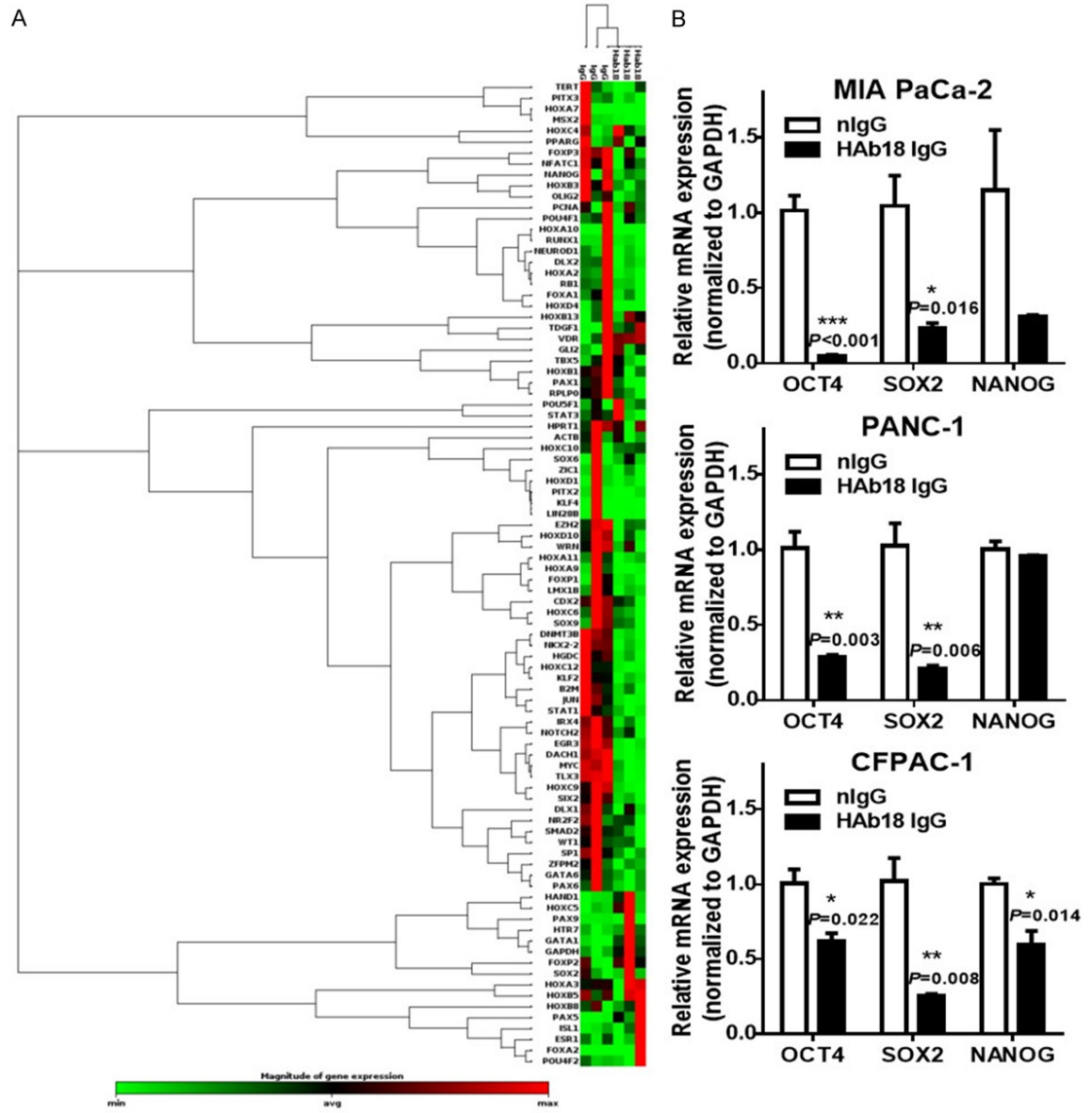

Figure 4. HAb18IgG inhibited the CSC stem cell transcription factors. A. Clustergram of genes expressed in MIA PaCa-2 cells treated with 10 μg/ml HAb18IgG or nIgG for 2 days. Data from non-supervised hierarchical clustering of the entire dataset to display a heat map with dendrograms indicating co-regulated genes across groups or individual samples. Changes in expression of individual genes are shown in **Table 2**. B. OCT4, SOX2 and NANOG mRNA expression in MIA PaCa-2, PANC-1 and CFPAC-1 cells treated with 10 μg/ml HAb18IgG or nIgG for 2 days. Levels were normalized to that of GAPDH.

shown in **Figure 5C** & **5D**, WP1066, a specific STAT3 inhibitor that decreased the ratio of ALDH$^+$ CSC subpopulations in MIA PaCa-2 alone, greatly enhanced this inhibition ratio from 23.13% in cells treated with HAb18IgG alone to 41.34% in cells treated with HAb18IgG plus WP1066. Similarly, WP1066 reinforced HAb18IgG-mediated the reduction of CSC subpopulations in PANC-1 cells, with an inhibition ratio that increased from 69.67% to 80.95%. Furthermore, STAT3 inhibition by WP1066 phenocopied the inhibitory effect of HAb18IgG into

Table 2. Human stem cell transcription factors affected by HAb18IgG (QIAGEN RT² Profiler™ PCR Array, Cat. no. PAHS-501Z)

Position	Gene Symbol	Fold Regulation	*p*-Value
A1	CDX2	-3.54	0.023853
A2	DACH1	-20.51	0.000957
A5	DNMT3B	-4.56	0.001173
A6	EGR3	-10.65	0.002894
A8	EZH2	-3.55	0.031319
B1	FOXP3	-2.73	0.000197
B2	GATA1	11.40	0.015772
B12	HOXB1	-3.28	0.022902
C2	HOXB3	-4.25	0.003706
C6	HOXC12	-6.05	0.008023
C10	HOXC9	-8.05	0.012903
C12	HOXD10	-4.35	0.028549
D3	IRX4	-7.12	0.012293
D5	JUN	-2.19	0.009078
D6	KLF2	-4.04	0.003574
D11	MYC	-5.23	0.000359
D12	NANOG	-2.47	0.044222
E2	NFATC1	-2.95	0.000693
E3	NKX2-2	-6.07	0.000268
E4	NOTCH2	-3.29	0.011823
E6	OLIG2	-5.35	0.018769
E7	PAX1	-6.00	0.021686
F8	SIX2	-14.29	0.026751
G1	SP1	-2.96	0.029264
G2	STAT1	-2.65	0.008213
G7	TLX3	-7.23	0.000739
G9	WRN	-2.36	0.021091

sphere formation and exhibited a significant decrease in the number of spheres compared with HAb18IgG alone (**Figure 5E**) (P=0.0009 and P=0.0004 for MIA PaCa-2 and PANC-1 cells, respectively). These results indicated that HAb18IgG eliminated pancreatic CSCs *via* the inhibition of CD44s-STAT3 signaling and STAT3 transcriptional activity.

Discussion

In this study, we showed that anti-CD147 HAb18IgG sensitized pancreatic cancer cells to chemoradiotherapy. Furthermore, HAb18IgG decreased colony and sphere formation and reduced CSC subpopulations and potentials by blocking CD44s-pSTAT3 signaling. Our data revealed a promising therapeutic role of HAb18IgG in suppressing pancreatic tumor initiation and overcoming post-chemoradiotherapy recurrence through the direct targeting of CSCs. Our study could widen the therapeutic application of anti-CD147 for fatal and incurable pancreatic cancer.

Due to its important roles in regulating CSC features and in promoting pSTAT3-mediated pancreatic tumor development, CD147 is an attractive therapeutic target. In our lab, anti-CD147 drug successfully prevented tumor recurrence post liver transplantation or radiofrequency ablation (metuximab) [18, 19] and was demonstrated to enhance chemosensitivity in NSCLC (matuzumab) [21]. Although one study showed that the combined treatment with anti-EMMPRIN and gemcitabine had an antagonistic effect in hypovascular tumors [22], the other two studies supported that the combination of anti-EMMPRIN or ^{90}Y-labeled 059-053 (fully human anti-CD147) with gemcitabine is a promising therapeutic option for pancreatic cancer [23, 24]. In addition, the combination of anti-EMMPRIN and anti-DR5 showed an additive effect on tumor suppression [25]. These studies indicated the therapeutic potential of anti-CD147 in cancer. However, the therapeutic potential of anti-CD147 was not investigated in pancreatic CSCs. In this paper, we demonstrated the therapeutic role of anti-CD147 in pancreatic cancer by targeting CSCs for the first time. Our study provided further supporting evidence for anti-CD147 sensitizing pancreatic cancer cells to chemoradiotherapy *via* targeting of CSCs.

Targeting CSCs can be done through antibodies, aptamers and peptide ligands as well as small molecules, RNA-based therapeutics and CRISPR/Cas9 technology [2]. In comparisons, antibodies have shown good targeting specificity, affinity, and preferable therapeutic efficiency with minimal toxicity, while the peptide, short interfering RNA or microRNA approaches have delivery problems. Therefore, antibody therapeutic agents are powerful with clinically proven therapeutic value and a leading product within the biopharmaceutical market [26]. Unfortunately, the number of clinical trials targeting CSCs is quite low. Among the 86 clinical trials targeting CSCs, only 12 use antibodies as therapeutic agents [27]. A key reason for the low rate of using antibodies, apart from the lack of specific markers, is the plasticity of the

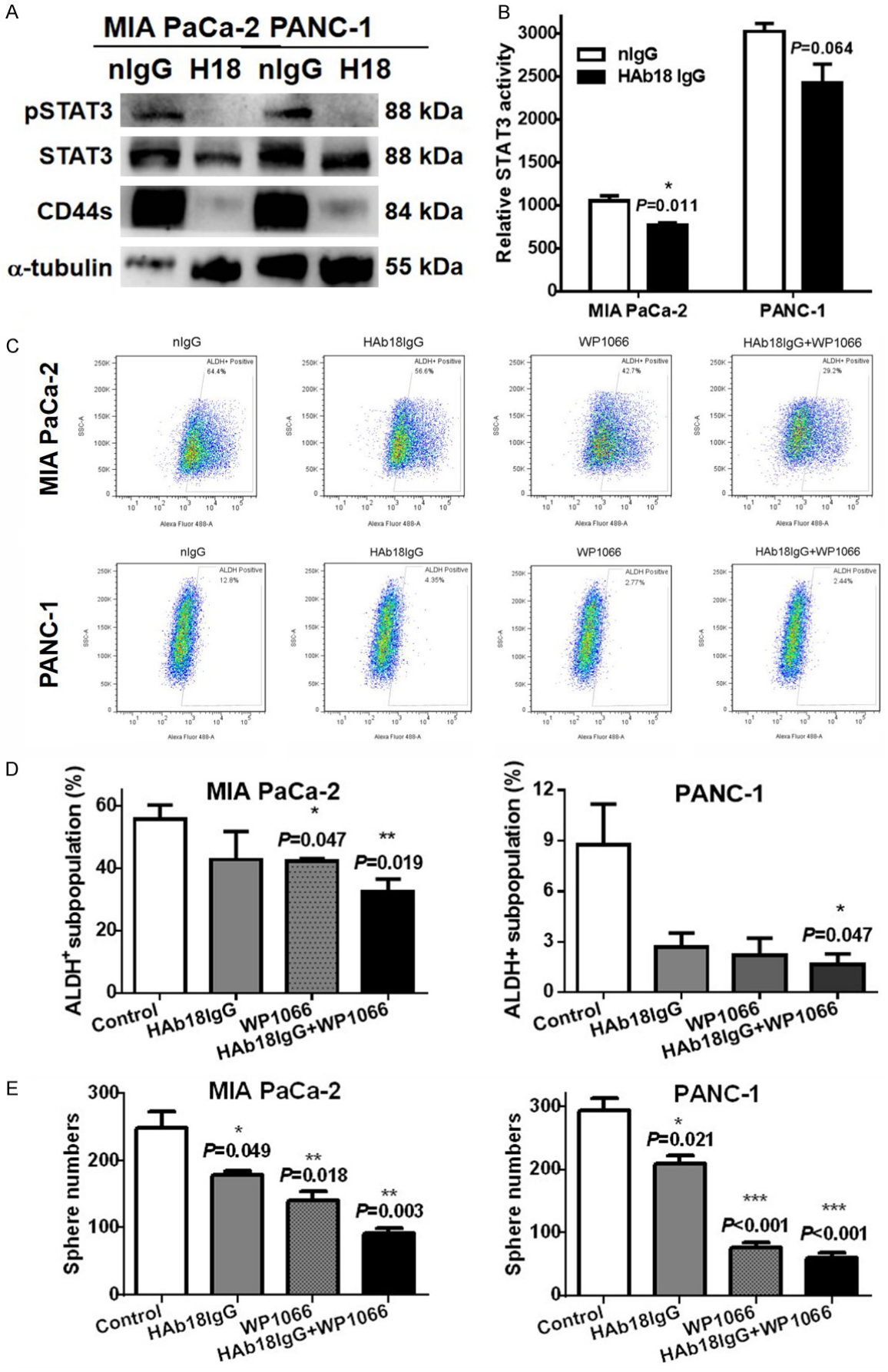
A
MIA PaCa-2 PANC-1
nIgG H18 nIgG H18
pSTAT3
STAT3
CD44s
α-tubulin
88 kDa
88 kDa
84 kDa
55 kDa
B
Relative STAT3 activity
nIgG
HAb18 IgG
P=0.064
*
P=0.011
MIA PaCa-2
PANC-1
C
nIgG
HAb18IgG
WP1066
HAb18IgG+WP1066
MIA PaCa-2
PANC-1
ALDH+ Positive
64.4%
56.6%
42.7%
29.2%
12.8%
4.35%
2.77%
2.44%
SSC-A
Alexa Fluor 488-A
D
MIA PaCa-2
ALDH+ subpopulation (%)
*
P=0.047
**
P=0.019
PANC-1
*
P=0.047
Control
HAb18IgG
WP1066
HAb18IgG+WP1066
E
MIA PaCa-2
Sphere numbers
*
P=0.049
**
P=0.018
**
P=0.003
PANC-1
*
P=0.021
***
P<0.001
***
P<0.001

Figure 5. HAb18IgG eliminated CSCs *via* CD44s-STAT3 signaling. (A, B) CD44s, pSTAT3 and STAT3 protein levels (A) or relative STAT3 transcriptional activity (B) in MIA PaCa-2 and PANC-1 cells treated with 10 μg/ml HAb18IgG(H18) or nIgG for 2 days. (C-E) $ALDH^+$ subpopulation ratios (C, D) or number of spheres (E) in MIA PaCa-2 and PANC-1 cells treated with HAb18IgG alone or in combination with STAT3 inhibitor WP1066 (5 μM).

CSCs, which is the transition of non-CSCs into CSCs [28]. Thus, antibodies that both recognize the surface antigen and have a functional influence on CSCs, such as the blockage of CSC plasticity transition, are urgently required. In addition to its linkage to CSC features, CD147 was recently found by us to promote the detachment-induced transversion of non-CSC into CSCs (data not shown). Then, the therapeutic effect of anti-CD147 is due not only to the direct elimination of CSCs but also to blocking the acquiring of the CSC properties.

Due to the heterogeneous of tumor and insufficient delivery, most antibodies alone are not necessarily curative. Therefore, an antibody-based strategy targeting CSCs, along with other chemotherapeutic drugs for the bulk of tumor cells, might be the best possible way to improve the outcome. In this paper, the therapeutic effects of chemoradiotherapy were improved further when the chemoradiotherapy agents were combined with HAb18IgG, although this result needs to be further studied before clinical application. Ideally, maintenance therapy with antibodies targeting CSCs when the residual tumor bulk is minimal would be the way forward in the long term.

In summary, we showed that anti-CD147 HAb18IgG sensitized pancreatic cancer cells to chemoradiotherapy by inhibiting the potential of CSCs and suppressing CD44s-pSTAT3 signaling. For application, HAb18IgG may be a promising therapeutic agent to suppress pancreatic tumor initiation and sensitize CSCs to chemoradiotherapy.

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Disclosure of conflict of interest

None.

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