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Pancreatic stellate cells enhance stem cell-like phenotypes in pancreatic cancer cells

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ABSTRACT

The interaction between pancreatic cancer cells and pancreatic stellate cells (PSCs), a major profibrogenic cell type in the pancreas, is receiving increasing attention. There is accumulating evidence that PSCs promote the progression of pancreatic cancer by increasing cancer cell proliferation and invasion as well as by protecting them from radiation- and gemcitabine-induced apoptosis. Recent studies have identified that a portion of cancer cells, called "cancer stem cells", within the entire cancer tissue harbor highly tumorigenic and chemo-resistant phenotypes, which lead to the recurrence after surgery or re-growth of the tumor. The mechanisms that maintain the "stemness" of these cells remain largely unknown. We hypothesized that PSCs might enhance the cancer stem cell-like phenotypes in pancreatic cancer cells. Indirect co-culture of pancreatic cancer cells with PSCs enhanced the spheroid-forming ability of cancer cells and induced the expression of cancer stem cell-related genes *ABCG2*, *Nestin* and *LIN28*. In addition, co-injection of PSCs enhanced tumorigenicity of pancreatic cancer cells *in vivo*. These results suggested a novel role of PSCs as a part of the cancer stem cell niche.

1. Introduction

Pancreatic ductal adenocarcinoma is characterized by a highly malignant phenotype such as rapid progression, early metastasis, and a limited response to chemotherapy and radiotherapy [1–3]. The abundant desmoplastic/stromal reaction is a characteristic feature of the majority of pancreatic cancers [4–8]. It has been recognized that the cells responsible for production of the desmoplastic reaction in pancreatic cancer are pancreatic stellate cells (PSCs) [4–10]. In normal pancreas, PSCs are quiescent and can be identified by the presence of vitamin A-containing lipid droplets in the cytoplasm. In response to pancreatic injury or inflammation, they are transformed ("activated") from their quiescent phenotype into myofibroblast-like cells, which express α-smooth muscle actin, actively proliferate, and produce extracellular matrix components such as type I collagen [9–12]. Over a decade, there is accumulating evidence that activated PSCs play a pivotal role in the development of pancreatic fibrosis in chronic pancreatitis and pancreatic cancer [4–12].

Emerging evidence has suggested that the capability of a tumor to grow and propagate is dependent on a small subset of cells within a tumor, termed cancer stem cells (CSCs) [13–16]. This CSC concept was established as a counterpart of normal tissue stem cells, which are indispensable for the normal tissue maintenance. The current consensus definition describes a CSC as a cell within a tumor that is able to self-renew and to produce the heterogeneous lineages of cancer cells that comprise the tumor [13–17]. Similar to the normal tissue stem cells, the existence of CSC niche was anticipated together with the tumor-specific tissue structures [13–15]. The subpopulations with $CD44^+CD24^+$epithelial-specific antigen (epithelial cellular adhesion molecule)$^+$ [16] or $CD133^+$ [17] properties in pancreatic cancer have been recognized as putative CSCs, because they can both self-renew and produce differentiated progeny. CSCs are highly tumorigenic, metastatic, and more resistant to conventional therapies [14–17]. Previous studies have shown that PSCs play critical roles in the progression of pancreatic cancer [4–8]. PSCs increased the proliferation and migration of pancreatic cancer cells, and protected them from gemcitabine- or radiation-induced apoptosis [4–8]. In addition, PSCs increased the metastasis of pancreatic cancer cells in an orthotopic model

Abbreviations: CSC, cancer stem cell; EMT, epithelial-to-mesenchymal transition; PSCs, pancreatic stellate cells; SV40, simian virus 40; hTERT, human telomerase reverse transcriptase.

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[6–8], suggesting that PSCs might serve as a part of the CSC niche. To address this issue, we here examined the effects of indirect co-culture with PSCs on the CSC-like phenotypes in pancreatic cancer cells *in vitro*, and co-injection of PSCs with pancreatic cancer cells in subcutaneous transplantation model mice.

2. Materials and methods

2.1. Cell line and cell culture

The human pancreatic cancer cell lines AsPC-1 and SUIT-2 [18] were obtained from American Type Culture Collection (Manassas, VA, USA) and Japanese Collection of Research Bioresources (Ibaraki, Osaka, Japan), respectively. Cells were maintained in DMEM supplemented with 10% heat-inactivated FBS, penicillin sodium, and streptomycin sulfate.

The immortalized human PSC line hPSC21-S/T was established by retrovirus-mediated gene transfer of simian virus 40 (SV40) T antigen and human telomerase reverse transcriptase (hTERT) into the human PSCs isolated from the resected pancreas tissue of a patient undergoing operation for pancreatic cancer, as previously described [19–21]. The retrovirus expression vector pMFGtsT [20] contains the partial coding region (~260 bp) of the SV40 T antigen. The retroviral packaging cells, Ψ-CRIP-MFGtsT, were grown to subconfluence, and human PSCs were treated for 12 h with the diluted culture supernatant of ψ-CRIP-MFGtsT cells in the presence of PDGF-BB (R&D Systems, Minneapolis, MN; at 25 ng/ml) and polybrene at 10 μg/ml. The gene transfer of hTERT was performed in a similar manner using the retrovirus vector pBABE-hygro-hTERT and the amphotropic packaging cell line ψ-CRIP-P131 [21]. Cells were maintained in Ham's F-12/DMEM (1:1) supplemented with 10% FBS, penicillin sodium, and streptomycin sulfate. This study was approved by the Ethics Committee of Tohoku University School of Medicine.

2.2. Indirect co-culture of pancreatic cancer cells and PSCs

Pancreatic cancer cells (1×10^5 cells/well) were seeded in 6-well culture plates (BD Biosciences, Bedford, MA) in DMEM supplemented with 10% FBS, penicillin sodium, and streptomycin sulfate. The hPSC21-S/T cells (1×10^5 cells/culture insert) were seeded into the culture inserts with 1.0 μm pore size (BD Bioscience) in Ham's F-12/DMEM (1:1) supplemented with 10% FBS, penicillin sodium, and streptomycin sulfate. The next day, the culture insets containing hPSC21-S/T cells were placed into the 6-well plates containing pancreatic cancer cells, and incubation was continued up to 3 days in DMEM supplemented with 10% FBS, penicillin sodium and streptomycin sulfate.

2.3. Sphere formation assay

Spheroid culture of AsPC-1 or SUIT-2 cells was performed as previously described [22]. Briefly, pancreatic cancer cells were seeded at a density of 500 cells/well in low-adhesion coated EZ-BindShut II (ASAHI TECHNO GLASS CORP, Tokyo, Japan) 6-well plates in serum-free medium supplemented with 10 ng/ml FGF (Life Technologies), 20 ng/ml EGF (Life Technologies) and 2.75 ng/ml selenium (Insulin-Transferrin-Selenium Supplement; Life Technologies). The next day, the culture insets containing hPSC21-S/T cells were placed into the 6-well plates containing pancreatic cancer cells. After 7 days, the number of formed spheroids was counted in randomly chosen 5 high power fields (×100 magnification).

Table 1
Sequences of the PCR primer sets.

Target	Forward primer (5′–3′)	Reverse primer (5′–3′)
ABCG2	AGATGGGTTTCCAAGCGTTCAT	CCAGTCCCAGTACGACTGTGACA
Snail1	TTCCAGCAGCCCTACGACCAG	GCCTTTCCCACTGTCCTCATC
Nestin	GGCGCACCTCAAGATGTCC	CTTGGGGTCCTGAAAGCTG
LIN28	GGTGAGTCAAAGGAACTCCAAC	TAATCATTTCACTCTGGCATGG
GAPDH	GGCGTCTTCACCACCATGGAG	AAGTTGTCATGGATGACCTTGGC

2.4. Cell growth assay

Cellular growth of AsPC-1 or SUIT-2 with or without PSCs was evaluated by direct cell counting. AsPC-1 or SUIT-2 cells (2×10^4 cells/well) were plated in 6-well plates and mono-cultured or co-cultured with hPSC21-S/T cells as described before. After 48 and 96 h, the cells were trypsinized and counted.

2.5. RNA extraction and real-time RT-PCR

Total cellular RNA was extracted from AsPC-1 or SUIT-2 cells using the mirVana™ miRNA Isolation Kit (Life Technologies, Carlsbad, CA). The real-time RT-PCR was performed using High-capacity RNA-to-cDNA™ Master Mix (Life Technologies), Fast SYBR Green Master Mix (Life Technologies) and StepOnePlus™ real-time PCR system (Life Technologies) according to the manufacturer's instructions. The primer sequences are shown in Table 1. The levels of *ABCG2*, *Snail*, *Nestin*, and *LIN28* expression in each sample were normalized by the respective, level of *GAPDH* expression, level as described before.

2.6. Subcutaneous transplantation of AsPC-1 cells with or without PSCs

Tumor formation *in vivo* was assessed in male athymic nude mice (ICR-nu) by subcutaneously injecting AsPC-1 cells (1×10^6 cells) with or without hPSC21-S/T cells (1×10^6 cells) suspended in 100 μl of sterile PBS. The tumor volume was measured every 7 days up to 3 weeks, and the volume of a tumor was calculated using the formula $V\,(\mathrm{mm}^3) = (L \times W^2)/2$ where L and W indicate the length and width of a tumor, respectively. All animal procedures were performed in accordance with National Institutes of Health Animal Care and Use guidelines.

2.7. Statistical analysis

The results were expressed as mean ± SE. Experiments were performed at least three times and similar results were obtained. Statistical analysis was performed using JMP (SAS Institute, Cary, NC). The differences between two groups were analyzed by Student's *t*-test. A p value less than 0.05 was considered statistically significant.

3. Results and discussion

3.1. Pancreatic cancer cells co-cultured with PSCs showed fibroblastic morphology

The human PSC line, hPSC21-S/T, immortalized with the retrovirus-mediated gene transfer of hTERT and SV40 large T antigen, expressed typical activation markers including α-smooth muscle actin, vimentin, type I collagen, and fibronectin (data not shown). The pancreatic cancer cells were mono-cultured or indirectly co-cultured with hPSC21-S/T cells, and the morphological alterations were examined under phase-contrast microscopy. Although mono-cultured cancer cells grew in monolayer cultures as epithelial

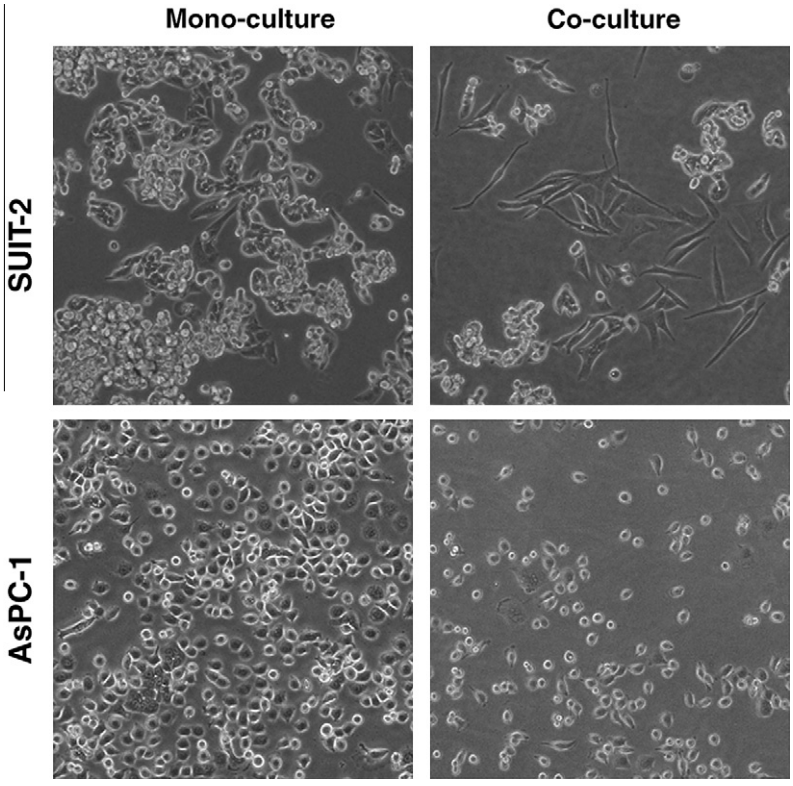

Fig. 1. Pancreatic cancer cells co-cultured with PSCs showed fibroblastic morphology. AsPC-1 or Suit-2 cells were mono-cultured or indirectly co-cultured with hPSC21-S/T cells for 72 h. The morphological alterations were examined under phase-contrast microscopy. Original magnification: ×200.

clusters, cells co-cultured with hPSC21-S/T cells showed loss of cell-to-cell contracts, spindle fibroblastic morphology, and cell scattering (Fig. 1). In addition, co-culture with hPSC21-S/T cells increased the expression of the mesenchymal marker vimentin but decreased that of the epithelial markers E-cadherin and cytokeratin19 in pancreatic cancer cells (data not shown). These results suggested that hPSC21-S/T cells promoted EMT in AsPC-1 and SUIT-2 pancreatic cancer cells [23]. Emerging evidence suggests that the acquisition of the EMT phenotype and the induction of CSC or a cancer stem-like cell phenotype are directly linked [24–26]. For example, forced overexpression of Notch-1 led to the acquisition of the EMT phenotype, resulting in increased capacity of CSC-self-renewal consistent with the up-regulation of CD44 and epithelial-specific antigen expression [24]. The induction of EMT by ectopic expression of either the Twist or Snail transcription factors generated stem cell-like cells [26].

3.2. Co-culture of pancreatic cancer cells with PSCs enhances spheroid-forming ability of pancreatic cancer cells

To examine the effect of PSCs on the CSC-related functions, we set up a spheroid culture. Pancreatic cancer cells were plated in stem cell-conditioned culture medium in 6-well plates at a density of 500 cells/well, which enabled the formation of colonies separated from each other. In this condition, cells grew as non-adherent, three-dimensional sphere clusters, called spheres. As shown in Fig. 2A, B, co-culture of AsPC-1 or SUIT-2 cells with hPSC21-S/T cells increased the number of formed spheroids compared with mono-culture. To exclude the contribution of enhanced cell proliferation in this finding, we assessed the proliferation of AsPC-1 and SUIT-2 cells with or without co-culture with hPSC21-S/T cells. Co-culture of pancreatic cancer cells with hPSC21-S/T cells did not affect the proliferation in pancreatic cancer cells (Fig. 2C). This is in disagreement with a previous study showing that PSCs stimulated the proliferation of pancreatic cancer cells *in vitro* [6]. In that study, a concentrated conditioned media of human PSCs increased the proliferation of BxPC3 and Panc-1 pancreatic cancer cells. The reason for the discrepancy remains unclear, but a possible explanation includes different experimental settings (indirect co-culture system vs. treatment with the concentrated conditioned medium) and different types of pancreatic cancer cells and PSCs employed. In our experimental settings, it was unlikely that the increased spheroid formation was due to the increased proliferation of pancreatic cancer cells.

3.3. Co-culture of pancreatic cancer cells with PSCs increased the expressions of CSC-related genes

We then examined the expression of several genes related to CSC functions, along with the EMT-inducing transcription factor Snail. In agreement with the morphological changes (Fig. 1), the expression of Snail-1 was increased in pancreatic cancer cells co-cultured with hPSC21-S/T cells than in mono-cultured cells (Fig. 3A). ABCG2 is a member of ATP-binding cassette transporters and is highly expressed in the CSC-containing side population cells [27]. An intermediate filament Nestin is a marker of pancreatic progenitor cells and contributes to the pancreatic cancer cell invasion and metastasis [28]. LIN28 is an RNA-interacting molecule and plays a crucial role in maintaining the embryonic stem cell functions [29]. The expression of these CSC-phenotype-related genes was increased by co-culture with hPSC21-S/T cells (Fig. 3B, D). These results indicate that indirect co-culture with PSCs regulated the expression of a panel of CSC-related genes in pancreatic cancer cells, although the underlying molecular mechanisms remain to be clarified.

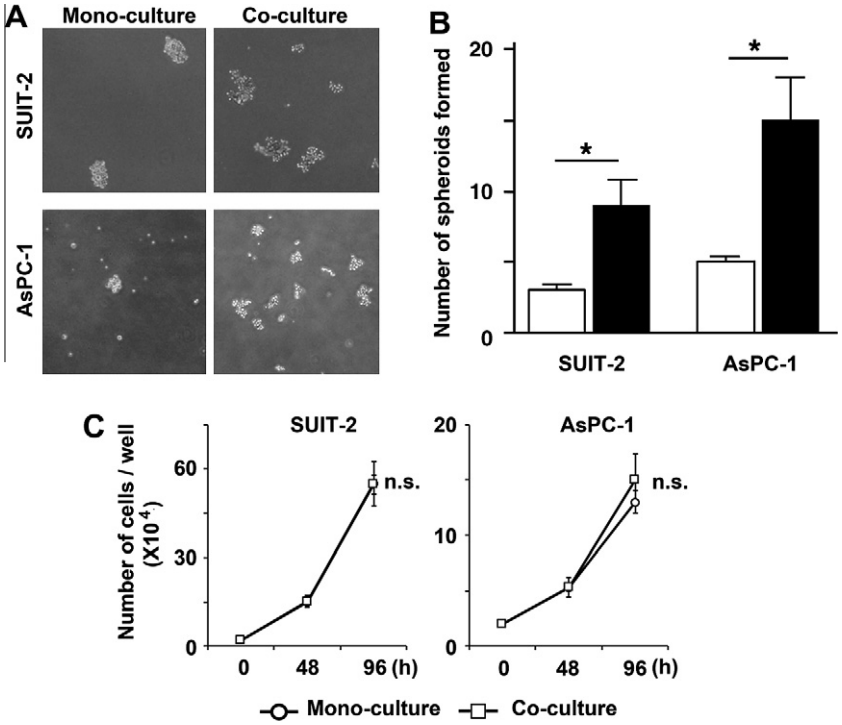

Fig. 2. Co-culture of pancreatic cancer cells with PSCs enhances spheroid-forming ability of pancreatic cancer cells. (A, B) AsPC-1 and Suit-2 cells were seeded at a density of 500 cells/well in low-adhesion coated 6-well plates in serum-free medium supplemented with EGF, FGF, and selenium. The next day, the culture insets containing hPSC21-S/T cells were placed into the 6-well plates containing pancreatic cancer cells. After 7 days, the number of formed spheroids was counted in randomly chosen 5 high power fields (A, ×100 magnification). (B) *indicates $p < 0.05$. Co-culture of AsPC-1 and SUIT-2 cells with PSCs significantly enhanced spheroid formation. (C) AsPC-1 and Suit-2 cells were mono-cultured or co-cultured with hPSC21-S/T cells. After 48 or 96 h, cell proliferation was examined by direct cell number counting. n.s.: not significant ($n = 3$). Co-culture of AsPC-1 and SUIT-2 cells with PSCs did not significantly alter cell proliferation.

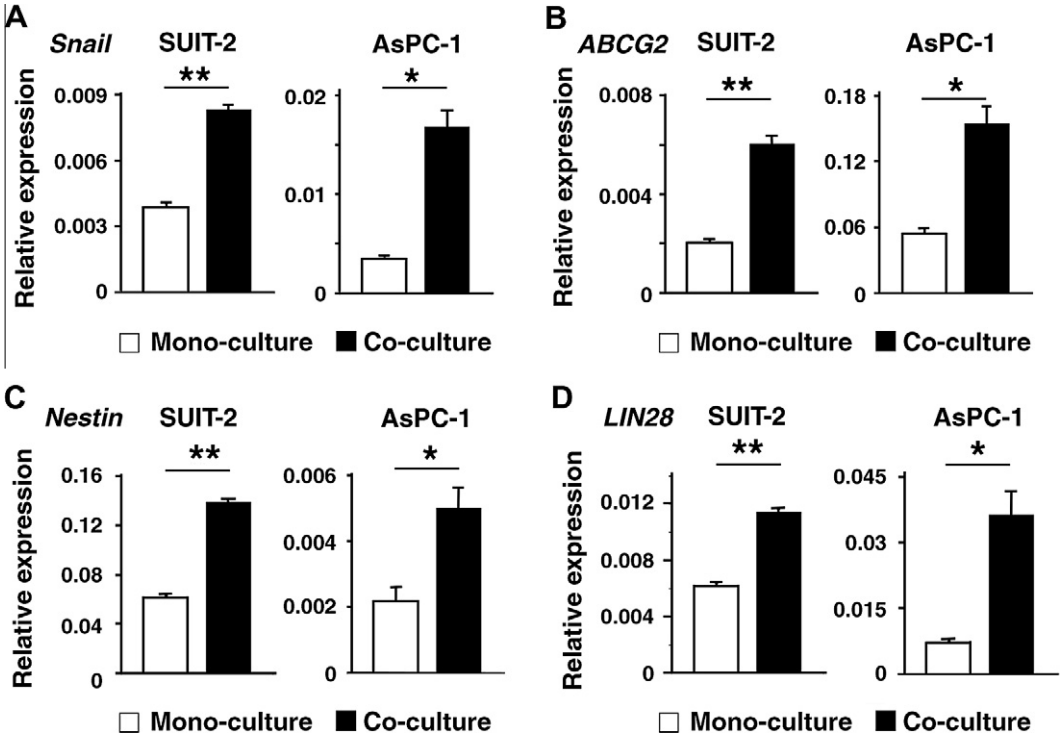

Fig. 3. Co-culture of pancreatic cancer cells with PSCs increased the expressions of CSC-related genes. AsPC-1 and Suit-2 cells were mono-cultured, or co-cultured with hPSC21-S/T cells. After 72 h, total RNA was extracted, and the levels of *Snail*, *ABCG2*, *Nestin*, and *LIN28* were examined by the real-time RT-PCR ($n = 3$ for each treatment). *$p < 0.05$; **$p < 0.01$.

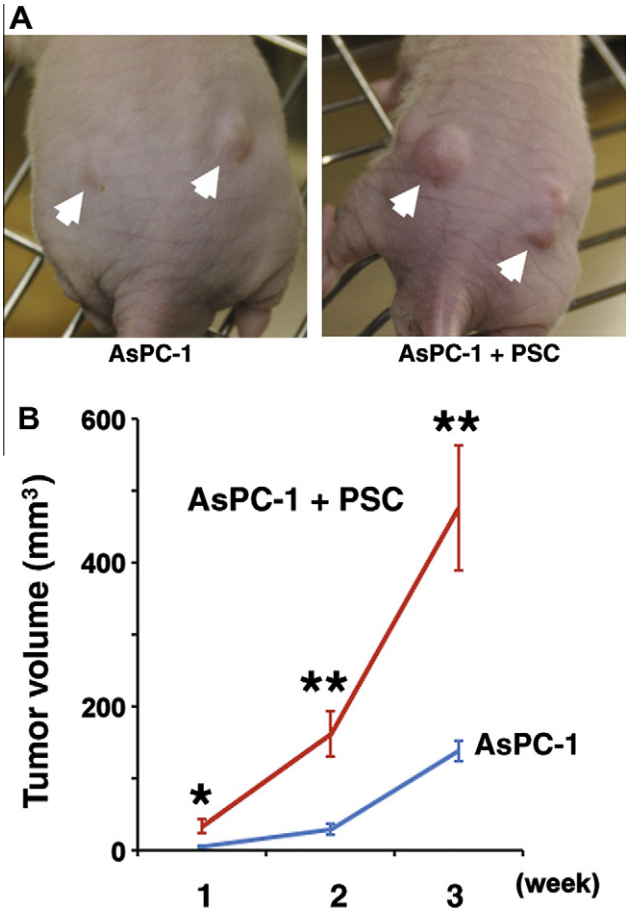

Fig. 4. PSCs support the growth of cancer cells *in vivo*. AsPC-1 cells were injected subcutaneously into nude mice either alone or together with hPSC21-S/T cells. Tumor formation *in vivo* was assessed in male athymic nude mice (ICR-nu) by subcutaneously injecting AsPC-1 cells (1×10^6 cells) with or without hPSC21-S/T cells (1×10^6 cells) suspended in 100 μl of sterile PBS. The tumor volume was measured every 7 days for up to 3 weeks after the injection. (A) Representative photographs of the subcutaneous tumors (arrows) at 2 weeks after the injection. The mice that received AsPC-1 + hPSC21-S/T cells developed larger tumors than those that received AsPC-1 cells alone. (B) The sizes of the tumors developed in mice injected with AsPC-1 + hPSC21-S/T cells ($n = 8$) were significantly larger than those that in mice injected with AsPC-1 cells only ($n = 7$). $*$ indicates $p < 0.05$, $**$ indicates $p < 0.01$.

3.4. Co-injection of PSCs enhanced tumorigenicity of pancreatic cancer cells in vivo

We finally examined whether hPSC21-S/T cells supported the growth of pancreatic cancer cells *in vivo*. AsPC-1 cells were subcutaneously injected alone or with hPSC21-S/T cells, and the sizes of the tumors were monitored for up to 3 weeks. Co-injection of hPSC21-S/T with AsPC-1 resulted in the formation of larger tumors (Fig. 4), indicating hPSC21-S/T enhanced the tumorigenicity of AsPC-1, which is a characteristic feature of CSCs [14–17]. Of note, injection of hPSC21-S/T cells alone did not develop tumors (data not shown). In agreement with this study, Hwang et al. reported that the presence of PSCs increased the incidence of tumor formation in an orthotopic nude mouse model when limited numbers of cancer cells were injected [6]. This finding further supports the notion that PSCs, as a part of the stem cell "niche", enhanced CSC-like phenotypes in pancreatic cancer cells, although the underlying mechanisms remain to be studied. A role for TGF-β might not be likely, because the neutralizing antibody against TGF-β did not inhibit PSCs-induced EMT in pancreatic cancer cells in a previous study [22].

Previous studies have shown that PSCs support the progression of pancreatic cancer by several mechanisms [4–12]. For example, PSCs increased the proliferation and migration of pancreatic cancer cells, protected them from gemcitabine- or radiation-induced apoptosis. In addition, it has been increasingly recognized that matricellular matrix proteins such as periostin, galectins, and tenascin-C, produced mainly by PSCs, create tumor-supportive microenvironments [11,12]. PSCs induced the resistance to conventional therapies [30]. Because PSCs play a critical role in the multiple steps of cancer progression, the interactions between PSCs and pancreatic cancer would serve as a novel therapeutic target for the treatment of pancreatic cancer. Indeed, Olive et al. [31] reported that inhibition of the Hedgehog signaling depleted tumor-associated stromal tissues and enhanced the delivery of chemotherapy in a mouse model of pancreatic cancer. Experiments along this line are underway in our laboratory. In conclusion, by using the indirect co-culture system, we here showed that PSCs enhanced the CSC-like phenotypes in pancreatic cancer cells. Our finding suggested a novel role of PSCs as a part of the cancer stem cell niche.

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