



Published in final edited form as:

*Pancreas*. 2019 March ; 48(3): 329–334. doi:10.1097/MPA.0000000000001249.

## Direct Interactions With Cancer-Associated Fibroblasts Lead to Enhanced Pancreatic Cancer Stem Cell Function

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### Abstract

**Objective:** Cancer-associated fibroblasts (CAFs) play an important role in the progression of pancreatic ductal adenocarcinoma (PDAC) by promoting tumor cell migration and drug resistance. We determined the impact of CAFs on PDAC cancer stem cells (CSCs).

**Methods:** Fibroblast cell lines from patients' tumors were co-cultured with PDAC cells and examined for clonogenic growth and self-renewal using colony forming assays and migration *in vitro*. Changes in the frequency of CSCs was determined by flow cytometry. The effect of integrin-Focal Adhesion Kinase (FAK) signaling on CAF-mediated clonogenic growth was evaluated using shRNAs against  $\beta 1$  integrin and FAK as well as a small molecule FAK inhibitor.

**Results:** Cancer-associated fibroblasts enhanced PDAC clonogenic growth, self-renewal, and migration that was associated with an increase in the frequency of CSCs. These fibroblast cells were activated by PDAC cells and increased collagen synthesis resulting in FAK activation in PDAC cells. Knock-down of  $\beta 1$ -Integrin and FAK or the inhibition of FAK kinase activity in PDAC cells abrogated the impact of CAFs on clonogenic growth.

**Conclusion:** Therefore, CAFs enhance PDAC clonogenic growth, self-renewal, and the frequency of CSCs through type I collagen production that enhances integrin-FAK signaling in PDAC cells.

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Conflict of Interest

Asma Begum, Ross McMillan, Yu-Tai Chang, Vesselin Penchev, NV Rajeshkumar, Anirban Maitra, Christopher Wolfgang, Zeshaan A. Rasheed, and William Matsui do not have competing financial interests.

## Keywords

pancreatic cancer; cancer stem cell; tumor microenvironment; reactive stroma; cancer-associated fibroblast

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## Introduction

Cancer stem cells (CSCs) have been prospectively identified in many diseases based on their increased tumor-initiating capacity, self-renewal potential, and the ability to phenotypically recapitulate the original tumor in the ectopic setting.<sup>1-3</sup> In PDAC, CSCs are highly migratory and found in metastatic lesions suggesting an additional role in disease progression.<sup>4</sup> A number of cell-intrinsic pathways regulating PDAC CSCs have been identified,<sup>5</sup> but the impact of extrinsic cell factors are not well understood.

The tumor microenvironment (TME) in pancreatic ductal adenocarcinoma (PDAC) is characterized by a dense desmoplastic reaction composed of stromal, immune, and endothelial cells as well as extracellular matrix (ECM) proteins. Changes in the TME are associated with poor clinical outcomes, and the activation of cancer-associated fibroblasts (CAFs) can impair intratumoral drug delivery by inducing fibrosis and facilitate metastatic disease by promoting the epithelial-mesenchymal transition (EMT) and tumor cell migration.<sup>6-8</sup> The reactive stroma has been found to impact stem cell functional properties in breast, lung, and colon carcinoma,<sup>9-11</sup> and we examined the impact of CAFs on PDAC CSCs.

We co-cultured CAFs and PDAC cells and found that direct cell to cell interactions enhanced tumor cell clonogenic growth, self-renewal, and migratory potential that was associated with an increased frequency of aldehyde dehydrogenase (ALDH) expressing PDAC CSCs. Type I collagen has been shown to increase PDAC tumor-initiating potential and self-renewal by signaling through  $\beta$ 1-Integrin and Focal Adhesion Kinase (FAK),<sup>12</sup> and we found that the inhibition of integrin-FAK signaling in PDAC cells partially abolished the impact of CAFs on these cells. In addition, PDAC cells enhanced the expression of type I collagen in CAFs. Therefore, the interaction between CAFs and PDAC cells may establish a positive feedback mechanism promoting stem cell features.

## MATERIALS AND METHODS

### Cell Lines and Xenografts

Primary cancer-associated fibroblasts (CAFs) were isolated from surgically resected primary tumors as previously described.<sup>13</sup> Normal human lung fibroblasts (nHLFs), Capan-1, and BxPC-3 cell lines were purchased from the American Type Culture Collection (Manassas, Va) and cultured in DMEM containing 10% fetal bovine serum (Sigma, St. Louis, Mo), 1% penicillin/streptomycin, and 2mM L-glutamine. Pancreatic cancer cells and CAFs were co-cultured at a 1:1 ratio for 7 or 14 days then washed with phosphate buffered saline (PBS), trypsinized, flow-sorted, and re-suspended in medium containing 10% FBS. Treatment with the FAK-inhibitor PF573228 (500nM; Sigma) was carried out for 7 days. The low passage patient-derived xenograft (PDX) JH102 has been previously described.<sup>14,15</sup> Conditioned

media from CAFs was collected after 7 days of culture and used at a final concentration of 50%.

### Plasmids

pLVX-IRES-mCherry and pLVX-IRES-zsGreen lentiviral vectors were purchased from Clontech Laboratories (Mountain View, Calif), and stable lines were generated following FACS sorting. Short hairpin RNA (shRNA) constructs against FAK (shFAK), or a scrambled control sequence was cloned into the Tet-pLKO-puro lentiviral vector (Addgene, Watertown, Mass).<sup>12</sup> The shRNA constructs against  $\beta$ 1-Integrin were cloned into the pLKO-puro vector. Cell lines expressing shRNA against FAK or  $\beta$ 1-Integrin were selected using puromycin (Thermo Fisher Scientific, Waltham, Mass).

### Clonogenic Assay

Tumor colony formation in methylcellulose was carried out as previously described.<sup>14,15</sup>

### Quantitative Real-time PCR Analysis

Total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) and reverse-transcribed with SuperScript III reverse transcriptase (Invitrogen, Carlsbad, Calif). Quantitative RT-PCR was carried out using TaqMan probes (Applied Biosystems, Foster City, Calif) for GAPDH (Hs99999905\_m1), COL1A1 (Hs00164004\_m1), SNAI1(Hs00195591\_m1), SNAI2 (Hs00161904\_m1), ZEB1 (Hs01566407\_m1), ZEB2 (Hs00207691\_m1), or CDH2 (Hs00983056\_m1).

### Flow Cytometry

Aldefluor staining was performed according to the manufacturer's protocol (Stemcell Technologies, Vancouver, Canada), and flow cytometry was carried out as previously described.<sup>14,15</sup> For intracellular staining, cells were fixed with 2% formalin for 10 minutes, followed by permeabilization with 1% NP40 for 10 minutes at room temperature. Cells were stained with rabbit monoclonal anti-pY397-FAK (Invitrogen), monoclonal alpha-Smooth Muscle Actin (M0851, DAKO-Agilent, Santa Clara, Calif), or rabbit polyclonal anti-FAK (Cell Signaling, Danvers, Mass) antibodies followed by the monoclonal anti-rabbit-APC antibody (Invitrogen).

### Migration Assay

Cell migration assay was carried out as previously described.<sup>14,15</sup>

### Statistics

Statistical differences between two groups were analyzed using the two-tailed, unpaired Student t-test (Version- Prism 6, GraphPad Prism Software, Inc.. La Jolla, Calif). *P* values < 0.05 were considered significant.

## RESULTS

### Cancer-associated Fibroblasts Enhance the Clonogenic Growth Potential of PDAC

Cancer-associated fibroblasts are a major cellular component of the reactive stroma and contribute to PDAC progression by secreting ECM proteins and cytokines.<sup>16</sup> To determine their impact on clonogenic tumor growth, CAF cell lines established from primary surgical specimens and stably expressing mCherry or zsGreen fluorescent proteins were co-cultured with PDAC cells.<sup>13</sup> Tumor cells were then isolated by FACS and plated in methylcellulose to quantify clonogenic growth potential (Supplemental Fig. 1A). Compared to Capan-1 and BxPC-3 cells cultured alone or with nHLFs, CAFs derived from multiple primary tumors significantly increased tumor colony formation by 1.3–2.5 fold (Fig. 1A;  $P < 0.05$ ). Similarly, PDAC cells isolated from a low passage PDX (JH102) formed significantly more colonies when co-cultured with CAFs (Fig. 1B;  $P < 0.001$ ). This enhanced clonogenic growth potential was cell-contact dependent as tumor cell colony formation was not significantly impacted by CAF-conditioned media (Supplemental Fig. 1B). Notably, increased colony formation was not due to changes in the proliferation (data not shown) of PDAC cells. The maintenance of clonogenic growth over time is required for disease relapse, and tumor colonies were harvested and serially replated to quantify the impact of CAFs on PDAC self-renewal. Although cells were not further exposed to CAFs, secondary colony formation by Capan-1 cells remained significantly increased  $> 1.3$  fold (Fig. 1C;  $P < 0.05$ ). Therefore, CAFs enhance PDAC clonogenic growth and self-renewal.

### Cancer-associated Fibroblasts Induce EMT and Facilitate PDAC Migration

The migratory potential of CSCs is increased compared to bulk tumor cells and suggests a role in metastatic PDAC progression.<sup>4</sup> Following co-culture with CAFs, the migration of PDAC cells was significantly increased by 1.3–2.1 fold (Fig. 2A, Supplemental Fig. 2A;  $P < 0.05$ ) similar to other reports in PDAC and other cancers.<sup>17–20</sup> In contrast, the treatment of PDAC cells with CAF-conditioned medium did not affect migration (Supplemental Fig. 2B) suggesting that direct interaction with CAFs was required. In various cancers, including PDAC, EMT contributes to metastasis by promoting cell invasion and migration.<sup>21–28</sup> Cancer stem cells may express features suggestive of EMT,<sup>29</sup> and we found that the co-culture of Capan-1 cells with CAFs decreased E-cadherin expression and increased the expression of genes such as *Snail*, *Slug*, *Zeb1*, *Zeb2*, and *N-cadherin* that are associated with EMT (Figs. 2B–D). Therefore, CAFs promote PDAC cell migration by inducing EMT.

### Cancer-associated Fibroblasts Enhance the Frequency of ALDH<sup>+</sup> PDAC CSCs

We previously demonstrated that highly clonogenic PDAC CSCs express aldehyde dehydrogenase (ALDH) activity.<sup>4</sup> Since co-cultures enhanced PDAC clonogenic growth and migration, the impact of CAFs on the frequency of ALDH<sup>+</sup> CSCs was examined. In Capan-1 and BxPC-3 cells, the frequency of ALDH<sup>+</sup> cells significantly increased by 1.6–8 fold following co-cultures with CAFs compared to PDAC cells cultured alone or with nHLFs (Fig. 3A, Supplemental Fig. 3A;  $P < 0.05$ ). Similarly, the frequency of ALDH<sup>+</sup> CSCs was increased by 2.3 fold in cells from the JH102 PDX when cultured with CAFs (Fig. 3C). Therefore, CAFs enhance the frequency of ALDH<sup>+</sup> PDAC CSCs.

## Co-cultured CAFs Induce Integrin-FAK Signaling in PDAC Cells

Activated CAFs express alpha-smooth muscle actin ( $\alpha$ SMA) and several ECM proteins, and their presence in primary PDAC tumors is associated with the poor outcomes.<sup>16,30</sup> Following co-culture with PDAC cells, the expression of  $\alpha$ SMA was increased (Fig. 4A, Supplemental Fig.4A) suggesting that their activation was induced by tumor cells. In addition, CAFs co-cultured with PDAC cells expressed significantly higher levels of type I collagen (Fig 4B;  $P=0.0002$ ). We previously demonstrated that type I collagen specifically increases tumor initiation and self-renewal potential by signaling through  $\beta$ 1-Integrin and activating FAK.<sup>12</sup> Following co-culture with CAFs, FAK activation, determined by phosphorylation at residue 397 (pY397), was increased in Capan-1 cells (Fig 4C). To determine the role of  $\beta$ 1-Integrin and FAK in the interaction between tumor and stromal cells, Capan-1 cells with shRNA against  $\beta$ 1-Integrin were co-cultured with CAFs and evaluated for clonogenic growth. Compared to cells with a control shRNA construct, the loss of  $\beta$ 1-Integrin expression abrogated the enhanced clonogenic growth of CAFs (Fig. 4D;  $P=0.05$ ). Similarly, treatment with a small molecule FAK inhibitor (PF573228) inhibited the enhanced clonogenic growth induced by CAFs. In our previous study, we also found that the loss of FAK expression inhibited CSCs to a greater extent than FAK kinase inhibition likely due to the loss of its role as a cellular scaffold.<sup>12</sup> Similarly, FAK knock-down significantly inhibited tumor colony formation even in the presence of CAFs (Fig 4E;  $P=0.002$ , Supplemental Fig. 4B). Therefore, both the kinase and non-kinase activities of FAK impact the interactions between CAFs and PDAC cells.

## DISCUSSION

In normal systems, the surrounding microenvironment regulates stem cell fate decisions that include proliferation, self-renewal, and differentiation. In many diseases, specific components of the TME, including specific cellular components, such as mesenchymal stem cells, stromal cells, immune cells, and endothelial cells have been found to regulate CSC self-renewal and differentiation.<sup>31</sup> In some cancers, direct cell contact or the production of cytokines by non-tumor cells within the TME can promote stem cell functional properties and the expansion of CSCs.<sup>10,32–34</sup> In PDAC, CAFs are a distinct part of the TME, and we found that these cells enhance the clonogenic growth and self-renewal of PDAC cells. Pancreatic CSCs are relatively more invasive and migratory than non-CSCs implicating a role in metastatic tumor dissemination, and previous studies have demonstrated that CAFs increase PDAC cell migration.<sup>35,36</sup> We found that CAFs increased the migratory potential of PDAC cells as well as the frequency of ALDH+ cells suggesting that this enhancement may be in part due to increased CSCs.

The activation of CAFs leads to the production of several ECM proteins that contribute to the desmoplastic reaction in PDAC. We previously demonstrated that type I collagen enhances stem cell properties in PDAC by signaling through  $\beta$ 1-Integrin and activating FAK.<sup>12</sup> In other diseases, ECM molecules produced by CAFs have also been found to induce stem cell properties.<sup>12,37</sup> Here we demonstrate that CAFs are activated by PDAC cells to express type I collagen that subsequently activates FAK in PDAC CSCs. Therefore, it is

possible that the bidirectional interactions between CAFs and PDAC cells ultimately lead to disease relapse and progression by stimulating CSCs.

Fibrosis produced by the reactive stroma may play an important role in PDAC therapeutic resistance by restricting adequate intratumoral levels of chemotherapeutic agents.<sup>38</sup> Attempts to clinically target the stromal cellular compartment by inhibiting the Hedgehog signaling pathway have not been successful.<sup>39</sup> Furthermore, inhibiting  $\alpha$ SMA resulted in more aggressive tumors in transgenic mice models, suggesting that the stroma may be protective in restricting tumor dissemination.<sup>40,41</sup> We found that the loss of  $\beta$ 1-Integrin or FAK activity in PDAC cells inhibited the impact of CAFs on clonogenic growth. Therefore, strategies to block the effects of the TME, such as FAK inhibitors,<sup>12,42</sup> rather than CAFs themselves may represent a novel therapeutic approach.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## ACKNOWLEDGMENTS

We thank Ada Tam, Hao Zhang and Lee Blosser of the Johns Hopkins Core Flow Cytometry facilities for their assistance with flow cytometry and cell sorting.

Grant support

Support was provided by The Pancreatic Cancer Action Network-AACR 10-70-25-RASH (ZAR), The V Foundation (ZAR), NIH R01CA150142 (WM), R01CA193887 (WM), and The Lustgarten Foundation (WM).

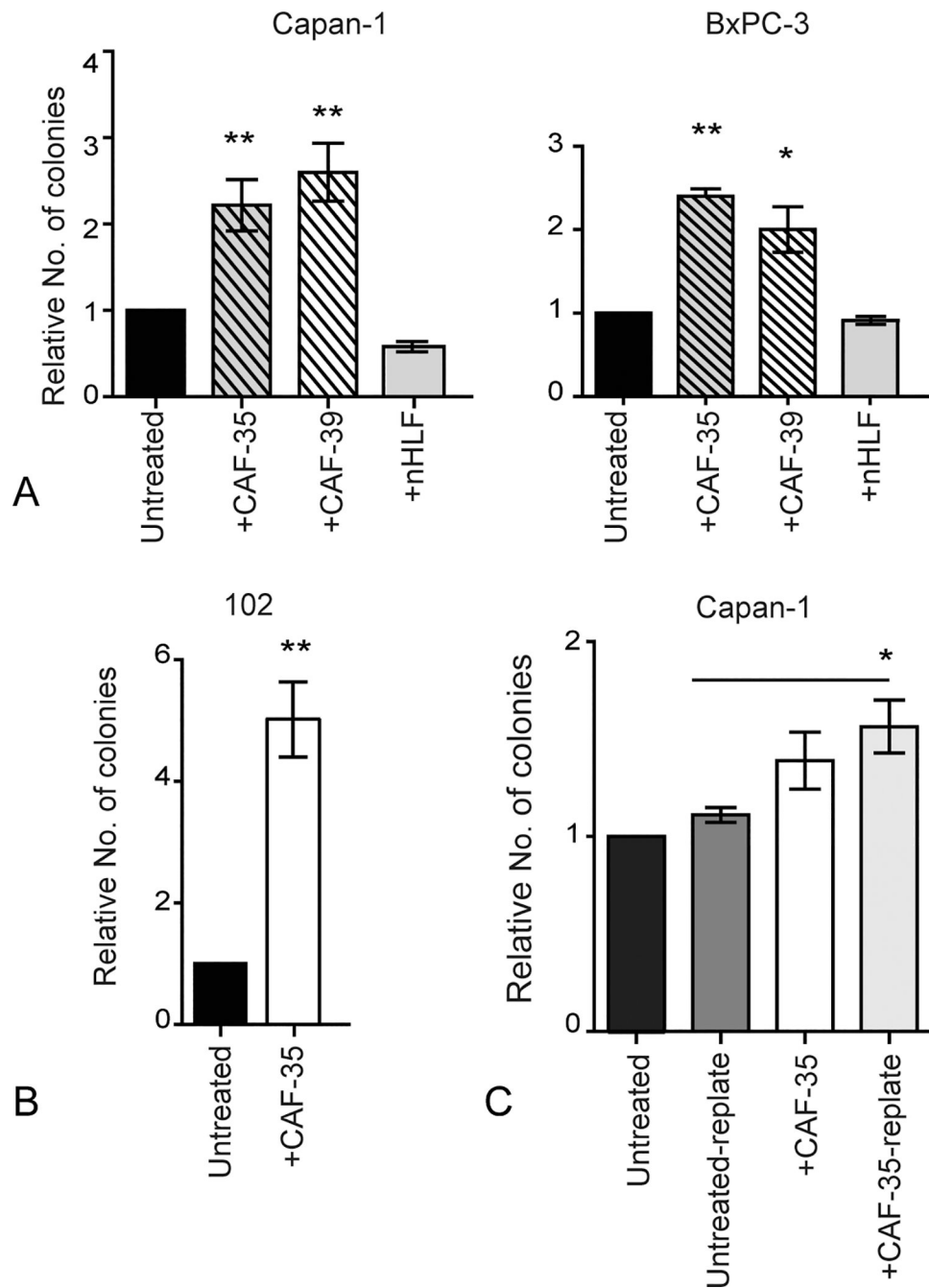
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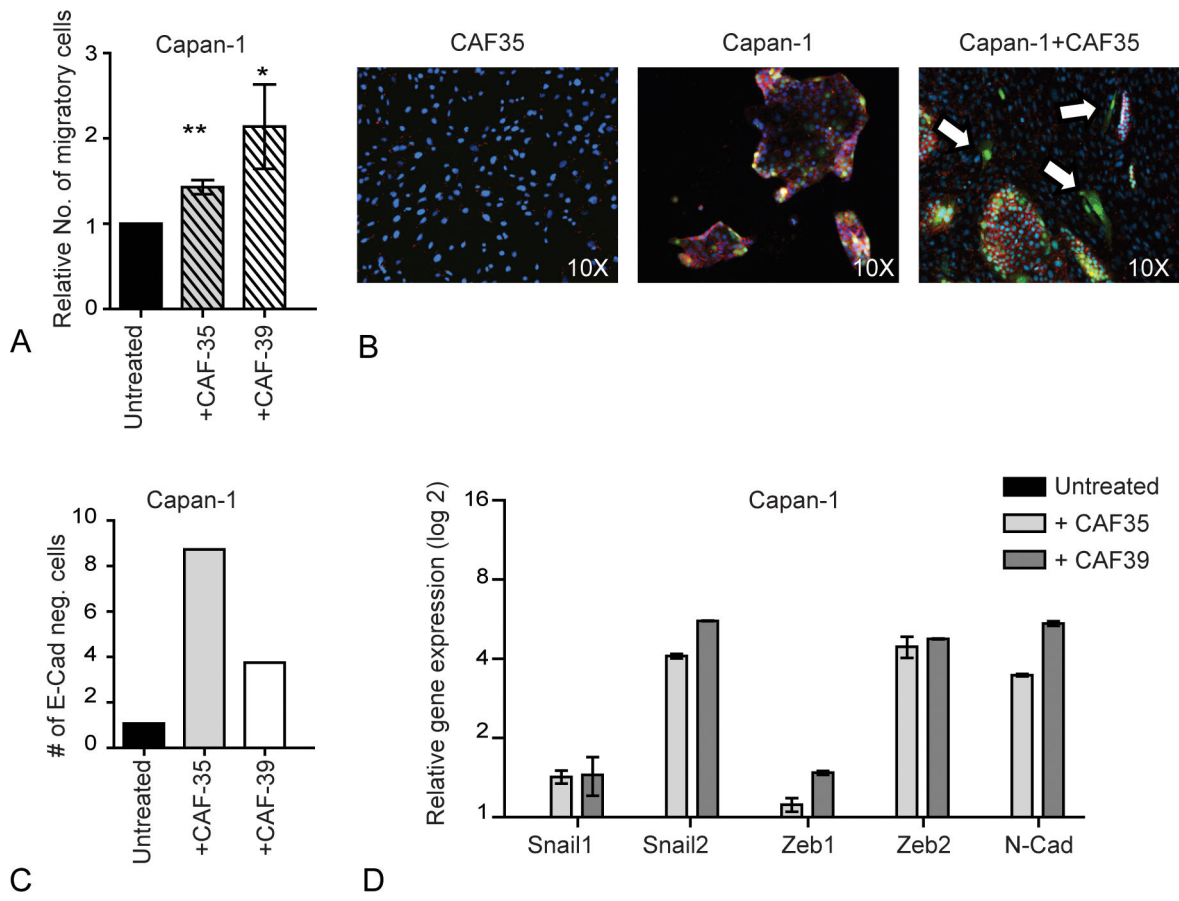
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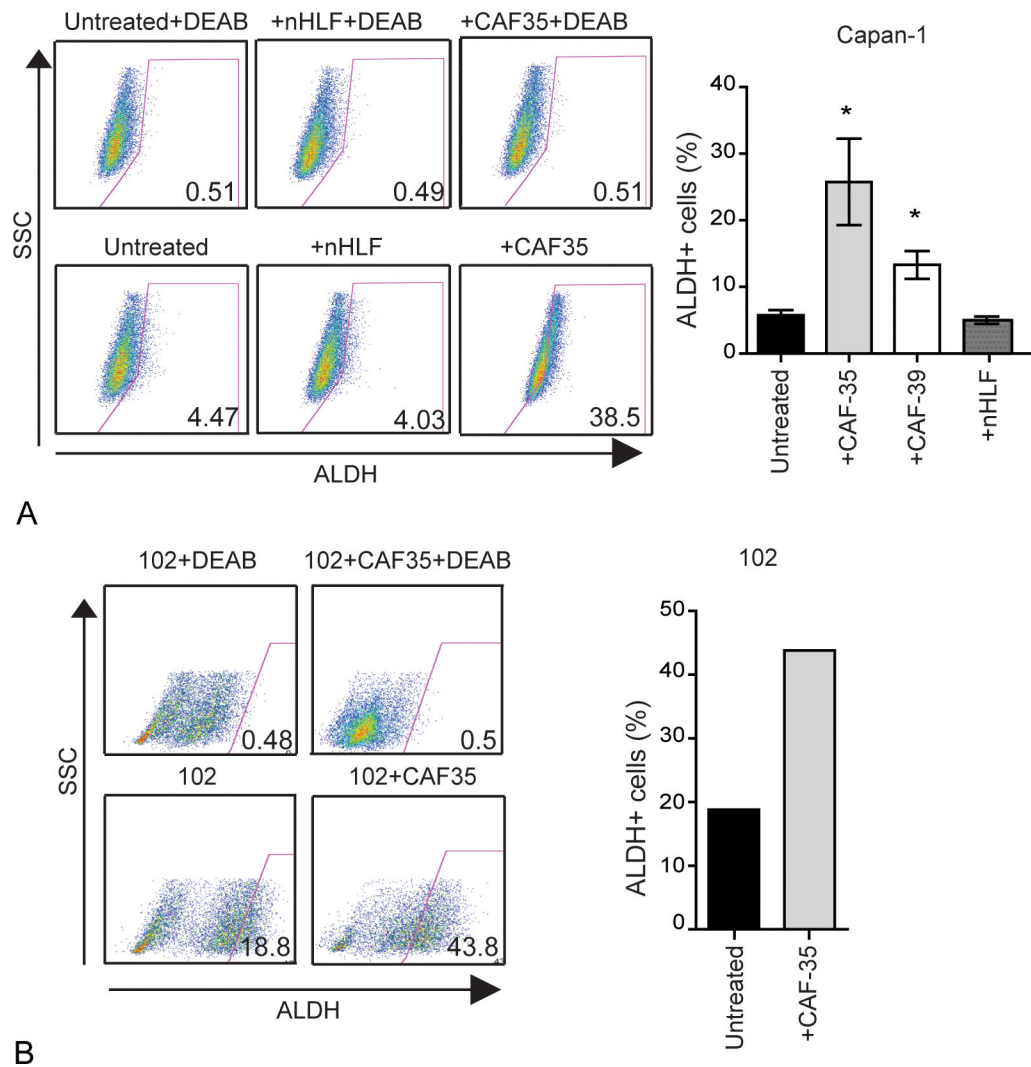


**FIGURE 1.** Cancer-associated fibroblasts enhance the clonogenic growth potential of PDAC. **A**, Colony formation by PDAC cell lines (Capan-1, and BxPC-3) and **B**, a PDX (JH102) cells following co-culture with CAFs or nHLFs for 7 days. Data represent the mean and SD of 4 experiments. \* $P < 0.05$ , \*\* $P < 0.005$ . **C**, Primary and secondary colony formation by Capan-1 cells cultured with or without CAFs for seven days. Data represent the mean and SD of 4 experiments. \* $P < 0.05$ .

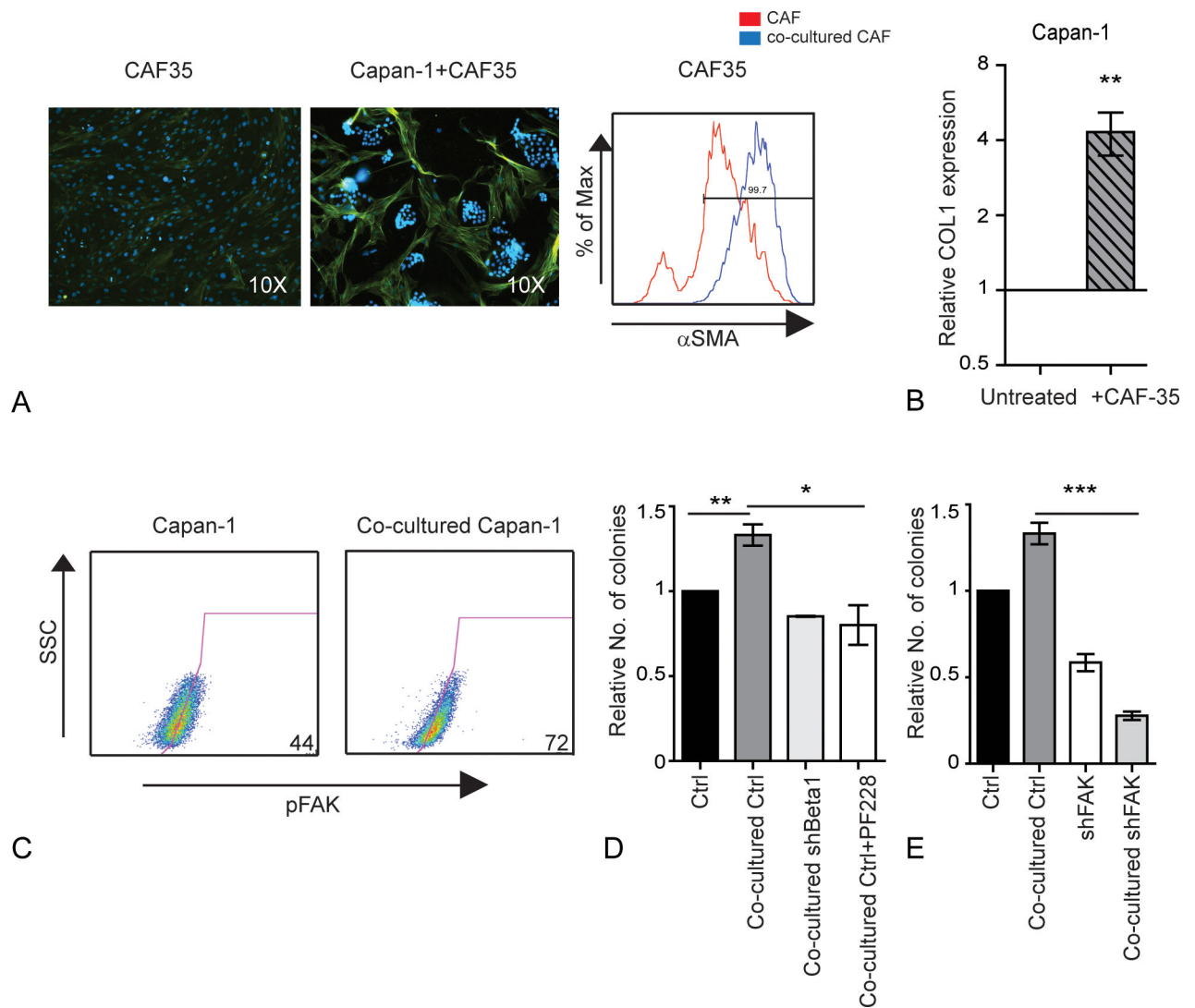


**FIGURE 2.**

Cancer-associated fibroblasts induce EMT and facilitate PDAC migration. **A**, *In vitro* migration of Capan-1 cells following culture with CAF-conditioned or control media for 7 days. Data represent the mean and SD of 4 experiments. \* $P < 0.05$ , \*\* $P < 0.001$ , \*\*\* $P < 0.0001$ . **B**, E-cadherin staining (red) of Capan-1 cells (GFP) following co-culture with CAFs for 7 days. Arrows indicate E-cadherin negative Capan-1 cells. **C**, The frequency of E-cadherin negative Capan-1 cells following culture with or without CAFs detected by flow cytometry. **D**, Relative mRNA expression of EMT associated genes in sorted Capan-1 cells following co-culture with CAFs. Data represent the mean and SD of 3 experiments.



**FIGURE 3.** Cancer-associated fibroblasts enhance the frequency of ALDH<sup>+</sup> PDAC CSCs. **A**, ALDH expression by Capan-1 cells following 7 days of co-culture with CAFs. Cells treated with DEAB were used as negative control for ALDH staining. Data represent the mean and SD of 3 experiments. \* $P < 0.05$ , \*\*\*  $P < 0.0001$ . **B**, The frequency of ALDH<sup>+</sup> cells was analyzed in patient-derived low passage PDX (102) following co-culture with CAF35.



**FIGURE 4.**

Cancer-associated fibroblasts express type I collagen and enhance integrin-FAK signaling in PDAC. **A**, αSMA expression of CAFs following co-culture with or without Capan-1 cells for 7 days. Left: immunostaining of αSMA (green) and nucleus-Hoechst 33342 (blue). Right: αSMA expression by CAF35 cells before and after co-cultured with Capan-1 cells. **B**, Relative mRNA expression of type I collagen by CAF35 cells following co-culture with Capan-1 cells. **C**, The frequency of pY397-FAK<sup>+</sup> Capan-1 cells following co-culture with CAFs. **D**, Colony formation by Capan-1 cells expressing a scrambled control (Ctrl) or β1 integrin (shBeta1) shRNA vectors or treated with PF573228 following co-culture with CAFs. Data represent the mean and SD of 3 experiments. \*\**P* < 0.001. **E**, Colony formation by Capan-1 cells expressing a scrambled control (Ctrl) or FAK (shFAK) shRNA following co-culture with CAFs. Data represent the mean and SD of 3 experiments. \**P* < 0.05; \*\**P* < 0.001, \*\*\**P* < 0.0001.