Cancer-Associated Fibroblasts in Pancreatic Cancer Are Reprogrammed by Tumor-Induced Alterations in Genomic DNA Methylation

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Abstract

Stromal fibrosis is a prominent histologic characteristic of pancreatic ductal adenocarcinoma (PDAC), but how stromal fibroblasts are regulated in the tumor microenvironment (TME) to support tumor growth is largely unknown. Here we show that PDAC cells can induce DNA methylation in cancer-associated fibroblasts (CAF). Upon direct contact with PDAC cells, DNA methylation of SOCS1 and other genes is induced in mesenchymal stem cells or in CAF that lack SOCS1 methylation baseline. Silencing or decitabine treatment to block the DNA methylation enzyme DNMT1 inhibited methylation of SOCS1.

In contrast, SOCS1 gene methylation and downregulation in CAF activated STAT3 and induced insulin-like growth factor-1 expression to support PDAC cell growth. Moreover, CAF facilitated methylation-dependent growth of PDAC tumor xenografts in mice. The ability of patient-derived CAF with SOCS1 methylation to promote PDAC growth was more robust than CAF without SOCS1 methylation. Overall, our results reveal how PDAC cells can reprogram CAF to modify tumor–stromal interactions in the TME, which promote malignant growth and progression. Cancer Res. 76(18); 5395–404. ©2016 AACR.

Introduction

The tumor microenvironment (TME) plays a complex role in supporting cancer initiation, progression, and metastasis (1–3). Pancreatic ductal adenocarcinoma (PDAC) with its characteristic fibrotic stroma compartment that occupies the vast majority of the tumor mass (4, 5) is an ideal disease model for the study of tumor–stroma interaction. Accumulated evidence has suggested that the malignant tumor stroma is a major obstacle to effective treatments of pancreatic cancer (6–9). However, experiments targeting stromal fibroblasts in mouse models of PDAC showed controversial results (10, 11), and highlighted the importance of investigating the specific molecular mechanisms on how stromal fibroblasts are regulated in the TME as a result of interaction with tumor cells. Particularly, when tumor formation is initiated or a metastatic tumor cell is homing to a distant organ, tumor cells need to change their surrounding environment, which could otherwise be naturally hostile to them. Although genetic alterations have been reported in cancer-associated fibroblasts (CAF) from breast and ovarian cancers (12, 13), such genetic mutations are extremely rare. It is more likely that the phenotypes of CAFs are regulated at the epigenetic level, as previously suggested by a genome-wide analysis of breast cancer stromal cells (14). By contrast, epigenetic regulation such as DNA methylation can be dynamically modulated through DNA methylation enzymes. Epigenetic regulation has been conceived to provide a dynamic and reversible modulation of stromal cells (15, 16) such as CAFs within the TME. This epigenetic footprint, particularly in the form of DNA methylation, can be stably passed through cell generations in high fidelity. Thus, through epigenetic regulation, the tumor cell, depending on its need, can create either a stable or dynamic TME that expresses growth factors to support the tumor growth.

In this study, we show that PDAC tumor cells are able to induce DNA methylation in CAFs. Tumor-induced DNA methylation in CAFs subsequently promotes the growth of PDAC xenografts in mice. This study thus reveals a novel mechanism that mediates the reprogramming of TME for promoting tumor growth.

Materials and Methods

Tissue specimens and cultures
Primary cultures of PDAC tumor cells, including 3.27T, 1.30T, 3.30T, 9.05T, 3.29T, and 7.07T, and CAFs, including...
2.15F, 10.29F, 5.10F, 2.01F, 7.02F, 10.09F, 9.07F, 9.28F, 5.10F, 3.16F, 7.09F, 3.05F, 3.27F, 1.30F, 3.30F, 9.05F, 2.29F, 7.12F, 1.23F, and 7.21F, were established from banked, surgically resected PDAC specimens between 2008 and 2014 and the Panc 10.05 cell line was established in 1998 in accordance with the Johns Hopkins Medical Institution Institutional Review Board (JHMI IRB)-approved protocols and authenticated by DNA and gene expression profiling as previously described (17, 18). These cell cultures were maintained in RPMI1640 (Life Technology) containing 10% FBS as described previously (19). Human mesenchymal stem cells (MSC) obtained from Texas A&M Health Science Center (http://medicine.tamhsc.edu/irm/irm/msc-distribution.html) were cultured in Alpha Modified Eagle Medium containing 10% premium selected FBS (Atlanta Biologicals) and passed for fewer than 6 months after resuscitation in 2013 and authenticated by DNA and gene expression profiling. Human cardiac fibroblasts (HNF) were from a dilated cardiomyopathy patient (20). Cells were harvested at the end of the 4-day treatment course for coculture or xenograft experiment.

Bisulfite conversion, methylation-specific PCR, and MethylSYBR PCR

Genomic DNA extraction from microdissected tissues or Dynabeads bound cells was performed by using QIAamp DNA Micro Kit (Qiagen). DNA from tumor or CAF cultures was extracted by Blood and Tissue DNase Kit (Qiagen). Extracted DNA was bisulfite modified with EZ DNA Methylation Kit (Zymo Research), which converted all unmethylated cytosines to uracils while leaving methylcytosines unaltered. Bisulfite-converted DNA was amplified by methylation-specific PCR (MSP) as described previously (21). The bisulfite-treated DNA was used to quantify expression levels of specific genes of interest by semi-quantitative RT-PCR (qPCR) in the presence of Methyl-SYBR Green qPCR core kit (Life Technology). qPCR was performed on the StepOnePlus Real Time PCR System (Life Technology) and analyzed by the stepOne software V2.1. The expression of specific genes of interest was quantified using the ΔΔCt method, with the ΔCt being calculated as ΔCt = Ctarget - Ccontrol. The expression of a known methylated control gene, such as DNMT1, DNMT3a, and DNMT3b, was used to normalize the methylation-specific PCR (MSP) data. The methylation ratio was calculated as (SOC1 volume of tumor/patient)/(SOC1 volume of normal tissue/patient).

Tumor-conditioned medium

Tumor-conditioned medium (TCM) for coculture or xenograft experiment was prepared by filtering Tumor-conditioned medium (TCM) was obtained by passing PDAC culture supernatant through a Corning sterile 50 mL filtration system with a 0.22 μm polyethersulfone membrane. Ten times concentrated TCM was obtained by centrifuging TCM at 3,500 g in Centricon Plus-70 Centrifugal Filter Units (EMD) for 30 minutes.

Fibroblast cell isolation

Fibroblast cells (CAF) and tumor cells were plated at a ratio of 1:3. After coculturing for 24 hours, fibroblast cell isolation was performed using the CELLection Biotin Binder Kit (Life Technology) and the sheep anti-human FAP biotinylated antibody (R&D Systems Inc.) according to the manufacturer's instructions.

Quantitative real-time RT-PCR

Total RNA was extracted from cells by RNeasy Micro Kit (Qiagen). cDNA was synthesized by Superscript First-Strand Synthesis Supermix Kit (Life Technology). Quantitative real-time RT-PCR (qPCR) was performed on the StepOnePlus Real Time PCR System (Life Technology) and analyzed by the Stepone software V2.1. The expression of SOCS1 was quantified using the Taqman probe system (Life Technology). The expression of DNMT1, DNMT3a, and DNMT3b, and IGF-1 was measured by SYBR Green-based qPCR. Information on primers and probes is provided in T S1.

RNA interference

RNA interference experiments were performed with the lentivirus encoding validated short hairpin RNA directed against SOCS1 (Thermo Scientific), DNMT1 (OriGene Technologies), or corresponding controls, as previously described (19). Transfection of MSCs with miR29b mimic (hsa-miR29b-3p; Ambion) or the control microRNA (negative control #1; Ambion) was performed according to the manufacturer's instructions.

In vivo tumorigenesis assays

NOD/SCID mouse strains were maintained and subjected to the experiments at 8 weeks old in accordance with the protocols approved by the Johns Hopkins Animal Care and Use Committee. For DAC treatment, mice were treated by intraperitoneal injection of 1 mg/g DAC of body weight for 5 days. Panc 10.05 tumor cells (2.5 × 10⁵) were injected subcutaneously, alone or mixed with 5 × 10⁵ MSCs. Tumor growth was monitored daily until tumor was palpable. Tumor-free survival was measured from the day of tumor inoculation to the day when tumor long axes was less than 2 mm. Then, tumor diameter was measured with calipers twice a week. Mice were euthanized at week 7 following tumor inoculation. The long (L) and short (S) axes of each tumor were measured on harvested tumors with calipers. Tumor volume (V) was calculated as V = (L × S²)/2.

DNA methylation and gene expression microarray analysis

Merged analysis of DNA methylation array and gene expression array was performed on the Illumina Infinium Methylation450K Beadarray (including CpG islands in both promoter regions and gene bodies) and the Illumina H-12 Expression Beadarray, respectively. Genes identified to be both more methylated and downregulated in MSCs or 09.05CAFs were subjected to the gene clustering analysis with a web-based tool, CROC (http://metagenomics.uv.es/CROC/; ref. 26).
Statistical analysis

Statistical analyses as indicated were performed using SPSS and GraphPad Prism software. The quantitative data were presented as the means ± SEM. A P-value <0.05 was considered to be statistical significance.

Results

SOCS1 is frequently methylated in pancreatic CAFs

To understand how CAFs in PDACs may be regulated through DNA methylation, we first sought to identify genes...
methylated in CAFs. We found 41 gene promoters that were reported to have DNA methylation in pancreatic adenocarcinoma by searching the Pubmeth Database that includes methylated genes validated by methylation-specific sequencing or PCR analyses in the literature. We then selected seven genes known to be expressed in or functionally relevant to the stromal fibroblasts of PDAC, including SPARC2, CCND2, SFRP2, RARB, PENK, SOCS1, and ST14 (Fig. 1A) as well as CDH1 as an epithelial gene control. With methylation-specific PCR (MSP), we checked the promoter methylation status of these eight genes in three matched primary PDAC neoplasm cultures and their associated fibroblasts, which were expanded in short-term cultures just to achieve adequate yield and purity. All genes except SOCS1 were either methylated in both PDACs and matched CAFs, or in PDACs but not in matched CAFs (Supplementary Table S2). Interestingly, none of the three PDACs were methylated in the SOCS1 promoter, whereas two of three CAFs showed SOCS1 promoter methylation (Fig. 1B). The SOCS1 gene encodes a member of the suppressor of cytokine signaling (SOCS) family (27–29). Subsequently, with quantitative MSP, we found that none of eight primary PDAC neoplasm cultures at early passages (<4 passages) had detectable SOCS1 promoter methylation, whereas 16 out of 20 CAFs demonstrated various levels of SOCS1 methylation. Consistently, both MSP and methylation pyrosequencing (Supplementary Table S3) showed SOCS1 methylation in the stroma microdissected from 15 out of 16 human PDAC specimens (Table 1). By contrast, none of the paratumoral normal tissues including both acinar cells and stroma showed SOCS1 methylation on the nucleotide sites tested with pyrosequencing. To compare the intratumor stroma specially to the stroma in the patumoral normal tissues, we examined the expression of SOCS1 in human PDAC specimens by the IHC staining (Fig. 1C) and found that SOCS1 expression is significantly decreased in the intratumoral stroma compared to the paratumoral stroma (Fig. 1D). In primary CAF cultures, the levels of SOCS1 mRNA expression appeared to be negatively related to the levels of promoter methylation in a statistically significant manner (Fig. 1E).

Induction of SOCS1 methylation in CAFs by interacting with tumor cells of PDAC

Next, we examined whether SOCS1 methylation in CAFs is preestablished or is induced by tumor–stroma interactions. We noticed that CAFs from some PDACs, such as 3.30CAFs from the Panc3.30 PDAC, did not have SOCS1 methylation. If SOCS1 methylation in CAFs is not preestablished, but is induced by tumor cells, we would anticipate the induction of SOCS1 methylation in 3.30CAFs cocultured with Panc1.30 tumor cells. To this end, we sought to examine whether SOCS1 methylation can be induced in human MSCs, which are thought to be the bone marrow-

![Figure 2](image-url)

The result did show that SOCS1 in 3.30CAFs was methylated and its mRNA expression was decreased after coculturing with 1.30Tumor (Fig. 2A and B).

As CAFs have already been exposed to tumor cells in vivo, we sought to examine whether SOCS1 methylation can be induced in human MSCs, which are thought to be the bone marrow-

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<tr>
<th>Gene</th>
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<td>Primary PDAC CAFs</td>
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Table 1. Summary of the results of SOCS1 methylation in multiple primary PDAC tumor cell cultures and primary CAFs analyzed by MSP and those in stroma dissected from PDAC tissues analyzed by pyrosequencing.
derived cell origin of CAFs (30). As shown in Fig. 2C, SOCS1 methylation was induced and SOCS1 mRNA expression was decreased in MSCs after coculture with Panc 10.05 tumor cells for 24 hours. As a control, the ST14 promoter remained unmethylated. By contrast, methylated SOCS1 could not be induced in MSCs by colon cancer cell lines such as SW620 (Fig. 2D). To confirm the initial MSP results of SOCS1 methylation in Fig. 1B, using another pair of matched PDAC tumor (Panc 09.05 tumor) and CAF (09.05CAF) cells that do not have baseline SOCS1 methylation, we found that SOCS1 methylation in 09.05CAF can be induced by Panc 10.05 tumor, but not its matched Panc 09.05 tumor cells (Fig. 2E). The same results were observed when MSC, 3.30CAF or 09.05CAF was cocultured with other human PDAC cell lines that are available to us and whose matched CAFs were found to have SOCS1 methylation (Fig. 2E). We also confirmed the MSP results (Fig. 1B and 2C) by more quantitative, pyrosequencing assays (Supplementary Fig. S1 and Table S4).

Induction of SOCS1 methylation in CAFs requires direct tumor–CAF contact

Next we sought to examine whether the tumor–stroma interaction that leads to the induction of SOCS1 methylation in CAFs or MSCs is mediated by paracrine signals or by direct cell–cell contact (Fig. 3). As shown in Fig. 3B, no SOCS1 methylation was induced in MSCs by TCM or 10 times concentrated TCM. When tumor cells and MSCs were separated by either 1 or 8 μmol/L semitransparent membrane. Panc 10.05 tumor cells seeded above the 1 μmol/L membrane could not induce SOCS1 methylation in MSCs seeded in the bottom chamber. Panc 10.05 tumor cells that migrated through the 8 μmol/L membrane could induce the SOCS1 methylation in MSCs on and after day 6. B, Panc 10.05T cells (bottom) on the 8 μmol/L-pore membrane migrated to the bottom chamber as seen on and after day 6. Panc 10.05T cells (top) were not able to migrate cross the 1 μmol/L-pore membrane. Scale bar, 20 μm.

**Figure 3.** Induction of SOCS1 methylation in MSCs requires direct cell–cell contact between tumor cells and MSCs. A, schematic illustration of the transwell system. B, no induction of SOCS1 methylation in MSCs cultured with fresh TCM, 10 times concentrated TCM, or in no contact coculture, where MSC and Panc 10.05 tumor cells were separated by a 1 μmol/L semitransparent membrane that tumor cells are not able to migrate through. C, MSCs and Panc 10.05 tumor cells were separated by either 1 or 8 μmol/L semitransparent membrane. Panc 10.05 tumor cells seeded above the 1 μmol/L membrane could not induce SOCS1 methylation in MSCs seeded in the bottom chamber. Panc 10.05 tumor cells that migrated through the 8 μmol/L membrane could induce the SOCS1 methylation in MSCs on and after day 6. B, Panc 10.05T cells (bottom) on the 8 μmol/L-pore membrane migrated to the bottom chamber as seen on and after day 6. Panc 10.05T cells (top) were not able to migrate cross the 1 μmol/L-pore membrane. Scale bar, 20 μm.

SOCS1 methylation was upregulated by DNMT1 overexpression in fibroblast

To examine whether DNMTs account for the induction of SOCS1 methylation, we examined the mRNA expression of DNMT1, 3a, and 3b, the three major enzymes responsible for DNA methylation in mammals (31, 32), in MSCs in comparison to MSCs cocultured with PDAC tumor cells. As shown in Fig. 4A, DNMT1 mRNA expression showed a significant increase in MSCs after coculture with tumor cells. By contrast, when DNMT1 in MSCs was knocked down by the shRNA of DNMT1 carried by lentivirus (Supplementary Fig. S2) inhibiting DNMT1 expression in MSCs (Fig. 4B), the methylation of SOCS1 in MSC could not be induced (Fig. 4C). Downregulation
Figure 4.

DNMT1-regulated SOCS1 methylation leads to the downregulation of SOCS1 expression and subsequent activation of STAT3 and induction of IGF-1 expression. Lentivirus-carried shRNA targeting DNMT1 (shDNMT1) was used to knockdown the expression of DNMT1 (Supplementary Fig. S2). GAPDH expression was used for normalization of all qPCR results. A, expression of DNMT1, DNMT3a, and DNMT3b measured by qPCR in MSCs before and after coculture with Panc 10.05 tumor (10.05T) for 24 hours was compared. B, DNMT1 expression in MSCs before and after coculture with 10.05T was compared with that in MSCs with knockdown of DNMT1. C, SOCS1 methylation examined by MethySYBR in MSCs before and after coculture with 10.05T was compared with that in MSCs with knockdown of DNMT1. D, SOCS1 expression in MSCs before and after coculture with 10.05T was compared with that in MSCs with knockdown of DNMT1. E, SOCS1 methylation in MSCs before and after coculture with 10.05T was compared with that in MSCs pretreated with DAC. F, IGF-1 expression measured by qPCR in MSCs before and after coculture with 10.05T was compared with that in MSCs with knockdown of DNMT1. G, MSCs were cocultured with Panc 10.05 tumor cells in an 8 mmol/L transwell system for 5 days as described in Fig. 3. Cells in the bottom chamber were fixed and subjected to a dual immunofluorescence staining with FITC-conjugated antibodies recognizing Pan-CK to mark epithelial tumor cells (green) and PE-conjugated antibodies either for STAT3 or for pSTAT3 (Tyr705; red), and counterstained with DAPI for nuclei (blue). Scale bar, 20 μm. H, SOCS1 methylation examined by MethySYBR in MSCs transfected with the control microRNA before and after coculture with 10.05T was compared with that in MSCs transfected with miR-29b. In A–F and H, comparisons were analyzed by a two-tail unpaired Student t test with P values shown.
of SOCS1 expression in MSCs after coculture with tumor cells was also reversed by DNMT1 knockdown (Fig. 4D). Consistently, methylation of SOCS1 in MSCs was inhibited by pretreating MSCs with a DNMTs inhibitor, DAC (Fig. 4E). These results suggest that the induction of SOCS1 methylation is not a spontaneous process, but a highly regulated, DNMT1-dependent process.

SOCS1 was also reversed by DNMT1 knockdown (Fig. 4D). Consistently, methylation of SOCS1 in MSCs was inhibited by pretreating MSCs with a DNMTs inhibitor, DAC (Fig. 4E). These results suggest that the induction of SOCS1 methylation is not a spontaneous process, but a highly regulated, DNMT1-dependent process.

STAT3 phosphorylation and IGF-1 expression were induced in CAFs after coculture with PDACs in a DNMT1-dependent manner

SOCS1 is a known suppressor of many pro-cancerous cytokines and growth factors (29). Therefore, we attempted to understand whether the induction of SOCS1 methylation in CAFs through tumor-stroma interaction also regulates this biological function of SOCS1. To this end, we examined the expression of the insulin-like growth factor 1 (IGF-1), a representative SOCS1 downstream pro-cancerous growth factor that is primarily secreted by fibroblasts (3, 33, 34). IGF-1 is chosen also because as its associated signaling pathway is among the pathways that are the most significantly upregulated in CAF or MSC upon coculture with PDAC tumor cells in the Ingenuity pathway analysis of the mRNA expression microarray data described later (Supplementary Fig. S3). As shown in Fig. 4E, IGF-1 mRNA expression in MSCs cocultured with tumor cells was increased by more than 100 times comparing to the single cultured MSCs. By contrast, this increase was diminished when DNMT1 was knocked down from MSCs.

It is known that the activation of the STAT family, particularly STAT3 and STAT5, mediates the suppressive role of SOCS1 in regulating the transcription of many pro-cancerous cytokines and growth factors including IGF-1 (35, 36). In the absence of SOCS1, STAT3 is phosphorylated and assumes a activated form (pSTAT3) to promote gene transcription. Thus, we examined STAT3 staining pattern in MSCs surrounding tumor cells. As shown in Fig. 4G, pSTAT3 nuclear staining was more obvious in MSCs immediately adjacent to tumor cells whereas pSTAT3 is not detectable in MSCs further away from tumor cells. It was previously reported that miR29b induces SOCS1 expression by promoting demethylation, leading to the reduced STAT3 phosphorylation (35). Thus, we hypothesized that miR29b serves as a negative regulator of tumor-induced SOCS1 methylation in MSCs. Consistent with this notion, as shown in Fig. 4H, SOCS1 methylation was not induced in the PDAC cocultured MSCs transfected with miR29b. Taken together, our results suggest that PDAC tumor cells induce DNA methylation of SOCS1 genes in CAFs, leading to the downregulation of SOCS1 expression and subsequently the activation of STAT3 and high expression of pro-cancerous growth factors such as IGF-1.

PDAC tumor cells induced DNA methylation in a global panel of clustered genes and caused their downregulation in CAFs

SOCS1 is unlikely the only gene that is methylated in CAFs as a result of tumor-stroma interaction. More likely, a global panel of genes, which are programmed to be unmethylated in fibroblasts or their origins such as MSCs under normal conditions, are reprogrammed to be methylated in CAFs upon interacting with tumor cells. A combined array analysis of DNA methylation and gene expression (GEO Accession No. GSE80369) showed that a panel of approximately 1,585 genes, including SOCS1, is both methylated and downregulated in MSCs upon interacting with Panc 10.05 tumor (Table 2 and Supplementary Table S5). A near complete overlapping panel of genes are both methylated and downregulated in 09.05CAF upon coculture with PDAC cells. Many of these genes do not appear to be distributed randomly, but are clustered, in a number of chromosomal regions (Table 2 and Supplementary Table S5), suggesting that, in addition to DNMT1, other chromatin remodeling mechanisms may also be involved in determining which clusters of genes in specific chromatin regions are simultaneously methylated and coregulated in CAFs upon interacting with PDAC cells. As shown in Supplementary Table S6, gene expression alterations in CAFs upon interacting with PDAC cells may also be mediated by other epigenetic mechanisms.

Tumor-induced DNA methylation and SOCS1 downregulation are important for stromal fibroblasts in supporting PDAC growth on mice

Fibroblasts were previously shown to promote tumor growth in a subcutaneous tumor model when coinjected with tumor cells (36, 37). We, therefore, investigated whether this pro-cancerous activity would be inhibited when methylation was blocked in CAFs. Panc 10.05 tumor cells were inoculated into NOD/SCID mice subcutaneously alone or with MSCs. Seven weeks later, xenografts formed by Panc 10.05 tumor plus MSCs were significantly bigger than those formed by Panc 10.05 tumor (Fig. 5A). However, when MSCs were pretreated with DAC for 4 days during the cell culture, they lost their capacity to promote xenograft growth. Similarly, if mice were pretreated with DAC 5 days before Panc 10.05 inoculation, the xenografts grew slower. This suggests that the stromal fibroblasts from the host mice lost the capacity to support xenograft growth due to the transient inhibition of DNA methylation (Fig. 5A and B). It is likely that the xenografts eventually grew in DAC-treated groups because the effect of transient DAC treatment stopped. However, the tumor-free survival of NOD/SCID mice with Panc 10.05 tumor co-inoculated with DAC-pretreated MSCs was significantly longer that of mice with Panc 10.05 tumor co-inoculated with untreated MSCs (Fig. 5C). The pro-cancerous effect of DAC-pretreated MSCs, measured by tumor volume (Fig. 5D and F), was restored when SOCS1 was knocked down by shRNA from MSCs (Supplementary Fig. S4). Even if the MSCs were pretreated with DAC, they were still able to promote the growth of Panc 10.05 tumor xenografts when SOCS1 was knocked down (Fig. 5D and E). These results suggest that DNA methylation in stromal fibroblasts is important for supporting PDAC tumor growth and also suggest that SOCS1 downregulation in stromal fibroblasts mediates the role of DNA methylation in supporting PDAC growth. Interestingly, the pro-cancerous effect of DAC-pretreated MSCs was not completely restored when SOCS1 was knocked down by shRNA from MSCs, as
measured by tumor-free survival and judged by the insignificant P value ($P = 0.1204$) comparing 10.05T+MSC-ctrl/DAC versus 10.05T+MSC-shSOCS1/DAC (Fig. 5E). Thus, other genes (Table 2) identified to be methylated in CAFs upon interacting with PDAC tumor cells may also be important for supporting PDAC growth.

Finally, we attempted to determine whether patient-derived CAFs may promote PDAC xenograft growth on mice. Panc 10.05 tumor were inoculated into NOD/SCID mice subcutaneously alone or with the patient-derived CAFs that were in sufficient quantity for this experiments. Among these CAFs, 9.28F showed no methylation in SOCS whereas 10.29F and 2.01F showed methylation (Fig. 1). The tumor-free survival of NOD/SCID mice co-inoculated with Panc 10.05 tumor and CAF 10.29F or 2.01F was significantly shorter than that of mice inoculated with Panc 10.05 tumor alone, whereas the tumor-free survival of NOD/SCID mice co-inoculated with Panc 10.05 tumor and CAF 9.28F was not significantly different from that of mice inoculated with Panc 10.05 tumor alone.

Figure 5. DNA methylation and SOCS1 downregulation in CAFs are critical for supporting PDAC growth on mice. A, volume measurements of xenograft tumors harvested at week 5 following inoculation of Panc 10.05 tumor in one representative experiment. 10.05T, mice inoculated with Panc 10.05 tumor alone ($n = 4$); 10.05T+Mice/DAC, DAC-pretreated mice inoculated with 10.05T ($n = 4$); 10.05T+MSC, 10.05T mixed with MSCs inoculated on untreated mice ($n = 4$); 10.05T+MSC/DAC, 10.05T mixed with MSCs pretreated with DAC ($n = 8$). B, tumor-free survival was compared between the 10.05T+MSC group ($n = 16$) and the 10.05T+MSC/DAC group ($n = 17$). C, tumors harvested from the experiment in A are shown. The red cycle indicates no tumor. Four biggest tumors are shown as representatives for the 10.05T+MSC/DAC group. D, volume measurements of xenograft tumors harvested at week 7 following inoculation of Panc 10.05 tumor. 10.05T+MSC-ctrl, 10.05T mixed with MSCs controlled for shRNA transduction ($n = 10$); 10.05T+MSC-ctrl/DAC, 10.05T mixed with MSCs controlled for shRNA transduction and pretreated with DAC ($n = 10$); 10.05T+MSC-shSOCS1/DAC, 10.05T mixed with MSCs transduced with shRNA targeting SOCS1 and pretreated with DAC ($n = 16$). E, tumor-free survival was compared between groups shown in D. 10.05T+MSC-ctrl ($n = 20$) vs. 10.05T+MSC-ctrl/DAC ($n = 20$), $P = 0.009$; 10.05T+MSC-shSOCS1/DAC ($n = 21$) vs. 10.05T+MSC-ctrl/DAC ($n = 20$), $P = 0.1204$. F, four largest tumors as representatives from each group in D are shown. G, tumor-free survival was compared between the groups of NOD/SCID mice inoculated with Panc 10.05 alone or in combination with different patient derived CAFs. 10.05T, mice inoculated with 10.05T alone; 10.05T+9.28F, mice inoculated with 10.05T and CAF 9.28F; 10.05T+10.29F, mice inoculated with 10.05T and CAF 10.29F; 10.05T+2.01F, mice inoculated with 10.05T and CAF 2.01F; CAF, a representative CAF sample alone. N = 10 per group. 10.05T+10.29F vs. 10.05T, $P = 0.017$; 10.05T+2.01F vs. 10.05T, $P = 0.044$; 10.05T+9.28F vs. 10.05T, $P = 0.217$. All experiments were repeated twice. A and D, comparisons were analyzed by a two-tail unpaired Student $t$ test; B, E, and G, comparisons were analyzed by the log-rank (Mantel–Cox) test.
free survival of NOD/SCID mice co-inoculated with Panc 10.05 tumor and CAF 9.28F was minimally shorter than that of mice with Panc 10.05 tumor inoculated alone (Fig. 5G). CAFs themselves did not form any tumor. This result suggested that the ability of patient-derived CAFs with SOCS1 methylation in promoting PDAC growth is stronger than that of CAFs without SOCS1 methylation.

Discussion

This is the first report demonstrating that CAFs within the TME are modulated by PDAC tumor cells through epigenetic regulation in the form of DNA methylation. By inducing methylation in CAFs, SOCS1 expression is suppressed, which leads to the activation of its downstream signaling pathways, such as the phosphorylation of STAT3. As a result, cytokines and growth factors such as IGF-1 are induced in the TME. Thus, cancer cells create a TME that supports their growth. This may be a novel and critical mechanism for tumor initiation, progression, and/or metastasis formation. Our findings also implicate that stromal fibroblasts at their baseline may be hostile to the tumorigenesis of PDACs if they are not modulated by tumor cells at the epigenetic level. Stromal fibroblasts modulated by tumor-induced methylation may subsequently reprogram the TME from an anticancerous one to a pronocancerous one. Hence, our findings are consistent with the recently published studies showing that general depletion of stromal fibroblasts, presumably prior to being modulated by tumor-induced methylation, facilitates PDAC development and metastasis in the mouse model (10, 11).

SOCS1 promoter hypermethylation and decreased expression of SOCS1 have been reported in pancreatic epithelial tumor cell lines and minimally dissected PDAC tissues (38). We did not observe SOCS1 hypermethylation in low-passage primary cultures of PDAC cells. It is possible that SOCS1 promoter hypermethylation had occurred spontaneously during the continuous culture of established PDAC cell lines.

DNA methylation is reversible, thus targeting methylation susceptible genes provide promising targets for cancer treatment and prevention. This study suggests that cell surface receptors mediate direct contact between tumor cells and CAFs. Such cell surface receptors, once identified, are ideal drug targets for modifying DNA methylation mechanisms specifically in CAFs. Both integrin and adheren pathways are significantly upregulated at the mRNA level in CAFs and MSCs upon coculture with PDAC tumor cells (Supplementary Fig. S3), supporting the investigation of both integrin and adheren families of cell surface molecules as candidates that may mediate direct contact between tumor cells and CAFs. Another future step is to determine whether methylation in stromal SOCS1 influences the clinical outcomes of patients with PDACs. Interestingly, the patients (3.30, 7.09, 9.28) whose CAFs did not show SOCS1 methylation all have an overall survival more than 3 years. This is consistent with our result with a limited sample number of patient-derived CAFs showing that CAFs without SOCS1 methylation did not appear to have the ability in promoting PDAC growth in mice in contrast to those with SOCS1 methylation. Therefore, the prognostic value of SOCS1 methylation in the stroma will need to be investigated in a larger number of stroma specimens dissected from PDACs.

We observed a global effect on gene methylation in CAFs upon interacting with PDAC tumor cells, whereas a previous study found very few methylated genes that responded to the 5-aza-deoxycytidine treatment in CAF cell lines (20). This discrepancy is likely attributed to the difference between primary CAF cultures in this study and immortalized CAF lines used in the previous study (20). Consistently, Shaky and colleagues reported expression changes of a large panel of genes that are reversible with DAC treatment in CAFs from a transgenic mouse model of pancreatic cancer (39). It was also recently reported that breast carcinoma cells altered the expression of genes, particularly ADAMTS1, in breast fibroblasts, not via promoter methylation, but via epigenetic alterations involving EZH2/H3K27 (40), suggesting that other epigenetic regulating mechanisms may be involved in reprogramming CAFs. In addition, although we show that colon cancer lines tested did not induce SOCS1 methylation in MSCs, we found that a similar tumor-induced methylation mechanism involving either the same or different genes expressed in stromal fibroblasts may exist for other types of malignancies. Moreover, a similar tumor-induced DNA methylation process may occur universally in other stromal cells including tumor infiltrating immune cells, thus highlighting the significance of our findings for further exploration.

Disclosure of Potential Conflicts of Interest

E.M. Jaffe reports receiving a commercial research grant from Corvus Pharmaceuticals, and reports other commercial research support from Bristol-Meyers Squibb, Aduro Biotech; he is a consultant/advisory board member for MedImmune. No potential conflicts of interest were disclosed by the other authors.

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