N-acetyl Cysteine Induces Quiescent-like Pancreatic Stellate Cells From an Active State and Attenuates Cancer-stroma Interactions

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Research

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Abstract

Background: Pancreatic stellate cells (PSCs) occupy the majority of the pancreatic cancer microenvironment, contributing to an aggressive behavior of pancreatic cancer cells (PCCs). Recently, anti-fibrotic agents have proven to be an effective strategy against cancer, but clinical trials have shown little efficacy and the driving mechanism remains unknown. N-acetyl-cysteine (NAC) is often used for cystic fibrosis. Pioglitazone, an agonist of peroxisome proliferator-activated receptor gamma, was often used for type II diabetes, but recently reported to inhibit metastasis of PCCs. However, few studies have focused on the effects of these two agents on cancer-stromal interactions.

Method: We evaluated the expression of α-smooth muscle actin (α-SMA) and the number of lipid droplets in PSCs cultured with or without NAC. We also evaluated changes in invasiveness and proliferation in PSCs and PCCs after NAC treatment. Using an indirect coculture system, we investigated changes in proliferation, invasiveness, and migration of PSCs and PCCs. Combined treatment effects of NAC and pioglitazone were evaluated in PSCs and PCCs. In vivo, PCCs and PSCs were subcutaneously injected into mice to evaluate tumor growth. We co-transplanted KPC-derived organoids and PSCs using a splenic xenografted mouse model and evaluated the effect of combination of NAC and pioglitazone.

Results: In vitro, NAC inhibited the proliferation, invasiveness, and migration of PSCs at a low concentration, but not those of PCCs. NAC treatment significantly reduced expression of α-SMA, collagen type I and fibronecrtin in PSCs. NAC-treated PSCs apparently present quiescent-like state with a high number of lipid droplets. Co-cultured PSCs and PCCs mutually promoted the proliferation, invasiveness, and migration of each other. However, these promotion effects were attenuated by NAC treatment. Pioglitazone maintained the NAC-induced quiescent-like state of PSCs, which were reactivated by PCC-supernatant, and enhanced chemosensitivity of PCCs. In vivo, administration of NAC to mice with subcutaneously implanted PCCs and PSCs significantly reduced tumor growth with less stromal components. The combination of NAC and pioglitazone suppressed liver metastasis in the 3D-organoid xenografted mouse model.

Conclusion: NAC suppressed activated PSCs and attenuates cancer-stromal interactions. NAC induces quiescent-like PSCs that were maintained in this state by pioglitazone treatment.

Introduction

Pancreatic ductal adenocarcinoma (PDAC), which is the ninth most common cancer, causes fourth common in cancer-related deaths in America (1). Although great strides have been made to explore the mechanism of pathogenesis in the past decade, the 5-year survival rate of PDAC is still less than 7% because of the high rate of resistance to chemotherapy and metastasis (2), which indicates that a better treatment strategy should be developed to improve the prognosis of PDAC.

There is a growing consensus has been accepted that the tumor microenvironment may be a significant contributing factor to the aggressive behavior of cancer and its resistance to chemotherapy (3). PDAC is
notable for its excessive desmoplasia that is caused predominately by pancreatic stellate cells (PSCs) (4), which are usually in a quiescent state containing a large amount of vitamin A stored in lipid vacuoles (5). They are activated and become myofibroblast-like cells by various growth factors secreted from pancreatic cancer cells (PCCs), which include platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) (6). As a result, activated PSCs express α-smooth muscle actin (α-SMA) and secrete several cytokines and chemokines, such as PDGF, TGF-β, and connective tissue growth factor (CTGF), which promote the invasion ability of PCCs (7). Activated PSCs also secrete an abundant extracellular matrix (ECM) inducing immunosuppression and chemoresistance (3, 8). Thus, targeting cancer-stroma interactions (PCCs-PSCs) appears to be a promising therapeutic strategy. It has been recently reported that inhibiting Hh signals in tumor stromal cells retards the growth and metastasis of PCCs (9). However, some research revealed that targeting tumor stroma can also promote cancer cell aggressiveness (10, 11). Therefore, owing to the controversial nature of stromal-targeting therapeutic approaches, the precise characteristics of PSCs are still under investigation.

N-acetyl-cysteine (NAC) is an aminothiol and synthetic precursor of de novo GSH synthesis (12). NAC is also commonly applied to ameliorate inflammation that occurs under pathological conditions such as chronic obstructive pulmonary disease, influenza, and idiopathic pulmonary fibrosis (13, 14). NAC also inhibits the activation of transcription factor activities like JNK, p38 MAPK, and NF-κB that regulate the expression of numerous genes (15). Besides, several reports indicate that NAC induces apoptosis in colon carcinoma cells (16). We have previously shown that some anti-fibrosis agents, such as pirfenidone and calpeptin, have the ability to suppress pancreatic cancer by disrupting cancer-stromal interactions (17, 18). Enhanced desmoplastic responses by coadministration of pirfenidone and NAC have been observed in HapT1-derived orthotopic tumors in a hamster model (19). However, there are few studies exploring the effects of NAC on cancer-stromal interactions or the functional alterations of PSCs in PDAC.

In this study, we hypothesized that NAC may be an effective anti-fibrosis against pancreatic cancer and alter the function of PSCs. The present data revealed that low concentrations of NAC suppressed activation of PSCs. NAC-treated PSCs had a quiescent-like state that was maintained by coadministration of NAC and pioglitazone, a ligand of peroxisome proliferator-activated receptor gamma (PPARγ) that used for type II diabetes previously (20). Activated PSCs significantly increased the proliferation and invasiveness abilities of PCCs. Additionally, PCCs also promoted the proliferation and migration abilities of PSCs, but these mutual promotion effects were effectively attenuated by NAC treatment. The effects of NAC were also investigated on xenografts mice which were co-implanted with PCCs and PSCs. Moreover, we used a splenic xenografts model to evaluate effect of a combined treatment with NAC and pioglitazone. Combined treatment with NAC and pioglitazone may be a novel therapy that targets cancer-stromal interactions.

### Materials And Methods

**Cell and reagents**
Human primary activated PSCs were harvested from surgical specimens of pancreatic cancer, established and maintained as described by Bachem et al. (21, 22), (23). The identity of the isolated PSCs was confirmed by their fibroblast-like morphology, positive staining for α-SMA and negative for cytokeratin 19 (CK19) (Fig. S1). Three primary cultures of PSCs were used within eight passages in this study. Three human PCC lines were also used in this study: PANC-1 (Riken Bioresource Center, Ibaraki, Japan), SUIT-2 and BxPC-3 (National Kyushu Cancer Center, Fukuoka, Japan). All cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and maintained at 37 °C with 10% CO₂. NAC and pioglitazone were purchased from Sigma-Aldrich (Tokyo, Japan). For in vitro research, NAC was dissolved in PBS at 100 mM and pioglitazone was dissolved in DMSO at 1 mM. All dilutions were stored at -20°C until use.

PDAC organoid culture

To establish PDAC organoids, we harvested and digested surgical specimens of PDAC patients with a Tumor Dissociation Kit (human, Cat#130-095-929; Miltenyi Biotec, CA, USA), and cultured them using the method as described previously (24). Briefly, the digested tumor tissue was integrated to a growth factor-reduced Matrigel (Cat# 356231; BD Bioscience, CA, USA), and finally cultured in a human complete medium at 37°C for 2 weeks.

Production of conditioned media (CM)

To exclude effects of supernatant stimulation between PCCs and PSCs, we prepared the conditioned media using supernatant from cultured PSCs and PCCs with an Amicon Ultra-15 30K Centrifugal Filter Device (Millipore, Germany) in accordance with the manufacturer’s protocol. In brief, subconfluent (75%) SUIT-2 cells, PSCs or 2.5 mM NAC-treated PSCs were cultured in serum-free DMEM for 48 hours and then 12 mL culture supernatant was collected into Filter Device, centrifuged at 5,000 g in a fixed-angle rotor for 30 minutes following recovered with 10 mL of 11.2 mM NaCl. The conditioned media (supernatant; SN) was recovered with removal of salts and stored at -20 °C until use.

Immunofluorescence staining

Cells were plated in glass-bottomed dishes (ibidi, Munich, Germany) at 1 × 10⁵ cells/well and treated with NAC or control (PBS) for 48 h, then fixed with −20 °C ethanol, blocked with 3% BSA in PBS and incubated with 10 mg/mL indicated primary antibodies at 4 °C overnight. These primary antibodies were used: anti-α-SMA (1:100), anti-CK19 (1:100), or anti-vimentin (1:100). The corresponding 10 mg/mL secondary antibodies carrying green- and red-fluorescent dye and 1 mg/mL nuclear DNA binding 4′,6-diamidino-2-phenylindole (DAPI; Dojindo, Kumamoto, Japan) were applied for protein marking. They were incubated at room temperature for 60 minutes, and then washed with 0.1% BSA, detected by a fluorescence microscope (BZ-X710; Keyence).

Cell proliferation assays
CellTiter-Glo assay (Promega; Madison, WI) was performed in accordance with the manufacturer’s protocol. PSCs or PCCs were seeded onto 96-well plates (Greiner Bio-One, Kremsünster, Austria) in triplicate at $1 \times 10^3$ cells/well with 100 µL fresh DMEM containing 10% FBS. To prevent stimulation effects, the medium was replaced and added with NAC, PBS or each SN indicated in the figure legends at 24 hours after seeding. For all cell proliferation assays, data are representative of three independent biological experiments.

Invasion and migration assays

The ability of migration and invasion in PSCs and PCCs were performed by counting the number of cells migrated or invaded through a Transwell System (8-mm pore size; Becton Dickinson, Franklin Lakes, NJ). For monoculture or co-culture migration and invasion assays, we seeded cells and treated them as previous described (40). For a collagen-coated invasion assay, membranes were coated with 100 µl collagen type I (354236; Corning, America), type IV collagen (354233; Corning), or Matrigel at 20 mg per well. To assess the effects of PSC and PCC supernatant stimulation on migration and invasion, separate batches of cells were cultured with each SN indicated in the figure legends. At 24 hours (for migration) and 48 hours (for invasion) after cell seeding, migrated or invaded cells were fixed with 70% ethanol, stained with hematoxylin and eosin, and counted in five random fields ($\times$100 magnification). For all migration and invasion assays, three experiments were independently conducted in triplicate.

Lipid droplet accumulation assay

Lipid droplet accumulation in PSCs was analyzed by staining with bodipy (#D-3922; Life Technologies) as described previously (25). After staining, images were captured in three random fields using fluorescence microscope.

qRT-PCR analysis

Total RNA of cells was extracted using a High Pure RNA Isolation kit (Roche, Mannheim, Germany). We performed qRT-PCR using a QuantiTect SYBR Green RT-PCR kit (Qiagen, Tokyo, Japan) and the CFX96 Real-Time PCR System (Bio-Rad Laboratories, Hercules, CA). 18S rRNA was used to normalize mRNA expression. All primers were purchased from Sigma-Aldrich, for details, see supplementary Table S1. Three experiments were independently conducted in triplicate.

Western blotting analysis

Total cellular proteins of PSCs and PCCs were prepared with PRO-PREP Protein Extraction Solution (InTron Biotechnology, Seongnam, Korea). Supernatant proteins were collected as described previously (25). Protein samples of cell lysates (20 mg) or supernatants (30 mg) were separated by 10% SDS PAGE on Mini-PROTEAN TGX Precast Gels (Bio-Rad Laboratories), followed by transfer to Trans-Blot Turbo Mini PVDF Transfer Packs (Bio-Rad Laboratories). The membrane was incubated at 4 °C overnight with primary antibodies (1:1000; supplementary Table S2) and then probed with secondary antibodies.
Immunoblot were developed by ECL western blotting detection reagent (Bio-Rad) and analyzed with the ChemiDoc XRS System (Bio-Rad Laboratories).

In vivo experiments

For in vivo experiments, we used 4-week-old female nude mice (Kyudo Co.) under the approval of Ethics Committee of Kyushu University. For subcutaneous transplantation experiments, PCCs (1 × 10^6) were suspended in 100 mL DMEM and subcutaneously transplanted into the left flank of mice, PSCs and PCCs (5 × 10^5, respectively) were implanted into the right flank of mice. One week after implantation (day 7), mice were intraperitoneally injected with 100 µl PBS (control, n = 6) or NAC (500 mg/kg, n = 6) once every two days for 4 weeks and then sacrificed on day 35. To explore the effects of combination with PLZ in an organoid model, nude mice underwent splenic implantation of PDAC organoids (1 × 10^5) with PSCs (1 × 10^5). One week after implantation (day 7), mice were injected intraperitoneally with control (100 µl PBS + 0.1 µl DMSO), NAC (500 mg/kg), PLZ (4 mg/kg) or cotreatment (NAC + PLZ) once every two days for 3 weeks and sacrificed on day 28. All orthotopic tumors and liver tissues were resected and weighed. We estimated tumor volume by the following formula: \(\pi/6 \times (L \times W \times W)\) (L = the largest tumor diameter, W = the smallest tumor diameter) (17). Mouse tumor tissues were prepared to 4-µm-thick paraffin sections for experimental validation at the tissue levels.

Immunohistochemistry

For immunohistochemistry (IHC) staining, the following primary antibodies were used: anti-\(\alpha\)-SMA (1:500), mouse monoclonal anti-CK 19 (1:100), and rabbit PCNA (1:500; abcam). Tissues were immunostained as described previously (26). Masson's trichrome staining were performed by ready-to-use kit (Sigma-Aldrich). To calculate \(\alpha\)-SMA-positive and PCNA-positive index, three random fields were counted by microscope (BZ-X710; Keyence). Serial sections were used for immunohistochemical staining.

Statistical analysis

For in vitro experiments, results are expressed as means ± SD. Significances between two groups were assessed by one-way analysis of the variance or the Student's t-test using GraphPad Prism 7.0 (GraphPad Software). Values of \(P < 0.05\) were considered as statistically significant in all analyses. Survival analyses were conducted using the Kaplan-Meier method. All experiments were performed at least three times with the exception of animal experiments.

Results

NAC inhibits the proliferation, migration, and invasion of PSCs rather than PCCs

First, we investigated the proliferation of both PSCs and PCCs by NAC treatment. In monoculture, NAC inhibited the proliferation of PSCs in a dose-dependent manner, and the IC_{50} of each primary culture of
PSCs was calculated (Fig. 1A; supplementary Fig.S2B–D). However, PCC proliferation was not inhibited by NAC at low concentrations (Fig. 1B; supplementary Fig.S2E–G). We found that PSC proliferation was inhibited effectively at 2.5 mM (0.46 mg/mL). Similarly, NAC also effectively inhibited the migration and invasion ability of PSCs, but not those of PCCs (Fig. 1C–F). These results indicate that NAC, which is inexpensive drug, can be used as an anti-tumor agent for pancreatic cancer by targeting PSCs. In following experiments in vitro, we used 2.5 mM NAC to investigate its specific effects on PSCs.

NAC decreases activation of PSCs and induces a quiescent-like state

Activated PSCs expressed a high level of α-SMA. After NAC treatment, the expression of α-SMA and fibronectin was decreased markedly (Fig. 2A, B). However, NAC did not decrease the expression of vimentin. Moreover, treatment with NAC increased the number of lipid droplets in PSCs (Fig. 2C), which indicated transition from activation to a quiescent-like state. These data indicate that NAC decreases the activity of PSCs.

To examine the characteristics of NAC-treated PSCs, we treated PSCs with a low concentration of NAC (2.5 mM) for 2 weeks and then cultured without NAC for another 2 weeks (Fig. 2D). The number of PSCs with spindle-like morphology was decreased significantly and the number of PSCs with star-like morphology was generally increased (Fig. 2D–F; supplementary Fig.S3B). PSCs treated with NAC on day 28 had more lipid droplets (supplementary Fig.S3A) and less expression of α-SMA compared with untreated PSCs (day 0) (Fig. 2G; supplementary Fig.S3E). Furthermore, flow cytometry showed that treatment with 2.5 mM NAC for 24 h increased the apoptosis rate of PSCs (supplementary Fig.S3D). To explore the morphological change of PSCs, we used time-lapse photography to record PSCs after treatment with a high concentration of NAC (6 mM) (supplementary Fig.S3C). Most PSCs exhibited an apoptosis-like morphology, namely evident shrinkage, at 24 hours.

NAC attenuates cancer-stroma interaction in pancreatic cancer

To explore the effects of NAC on cancer-stroma interaction, SUIT-2 supernatant (SUIT-SN), normal PSCs supernatant (PSC-SN), and NAC-treated PSC supernatant (NAC-PSC-SN) were prepared. We assessed the ability of proliferation, migration, and invasiveness in PCCs and PSCs after each supernatant stimulation (Fig. 3A; supplementary Fig.S4A). PSC-SN increased these abilities of PCCs (Fig. 3B; supplementary Fig.S4B, C), while SUIT-SN also promoted these abilities of PSCs (Fig. 3C, Fig. 3F, G). We also performed indirect coculture transwell assays using PCCs and PSCs (Fig. 3A). PSCs clearly promoted the migration and invasiveness of PCCs (Fig. 3D, E). However, these promotion effects were attenuated by NAC. NAC-treated PSCs did not show enhanced proliferation, migration, or invasiveness (Fig. 3B, D, and E). Mutually, PSCs activity was also inhibited regardless of PCC-SN treatment (Fig. 3C, F, and G).

In the proliferation assay, PCCs treated with PSC-SN and 5 mM NAC demonstrated no significant difference from PCCs treated with only PSC-SN (Fig. 3B). SN derived from NAC-treated PCCs still enhanced the proliferation of PSCs (Fig. 3C). These results indicate that NAC attenuates cancer-stroma interactions by targeting PSCs.
Characteristic changes in PSCs induced by NAC treatment in cancer-stroma interaction

To determine the secreted factors related to the inhibitory effects of NAC on cancer-stroma interactions, we evaluated mRNA and protein expression levels of those factors in PSC-SN and whole PSC cell lysates (Fig. 4A). Treatment with NAC significantly reduced the mRNA and protein levels of most secreted factors, especially PDGF-A, PDGF-B, TGF-β, CTGF, VEGF, α-SMA, FN, and collagen type I, but not collagen IV. Exposure to PCC-SN considerably increased the expression of PDGF-A, IL-6, CTGF, MMP2, α-SMA, FN, but NAC still effectively inhibited their expression. PCC-SN dramatically decreased collagen IV in supernatants from PSCs, although there was no significant change of collagen IV in whole cell lysate. NAC inhibited the decrease of collagen IV in supernatants of PSCs treated with PCC-SN (Fig. 4A). These inhibitory effects were also observed by immunofluorescence staining (Fig. 4B). Compared with normal Matrix- and collagen type I-coated gels, the collagen type IV-coated gel showed a reduced number of invaded PCCs (Fig. 4C), which indicated that collagen type IV may have an anti-invasion function that was not affected by NAC. These findings suggest that NAC is an effective agent that targets tumor-stroma interactions.

To assess the mechanism mediated by NAC, we investigated several signaling pathways associated with activation of PSCs. Activities of PI3K-AKT and NF-κB signaling pathways in PSCs were significantly decreased after NAC treatment. However, there was no change in ERK pathway activity (Fig. 5A). Exposure to PCC-SN increased the activities of PI3K-AKT and NF-κB signaling pathways in NAC-treated PSCs (supplementary Fig.S5A). We also found that the expression of peroxisome proliferator-activated receptor gamma (PPARγ), a nuclear receptor that regulates fatty acid storage and glucose metabolism (27), was significantly decreased (Fig. 5A).

Combined NAC with PLZ suppresses reactivation of PSCs and enhances chemotherapy sensitivity of PCCs

We evaluated the therapeutic efficiency of the combination of NAC and pioglitazone (PLZ; a ligand of PPARγ). When treated with NAC, PLZ, or both, a morphological change was observed in PSCs (supplementary Fig.S5B). PLZ (20 µM) inhibited neither the proliferation of NAC-treated PSCs directly (Fig. 5C) nor the migration and invasiveness of PSCs (supplementary Fig.S5C). NAC-treated PSCs were reactivated by SUIT-2-SN (Fig. 5B, C). However, when cotreated with 20 µM PLZ, PSCs maintained their inactive state.

The expression of PPARγ was also increased in PCCs after NAC treatment (Fig. 5D). Although low concentrations of PLZ or NAC alone did not inhibit the viability of PCCs, their cotreatment remarkably restricted the proliferation, migration, and invasiveness of PCCs (Fig. 5E, F; supplementary Fig.S5D). These results indicate that cotreatment of NAC and PLZ may be a promising new approach for cancer treatment.

NAC inhibits subcutaneous tumor growth in mice
To evaluate the effects of NAC on cancer-stroma interactions in vivo, we implanted SUIT-2 cells \(1 \times 10^6\) alone into the left flank of mice and co-implanted SUIT-2 cells with PSCs \(5 \times 10^5\), respectively) into the right flank of same mice (Fig. 6A). At day 7, NAC, or PBS (control) were injected intraperitoneally into the mice for 4 weeks. In the group without treatment, tumors in right flank (SUIT-2 + PSC) were much larger than left flank (SUIT-2 alone) (Fig. 6A, B). Although the left flank showed few \(\alpha\)-SMA-positive cells, probably originated from the host, the tumors in the right flank showed much more numbers of \(\alpha\)-SMA-positive cells and collagen fiber than left flank (Fig. 6C–F). Treatment with NAC significantly suppressed the growth of tumors in both flanks compared with those of the control group (Fig. 6A, B). Compared to control group, NAC decreased the collagen fiber areas and \(\alpha\)-SMA positive index in both flanks (Fig. 6C–E). NAC also reduced the PCNA index in right flank but not in left flank compared with control group (Fig. 6F). These findings suggest that NAC suppresses tumor formation by targeting stromal cells in pancreatic cancer.

Combined treatment of NAC with PLZ inhibits metastasis in KPC cancer organoid cells co-implanted with PSCs

We previously established pancreatic cancer organoids for 2 weeks using PDAC cells derived from the KPC mouse (Fig. 7A). Compared with normal 2D-cultured cells, the implanted tumor showed more collagen fiber areas and more \(\alpha\)-SMA-positive index in splenic xenograft models with 3D-organoids (supplementary Fig.S6C-F). Thus, we co-implanted KPC cancer-derived organoids with KPC-derived PSCs into the spleen of nude mice and treated mice intraperitoneally with the vehicle, NAC, PLZ or a combination for three weeks. On day 28, the mice were sacrificed and their liver metastases were harvested and evaluated (Fig. 7B-C). Compared with controls, the number of metastatic nodules (15 vs. two, average) in the liver (Fig. 7D) (liver volume: 2.61 vs. 1.41 cm\(^3\), average; liver weight: 2.12 vs. 1.13 g, average; Fig. 7E) was decreased significantly in the combined treatment group, although NAC or PLZ alone also decreased the number of metastatic nodules (Fig. 7D). The number of peritoneal disseminated nodules was also significantly decreased after the combined treatment (Supplementary Fig.S6B). These findings indicate that NAC suppresses metastasis of PDAC organoids, and combined treatment of NAC with PLZ has cooperative effects on inhibiting tumors metastasis.

**Discussion**

In this study, we investigated the effects of NAC on PCCs and PSCs, as well as its functional effect on pancreatic cancer-stroma interactions. We found that PCCs efficiently increased the proliferation, migration, and invasion of PSCs. PSCs also increased these abilities of PCCs. Through these tumor-stroma interactions, PCCs showed more rapid growth and higher malignancy. However, NAC strongly suppressed the activities of PSCs and decreased the production of growth factors at a low concentration rather than those of PCCs and attenuated the protumorigenic effects promoted by cancer-stroma interactions. NAC-treated PSCs were reactivated after exposure to PCC-SN, but cotreatment with NAC and PLZ maintained their quiescent-like state. Thus, NAC enhanced the therapeutic effect of PLZ at low concentration in PCCs. This is the first report showing that NAC-induced quiescent-like PSCs from an
activated state were maintained by combined treatment with PLZ. This combination therapy may induce conversion from protumorigenic PSC to anti-tumorigenic PSCs, which leads to remodeling the tumor microenvironment of pancreatic cancer.

Current studies have focused mainly on suppressing pancreatic cancer desmoplasia or disrupting cancer-stroma interactions. Previously, agents such as pirfenidone and calpeptin have been used to suppress desmoplasia, which made PCCs more sensitive to gemcitabine (16, 17). Moreover, Sujit, et al. combined pirfenidone and NAC to decrease tumor growth in orthotopic tumor models via inhibition of TGF-β and ROS in PSCs (19). Thus, anti-fibrotic and anti-NAF therapeutic strategies appear to have a great effect in anti-tumor therapy. However, the exact mechanism underlying their combined effect and the functional or state alteration of NAC-treated PSCs were yet to be investigated. In the present study, NAC treatment inhibited activated PSCs and significantly decreased the growth factors and prominent ECM via alterations of multiple pathways. Our data also revealed that NAC not only decreased desmoplasia, but also induced a quiescent-like state in PSCs. Quiescent or resting PSCs are star-shaped stellate cells that are activated when needed (28). After external stimulation such as stress, reactive oxygen species (ROS), chemokines or growth factors, quiescent fibroblasts can reversibly transfer to normal activated fibroblasts (NAFs), which express α-SMA and vimentin and present spindle-shaped morphology (28). These fibroblasts show high expression of α-SMA, which is why they also called myofibroblasts (29).

It has been well reported that multiple signaling pathways and molecules are involved in the activation and pro-tumor cell functions of PSCs, including PI3k-AKT, NF-κB, and ERK1/2 signaling pathways (30–34). The change of these signaling pathway underlying NAC-treated PSCs were also investigated in this study. We found that PCCs increased activation of PI3K-AKT and NF-κB signaling pathways in PSCs derived from tumor tissue via cancer-stroma interactions. The present data also revealed that NAC treatment significantly suppressed activated PI3K-AKT and NF-κB pathways, but not of the ERK1/2 signaling pathway in PSCs. Moreover, we found that expression of PPARγ was increased in PSCs treated with NAC and that cotreatment with NAC and PLZ, a ligand of PPARγ, significantly inhibited not only PI3K-AKT and NF-κB pathways, but also activation of ERK1/2 in PSCs. The expression level of PPARγ decreases markedly during activation of hepatic stellate cells (35). These findings suggest that administration of PLZ maintains PSCs in a quiescent state induced by NAC.

Previously, PLZ was often used for type II diabetes, but recently identified as a potential therapeutic agent to inhibit proliferation and metastasis of PCCs (36). However, the treatment of a high concentration of PLZ was reported to increase the risk of bladder cancer (37). In this study, we administrated PLZ with NAC at a safe concentration in coculture or coimplantation of PSCs and PCCs and observed promising
therapeutic effects. These data suggest that NAC increases sensitivity to PLZ in PCCs, although it cannot inhibit PCCs directly. Thus, cotreatment with NAC and PLZ may exert its therapeutic effects in both PSCs and PCCs.

We found that NAC inhibited the secretion of most ECM proteins, but expression of collagen type IV, one of the major components of the basement membrane (BM), was maintained even in NAC-treated PSCs. BM is a sheet-like structure that separates ductal epithelial cells from the surrounding stroma. PCCs also increase the secretion of various Matrix metalloproteinases (MMP) to decompose the BM structure for their invasion. (38). The BM primarily consists of collagen IV that is degraded by extracellular proteases such as MMP2 and MMP9 (39). We previously reported that PSCs secreted MMP2 and induced the BM destruction in cancer-stromal microenvironment (24). The present results showed that NAC decreased the expression of MMP2 in PSCs, which possibly maintained the BM structure and impeded PCCs invading into stroma. These data indicate that the microenvironment in pancreatic cancer was altered to an anti-tumor microenvironment by NAC treatment. To clarify the precise mechanism induced by the changes in collagen type IV and MMP-2 expression when PSCs were treated with NAC, further studies will be performed in the future.

Tumor organoid is considered as a brand-new model tool in biomedical research. In a previous study, we demonstrated that an ERK inhibitor reduces the number of metastasis in xenografts formed by tumor organoids (40). In the present study, we performed in vivo xenograft experiments using KPC mouse-derived cancer organoids that represent the microenvironment in PDAC, including PSCs. PSCs cocultured with organoids from pancreatic cancer induced excessive desmoplasia compared with coculture with PCCs, which indicated that this organoid model is of great benefit to investigate the therapeutic effect of combined NAC and PLZ treatment.

Conclusion

In summary, the present data indicated that NAC treatment decreases the activity of PSCs and attenuates cancer-stroma interactions. Moreover, the combination of NAC and PLZ maintains the quiescent-like state of PSCs, which leads to enhanced therapeutic effect on tumor stromal components. Furthermore, NAC makes PCCs more sensitive to PLZ treatment. Taken together, the present data suggest that combination therapy of NAC and PLZ is a promising treatment that targets for both stromal and cancer cells in pancreatic cancer.

Abbreviations

PSC
Pancreatic stellate cell; PDAC: Pancreatic ductal adenocarcinoma; PCC: Pancreatic cancer cell; NAC: N-acetyl-cysteine; PPARγ: Peroxisome proliferator-activated receptor gamma; IC50: 50% inhibitory concentration; PLZ: Pioglitazone; KPC: LSL-Kras G12D/++; LSL-Trp53R172H/++; Pdx-1-Cre; ERK: Extracellular
signal-regulated kinase; PI3K:Phosphatidylinositol-3 kinase; SN:supernatant; MMP:Matrix metalloproteinases

**Declarations**

**Ethics approval and consent to participate**

This study was approved by the Kyushu University Institutional Review Board (Fukuoka, Japan). The use of surgical specimens was approved by the Ethics Committee of Kyushu University (Fukuoka, Japan) and conducted in accordance with the Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government and Declaration of Helsinki. All animal procedures and care were conducted in accordance with institutional guidelines and in compliance with national and international laws and policies.

**Consent for publication**

Not applicable.

**Availability of data and material**

All data generated or analyzed during this study are included either in this article or in the Additional file.

**Competing interests**

The authors have declared that no competing interests exists.

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**Author contributions**

HF designed the study, conducted experiments, acquired, and analyzed data, and wrote the manuscript. NS, TK, CI, KS, NI, SN, KN, and KM discussed and revised the manuscript; TM, KO, and MN were responsible for conception and supervision of the study and wrote the manuscript. All authors revised drafts and approved the final version of the manuscript.

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