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Pancreatic Stellate Cells Have Distinct Characteristics From Hepatic Stellate Cells and Are Not the Unique Origin of Collagen-Producing Cells in the Pancreas

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Objectives: The origin of collagen-producing myofibroblasts in pancreatic fibrosis is still controversial. Pancreatic stellate cells (PSCs), which have been recognized as the pancreatic counterparts of hepatic stellate cells (HSCs), are thought to play an important role in the development of pancreatic fibrosis. However, sources of myofibroblasts other than PSCs may exist because intensive studies of liver fibrosis have uncovered myofibroblasts that did not originate from HSCs. This study aimed to characterize myofibroblasts in an experimental pancreatic fibrosis model in mice.

Methods: We used transgenic mice expressing green fluorescent protein via the collagen type I $\alpha 1$ promoter and induced pancreatic fibrosis with repetitive injections of cerulein.

Results: Collagen-producing cells that are negative for glial fibrillary acidic protein (ie, not derived from PSCs) exist in the pancreas. Pancreatic stellate cells had different characteristics from those of HSCs in a very small possession of vitamin A using mass spectrometry and a low expression of lecithin retinol acyltransferase. The microstructure of PSCs was entirely different from that of HSCs using flow cytometry and electron microscopy.

Conclusions: Our study showed that characteristics of PSCs are different from those of HSCs, and myofibroblasts in the pancreas might be derived not only from PSCs but also from other fibrogenic cells.

Key Words: pancreatic fibrosis, chronic pancreatitis, myofibroblast, pancreatic stellate cell, collagen, vitamin A

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Excessive amounts of extracellular matrix (ECM) cause organ fibrosis due to the loss of balance between ECM synthesis and degradation. Chronic pancreatitis is characterized by inflammation and the replacement of parenchymal cells with fibrotic tissue, which leads to functional alterations such as debilitating exocrine and occasional endocrine insufficiency. The incidence of chronic pancreatitis is increasing,¹ and patients with chronic pancreatitis also have a substantially increased risk for pancreatic cancer, which is commonly known as an abundant fibrotic component associated with chemoresistance.^{2–5}

To date, there is no approved treatment of pancreatic fibrosis. Determining the mechanism of pancreatic fibrosis is essential to develop a new therapeutic approach. Collagen-producing myofibroblasts are potential therapeutic targets of organ fibrosis because they directly contribute to the development of fibrosis via ECM synthesis. Despite the recognition of its enormous importance, the origin of myofibroblasts in pancreatic fibrosis is still unknown.

It has been suggested that activated pancreatic stellate cells (PSCs) play an important role in the development of pancreatic fibrosis. Pancreatic stellate cells are activated by a variety of inflammatory mediators such as transforming growth factor β and interleukins 1, 6, and 10, then they transdifferentiate into myofibroblasts and produce ECM, including many types of collagen, laminin, and fibronectin, while releasing some degradation enzymes such as matrix metalloproteinases.^{6–9}

Quiescent PSCs are fat-storing cells and exhibit autofluorescence due to cytoplasmic lipid droplets containing vitamin A.^{6,7} Pancreatic stellate cells that are isolated using density gradient centrifugation are in a quiescent state and express intermediate filament proteins such as desmin and glial fibrillary acidic protein (GFAP).¹⁰ Primary PSCs become activated after a few days in culture, which is characterized by the loss of lipid droplets, transdifferentiation into a myofibroblast-like phenotype, and the expression of α -smooth muscle actin (α -SMA).⁶ Then activated PSCs secrete some ECM proteins on a culture plate. A small contribution of bone marrow–derived cells to the PSCs population has been reported,^{11–13} but the origin of PSCs remain unresolved.

In liver fibrosis, recent studies have shown that the origins of myofibroblasts other than hepatic stellate cells (HSCs) exist, such as portal fibroblasts, fibrocytes derived from bone marrow cells, or epithelial cells (ie, hepatocytes and cholangiocytes) undergoing epithelial-mesenchymal transition.^{14–19} However, there is still heated debate about this issue, and it remains controversial. In light of the history of pancreatic fibrosis research, which has been similar to liver fibrosis research in many aspects, it is plausible that there are myofibroblasts originating from non-PSC sources as well, and this possibility has not been as intensely debated as the HSC origins in liver fibrosis. Collagen–green fluorescent protein (Coll-GFP) mice, in which GFP is expressed using the

collagen type I $\alpha 1$ (Col1 $\alpha 1$) promoter, are excellent tools for identifying collagen-producing cells in vivo. Indeed, several studies efficiently used this strain to tag collagen-producing cells in fibrosis research of several organs such as the liver,^{18–21} the kidneys,²² and the lungs.²³ However, no studies of pancreas fibrosis have used this strain.

Once advanced fibrosis occurs, pancreatic endocrine and exocrine functions are irreversibly impaired, and patients with pancreatic fibrosis have high morbidity from pancreatic cancer. Therefore, determining the mechanism of pancreatic fibrosis progression is key for improving the prognosis of patients with chronic pancreatitis. This study aimed to investigate the characteristics of myofibroblasts in an experimental pancreatic fibrosis model by taking advantage of Coll-GFP mice.

MATERIALS AND METHODS

Animals

Coll-GFP mice with C57BL/6 background were kindly provided by Dr. David Brenner of the University of California, San Diego, and the mice were housed in our specific pathogen-free animal facility. To induce pancreatic fibrosis, the male mice were given 6 intraperitoneal injections of the cholecystokinin analog cerulein (Sigma-Aldrich, St Louis, Mo) at a dose of 50 μ g/kg at hourly intervals for 3 days a week for 6 weeks. They were killed 72 hours after the last injection.²⁴ The Coll-GFP control mice were injected with physiological saline in the same way. The animal protocols were approved by the Animal Research Committee of Kyoto University, and all experiments were conducted in accordance with the *Guidelines for the Care and Use of Laboratory Animals* from the National Institute of Health.

Histological Examination

Formalin-fixed, paraffin-embedded specimens were sectioned at a 4- μ m thickness and were stained with hematoxylin and eosin (H&E) and Sirius red.

Immunohistochemistry

Formalin-fixed, paraffin-embedded sections were retrieved by incubation in citrate buffer at 90°C for 20 minutes. They were incubated with a primary antibody recognizing GFAP (no. 556330; BD Biosciences, San Jose, Calif) at a 1:50 dilution at 4°C overnight and then incubated with EnVision+ System horseradish peroxidase-labeled polymer (Dako, Glostrup, Denmark) and counterstained with hematoxylin.

Immunofluorescence

For immunofluorescence, the specimens were fixed with 4% paraformaldehyde, followed by embedding in optimal cutting temperature compound, and were sliced at a 2- μ m thickness. The antigens were retrieved by incubation in citrate buffer at 90°C for 20 minutes. The sections were incubated with a primary antibody recognizing α -SMA (no. ab5694; Abcam, Cambridge, UK) at a 1:100 dilution and GFAP (no. 556330; BD Biosciences) at a 1:50 dilution at 4°C overnight and were then incubated with a secondary Alexa Fluor 594 antibody (antirabbit no. 11012, antimouse no. 11005; Invitrogen, Carlsbad, Calif) at a 1:200 dilution at room temperature for 1 hour. Collagen GFP expression was detected using fluorescent microscopy.

Cell Isolation

Pancreatic nonparenchymal cells (NPCs) were isolated from Coll-GFP mice pancreata using a previously described density gradient centrifugation method.^{6,7,11,25} In brief, the pancreata were resected and minced with scissors and then digested with 0.03% collagenase (Wako Pure Chemical Industries, Osaka, Japan), 0.02% pronase (Kaken, Shizuoka, Japan), and 0.8% DNaseI (Roche, Mannheim, Germany) in Hank balanced salt solution for 25 minutes at 37°C. Digested tissue was filtered, and the cells were washed and resuspended in 8 mL of Gey balanced salt solution containing 0.3% bovine serum albumin. The cell suspension was mixed with 9 mL of 28.7% (wt/vol) Nycodenz (Nycomed Pharm, Oslo, Norway) solution. The Nycodenz gradient was prepared by layering the cell suspension in Nycodenz underneath 6 mL of Gey solution. The gradient was centrifuged at 1400g for 20 minutes. The cells were gathered just above the interface of the Nycodenz solution and the aqueous buffer and were maintained in Ham F-12/Dulbecco modified Eagle medium (1:1) supplemented with 10% fetal bovine serum and antibiotics.

Hepatic stellate cells were also isolated from Coll-GFP mice livers using a previously described method.²⁵ In brief, the livers were perfused and digested with pronase and collagenase. The cell suspension was centrifuged using a 9.7% Nycodenz cushion. The HSC-enriched band was transferred into Dulbecco modified Eagle medium, supplemented with 10% fetal bovine serum and antibiotics, and the HSCs were incubated. Hepatic NPCs were isolated using a 15% Nycodenz solution in the same manner as the HSCs.

Immunofluorescent Cell Staining

Isolated cells were fixed with 4% paraformaldehyde and permeabilized with 0.05% Triton X-100 (Sigma-Aldrich). The cells were then incubated at room temperature for 1 hour with a

TABLE 1. List of Primers for RT-PCR

AQ2 Genes	Forward Primer (5'–3')	Reverse Primer (5'–3')
<i>coll1a1</i>	GCT CCT CTT AGG GGC CAC T	CCA CGT CTC ACC ATT GGG G
<i>α-SMA</i>	GTC CCA GAC ATC AGG GAG TAA	TCG GAT ACT TCA GCG TCA GGA
<i>Gfap</i>	AGA TCG CCA CCT ACA GGA AA	TTT CTC GGA TCT GGA GGT TG
<i>Lrat</i>	TAT GGC TCT CGG ATC AGT CCA	AAT CCC AAG ACA GCC GAA GC
<i>plin2</i>	TTT AAC AGT CGT TCT GGC CG	CTC ATC ACC ACG CTC TGT TG
<i>rbbp1</i>	CCA AGT TGG GAA GGA GTT TG	TCT CCC TTC TGC ACA CAC TG
<i>18s</i>	AGT CCC TGC CCT TTG TAC ACA	CGA TCC GAG GGC CTC ACT A

RT-PCR indicates reverse transcription polymerase chain reaction.

primary antibody recognizing α -SMA (no. ab5694; Abcam) at a 1:200 dilution or GFAP (no. ab48050; Abcam) at a 1:200 dilution and were then incubated at room temperature for 1 hour with a secondary Alexa Fluor 594 antibody (no. 11012; Invitrogen) at a 1:200 dilution. Nuclei were stained with DAPI.

Intracellular Lipid Staining

Intracellular lipid droplets were stained with Oil Red O and counterstained with hematoxylin.

Quantitative Reverse Transcription Polymerase Chain Reaction

Total RNA was prepared from isolated cells using the RNeasy Mini Kit with on-column DNA digestion (Qiagen, Tokyo, Japan) and was reverse transcribed to complementary DNA using the

Omniscript RT Kit (Qiagen, Valencia, Calif). A quantitative real-time reverse transcription polymerase chain reaction was performed using an SYBR Green Master reaction mix on a StepOnePlus system (Applied Biosystems, Foster City, Calif). Gene expression levels were calculated after normalization to 18S by using a standard curve-based method. The primer sequences are shown in Table 1.

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Flow Cytometry

Flow cytometry was based on the simultaneous detection of GFP and vitamin A (autofluorescent signals were detected by an ultraviolet laser at 375 nm) in Coll-GFP mice. Analysis of the cells isolated from Coll-GFP mice was performed using fluorescence-activated cell sorting (FACS) Aria (BD Biosciences).

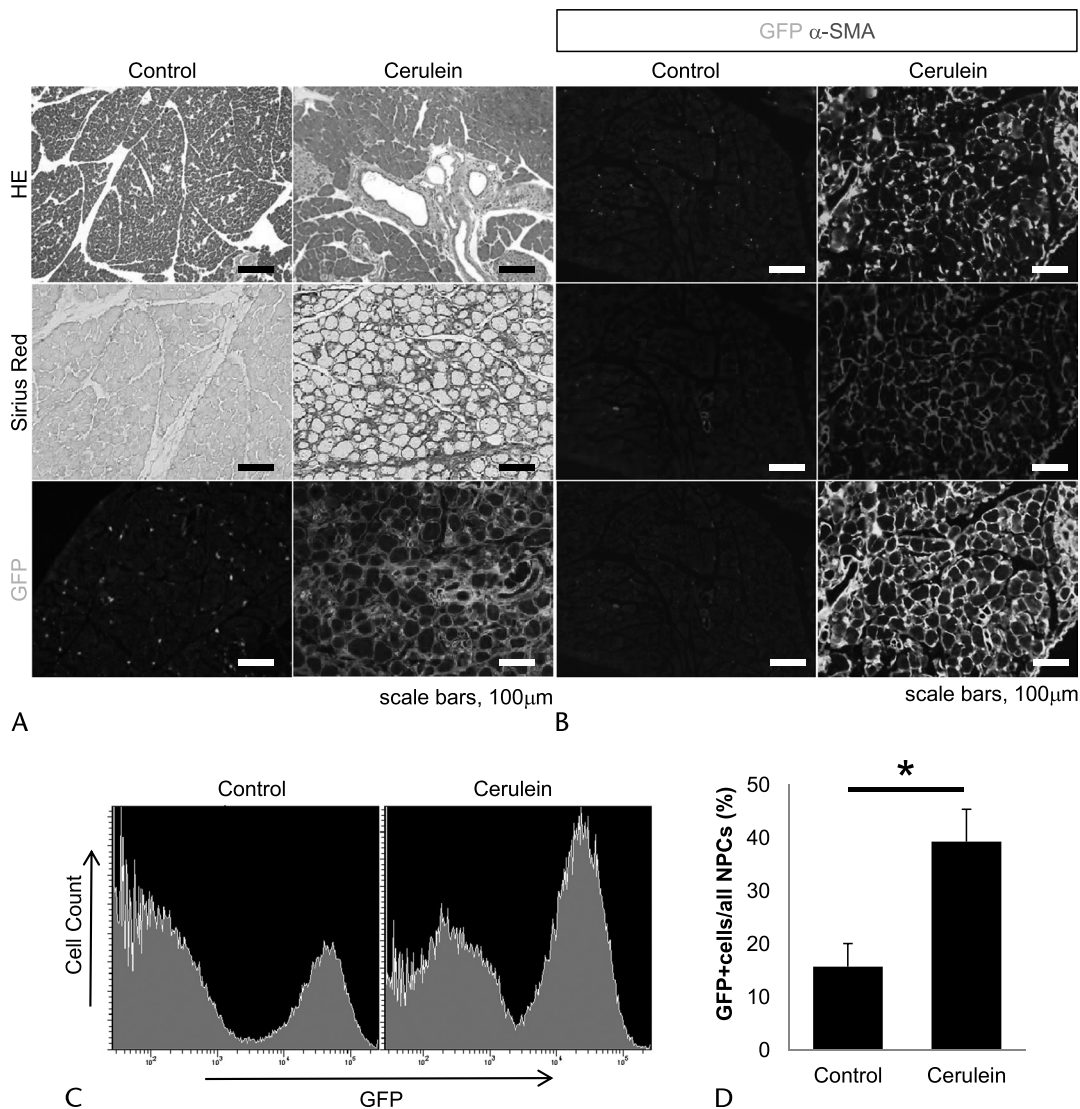


FIGURE 1. Repetitive injections of cerulein-induced pancreatic fibrosis with a robust increase in collagen-producing myofibroblasts in mice. A, In the pancreatic sections of treated mice, H&E and Sirius red staining showed a decrease in acinar cells and the development of remarkable fibrosis (right middle panel). The number of collagen-producing cells increased, which was demonstrated by the appearance of GFP-positive areas along fibrotic septa (right lower panel). B, In fluorescent immunostaining, GFP-positive areas overlapped with the positive areas for α -SMA. C and D, FACS analysis revealed an increase in the GFP-positive fraction after cerulein treatment (control, 15.7% $\pm$ 4.4%; cerulein, 39.3% $\pm$ 6.1%; $P < 0.05$). **Editor's note:** A color image accompanies the online version of this article.

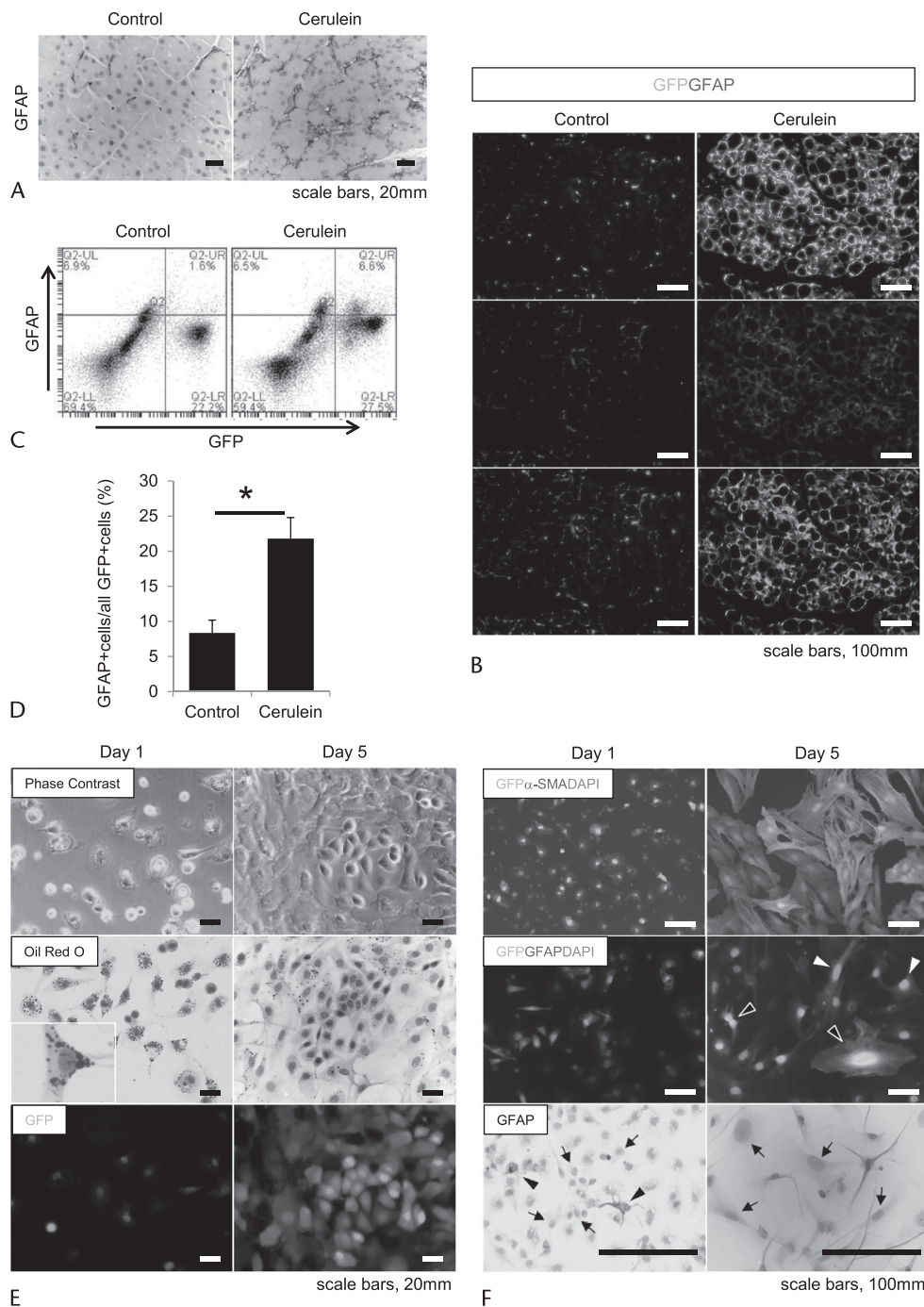


FIGURE 2. GFAP-positive PSCs were not the only cellular source of collagen-producing myofibroblasts in pancreatic fibrosis. A, In the treated pancreas, the number of GFAP-positive cells markedly increased. B, However, double immunostaining for GFAP and GFP in the treated pancreata demonstrated that only a small minority of GFP-positive cells overlapped with the GFAP-positive cells. C and D, Flow cytometry analysis showed an increase in the ratio of GFAP and GFP double-positive cells to all GFP-positive cells ($8.3\% \pm 1.8\%$ in untreated pancreas, $21.8\% \pm 3.0\%$ in fibrotic pancreas; $P < 0.05$). E, Morphological studies of the isolated pancreatic NPCs revealed that some of the isolated cells were spindle shaped with abundant cytoplasm and had cytoplasmic lipid droplets that were stained with Oil Red O, which are all known characteristics of PSCs (left upper and middle panels). There were few GFP-positive PSCs on the first day of culture (left lower panel). After a few days in culture, the lipid-storing cells lost their lipid droplets and changed their morphology into a myofibroblast-like morphology (right upper and middle panels). F, After 5 days in culture, the cells intensely expressed α -SMA and GFP (upper panels). A double-immunofluorescence study for GFP and GFAP in cells cultured for 5 days showed GFP-positive cells consisting of both GFAP-positive and GFAP-negative cells (right middle panel; GFP+/GFAP+, black arrowhead; GFP+/GFAP-, white arrowhead). The immunocytochemistry results revealed that round-shaped cells apparently showed a lack of GFAP expression (left lower panel, arrow), making a clear contrast to GFAP-positive PSCs (left lower panel, arrowhead), and there were also GFAP-positive and GFAP-negative cells in the 5-day culture (right lower panel, arrow). **Editor's note:** A color image accompanies the online version of this article.

In the analysis of GFAP and GFP, pancreatic NPCs isolated from Coll-GFP mice were fixed with Cytofix Buffer (BD Biosciences) for 20 minutes at 4°C, permeabilized with Phosflow Perm/Wash Buffer (BD Biosciences), and then stained with Alexa Fluor 647 anti-GFAP (no. 561470; BD Biosciences) and Alexa Fluor 488 anti-GFP (no. 338007; BioLegend, San Diego, Calif.).

Electron Microscopy

AQ3

The specimens were fixed in 4% paraformaldehyde with 0.3% glutaraldehyde, and sections were observed using transmission electron microscopy (TEM; Hitachi H-7650). For immunogold staining, pancreata were sliced with a freezing microtome and were incubated with a primary anti-GFP antibody (no. 11122; Invitrogen) at a 1:200 dilution overnight at 4°C; the slices were then incubated with Nanogold antirabbit IgG (no. 2004; Nanoprobes, Yaphank, NY) at a 1:100 dilution overnight at 4°C. Silver enhancement was performed using the Silver Enhancement Kit (no. 2012; Nanoprobes).²⁶

Untargeted Global Lipidome Analysis

For comprehensive intracellular lipid analysis, we obtained lipid extracts from primary mouse pancreatic NPCs, HSCs, and hepatocytes (1×10^5 cells) using the Bligh and Dyer method.²⁷ The lipid extracts were reconstituted in 50% isopropanol/25% acetonitrile/25% water and were diluted to the optimal concentrations. Chromatographic separation was carried out using ultraperformance liquid chromatography (UPLC H-Class; Waters, Milford, Mass) with an ACQUITY UPLC CSH C18 column (130 Å, 1.7 µm, 2.1 mm × 100 mm; Waters) at 55°C. The mobile phases A and B were 60% acetonitrile/10 mM ammonium formate/0.1% formic acid and 90% isopropanol/10% acetonitrile/10 mM ammonium formate/0.1% formic acid, respectively. The gradient program was as follows: 40% to 43% B in 2 minutes (curve 6), 43% to 50% B in 0.1 minute (curve 1), 50% to 54% B in 9.9 minutes (curve 6), 54% to 70% B in 0.1 minute (curve 1), 70% to 99% B in 5.9 minutes (curve 6), 99% to 40% B in 0.1 minute (curve 6), and then 40% B for 1.9 minutes. The flow rate was set to 0.4 mL/min. The eluates from UPLC were infused online to a Xevo G2-S Q-ToF mass spectrometer (Waters) used in the electrospray ionization–positive mode. Data sets were acquired in continuum/sensitivity mode using MSe technology (Waters).

AQ4 The acquired data sets were imported to Progenesis QI software (Nonlinear Dynamics, New Castle upon Tyne, UK) for the relative quantification of peaks/compounds. The results were exported to

AQ5 EZinfo software (Waters) to carry out the latent structure–discriminant analysis with Pareto scaling for the global comparison of lipidome compositions.

Quantification of Retinyl Palmitate

For the absolute quantification of retinyl palmitate, fat-soluble vitamins were extracted from the cells using a previously described method.²⁸ Briefly, we added 1 mL of 2-propanol (Wako Pure Chemical Industries) that contained 20 pmol of trans-β-apo-8'-carotenal as an internal standard (IS) to each sample (1×10^5 cells). Then, fat-soluble vitamins were extracted with 6 mL of *n*-hexane (Junsei Chemical, Tokyo, Japan). The mixtures were sonicated 3 times for 1-minute intervals in an ultrasonic bath and were vigorously mixed with a vortex. The samples were then centrifuged at 1800g for 10 minutes. Next, 1 mL of each sample was collected, and the solvent was evaporated in the dark under a gentle stream of nitrogen. A dilution series of standards was prepared by the extraction of mixtures of retinyl palmitate (0–500 µmol), retinol (0–500 µmol; Sigma), and IS (20 pmol), instead of cell pellets. The extracts were diluted in 50% 2-propanol/25% acetonitrile/25%

methyl tert-butyl ether and were injected into a Nexera ultrahigh-performance liquid chromatography (UHPLC) system (Shimadzu, Kyoto, Japan). A Shim-Pack XR-ODS II column (75 × 2.2 mm; Shimadzu) was used at 55°C. The mobile phases A and B were 60% acetonitrile/10 mM ammonium formate and 90% isopropanol/10% acetonitrile/10 mM ammonium formate, respectively. The gradient program was as follows: 20% B for 0.01 minute, 20% to 40% B in 11.99 minutes, 40% to 70% B in 0.01 minute, 70% to 99% B in 5.99 minute, 99% to 20% B in 0.01 minute, and then 20% B for 1.99 minutes. The eluates from UHPLC were infused online into an LC/MS-8030 plus system (Shimadzu) equipped with an APC ion source used at positive polarity. The response of retinyl palmitate and IS was monitored with transitions 269.10 > 93.00 and 417.40 > 325.20, respectively. The matrix effects of extracts from pancreatic quiescent NPCs (day 1), pancreatic activated NPCs (day 5), quiescent HSCs (day 1), and activated HSCs (day 5) were negligible and were not different among the cell types.

AQ6

Statistical Analysis

Data are expressed as mean ± SE. Differences in the measured variables between each group were assessed by using a Mann-Whitney *U* test. JMP 10 (SAS Institute, Cary, NC) was used for all statistical analyses.

RESULTS

Repetitive Injections of Cerulein Induced Pancreatic Fibrosis With a Robust Increase in Collagen-Producing Myofibroblasts in Mice

Pancreatic fibrosis was induced with repetitive injections of the cholecystokinin analog cerulein in Coll-GFP mice. In the pancreatic sections of cerulein-treated mice, H&E and Sirius red staining showed a decrease in acinar cells and the development of remarkable fibrosis (Fig. 1A, right middle panel). The number of collagen-producing cells was markedly increased, which was demonstrated by the appearance of GFP-positive areas along fibrotic septa (Fig. 1A, right lower panel). In the fluorescent immunostaining, GFP-positive areas overlapped with the positive areas for α-SMA, which is a universal marker for myofibroblasts (Fig. 1B). The FACS analysis of isolated pancreatic NPCs revealed an increase in the GFP-positive fraction after cerulein treatment (control, 15.7% ± 4.4%; cerulein, 39.3% ± 6.1%; Fig. 1C, D).

F1

GFAP-Positive PSCs Were Not the Only Cellular Source of Collagen-Producing Myofibroblasts in Pancreatic Fibrosis

In cerulein-treated pancreas samples, GFAP-positive cells (GFAP is a typical marker for PSCs) markedly increased (Fig. 2A). However, double immunostaining for GFAP and GFP in the treated pancreas samples showed only a small minority of GFP-positive cells overlapping with GFAP-positive cells (Fig. 2B). The intracellular flow cytometry analysis of the NPC fraction isolated from the pancreata showed an increase in the ratio of GFAP and GFP double-positive cells among all GFP-positive cells (8.3% ± 1.8% in the untreated pancreata, 21.8% ± 3.0% in the fibrotic pancreata; Fig. 2C, D). These results suggested that, in addition GFAP-positive cells consistent with PSCs, GFAP-negative collagen-producing cells existed and contributed to the pool of myofibroblasts in the pancreas.

F2

The morphological studies of the isolated pancreatic NPCs revealed that some of the isolated cells were spindle shaped with abundant cytoplasm and had cytoplasmic lipid droplets that were

stained with Oil Red O; all these features are known characteristics of PSCs (Fig. 2E, left upper and middle panels). There were few GFP-positive PSCs on the first day of cell culture (Fig. 2E, left lower panel). Simultaneously, we observed a cell population with distinct morphology from PSCs. These cells were round shaped with scant cytoplasm as observed via phase contrast and lacked cytoplasmic lipid droplets as observed via Oil Red O staining. During the first few days in culture, the lipid-storing cells lost their lipid droplets and changed their morphology into myofibroblast-like cells (Fig. 2E, right upper and middle panels). A time-lapse study clearly showed a change in cell morphology and GFP expression, and there seemed to be at least 2 types of GFP-positive cells (Supplemental Digital Content 1, <http://links.lww.com/MPA/A605>, and Supplemental Digital Content 2, <http://links.lww.com/MPA/A606>). After 5 days in culture, the cells highly

expressed α -SMA and GFP (Fig. 2F, upper panels). On the other hand, the round-shaped cells maintained their morphology at even 2 weeks later. A double-immunofluorescence study for GFP and GFAP in cells cultured for 5 days showed that GFP-positive cells consisted of both GFAP-positive and GFAP-negative cells (Fig. 2F, right middle panel; GFP+/GFAP+, black arrowhead; GFP+/GFAP-, white arrowhead). As shown via immunocytochemistry, round-shaped cells apparently showed a lack of GFAP expression (Fig. 2F, left lower panel, arrow), making a clear contrast to GFAP-positive PSCs (Fig. 2F, left lower panel, arrowhead). There were also GFAP-positive and GFAP-negative cells in the 5-day culture (Fig. 2F, right lower panel, arrow). Our immunofluorescent study on isolated pancreatic cells that were activated on a culture plate showed that among the GFP-positive cells morphologically consistent with stellate cells, $42.2\% \pm 24.4\%$

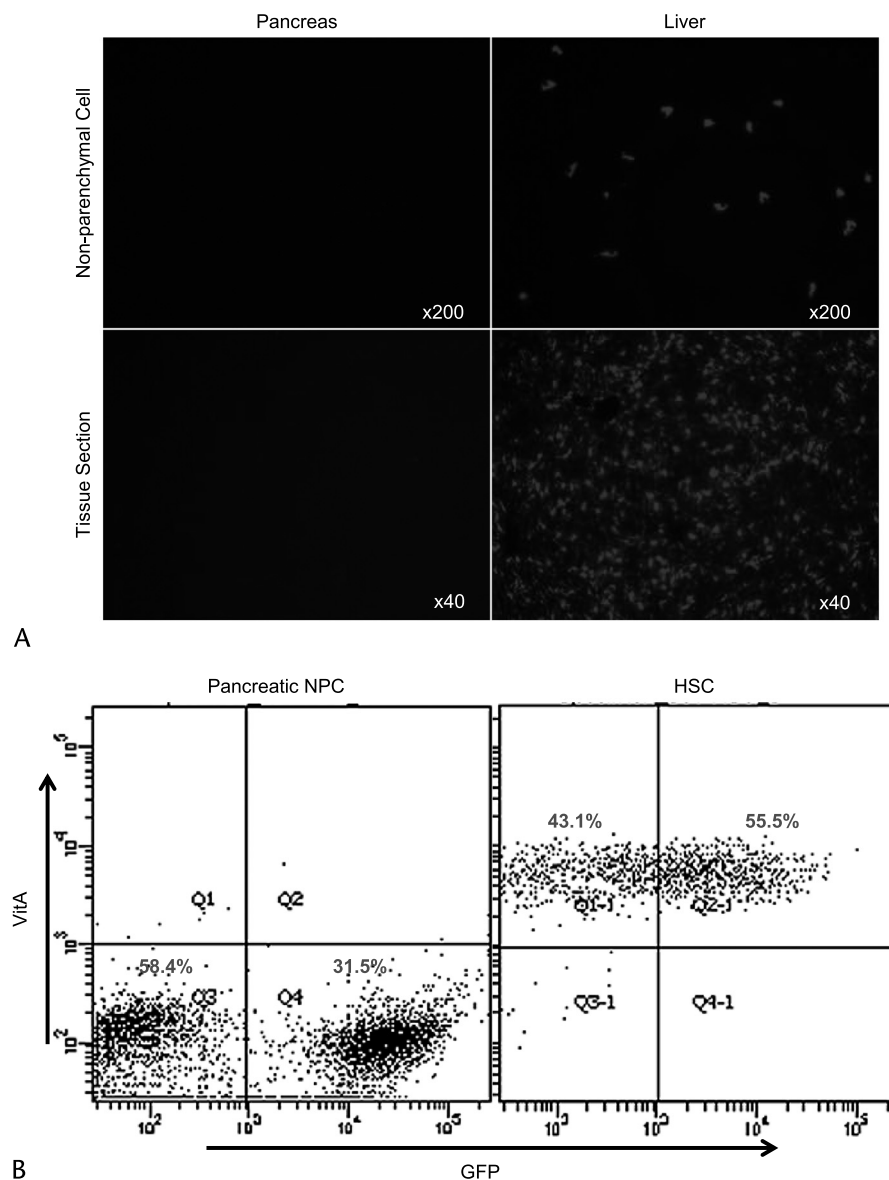


FIGURE 3. Pancreatic stellate cells did not generate vitamin A autofluorescence. A, Although we identified lipid droplets in the cytoplasm of PSCs, unlike HSCs, vitamin A autofluorescence was undetectable in the PSCs. We successfully detected vitamin A autofluorescence in the isolated HSCs and in the liver sections but failed to detect it in the pancreas sections using fluorescent microscopy. Vitamin A autofluorescence signal was also detected using flow cytometry in the isolated HSCs and in the activated HSCs (GFP-positive HSCs). B, Using the same method, we did not observe any autofluorescence-positive PSCs. **Editor's note:** A color image accompanies the online version of this article.

were GFAP positive. Taken together, these results indicate that collagen-producing myofibroblasts in pancreatic fibrosis consist of at least 2 distinct cell populations: one with characteristics of PSCs and another that seem to be a non-PSC population. The small round cells were negative for CD45 (data not shown), which is a well-known marker of fibrocytes.

PSCs Did Not Generate Vitamin A Autofluorescence

Although we identified lipid droplets in the cytoplasm of PSCs, unlike HSCs, vitamin A autofluorescence was undetectable [F3] in the PSCs (Fig. 3A). We successfully detected vitamin A autofluorescence, which was characterized by a rapidly fading fluorescent signal after excitation with ultraviolet light, in the isolated HSCs and in the liver sections, but we failed to detect it in the pancreas sections using fluorescent microscopy (Fig. 3A).

The vitamin A autofluorescence signal was also detected using flow cytometry in isolated HSCs and in activated HSCs (GFP-positive HSCs). Using the same method, we did not observe any autofluorescence-positive PSCs (Fig. 3B).

Pancreatic NPCs Contained Only Trace Amounts of Retinyl Palmitate

First, we performed a comprehensive intracellular lipid analysis. The extracts from primary pancreatic NPCs, HSCs, and hepatocytes were subjected to global lipidome analysis using UPLC-quadrupole time-of-flight mass spectrometry. The obtained data sets were subjected to supervised multivariate data analysis (projection to latent structure–discriminant analysis) to compare the lipid compositions of the primary pancreatic NPCs, HSCs, and hepatocytes. As shown in Figure 4A, the lipid composition of each [F4] primary cell type was markedly different. The peaks/compounds

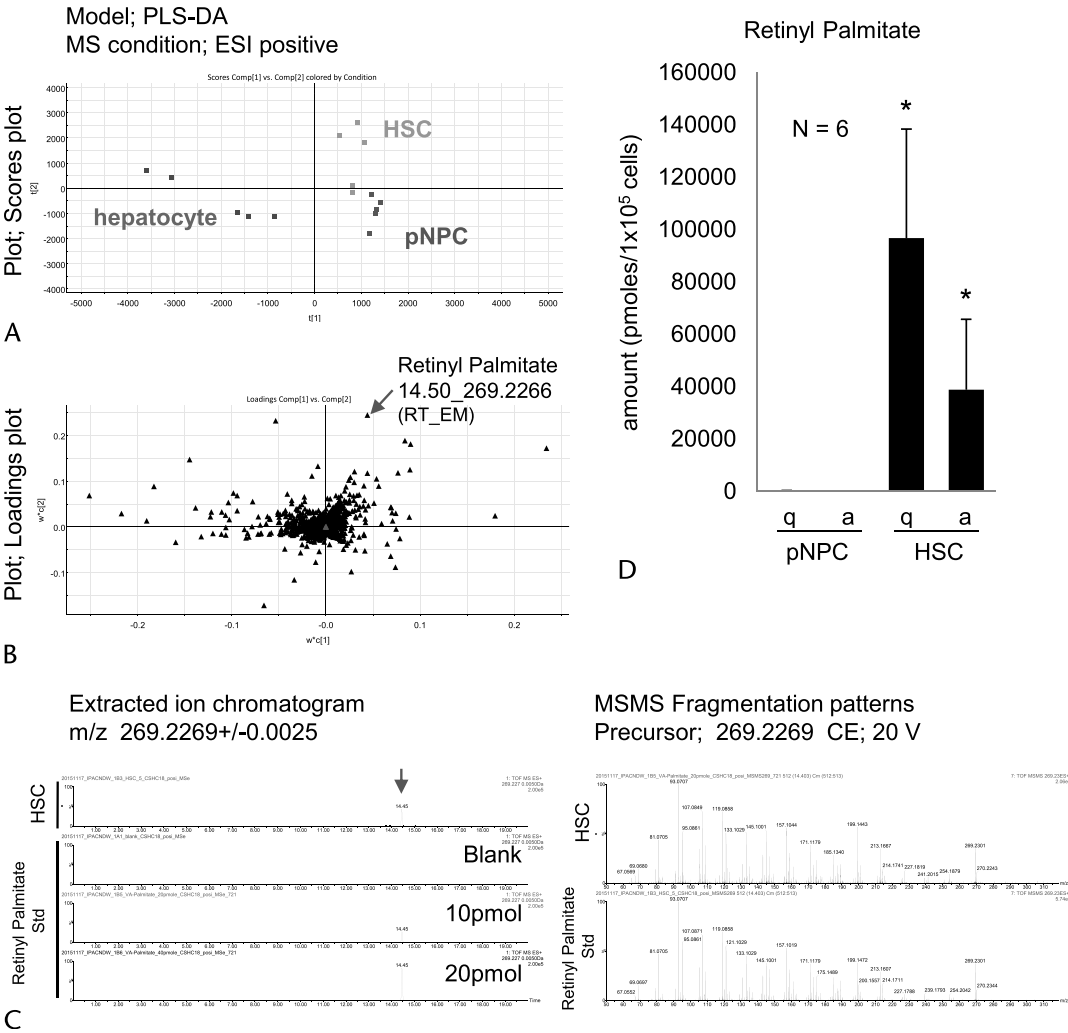


FIGURE 4. Pancreatic NPCs contained only trace amounts of retinyl palmitate. The extracts from primary pancreatic NPCs, HSCs, and hepatocytes were subjected to the global lipidome analysis using UPLC-quadrupole time-of-flight mass spectrometry. A, The lipid composition of each primary cell type was markedly different. B, Among the peaks/compounds that were significantly different between the cell types, one of the compounds had a retention time of 14.50 minutes and an exact mass m/z of 269.2266 that was characteristic of HSCs (arrow). The compound was tentatively identified as retinyl palmitate, and the identification was confirmed by the analysis of a retinyl palmitate standard and the cellular extracts under the same conditions. C, The peak retention times and MSMS fragmentation patterns of the retinyl palmitate standard and the cellular extracts were identical. D, UHPLC-triple quadrupole mass spectrometry revealed that the pancreatic NPCs contained only trace amounts of retinyl palmitate, which was approximately five-hundredths of the amount of the HSCs. * $P < 0.05$ compared with quiescent pNPC. pNPC, pancreatic NPC. **Editor's note:** A color image accompanies the online version of this article.

were significantly different between cell types. One of the compounds had a retention time of 14.50 minutes and an exact mass m/z of 269.2266 that was highly characteristic of HSCs (Fig. 4B, arrow). Judging from the observed retention time and the exact mass in comparison to our local database, the compound was tentatively identified as retinyl palmitate. Prior studies have shown that retinyl palmitate is the most abundant retinyl ester in HSCs.²⁹ Retinoids (vitamin A and its metabolites) present in the body include retinyl ester, retinol, retinal, and retinoic acid. The identification was confirmed by the analysis of a retinyl palmitate standard and the cellular extracts under the same condition. The peak retention times and the MS/MS fragmentation patterns of the retinyl palmitate standard and the cellular extracts were identical (Fig. 4C). Next, we determined the absolute quantity of retinyl palmitate to evaluate the difference in pancreatic NPCs and HSCs. The extracts from quiescent pancreatic NPCs and HSCs (day 1 in culture) and activated pancreatic NPCs and HSCs (day 5 in culture) were quantified using UHPLC–triple quadrupole mass spectrometry. It was revealed that pancreatic NPCs contained only trace amounts of retinyl palmitate, which was approximately five hundredths of the amount of HSCs (Fig. 4D).

AQ8 MS/MS fragmentation patterns

Pancreatic NPCs Expressed Only Trace Amount of Lrat

We compared the messenger RNA (mRNA) expression of genes involved in retinol and lipid metabolism between pancreatic NPCs and HSCs in quiescent status (day 1 in culture) and activated status (day 5 in culture; Fig. 5).

F5

Messenger RNA expression of *Col1α1* and *α-SMA* increased with activation in both pancreatic NPCs and HSCs. Messenger RNA expression of lecithin retinol acyltransferase (*Lrat*), which encodes the *Lrat* that catalyzes the esterification of retinol, is an essential reaction for vitamin A metabolism in the liver, and the amount of *Lrat* in pancreatic NPCs was approximately one hundredth of the amount in HSCs, whereas pancreatic NPCs expressed *RBP1* and *Plin2*. This result corresponded with the lack of vitamin A autofluorescence in pancreatic NPCs.

Microstructure of the PSCs Was Entirely Different From the Microstructure of the HSCs Measured Using Flow Cytometry and Electron Microscopy

Using flow cytometry, the GFP-positive hepatic non-parenchymal fraction can be divided into 2 fractions with side-scattered light (SSC). The fractions included the HSC fraction and the other cell fraction (Fig. 6A). This result indicated that the abundant lipid droplets in the HSCs led to a complex internal structure that resulted in a high SSC value. On the other hand, GFP-positive pancreatic NPCs were not separated by SSC and FSC, which suggested that the few lipid droplets in PSCs did not increase the complexity of the internal cell structure (Fig. 6A).

F6

We performed TEM to evaluate the microstructure of the PSCs and the HSCs. We successfully identified GFP-positive cells using TEM with immunogold labeling (Fig. 6B). The morphology of the isolated PSCs and the organ sections was distinct from the morphology of the HSCs in that the number of lipid droplets in the PSCs was smaller, and these droplets were smaller than the droplets in the HSCs (Fig. 6B), which was consistent with the difference in the lipid-droplet content between HSCs and PSCs.

DISCUSSION

Pancreatic stellate cells, which are generally recognized as the pancreatic counterparts of HSCs, and HSCs have not been characterized. In addition, sources of collagen-producing myofibroblasts

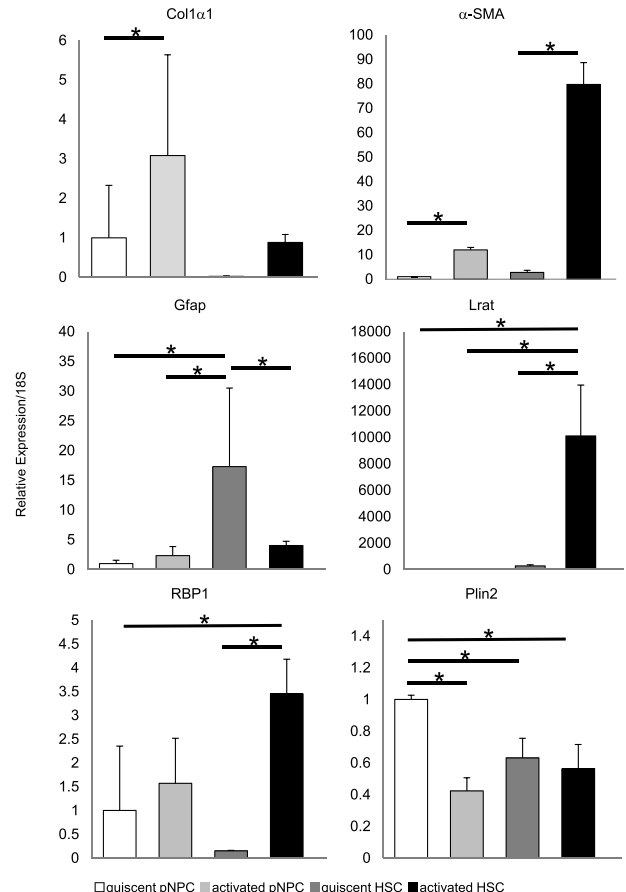


FIGURE 5. Pancreatic NPCs expressed only trace amount of *Lrat*. We compared mRNA expression of genes involved in retinol and lipid metabolism between the pancreatic NPCs and HSCs in quiescent status (day 1 in culture) and activated status (day 5 in culture). * $P < 0.05$.

in pancreatic fibrosis other than PSCs may exist, which have not been discussed as extensively as in the research of liver fibrosis. Our study focused on the further characterization of PSCs and collagen-producing cells in a chronic pancreatitis model in mice.

We induced pancreatic fibrosis with repetitive injections of cerulein. The cerulein-induced pancreatitis model has been widely used, and the supraphysiologic stimulation is directly responsible for persistent inflammation in the pancreas with the secretion of lipase, amylase, or proteases and bicarbonate in the pancreatic juice.³⁰

In the pancreatic sections of cerulein-treated mice, the number of GFP- and GFAP-positive cells markedly increased, which was consistent with current knowledge indicating that PSCs are the primary source of collagen-producing cells in pancreatic fibrosis. However, we also observed abundant GFAP-negative and GFP-positive cells, which suggests the existence of fibrogenic cells other than PSCs.

We isolated pancreatic NPCs from Coll-GFP mice and cultured them in plastic dishes to activate the cells. After 5 days in culture, we observed 2 types of GFP-positive cells that were morphologically distinct from each other. The spindle-shaped cells were characterized as PSCs because of their typical morphology and cytoplasmic lipid droplets. The small round cells, which were morphologically distinct from PSCs, may be regarded as collagen-producing cells other than PSCs.

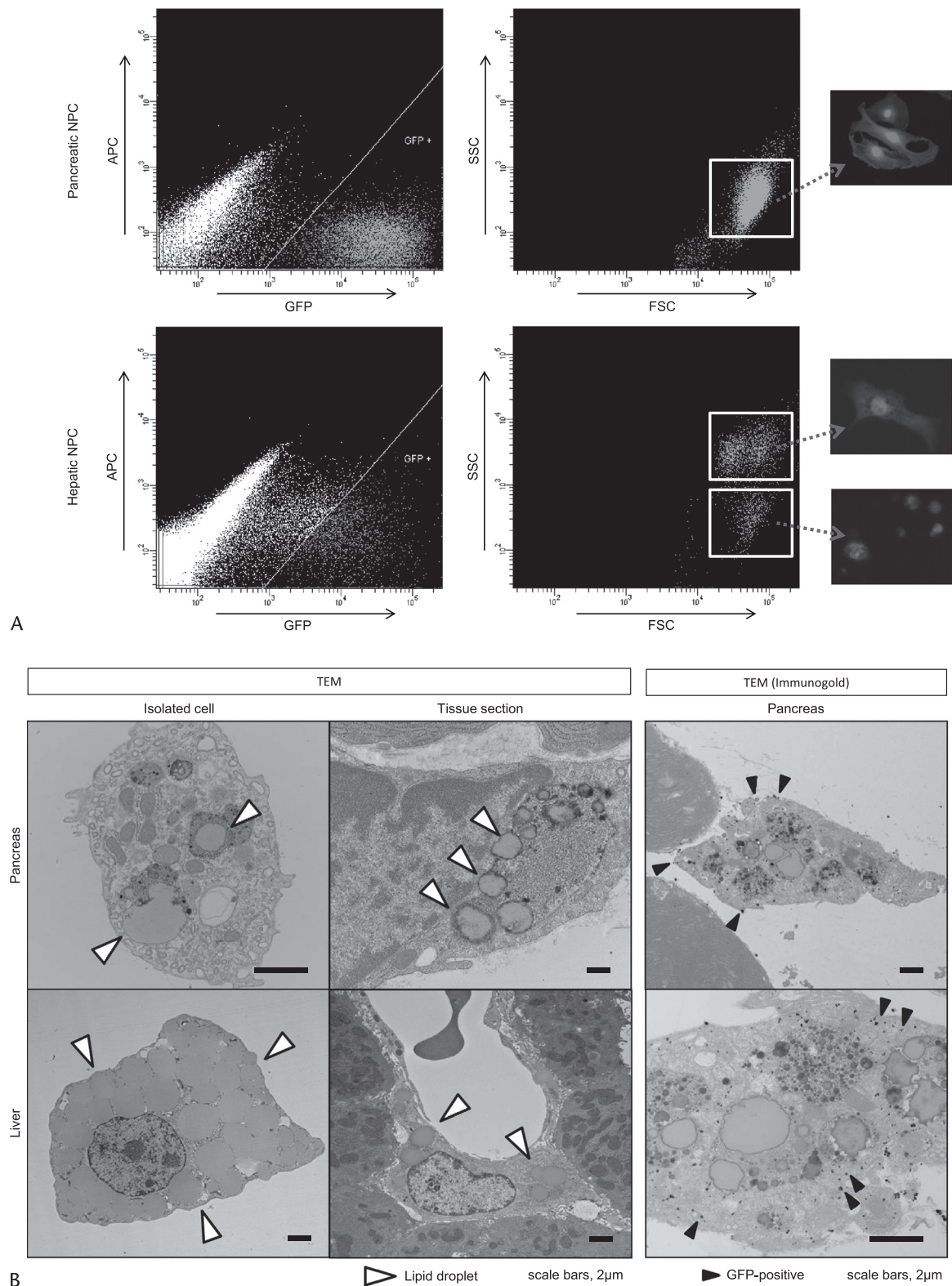


FIGURE 6. Microstructure of the PSCs was entirely different from the microstructure of the HSCs measured using flow cytometry and electron microscopy. Green fluorescent protein–positive hepatic nonparenchymal fractions were divided into HSCs and other cells using SSC. A, However, in the pancreatic NPCs, only one GFP-positive group containing PSCs was sorted by SSC, and the SSC levels were lower than the levels in the HSCs. B, Using TEM, the cell morphology of the isolated PSCs and the organ sections was distinct from the morphology of the HSCs in that the number of lipid droplets in the PSCs was smaller, and these droplets were smaller than the droplets in the HSCs. We identified GFP-positive AQP9 cells using TEM with immunogold labeling. **Editor’s note:** A color image accompanies the online version of this article.

In a flow cytometry analysis of NPCs from cerulein-treated mice, more GFP-positive cells than the cells from control mice were sorted, and only 20% of those cells were positive for GFAP. This result also suggested that cells other than the PSCs trans-differentiated into myofibroblasts. Previous studies showed that CD34-positive fibrocytes were detected in the tissue specimens from patients with chronic pancreatitis,^{31,32} and evidence indicated that CD45- and Col1 α 1-positive fibrocytes contributed to pancreatic fibrosis in mice.^{32,33} However, our immunofluorescent staining showed that the small round, GFP-positive cells were negative for CD45 *in vitro*. Studies on liver fibrosis have demonstrated that the origins of collagen-producing cells other than HSCs include fibrocytes and portal fibroblasts (characterized by positivity for mesothelin and Thy1).¹⁹ Thus, we considered the possibility that GFP-positive, small, and round-shaped cells that were morphologically distinct from PSCs were the counterpart of portal fibroblasts in the pancreas. However, immunofluorescent studies showed that these cells were negative for mesothelin and Thy1 (data not shown). Therefore, these cells were distinct from fibrocytes or portal fibroblasts and were presumably collagen-producing cells that had not yet been recognized in the pancreas.

It is a matter of interest which cell type is the major producer of collagen. Figure 2C and D demonstrates that approximately one-fifth of collagen-producing cells were GFAP positive. On the basis of our observation that approximately 40% of morphologically assumed PSCs on the culture were GFAP positive, we might be able to estimate that approximately 50% of the collagen-producing cells were derived from PSCs in this model. Therefore, we assume that the contribution of collagen-producing cells derived from non-PSCs is not negligible, although the contribution of each cell type to the source of collagen-producing cells can vary depending on the fibrosis models, as is seen in the liver fibrosis model.¹⁹ Surprisingly, our study showed that the lipid droplets of primary PSCs lacked vitamin A autofluorescence, which was an established and useful stellate cell marker. Our system successfully detected autofluorescence in HSCs, which excluded the possibility that the sensitivity of our system was not high enough to detect the autofluorescence of vitamin A. The FACS analysis consistently showed the presence of vitamin A autofluorescence in HSCs and its absence in pancreatic NPCs.

The lipid composition analysis revealed that the pancreatic NPCs had only one-hundredth of the amount of retinyl palmitate (vitamin A palmitate) in HSCs, which supported our result showing that PSCs did not exhibit detectable autofluorescence. The content of retinyl palmitate in the pancreatic NPCs was much less than the content in the activated HSCs, which lost their cytoplasmic vitamin A when their activation occurred. The relative mRNA expression of Lrat, which is an enzyme that catalyzes the formation of retinyl ester in pancreatic NPCs, was much lower than Lrat expression in the HSCs. This result was a sharp contrast to the comparable expression of Plin2, which is also known as adipose differentiation-related protein.^{34,35} between pancreatic NPCs and HSCs. Plin2 served to form lipid droplets, whereas Lrat allowed for the formation of retinyl ester. The comparable expression of Plin2 and the low expression of Lrat in the pancreatic NPCs compared with the HSCs agreed with our finding that the PSCs had cytoplasmic lipid droplets but did not have detectable vitamin A autofluorescence. Indeed, a previous study by Liu and Gudas³⁶ demonstrated that Lrat deficiency did not affect retinyl palmitate content in the pancreas, which suggested that Lrat was scarcely expressed and played only a minor role in the pancreas.

Fifty percent to 80% of the total body vitamin A is stored in the liver, in the form of fatty acid esters in the lipid droplets of HSCs.^{37,38} There are several studies that examined the role of vitamin A in the liver,^{35,39,40} and only a few reports of vitamin A

in the pancreas demonstrated retinol-induced quiescence in PSCs.^{41–44} Our study showed that PSCs possessed a very small amount of retinyl palmitate. Therefore, further research is needed to clarify the relationship between PSCs and vitamin A.

We showed that hepatic GFP-positive NPCs were divided into 2 fractions based on SSC with flow cytometry. Side-scattered light intensity is proportional to the granules of cells and the complexity of their structure. The SSC high fraction represented HSCs because of their complicated intracellular structure. On the other hand, pancreatic GFP-positive nonparenchymal fraction cells were not partitioned by SSC, and their SSC intensity was lower than that of the HSCs. This finding prompted us to explore the difference in the microstructures of PSCs and HSCs. An immunoelectron microscopic technique identified GFP-positive cells with lipid droplets on the pancreas sections. Their morphology was distinct from HSCs in that the number of lipid droplets was apparently smaller, and the lipid droplets were smaller as well. This result and the flow cytometry analysis showed a difference in their organelles, which mostly included lipid droplets, between PSCs and HSCs.

Our study has limitations; some analyses were done on pancreatic NPCs, not on purified PSCs. In fact, we isolated pancreatic NPCs using the same method that has been reported as an isolation method for PSCs, in which 15.2% Nycodenz was used to float up the targeted cells.¹¹ As a result, we noticed that the isolated cells included not only PSCs but also other NPCs. For HSC isolation, 9.7% Nycodenz is the standard, because HSCs are light enough to be floated with 9.7% Nycodenz. When we applied 9.7% for PSC isolation, no cells were obtained, indicating that PSCs are heavier than 9.7% Nycodenz. This is consistent with our finding that PSCs have less lipid droplets and therefore are heavier than HSCs. In fact, 15.2% is comparable to the density that we use for NPC isolation from the liver. From these observations, we concluded that PSCs cannot be separated from the other NPCs in the pancreas only by using the density gradient method; therefore, we could not avoid analyzing NPCs and were not analyzing only purified PSCs.

Since PSCs were found in 1998,^{6,7} pancreatic fibrosis research has expanded. Several studies have discussed the relationship between PSCs and pancreatic fibrosis, but few reports have mentioned collagen-producing cells other than PSCs. In previous studies of pancreatic fibrosis, many researchers have followed the research of liver fibrosis and HSCs, and they developed many studies focused on PSCs. Our study showed not only the possibility of collagen-producing cells other than PSCs but also different characteristics between mouse primary PSCs and HSCs in morphological and functional aspects, although the previous report showed that the expression profiles of human primary PSCs and HSCs displayed a great extent of similarity.⁴⁵

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