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Activated Ductal Proliferation Induced by *N*-Nitroso*bis* (2-oxopropyl)amine in Fat-infiltrated Pancreas of KK-A^y Mice

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Abstract. *Background/Aim: Our aim was to investigate whether tissue with fatty infiltration within the lobes of the pancreas (scattered FI) is sensitive to carcinogen-induced pancreatic ductal proliferation. Materials and Methods: Seven-week-old female C57BL/6J, C57BL/6J-A^y, KK-A^y, and ICR mice were subcutaneously treated with N-nitrosobis(2-oxopropyl) amine at a dose of 80 mg/kg body weight, and the differences in damage-induced cell proliferation and their biochemical data were compared 2 days after. Results: Scattered FI in the pancreas was obvious only in KK-A^y mice, which have high serum lipid, leptin and insulin levels, and cell proliferation both in pancreatic and common bile ducts was enhanced only in KK-A^y mice by the carcinogen treatment. Conclusion: Scattered FI in the pancreas per se can be an important factor for carcinogenesis. The genetic background causing scattered FI of the pancreas should be further investigated.*

Epidemiological studies demonstrated that pancreatic cancer is associated with age, obesity, and type 2 diabetes mellitus (T2DM), which are also risk factors of fatty infiltration (FI) in the pancreas (1, 2). We previously showed there to be a positive correlation in humans between FI in the pancreas and pancreatic cancer in histopathological analysis, even after adjustment for sex, age, body mass index (BMI) and prevalence of DM (3). In addition, we showed that Syrian golden hamsters manifesting hyperlipidemia have FI of the pancreas, and the degree is further exacerbated with increasing age and treatment with the pancreatic carcinogen *N*-nitroso*bis*(2-oxopropyl)amine (BOP) (4). In contrast, ICR mice (5) and Otsuka Long-Evans Tokushima Fatty (OLETF) rats, rat models of T2DM accompanied by hypertriglyceridemia (6), do not develop pancreatic ductal adenocarcinomas (PDACs) on BOP treatment. It was notable that OLETF rats did not develop the same degree of FI in the pancreas as that observed in Syrian golden hamsters. Thus, the data indicate that hyperlipidemia or hyperinsulinemia in T2DM is not enough to increase susceptibility to pancreatic carcinogenesis.

FI of the pancreas is characterized by adipocyte distribution, adipocyte infiltration of the area around great vessels, and scattered FI, *i.e.* the replacement of acinar cells by adipocytes within lobules in a scattered pattern (7). Hamsters have more scattered FI in the pancreas than do mice or rats. Pancreatic intraepithelial neoplasia lesions have been reported to be associated with intralobular fat (odds ratio=17.86; 95% confidence interval=4.935-88.12) in humans (8).Therefore, the distribution of pancreatic FI could be an important phenomenon involved in sensitivity to pancreatic carcinogens. Recently, we showed that KK-A^y mice, a T2DM mouse model including two genetic characteristics of KK/TaJcl (KK) mice, a T2DM model, and C57BL/6J-A^y mice, which carry the Agouti yellow (A^y) gene, have severe hyperphoagia, hyperinsulinemia and dyslipidemia, and are highly susceptible to azoxymethane-induced colon carcinogenesis (9). In the study, we found that large adipocytes were scattered within the lobules of the pancreas of KK-A^y mice at 17 weeks of age, similar to that in the pancreas of hamsters. On the other hand, fewer adipocytes were observed in the lobules of C57BL/6J-A^y mice, and only small adipocytes were observed in the lobules of ICR mice. Scattered FI was rarely observed in C57BL/6J mice at the same age (9). However, it remains to be clarified whether the scattered FI in the pancreas *per se*, is an important factor for pancreatic carcinogenesis.

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Altered cell proliferation is a characteristic of neoplastic development. High-dose BOP treatment has been reported to increase DNA synthesis in pancreatic ductal cells in hamsters (10, 11). In the present study, we examined the effect of BOP treatment on pancreatic ductal cell proliferation in KK-A^y mice.

Materials and Methods

Animals. Female C57BL/6J and C57BL/6J-A^y mice and Syrian golden hamsters were obtained from Japan SLC (Shizuoka, Japan), and ICR and KK-A^y mice from CLEA Japan (Tokyo, Japan). All animals were acclimated to laboratory conditions for 1 week. They were fed a standard diet, CE-2 (total calories, 3.43 kcal/g; CLEA Japan). Food and water were available *ad libitum*. The experimental protocol was carried out in accordance with the guidelines for Animal Experiments at the National Cancer Center, and was approved by the Institutional Ethics Review Committee for Animal Experimentation (Approval numbers: T09-057, T07-012).

Animal experiments. Six or seven out of 12 or 13 mice of each strain were injected subcutaneously with BOP (Nacalai Tesque, Kyoto, Japan) at a dose of 80 mg/kg body weight (BW) at 7 weeks of age, whereas the remaining six mice of each type received saline as a vehicle control. Two days after BOP injection, the mice were sacrificed under deep anesthesia. Blood samples were collected in all cases, and the pancreas, liver, lungs and heart were carefully examined macroscopically and then fixed in 10% phosphate-buffered formalin (pH 7.4). Two female Syrian golden hamsters were injected subcutaneously with BOP at a dose of 70 mg/kg BW at 9 weeks of age, whereas two hamsters received saline or DMSO as vehicle control. Two days after BOP injection, the hamsters received intraperitoneally Bromodeoxyuridine (BrdU) at a dose of 20 mg/kg BW and were sacrificed under deep anesthesia after 2 hours of the injection. Paraffin-embedded organs were sectioned and stained with hematoxylin and eosin for assessment of histopathological features including FI of the pancreas. There are two types of FI, scattered type, with adipocyte marbling within the lobes, and accumulated type, with adipocytes accumulated around vessels. The area of scattered FI in pancreatic lobes was estimated as 0-1% (−), 1-2% (±), 2-5% (+) or >5% (++). For analysis of ductal cell proliferation, pancreatic tissues were fixed between filter papers and dissected vertically across the main ducts into four, four and eight sections at 2 mm intervals in the head, gastric lobe and splenic lobe of the pancreas, respectively, and then embedded in paraffin.

Immunohistochemical analysis. In Ki-67 immunostaining of mouse pancreatic sections, 5-μm-thick sections of paraffin-embedded tissue blocks were autoclaved for antigen retrieval in 10 mM citrate buffer (pH 6.0) for 10 min and treated with a polyclonal antibody against the proliferative marker Ki-67 at a dilution of 1:200 (Novocastra, Newcastle, UK). As the secondary antibody, biotinylated anti-rabbit IgG (H+L) raised in a goat, affinity purified, (Vector Laboratories Inc., Burlingame, CA, USA) was employed at 200× dilution. In BrdU immunostaining of hamster pancreatic sections, 5-μm-thick sections of paraffin-embedded tissue blocks were deparaffinized and incubated for DNA hydrolysis in 4 M HCl for 30 min. After neutralization and blocking, the tissue sections were treated with a mouse monoclonal antibody against BrdU at a dilution of 1:300 (Dako, Kyoto, Japan). As the secondary antibody, affinity purified biotinylated anti-mouse IgG (H+L) raised in a horse (Vector Laboratories Inc., Burlingame, CA, USA) was employed at 200× dilution. Staining was carried out using avidin-biotin-peroxidase reagents (Vectastain Elite; Vector Laboratories Inc.), 3,3'-diaminobenzidine and hydrogen peroxide.

The number of Ki-67-positive nuclei in pancreatic ductal cells (251 to 1172 cells, depending on the size of each pancreas) and in common bile ductal cells (40 to 969 cells) were counted and the results were expressed as a percentage of the total cells examined. The numbers of BrdU-positive nuclei in pancreatic ductal cells (4980 to 15889 cells) and in common bile ductal cells (269 to 2082 cells) were counted and the results were expressed as a percentage of the total cells examined.

Biochemical analysis. The levels of serum insulin (Millipore Corp., Billericay, MA, USA) and leptin (BioVendor, Brno, Czech Republic) were examined using enzyme-linked immunoassay kits in accordance with the manufacturer's instructions. The levels of serum triglycerides (TGs) and total cholesterol (TC) were analyzed using the FUJI Dri-Chem system (FUJI Film Medical, Tokyo, Japan). The blood glucose levels of the mice were measured using an automatic blood glucose meter (Medisafe-mini GR-102; Terumo, Tokyo, Japan).

Statistical analysis. Data for continuous variables are shown as the mean±SD. Two-sided *p*-values of less than 0.05 were considered to indicate statistical significance. All statistical analyses were carried out using the JMP 13 software package (SAS Institute, Inc., Cary, NC, USA).

Results

Histopathology of pancreas and biochemical data in each mouse strain. A considerable number of scattered adipocytes within the exocrine pancreas in addition to adipocyte accumulation around the vessels was apparent in obese diabetic KK-A^y mice at 7 weeks of age (Figure 1). The serum levels of TG, TC, insulin, and leptin were already high in KK-A^y even at this young age (Table I). These changes are similar to those in Syrian golden hamsters at 14 weeks of age observed in our previous study (4). On the other hand, almost no or a little FI was observed in the pancreas of C57BL/6J, C57BL/6J-A^y and ICR mice. Adipocyte accumulation around the great vessels of the pancreas was observed markedly in KK-A^y mice, and to some extent in C57BL/6J-A^y and ICR mice. Moreover, serum TG, insulin and leptin levels were low in C57BL/6J, C57BL/6J-A^y and ICR mice.

Blood glucose levels were significantly reduced with BOP treatment in all four mouse strains (Table I). With BOP treatment, serum TG levels were significantly reduced in ICR and KK-A^y mice and the BW values and serum insulin levels were significantly reduced in ICR and KK-A^y mice. The serum leptin level was significantly increased in ICR mice by BOP treatment. Serum TC levels were significantly increased with BOP treatment in all four mouse strains.

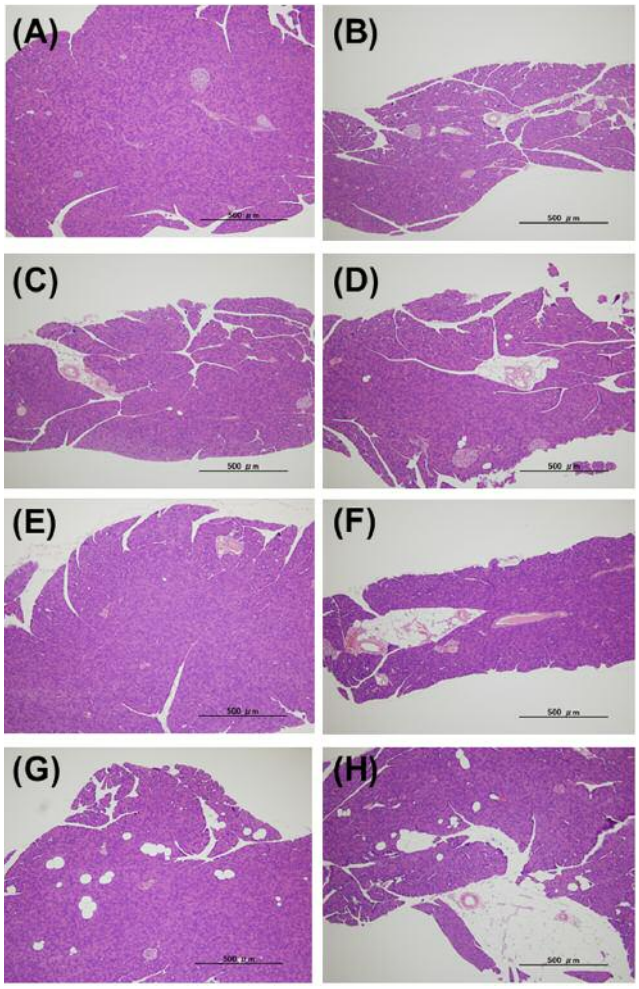

Figure 1. *Histopathological sections of the pancreas of C57BL/6J (A and B), C57BL/6J-A^y (C and D), ICR (E and F) and KK-A^y (G and H) mice at 7 weeks of age, stained with hematoxylin and eosin.*

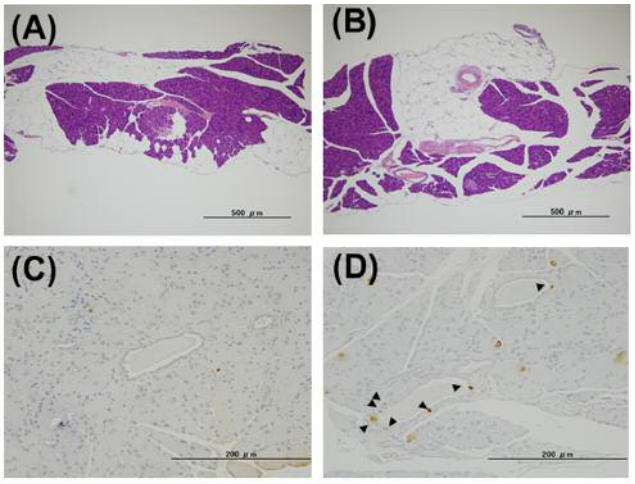

Figure 2. *Pancreatic tissue sections of Syrian golden hamsters. A, B: Histopathological sections of the pancreas of Syrian golden hamsters, at 9 weeks of age, stained with hematoxylin and eosin. C, D: Histopathological sections of the pancreas containing pancreatic ducts of Syrian golden hamsters injected with saline (C) or N-nitrosobis(2-oxopropyl)amine (D) and stained immunohistochemically for BrdU incorporation. Arrow heads show positively-stained ductal cell nuclei.*

High ductal cell proliferation induced by carcinogen treatment in KK-A^y mice. To validate our hypothesis that BOP treatment enhances the proliferation of ductal cells in the pancreas of KK-A^y mice, KK-A^y mice at 7 weeks of age were treated with BOP and then sacrificed 2 days after injection. We examined the cell proliferative marker Ki-67 by immunostaining to determine whether the pancreatic ducts and common bile ducts showed reactivity to BOP. In the hamster pancreas, proliferating ductal cells were markedly increased at 2 days after BOP treatment with a dose of 70 mg/kg BW (Table II and Figure 2). In the pancreatic ducts, the ratio of Ki-67-positive cells to total ductal cells was significantly increased by BOP treatment in KK-A^y mice, but not in C57BL/6J, C57BL/6J-A^y or ICR mice (Table III and Figure 3). On the other hand, the ratio of Ki-67-positive cells in the common bile ducts was significantly increased by BOP treatment in KK-A^y and C57BL/6J-A^y mice, and tended to increase in ICR mice (Table III and Figure 4). However, effects of BOP treatment on FI in the pancreas were not apparent in all four mouse strains.

Discussion

The present study demonstrated that BOP treatment enhanced the proliferation of ductal cells in the pancreas of KK-A^y mice, but not of C57BL/6J, C57BL/6J-A^y and ICR mice. Similarly, enhancement of cell proliferation in the pancreatic ducts by BOP treatment has also been observed in Syrian golden hamsters (11). Adipocytes scattered within the exocrine pancreas were observed in Syrian golden hamsters and KK-A^y mice, while FI was only observed around the great vessels in the pancreas of C57BL/6J mice fed a high fat diet (unpublished data). Taken together, our data suggest that scattered adipocytes and adipocytes existing around the great vessels in the pancreas are generated through a distinct mechanism.

It has been reported that lipotoxicity caused by a high TG content induces inflammatory responses and necrosis in pancreatic acinar cells *in vitro* (12, 13). It has also been shown that c-Myc activity is required for growth and maturation of the exocrine pancreas and for the transdifferentiation of acinar cells into adipocytes in mice (14). Thus, pancreatic tissue containing scattered adipocytes might be more sensitive to acinar cell damage due to lipotoxicity and other genetic factors. Moreover, scattered FI may reflect acinar cell death

Table I. *Characteristics and fatty infiltration (FI) in the pancreas in each mouse strain at 7 weeks of age.*

Mouse strain	Age (weeks)	Gender	N	Treatment	BW (g)	Blood glucose (mg/dl)	Serum TG (mg/dl)	Serum TC (mg/dl)	Serum insulin (ng/ml)	Serum leptin (ng/ml)	Scattered FI of the pancreas
C57BL/6J	7	Female	6	Saline	16.5±1.1	166±21	94±33	50±6.4	2.7±0.4	2.8±2.1	– - ±
			6	BOP	15.4±1.7	84±78^{c}	107±42	241±109^{d}	3.4±1.0^{c}	7.5±6.6	– - ±
C57BL/6J-*A^{y}*	7	Female	6	Saline	18.1±1.0^{a}	184±14	123±28	66±9.6^{b}	2.8±0.4	4.7±5.4	±
			6	BOP	16.3±0.9^{c}	90±28^{d}	73±65	184±71^{d}	3.1±0.4^{c}	25.0±25.5	±
ICR	7	Female	6	Saline	29.0±1.0^{b}	188±17	151±43^{a}	81±17^{b}	4.2±3.8	4.1±3.0	– - ±
			7	BOP	27.0±0.5^{bc}	69±30^{d}	62±15^{ad}	244±136^{b}	3.7±0.6^{d}	16.6±13.2^{bc}	– - ±
KK-*A^{y}*	7	Female	6	Saline	34.5±1.1^{b}	250±73^{a}	292±86^{b}	111±13^{b}	26.0±12.8^{b}	120.2±30.0^{b}	+ - ++
			7	BOP	31.2±1.0^{bd}	57±23^{d}	101±56^{d}	373±77^{d}	13.5±2.6b^{c}	137.8±56.9^{b}	+ - ++

BOP, *N*-Nitroso*bis* (2-oxopropyl) amine; TC, total cholesterol; TG, tryglycerides. Data are shown as the mean±SD. Significantly different at $^{a}p<0.05$ and $^{b}p<0.01$ *vs*. C57BL/6J mice treated the same way; $^{c}p<0.05$ and $^{d}p<0.01$ *vs*. saline-treated. The area of scattered FI in pancreatic lobes was estimated as 0-1% (–), 1-2% (±), 2-5% (+) or >5% (++).

Table II. *The area of scattered fatty infiltration (FI) in the pancreas and ductal proliferation caused by N-nitrosobis (2-oxopropyl) amine (BOP) treatment in Syrian golden hamsters.*

Species	Strain	Age (weeks)	Gender	N	Treatment	BW (g)	Scattered FI in the pancreas	Proportion of BrdU-positive cells	
								Pancreatic duct	Common bile duct
Hamster	Syrian golden	9	Female	2	Saline or DMSO	115.8±0.6	+ - ++	0.38±0.16%	0.47±0.42%
				2	BOP	100.1±0.1	+ - ++	2.67±1.29%	4.12±2.60%

BW, Body weight; BrdU, bromodeoxyuridine; DMSO, dimethyl sulfoxide. Data are shown as the mean±SD. The area of scattered FI in pancreatic lobes was estimated as 0-1% (–), 1-2% (±), 2-5% (+) or >5% (++).

Table III. *Ductal proliferation caused by N-nitrosobis (2-oxopropyl) amine (BOP) treatment in each mouse strain.*

	Pancreatic ducts (%)				Common bile ducts (%)			
Treatment	C57BL/6J	C57BL/6J-A^{y}	ICR	KK-A^{y}	C57BL/6J	C57BL/6J-A^{y}	ICR	KK-A^{y}
Saline	4.4±2.9	4.3±1.1	4.5±2.8	3.4±1.5	5.9±5.6	2.8±1.1	6.3±4.3	1.6±1.2
BOP	2.4±0.8	3.2±1.5	3.6±1.9	7.9±3.5^{ab}	4.9±7.0	13.9±0.4^{c}*	20.0±25.8	36.5±13.6^{ac}

The data are the ratio of Ki-67-positive cells to total ductal cells in the pancreatic sections. Significantly different at $^{a}p<0.01$ *vs*. C57BL/6J mice treated with BOP; $^{b}p<0.05$ *vs*. saline-treated; $^{c}p<0.01$ *vs*. saline-treated. *There were common bile ducts in the pancreatic sections of only two mice.

or transdifferentiation after damage. In addition, sensitivity to cell damage could contribute to induce cell proliferation after exposure to carcinogens.

Recent evidence suggests that ectopic fat accumulation produces certain adipocytokines that induce cell proliferation (15, 16). In the present study, the serum levels of TG, TC, insulin, and leptin were high in KK-*A^{y}* even at a young age. Adipocytokines secreted from accumulated adipocytes may further enhance ductal cell proliferation. We previously showed that pancreatic leptin mRNA expression and serum leptin levels were higher in BOP-treated hamsters fed a high fat diet than those fed a standard diet, and PDACs developed only in BOP-treated hamsters fed a high fat diet at 14 weeks of age, at an incidence of 67% (4). Thus, local release of leptin from adipocytes in an adipocyte-rich microenvironment and increase of serum leptin level appear to be correlated with PDAC development. Increased cell proliferation in the common bile duct of BOP-treated KK-*A^{y}* mice may also increase the risk of bile duct carcinoma. Indeed, BOP-treated hamsters develop bile duct carcinomas in addition to PDACs (4).

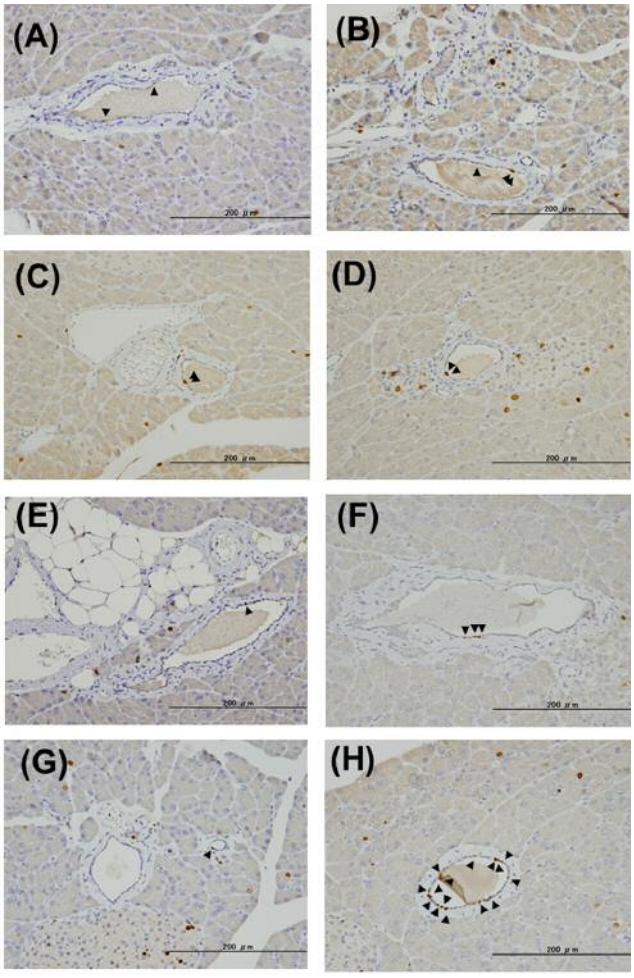

Figure 3. *The ratio of Ki-67-positive cells in the pancreatic ducts was significantly increased by N-nitrosobis(2-oxopropyl)amine treatment in KK-A^y mice. Histopathological sections of the pancreas showing pancreatic ducts of C57BL/6J (A and B), C57BL/6J-A^y (C and D), ICR (E and F) and KK-A^y (G and H) mice injected with saline (A, C, E, G) or N-nitrosobis(2-oxopropyl)amine (B, D, F, H) stained immunohistochemically for Ki-67. Arrowheads show positively-stained ductal cell nuclei.*

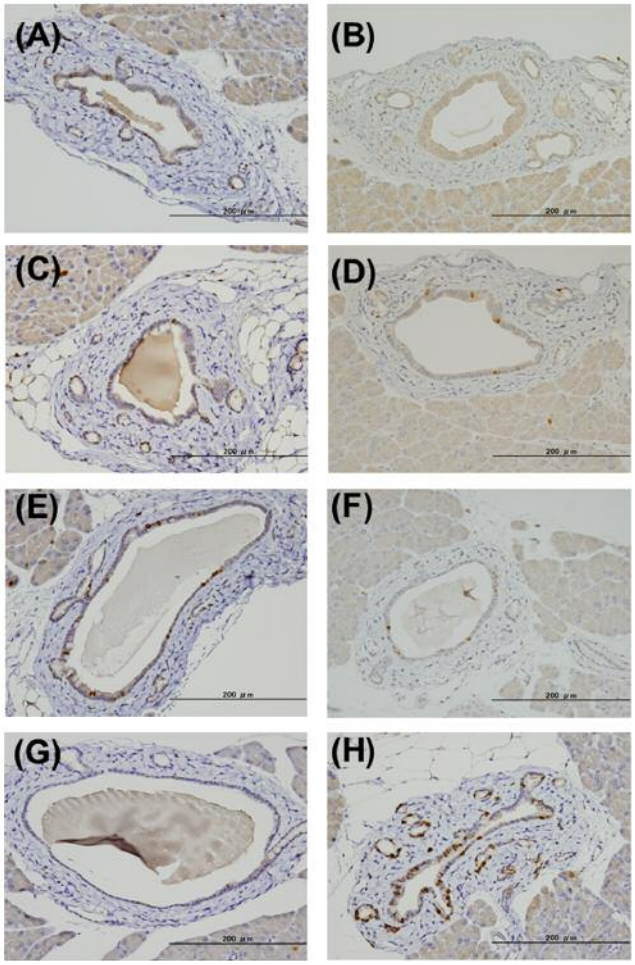

Figure 4. *The ratio of Ki-67-positive cells in the common bile ducts was significantly increased by N-nitrosobis(2-oxopropyl)amine treatment in KK-A^y and C57BL/6J-A^y mice. Histopathological sections of the pancreas showing common bile ducts of C57BL/6J (A and B), C57BL/6J-A^y (C and D), ICR (E and F) and KK-A^y (G and H) mice injected with saline (A, C, E, G) or N-nitrosobis(2-oxopropyl)amine (B, D, F, H) stained immunohistochemically for Ki-67.*

Several possible mechanisms underlying the development of FI in the pancreas can be speculated. It has been shown in animal experimental models that FI can be induced in the pancreas by obstruction of the pancreatic duct or vasculature (17, 18). Smits *et al*. has shown that FI or non-alcoholic fatty pancreas disease represents fat accumulation associated with obesity or metabolic syndrome, while fatty replacement represents replacement of adipocytes induced by death of acinar cells (19). The distribution of pancreatic scattered FI in patients with pancreatic cancer (3) is similar to that observed in the pancreas of hamsters (4) and KK-A^y mice. Genetic backgrounds or environmental factors for the development of pancreas with scattered adipocytes *per se* in hamsters or KK-A^y mice should be investigated and considered for use in the prevention of pancreatic cancer.

We spent much time and effort in the attempt to develop pancreatic tumors in KK-A^y mice treated with BOP, but failed (data not shown). Some researchers have also tried to develop pancreatic tumors in mice or rats treated with BOP, but succeeded only in hamsters. We have shown that BOP treatment led to development of pancreatic tumors with increase of FI in the pancreas in Syrian golden hamsters (4). In the present study, BOP treatment did not change FI in the pancreas of KK-A^y mice. In young/adult KK-A^y mice, the islet size in the pancreas was found to significantly increase (20, 21), although pancreatic islet pathology in T2DM is

characterized by reduced beta-cell function and mass. In our study, the number of islets was significantly increased in KK-A^y mice treated with BOP at 46 weeks of age (data not shown). Thus, the function of islet cells may have been maintained even in aged KK-A^y mice. BOP treatment increased serum TC in all four mouse strains. Acute liver toxicity and excess stress caused by BOP treatment may increase serum TC, but the mechanism remains to be clarified. Our findings in the present study indicate that KK-A^y mice resemble Syrian golden hamsters in developing scattered FI of the pancreas and sensitivity to BOP-induced ductal cell proliferation, but there are still other contributing factors, *i.e.* species differences, in the development of pancreatic cancer.

To initiate pancreatic carcinogenesis, *K-ras*-activating mutations are necessary, and transgenic mouse models expressing *K-ras* mutants in the pancreas develop pre-neoplastic pancreatic lesions. Thus, the introduction of a genetic background of severe FI of the pancreas to pancreas-specific *K-ras* mutant mouse models would increase pancreatic cancer development. Indeed, there is a report that concurrent pigment epithelium-derived factor deficiency and *K-ras* mutation induce invasive pancreatic cancer and adipose-rich stroma in mice (22). The genetic background of non-insulin-dependent DM in KK-A^y mice is polygenic (23), and further study to identify the causative genes of scattered type of FI of the pancreas in KK-A^y mice as well as Syrian golden hamsters is needed.

In conclusion, our study indicated that scattered FI in the pancreas *per se* could be a risk factor for pancreatic damage and may play an important role in early-phase pancreatic carcinogenesis. Identification of the genetic background causing scattered FI of the pancreas would be helpful in clarifying the involvement of FI in pancreatic carcinogenesis.

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Conflicts of Interest

The Authors have no potential conflicts of interest to disclose.

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