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Pancreatic adipocytes mediate hypersecretion of insulin in diabetes-susceptible mice

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ABSTRACT

Objective: Ectopic fat accumulation in the pancreas in response to obesity and its implication on the onset of type 2 diabetes remain poorly understood. Intermittent fasting (IF) is known to improve glucose homeostasis and insulin resistance. However, the effects of IF on fat in the pancreas and β-cell function remain largely unknown. Our aim was to evaluate the impact of IF on pancreatic fat accumulation and its effects on islet function.
Methods: New Zealand Obese (NZO) mice were fed a high-fat diet ad libitum (NZO-AL) or fasted every other day (intermittent fasting, NZO-IF) and pancreatic fat accumulation, glucose homoeostasis, insulin sensitivity, and islet function were determined and compared to ad libitum-fed B6.V-*Lep*$^{ob/ob}$ (ob/ob) mice. To investigate the crosstalk of pancreatic adipocytes and islets, co-culture experiments were performed.
Results: NZO-IF mice displayed better glucose homeostasis and lower fat accumulation in both the pancreas (−32%) and the liver (−35%) than NZO-AL mice. Ob/ob animals were insulin-resistant and had low fat in the pancreas but high fat in the liver. NZO-AL mice showed increased fat accumulation in both organs and exhibited an impaired islet function. Co-culture experiments demonstrated that pancreatic adipocytes induced a hypersecretion of insulin and released higher levels of free fatty acids than adipocytes of inguinal white adipose tissue.
Conclusions: These results suggest that pancreatic fat participates in diabetes development, but can be prevented by IF.

1. Introduction

The worldwide prevalence of obesity has nearly tripled since 1975 with 13% of adults obese and 39% overweight in 2016 and a growing prevalence among children (WHO, 2018). This constitutes a major health concern as obesity is associated with several co-morbidities including diabetes, cancer, and cardiovascular diseases. In obesity, the circulating levels of non-esterified fatty acids (NEFA) and triacylglycerol (TG) are elevated in combination with a limited adipose tissue capacity. This results in an ectopic fat storage in non-adipose tissue organs, such as liver, skeletal muscle or pancreas [1,2]. The elevated fat deposition in the liver, the non-alcoholic fatty liver disease (NAFLD) has been well studied over the last decades. However, this is not the case for the pancreas, even if it seems to be more susceptible to fat accumulation in comparison to the liver [3]. The first description of excessive fat storage in the pancreas was reported by Ogilvie in 1933 [4]. Later, several terms were used to qualify pancreatic fat accumulation. In 2011, Smits and van Geenen defined pancreatic steatosis in association with obesity and metabolic syndrome as non-alcoholic fatty pancreas disease (NAFPD) [5]. Animal and human studies have shown that both NAFPD and NAFLD very frequently co-exist [6–8]. Insulin resistance plays an important role in the development of NAFPD, NAFLD and type 2 diabetes, but pancreatic fat infiltration might lead to more rapid progression of diabetes through other mechanisms such as a decrease in β-cell number and function [2,3]. However, despite in vitro and animal studies were able

Abbreviations: AL, ad libitum; APCs, adipogenic precursor cells; AUC, area under the curve; FITC, fluorescein isothiocyanate; GSIS, glucose-stimulated insulin secretion; HFD, high-fat diet; IF, intermittent fasting; ipITT, intraperitoneal insulin tolerance test; iWAT, inguinal white adipose tissue; KRBH, Krebs-Ringer bicarbonate HEPES buffer; NAFLD, non-alcoholic fatty liver disease; NAFPD, non-alcoholic fatty pancreas disease; NEFA, non-esterified fatty acids; NZO, New Zealand Obese; ob/ob, B6.V-*Lep*$^{ob/ob}$; OGTT, oral glucose tolerance test; TG, triacylglycerol.

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to show that NAFPD plays a role in diabetes development via effects on insulin resistance and β-cell dysfunction, the data in humans remain inconclusive [2,9,10].

As overfeeding leads to the development of obesity, insulin resistance, type 2 diabetes and ectopic fat accumulation, dietary interventions limiting energy intake such as intermittent fasting (IF) could be used to prevent and/or treat these conditions. In rodents, IF improves some gluco-metabolic, cardiovascular and neuronal parameters [11,12]. Recent trials aim to confirm this in human subjects [13]. However, all these emerging statements need to be validated and the mechanisms behind the improvement of general health by IF remain poorly understood.

We have recently shown that IF prevents the onset of diabetes in New Zealand Obese (NZO) mice, a mouse model for obesity and type 2 diabetes. This was associated with changes in lipid droplet proteome and levels of some diacylglycerols in the liver and skeletal muscle [14]. As NAFPD, NAFLD and type 2 diabetes are often associated, we followed two approaches: (1) we compared hepatic and pancreatic fat content in two obese mouse models which differ in their diabetes-susceptibility and (2) tested whether IF has beneficial effects on pancreatic fat accumulation.

2. Material and methods

2.1. Animals

Male NZO/HlBomDife and B6.V-*Lep*$^{ob/ob}$ (ob/ob) mice from our own breeding (German Institute of Human Nutrition Potsdam-Rehbruecke, Nuthetal, Germany) were kept at a temperature of 20 ± 2 °C with a 12:12 h light-dark cycle and ad libitum access to drinking water. After weaning, mice were single caged and fed a high-fat diet (HFD, 49 kcal % from carbohydrates, 33 kcal% from fat, and 18 kcal% from protein, S8022-E080 unsat. FA, Ssniff, Soest, Germany) ad libitum (NZO-AL and ob/ob) until the age of 9 weeks. Starting at 4 weeks of age, a subgroup of NZO mice received food only every other day with constant access to drinking water (intermittent fasting, NZO-IF). The measurements of glucose tolerance, insulin tolerance and the islet isolation were performed following to a feeding day. All experiments with mice were approved by the ethics committee of the State Office of Environment, Health and Consumer Protection (Federal State of Brandenburg, Germany).

2.2. Body weight, body composition and blood glucose measurements

Body weight and blood glucose levels were measured weekly in the morning. At 3, 6 and 9 weeks of age, blood samples were collected and body composition was assessed by nuclear magnetic resonance (EchoMRI-100H, EchoMRI LLC, Houston, TX, USA).

2.3. Glucose and insulin tolerance tests

At the age of 7 weeks, mice were fasted for 6 h and received either an oral glucose bolus (2 g/kg body weight) for oral glucose tolerance tests (OGTT) or an intraperitoneal insulin injection (1 IU/kg body weight) for insulin tolerance tests (ipITT). Blood was sampled from the tail vein at indicated time points for blood glucose and plasma insulin detection.

2.4. Plasma parameters, total pancreatic insulin, pancreatic and hepatic triacylglycerol levels

Plasma insulin, TG, glycerol and NEFA levels were determined as described before [15]. Total plasma cholesterol was measured using the Cholesterol liquicolor kit (Human Diagnostics Worldwide, Wiesbaden, Germany). Circulating adiponectin and leptin levels were assessed as described before [16]. To examine pancreatic insulin and TG concentrations, pancreases were grounded in liquid nitrogen and divided into two parts. One part was homogenized in ice-cold acidic ethanol (0.18 mol/L HCl) and incubated overnight at 4 °C. After centrifugation (5000 ×*g*, 15 min), insulin concentrations were measured by ELISA (80-INSMSH-E01 and 80-INSMSU-E01, Alpco Diagnostics, Salem, USA). The other part was homogenized (10 mmol/L NaH_2PO_4, 1 mmol/L EDTA, 1% Polyoxyethylene (10) tridecyl ether, pH 7.4), incubated for 5 min at 70 °C while shaking, placed on ice for 5 min and centrifuged (13,000 ×*g*, 10 min). TG concentrations from pancreases and liver samples were determined using a kit (TR210, Randox Laboratories, Crumlin, UK).

2.5. Isolation of pancreatic adipogenic precursor cells and stromal vascular fraction from inguinal white adipose tissue

Flow cytometric isolation was performed based on procedures as described before [17]. In brief, minced pancreas or inguinal white adipose tissue (iWAT) were digested at 37 °C for 1 h in 1 mg/mL and 2 mg/mL of collagenase type II (Worthington Biochemical Corporation, Lakewood, NJ, USA) solution, respectively. Pancreas and iWAT samples were centrifuged at 100 ×*g* and 290 ×*g*, respectively. Cells were stained with antibodies as follows: Anti-Mouse Ly-6A/E (Sca-1) (Clone: D7; eBioscience), Anti-Mouse CD45 FITC (Clone: 30-F11; eBioscience); Anti-Mouse CD31 (PECAM-1) FITC (Clone: 390; eBioscience) using protocols described before [17]. Cells were sorted for Sca-1 (positive selection) and CD45/CD31 (negative selection) to purify adipogenic precursor cells (APCs). After sorting, pancreatic APCs and the stromal vascular fraction from iWAT were plated and cultured for one week as described [17]. Subsequently, cells were seeded on cell culture inserts (353103, Corning Incorporated, Corning, NY, USA) for three days followed by adipogenic differentiation [17].

2.6. Co-culture experiments and glucose-stimulated insulin secretion

Pancreatic islets of 9-week-old mice were isolated as described [18] and co-cultured with differentiated adipocytes derived from pancreas APCs or iWAT stromal vascular fraction in RPMI 1640 medium (PAA Laboratories, Pasching, Austria) containing 10% FCS GOLD (PAA Laboratories) and 1% penicillin/streptomycin for 48 h. Afterwards, islets were equilibrated in Krebs-Ringer bicarbonate HEPES buffer (KRBH, 115 mmol/L NaCl, 4.5 mmol/L KCl, 2.6 mmol/L $CaCl_2$, 1.2 mmol/L KH_2PO_4, 1.2 mmol/L $MgSO_4$, 20 mmol/L $NaHCO_3$, 10 mmol/L HEPES, 0.2% BSA, pH 7.4) for 1 h under low glucose conditions (2.8 mmol/L) and consecutively transferred to 2.8 mmol/L glucose and 20 mmol/L glucose for 1 h each to detect glucose-stimulated insulin secretion (GSIS). Insulin concentrations in the supernatants were measured and islets were transferred to 1 mL ice-cold acidic ethanol to extract residual insulin (80-INSMSH-E01, Alpco Diagnostics).

2.7. Co-culture experiments with 3T3-L1 cells and islets of C57BL/6J mice

3T3-L1 pre-adipocytes or 3T3-L1 adipocytes were grown in cell culture inserts as described above and co-cultured with islets isolated from 9-week-old C57BL/6J mice for 48 h before insulin secretion was measured as described in Section 2.6.

2.8. Islet perifusion

Sixty freshly isolated pancreatic islets per mouse were used for perifusion experiments. Islets were equilibrated in KRBH under low glucose conditions (2.8 mmol/L) for 30 min and subsequently transferred into perifusion chambers. After a 10 min adaption phase, GSIS was recorded with a continues flow of 0.5 mL/min under low glucose, high glucose (20 mmol/L), low glucose and 40 mmol/L KCl conditions for 18, 35, 30 and 20 min, respectively. Insulin levels of the

perifusates were determined and normalized to residual insulin content (80-INSMSU-E01, Alpco Diagnostics).

2.9. Pancreas histology, immunofluorescence and image analysis

For the determination of pancreatic adipocytes and islet mass, 4 μm paraffin sections were deparaffinized, rehydrated, and stained with Mayer's hematoxylin (1 min, Bio-Optica, Milano, Italy) and eosin (45 s, Sigma-Aldrich, St Louis, MO, USA), followed by dehydration. Sections were recorded with a MIRAX MIDI scanner (Zeiss, Oberkochen, Germany) and adipocytes as well as islet mass were analyzed using the MIRAX Viewer software.

For insulin glucagon co-staining, primary antibodies against insulin (1:50,000, I2018, Sigma-Aldrich) and glucagon (1:2000, A0565, Agilent, Santa Clara, CA, USA) were applied for 1 h at room temperature and overnight at 4 °C, respectively. Alexa Fluor conjugated secondary antibodies were applied for 1 h at room temperature (1:200, A11017 and A11035, Invitrogen, Karlsruhe, Germany). Nuclei were stained with DAPI. Images were recorded using the Keyence BZ-9000 fluorescent microscope and the BZ-II Analyser software (Keyence International, Mechelen, Belgium).

2.10. Statistical analysis

All data are expressed as mean ± SEM. Statistical analysis was performed using Student's *t*-test, one-way ANOVA with Tukey's multiple comparisons test or two-way ANOVA with Sidak's multiple comparisons test (GraphPad Prism Version 6.07, GraphPad, La Jolla, CA, USA). If not otherwise stated, statistical significance is expressed as $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

3. Results

3.1. Intermittent fasting reduced body weight gain and fat mass in NZO mice

HFD-fed male NZO and ob/ob mice were subjected to different feeding regimens. NZO-AL and ob/ob mice were fed ad libitum while NZO-IF mice had access to food ad libitum only every other day. NZO-IF and ob/ob mice exhibited lower body weights in comparison to NZO-AL mice (Fig. 1A). This was significant from 5 weeks of age, thus already one week after the beginning of the IF procedure. NZO-IF animals also displayed lower body fat mass and slightly higher body lean mass compared to NZO-AL and ob/ob mice (Fig. 1B and C). At the end of the protocol, NZO-AL animals had increased pancreas and spleen weights compared to NZO-IF and ob/ob groups. NZO-IF animals were lighter and had a reduction in selected fat depots and visceral white adipose tissue masses in comparison to NZO-AL and ob/ob animals, and in heart and kidney weights compared to NZO-AL animals. Ob/ob mice displayed higher weight of liver, white and brown adipose tissue as well as lower weight of heart, kidney and gastrocnemius than both NZO-AL and NZO-IF groups (Table 1).

3.2. Intermittent fasting prevented the development of hyperglycemia and hyperinsulinemia and enhances circulating lipids

NZO-IF mice showed significantly lower blood glucose levels than NZO-AL mice from 6 weeks of age until the end of the protocol (Fig. 1D) and lower plasma insulin levels than ob/ob mice at 9 weeks of age (Fig. 1E). NZO-IF animals displayed significantly lower plasma leptin concentration in comparison to their NZO-AL littermates (Table 2). NZO-AL mice had higher circulating levels of TG compared to NZO-IF and ob/ob. Total cholesterol levels were significantly lower in

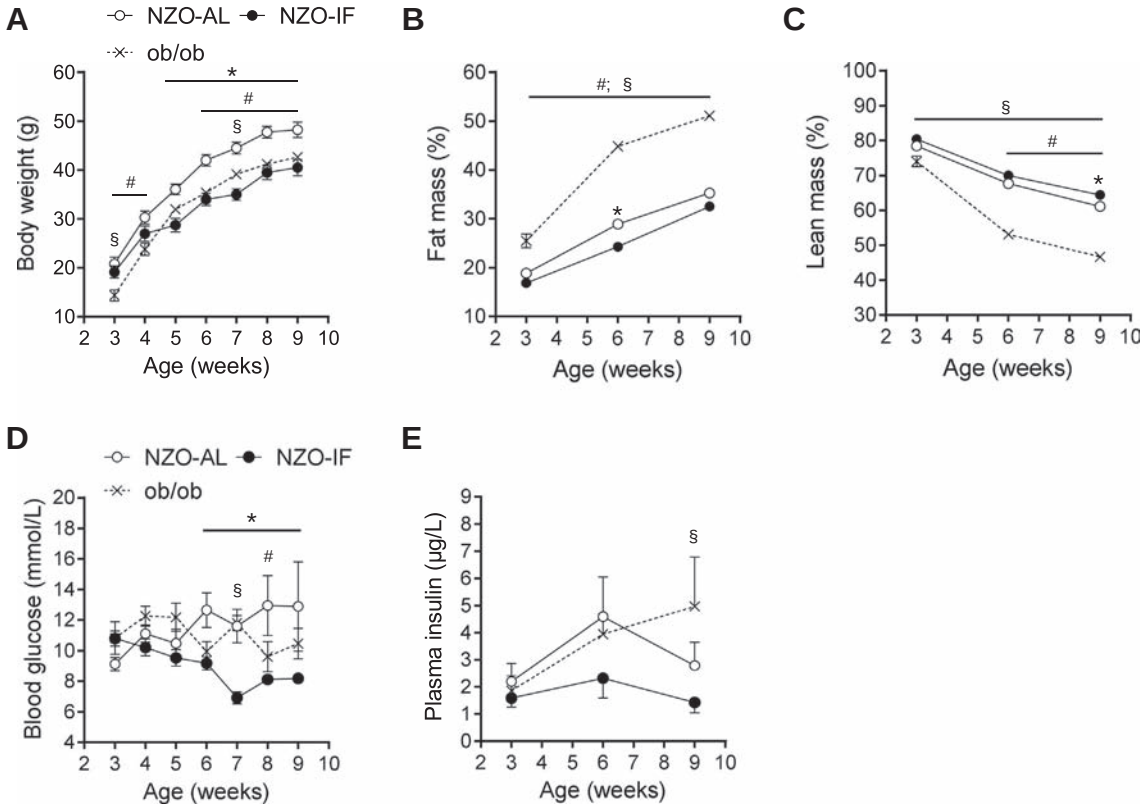

Fig. 1. Intermittent fasting improves body composition and glucose homeostasis in NZO mice. (A) Body weight, (B) fat mass, (C) lean mass, (D) blood glucose levels, and (E) plasma insulin concentrations of NZO-AL, NZO-IF and ob/ob mice fed a high-fat diet at indicated time points. Data are mean ± SEM, $n = 8$–11 mice per group, $^{*}P < 0.05$ (NZO-AL vs. NZO-IF), $\#P < 0.05$ (NZO-AL vs. ob/ob), $\S P < 0.05$ (NZO-IF vs. ob/ob) by two-way ANOVA with Sidak's multiple comparisons test.

Table 1
Body weight and organ tissue weights of 9-week-old NZO-AL, NZO-IF and ob/ob mice.

	NZO-AL	NZO-IF	ob/ob	*P* value
Body weight (g)	45.6 ± 1.0	39.2 ± 1.0	45.2 ± 1.4	*, §
Pancreas (g)	0.28 ± 0.01	0.23 ± 0.01	0.23 ± 0.01	*, #
Liver (g)	1.54 ± 0.05	1.38 ± 0.04	3.40 ± 0.18	#, §
Fat (g)	3.02 ± 0.12	2.48 ± 0.15	4.12 ± 0.16	*, #, §
Visceral fat (g)	1.09 ± 0.05	0.79 ± 0.06	1.29 ± 0.09	*, #, §
Spleen (g)	0.111 ± 0.008	0.084 ± 0.003	0.075 ± 0.005	*, #
Heart (g)	0.169 ± 0.003	0.143 ± 0.002	0.110 ± 0.003	*, #, §
Kidney (g)	0.264 ± 0.007	0.245 ± 0.006	0.164 ± 0.004	#, §
Gastrocnemius (g)	0.097 ± 0.003	0.093 ± 0.003	0.080 ± 0.004	#, §
BAT (g)	0.34 ± 0.03	0.38 ± 0.02	0.53 ± 0.04	#, §

Data are mean ± SEM (n = 16–17). Fat mass was calculated as the sum of subcutaneous, gonadal and visceral fat depots. *P < 0.05 (NZO-AL vs. NZO-IF), #P < 0.05 (NZO-AL vs. ob/ob), §P < 0.05 (NZO-IF vs. ob/ob) by one-way ANOVA with Tukey's multiple comparisons test.

NZO-IF and higher in ob/ob group while plasma glycerol concentration was increased in NZO-AL and decreased in ob/ob mice. Plasma NEFA were significantly lower in ob/ob animals compared to both other groups (Table 2).

3.3. Intermittent fasting improved glucose tolerance and insulin sensitivity

Glucose and insulin tolerance tests were performed at 7 weeks of age. During OGTT, NZO-IF mice displayed lower blood glucose concentrations before and 15 min after glucose administration in comparison to NZO-AL and ob/ob mice. However, from time point 30 min until the end of the test, NZO-IF and ob/ob mice had similar blood glucose levels while NZO-AL animals showed increased blood glucose levels (Fig. 2A, left panel). The area under the curve (AUC) of the OGTT was higher in the NZO-AL mice than in the two other groups (Fig. 2A, right panel). The insulin values during the OGTT showed a marked difference between the strains. They were about 5-fold higher in ob/ob than in NZO mice, which showed lower insulin concentrations in response to IF (Fig. 2B). Plasma insulin concentrations of NZO-AL were between 1.37 and 1.84 μg/L, of NZO-IF much lower with 0.37 to 0.79 μg/L, pointing towards improved insulin sensitivity in response to intermittent fasting. In fact, MATSUDA index calculated based on basal blood glucose and insulin levels, was significantly higher in NZO-IF than in NZO-AL and ob/ob mice (Fig. 2C). Also during ipITT, NZO-IF animals displayed lower blood glucose concentrations before and 30, 60 and 120 min after insulin injection (Fig. 2D, left panel), resulting in a lower AUC during the test in comparison to both NZO-AL and ob/ob groups (Fig. 2D, right panel). The transiently elevated blood glucose concentration of NZO mice 15 min after insulin treatment might be a result of stress induced by handling during the insulin application. Taken together, these data confirm that IF improves glucose tolerance and insulin sensitivity in NZO mice and show that ob/ob mice exhibit a similar glucose clearance as NZO-IF mice.

Table 2
Circulating cytokines and plasma lipids of 9-week-old NZO-AL, NZO-IF and ob/ob mice.

	NZO-AL	NZO-IF	ob/ob	*P* value
Adiponectin (ng/ml)	6782 ± 245	6626 ± 399	5212 ± 331	#, §
Leptin (pg/ml)	68,133 ± 5983	42,292 ± 3004	*N/A*	P < 0.001
Triacylglycerol (μg/ml)	1037 ± 64	446 ± 24	404 ± 34	*, #
Glycerol (μg/ml)	727 ± 34	632 ± 21	467 ± 25	*, #, §
Cholesterol (mg/dl)	185 ± 6	162 ± 5	215 ± 10	*, #, §
NEFA (mmol/L)	1.15 ± 0.05	1.03 ± 0.04	0.67 ± 0.04	#, §

Data are mean ± SEM (n = 13–18). *P < 0.05 (NZO-AL vs. NZO-IF), #P < 0.05 (NZO-AL vs. ob/ob), §P < 0.05 (NZO-IF vs. ob/ob) by one-way ANOVA with Tukey's multiple comparisons test. For leptin values, statistical analysis was performed using Student's t-test.

3.4. Intermittent fasting reduced fat in pancreas and liver

In order to test if NZO and ob/ob mice differ in their pancreatic fat content and if IF can reduce it, we evaluated both the total TG and the insulin concentrations from the whole organ. Pancreas TG levels were significantly lower in NZO-IF and ob/ob animals in comparison to NZO-AL ones (Fig. 3A). The total insulin content in the pancreas was similar between NZO-AL and NZO-IF groups but significantly higher in the ob/ob group (Fig. 3B). In the liver, the TG concentration was significantly lower in NZO-IF animals while it was higher in ob/ob than in NZO-AL mice (Fig. 3C). The hematoxylin/eosin staining of pancreas slices shows an accumulation of adipocytes in the tissue of NZO-AL mice and only a few adipocytes in NZO-IF and ob/ob pancreases (Fig. 3D, upper panel). The quantification of adipocytes revealed a lower adipocyte area in ob/ob and NZO-IF in comparison to the NZO-AL pancreas (Fig. 3D, lower panel). These data show that diabetes-susceptible mice exhibit higher fat content in the pancreas, whereas diabetes-resistant mice store more fat in the liver. In addition, IF reduces both, hepatic and pancreatic fat.

The staining of glucagon and insulin of pancreatic sections of 9-week-old NZO-AL, NZO-IF and ob/ob mice showed comparable islet architecture. However, the islets of ob/ob mice appear to be larger with a higher number of β-cells (Fig. S1). The quantification of islet mass in pancreatic sections confirmed that ob/ob mice exhibited an elevated islet area per pancreas in comparison to NZO mice (Fig. 3E).

3.5. Intermittent fasting protected islets from insulin hypersecretion

In order to determine if intermittent fasting has effects on the first- and the second-phase of insulin secretion, we analyzed freshly isolated islets from NZO-AL, NZO-IF and ob/ob mice with a perifusion system. The first-phase insulin secretion was similar in NZO-AL and ob/ob islets but lower in NZO-IF islets, whereas the second-phase insulin secretion of NZO-IF and ob/ob islets was similar (Fig. 4A). The low insulin secretion of NZO-IF islets confirms the low insulin concentrations detected during the glucose tolerance test (Fig. 2B), and might be a consequence of the improved glucose homeostasis and insulin sensitivity in response to intermittent fasting. During the second-phase and even after reducing the glucose concentration to 2.8 mmol/L, NZO-AL islets did not reduce their insulin secretion in the same manner as NZO-IF and ob/ob islets (Fig. 4A). Thus, the overall insulin secretion during the perifusion experiment, assessed by the AUC, was significantly reduced in NZO-IF islets and tended to be lower in ob/ob islets as compared to NZO-AL (P = 0.053) (Fig. 4B).

3.6. Adipocytes modified islet insulin secretion

To clarify whether adipocytes affect insulin secretion, we first co-cultured 3T3-L1 adipocytes with islets from B6 mice for 2 days and subsequently measured insulin secretion under low (2.8 mmol/L) and high (20 mmol/L) glucose concentrations. Without co-culture, B6 islets secreted very low levels of insulin at low glucose conditions; insulin release increased 17-fold at high glucose concentrations. Co-culture experiments with 3T3-L1 pre-adipocytes showed a similar response. Interestingly, after co-culture with 3T3-L1 adipocytes, basal as well as high glucose-stimulated insulin secretion significantly increased but the effect of glucose stimulation (2.6-fold) was smaller than without co-culture (Fig. 5A).

Next, adipogenic precursor cells (APCs) were isolated from pancreases of B6 mice by flow cytometric sorting (Fig. 5B) and adipocytes from iWAT stromal vascular fraction were taken and cultured as well. In parallel, islets were isolated from NZO-AL, NZO-IF and ob/ob mice and recovered for 2 days in order to wash out the effects of the fatty pancreas. The islets were divided in 3 subgroups: The first subgroup of islets of NZO-AL, NZO-IF and ob/ob mice was cultured in standard conditions, the second subgroup was co-cultured with

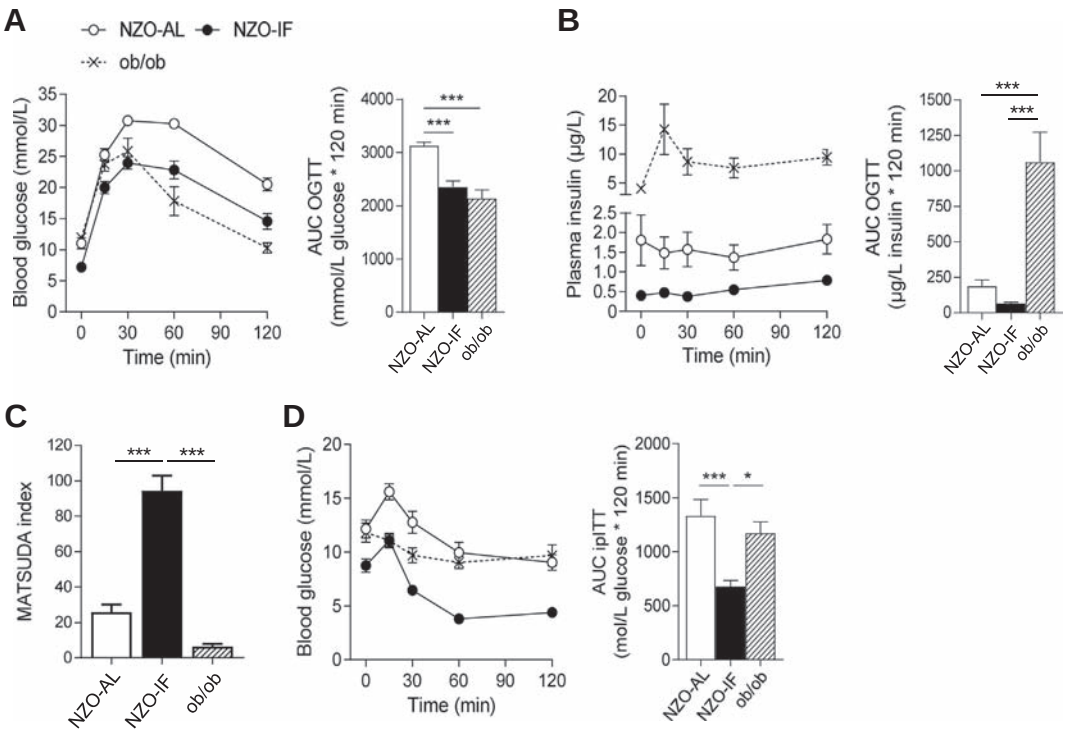

Fig. 2. Intermittent fasting ameliorates glucose tolerance and insulin sensitivity in NZO mice. (A) Blood glucose and (B) plasma insulin levels during OGTT with respective area under the curves (AUCs) and (C) MATSUDA indices. (D) Blood glucose levels during ipITT with respective AUC. Data are mean ± SEM, $n = 9–12$ mice per group.*$P < 0.05$, ***$P < 0.001$ by one-way ANOVA with Tukey's multiple comparisons test.

differentiated APCs from the pancreas, the third subgroup with differentiated adipocytes from iWAT (Fig. 5C). In the first subgroup (no co-culture), the islet insulin secretion during GSIS was similar between the three groups (Fig. 5D, left panel), demonstrating that culture conditions washed out the differences in insulin secretion as detected by the perifusion of freshly isolated islets (Fig. 4). However, after 2 days of co-culture with pancreatic adipocytes or iWAT adipocytes, islets from NZO-AL, NZO-IF and ob/ob animals displayed a much higher insulin secretion already at the basal state (2.8 mmol/L glucose) (Fig. 5D, left panel). The insulin secretion was even higher than at 20 mmol/L glucose (Fig. 5D, right panel). The co-culture with pancreatic and iWAT adipocytes affected NZO-AL and NZO-IF islets more than ob/ob islets which displayed overall a lower insulin secretion during the GSIS (Fig. 5D). However, NZO-AL and NZO-IF islets showed the same pattern and levels of insulin secretion under the different conditions, demonstrating that IF did not change the insulin secretion of the islets after a 2-day recovery. As the co-culture of B6 islets with 3T3-L1 adipocytes resulted already in a lower degree of glucose-stimulated insulin secretion, we believe that the lack of an induction of glucose-stimulated insulin secretion in islets of obese mice is a consequence of a severe islet insulin resistance which might be induced by the elevated secretion of fatty acids from adipocytes. Accordingly, B6 islets which were co-cultured with adipocytes derived from pancreatic APCs showed an elevated insulin secretion at 2.8 mmol/L glucose, and 20 mmol/L of glucose was not sufficient to potentiate this reaction (Fig. S2). We measured the NEFA released by the pancreatic and iWAT adipocytes into the medium after 2 days of co-culture and detected significantly higher NEFA concentrations in the co-culture medium of pancreatic adipocytes than in the medium of adipocytes derived from iWAT (Fig. 5E). Taken together, these data indicate that adipocytes that are in close proximity to islets induce insulin hypersecretion.

4. Discussion

In this study, we demonstrate that (i) fat accumulation in the pancreas is associated with diabetes development and β-cell dysfunction, (ii) adipocytes in close proximity to islets induce a hypersecretion of insulin, (iii) pancreatic adipocytes are particularly harmful for the islet function, and (iv) intermittent fasting reduces pancreatic fat cell accumulation and prevents the onset of diabetes.

For the first time to our knowledge, we show that the reduction of pancreatic fat accumulation participates in the prevention of diabetes in response to intermittent fasting. As already suggested by some previous studies in other animal models [11–13], we could prove that intermittent fasting is an effective strategy to prevent diabetes also in NZO mice [14]. In these diabetes-prone mice intermittent fasting resulted in a significant reduction of body weight, blood glucose and insulin levels, decreased fat mass and improved glucose tolerance and insulin sensitivity. During the OGTT, NZO mice under IF displayed similar blood glucose levels as ob/ob mice which were protected from diabetes [19]. The improved glucose tolerance in NZO-IF mice occurred despite a lower insulin content in comparison to ob/ob mice, which was assessed by the total pancreatic insulin. This suggests that intermittent fasting can normalize the glucose tolerance and improve the β-cell function of NZO mice to a similar extent as reached by ob/ob mice via inducing β-cell proliferation [19].

To date, most studies which link ectopic fat accumulation and diabetes suggest that insulin resistance is a common pathway for the development of NAFPD, NAFLD and diabetes [2]. However, other mechanisms than insulin resistance, such as a reduction in β-cell number and function, could also be responsible for the rapid progression of diabetes with NAFPD. Previous studies on this matter are inconclusive on whether pancreatic fat causes lipotoxicity to the β-cells or whether NAFPD is rather a marker of β-cell dysfunction [20,21]. Besides, the association between NAFPD and insulin resistance is controversial. It seems that NAFPD could be positively associated to insulin resistance only when NAFLD is also occurring [22,23] and that insulin resistance could be more related to visceral WAT and hepatic fat instead of pancreatic fat [2,24,25]. In this study, we wanted to clarify whether fat accumulation in the pancreas can be linked to an elevated diabetes risk. Diabetes-prone NZO-AL animals, which are insulin resistant and glucose intolerant, display high amounts of fat in the pancreas and in the liver. In contrast, diabetes-

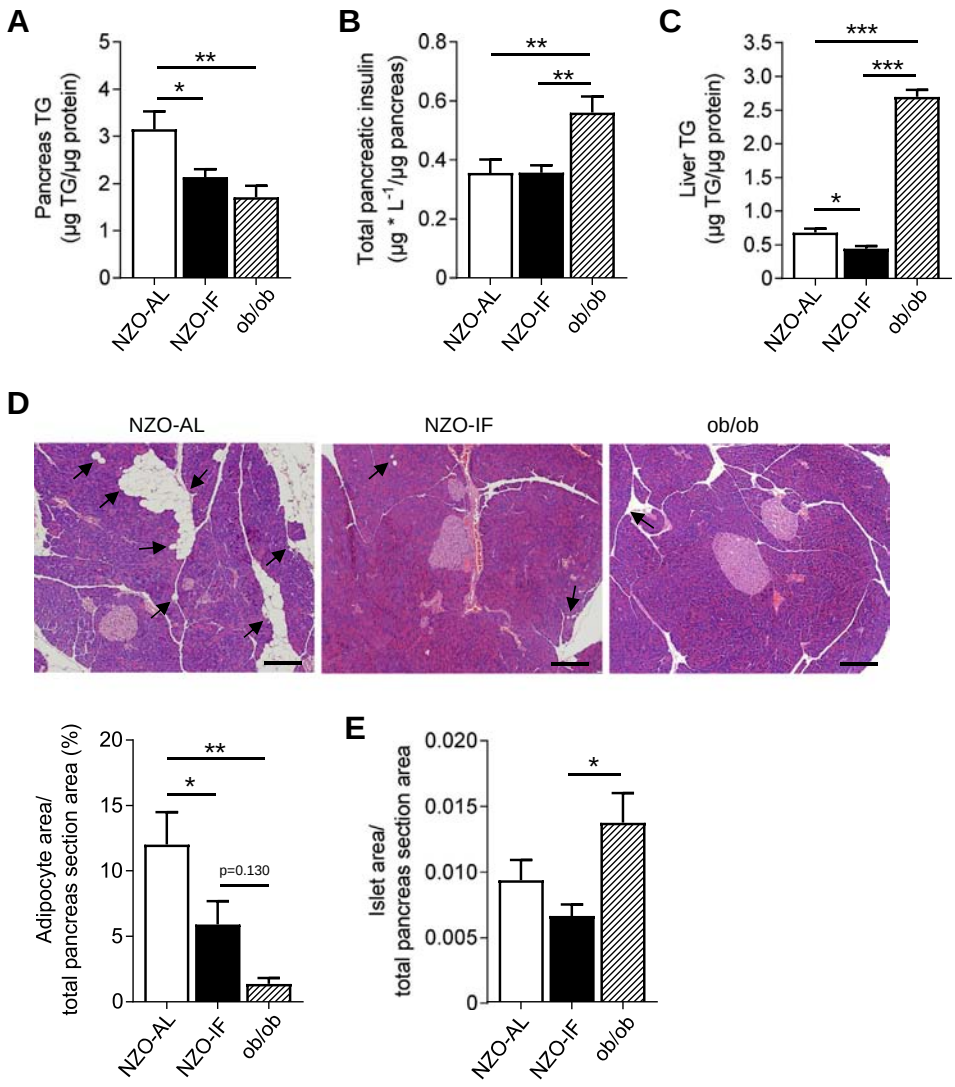

Fig. 3. Diabetes-prone and diabetes-resistant mice exhibit different ectopic lipid accumulations in pancreas and liver. (A) Pancreas TG levels, (B) total pancreatic insulin concentrations and (C) liver TG levels of 9-week-old mice. (D) Representative H&E stains for the determination of pancreatic adipocytes and quantification of adipocytes and islet area in (E). Adipocytes are indicated by the arrows. Scale bars, 200 µm. Data are mean ± SEM, $n = 7–10$ for A, $n = 9–11$ for B, $n = 15–16$ for C, and $n = 4–6$ for D and E, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by one-way ANOVA with Tukey's multiple comparisons test.

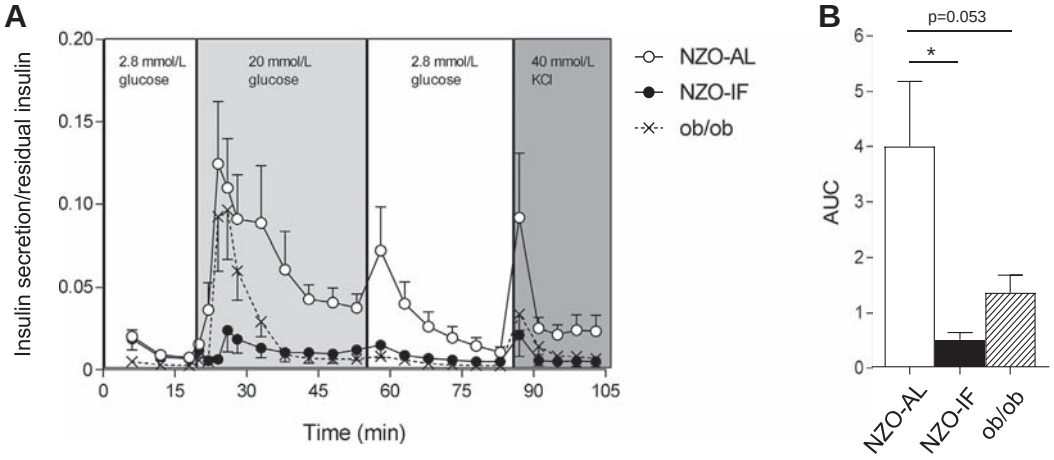

Fig. 4. Hypersecretion of insulin of islets from diabetes-prone NZO mice. (A) Glucose-stimulated insulin secretion of freshly isolated pancreatic islets of 9-week-old NZO-AL, NZO-IF and ob/ob mice at indicated time points measured during perifusion experiments. (B) AUC of results shown in A. Data are mean ± SEM, $n = 5$ mice per group, $^{*}P < 0.05$ by one-way ANOVA with Tukey's multiple comparisons test.

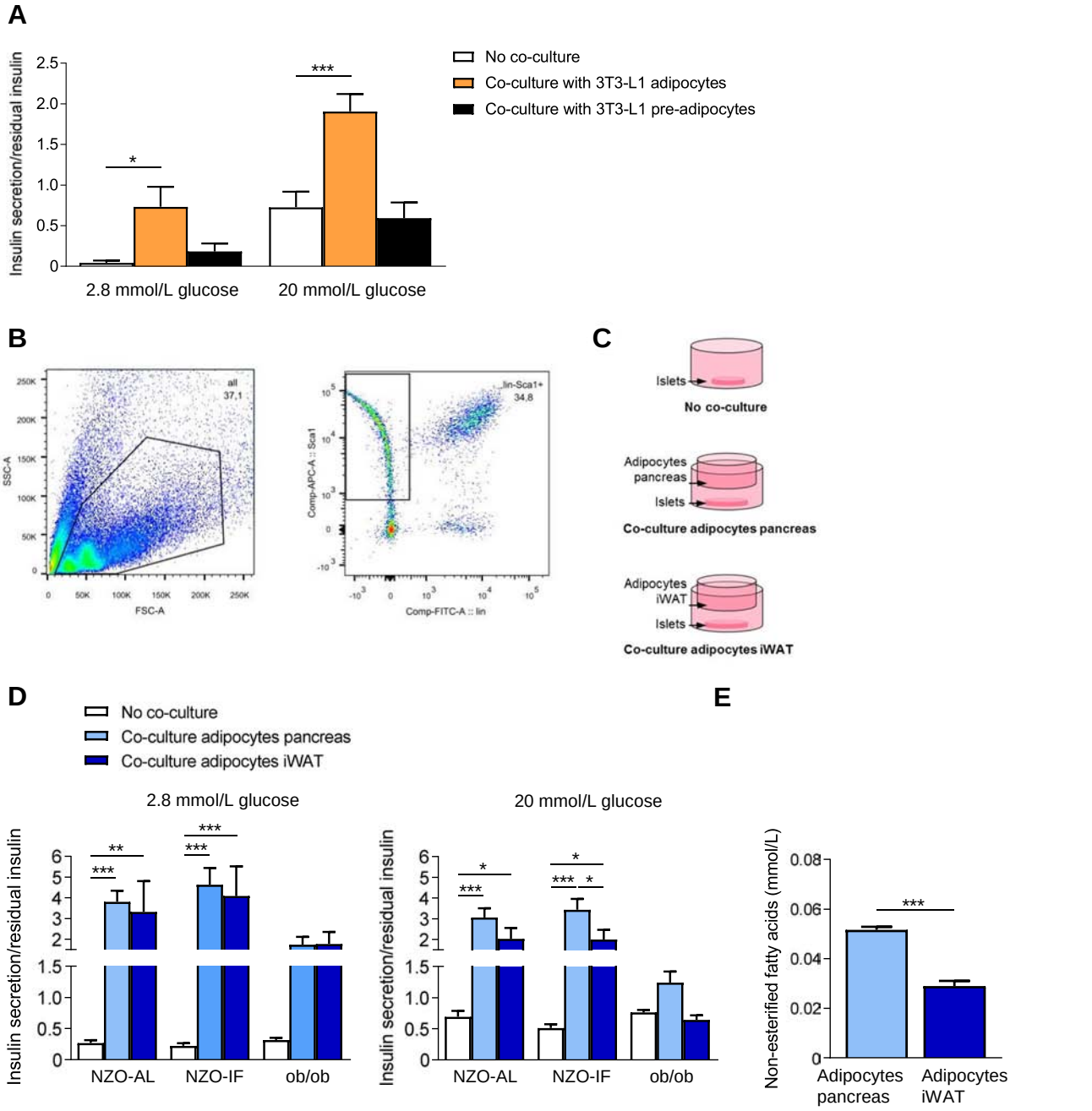

Fig. 5. Specificity of pancreatic adipocytes in the impairment of islet insulin secretion. (A) Glucose-stimulated insulin secretion of islets from 9-week-old B6 mice without co-culture (white bars, $n = 5$–6) or after 2 days of co-culture with mature 3T3-L1 adipocytes (orange bars, $n = 5$) or 3T3-L1 pre-adipocytes (black bars, $n = 4$–5) at basal (2.8 mmol/L) and high (20 mmol/L) glucose conditions. (B) Flow cytometric separation and FACS analysis of CD31/CD45-negative cells by Sca1 selection. (C) Schematic representation of the culture conditions used in D. (D) Islets of NZO-AL, NZO-IF and ob/ob mice were cultured separately (white bars, $n = 14$–15 for NZO islets, $n = 5$ for ob/ob islets) or co-cultured for 2 days with pancreatic adipocytes (light blue bars, $n = 7$–10 per group) or with adipocytes derived from iWAT (dark blue bars, $n = 2$–3 per group). Insulin secretion was measured at basal glucose (2.8 mmol/L, left panel) and high glucose conditions (20 mmol/L, right panel). Data are mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by two-way ANOVA with Sidak's multiple comparisons test. (E) Concentrations of NEFA detected in the medium after co-culture. Data are mean ± SEM, $n = 8$–9, $^{***}P < 0.001$ by Student's *t*-test.

resistant ob/ob animals are insulin resistant, exhibit a severe hepatosteatosis, but showed only low fat accumulation in the pancreas and at the same time a better glucose tolerance than NZO mice. This fact indicates that fatty pancreas is a stronger risk factor for type 2 diabetes than fatty liver. Interestingly, when NZO mice were put on an intermittent fasting intervention, their pancreatic fat decreased, as well as their hepatic fat accumulation, which improved glucose tolerance and insulin sensitivity. Thus, our data indicate that insulin resistance is responsible for fatty liver while pancreas steatosis induces β-cell dysfunction presumably via an adipocyte islet cell crosstalk.

Among others, circulating lipids and cytokines are factors known to influence β-cell function in type 2 diabetes [26]. Thus, high concentrations of circulating NEFA, derived from lipoprotein hydrolysis or adipocyte lipolysis, have been shown to impair β-cell function [26–28]. In our study, NZO-AL mice have increased circulating levels of TG, NEFA and glycerol, pointing towards an elevated lipolysis. In contrast, NZO-IF and ob/ob mice display lower plasma lipid concentrations. These results confirm the negative effect of circulating TG, NEFA, and glycerol on β-cell function as only the NZO-AL mice developed type 2 diabetes. In the same way, some cytokines released by adipose tissue (adipokines)

or the liver (hepatokines) can also modulate β-cell function [22,26]. Two major adipokines play a role in type 2 diabetes and islet function: adiponectin, described to exert positive effects on the β-cell, and leptin, which contributes to β-cell failure [29–31]. Plasma adiponectin concentrations were significantly lower in ob/ob mice in comparison to both NZO groups. This is consistent with previous reports indicating that ob/ob mice which are leptin-deficient have low plasma adiponectin levels [32]. According to their reduced fat mass, NZO-IF animals displayed significantly lower levels of plasma leptin in comparison to their NZO-AL littermates, which is in line with their improvement in glucose homeostasis.

We next asked the question whether IF, which improved glucose homeostasis and reduced ectopic fat accumulation, is sufficient to recover islet function. To this end, we examined the insulin secretion of freshly isolated islets, which should still carry the memory of the organ/body environment. Ob/ob islets secreted more insulin than NZO-IF islets during the first phase, but insulin secretion was comparable in the second phase in both groups. In contrast, islets from NZO-AL mice did not reduce their insulin secretion after the first phase and after lowering the glucose concentration as it was detected for ob/ob islets. Thus, NZO islets show a hypersecretion which could result in a later secretory decline (due to exhaustion) and diabetes development as it was recently shown for children with high circulating NEFA [33]. Another recent study showed that lowering endogenous FA levels in β-cell medium, immediately reduced their insulin secretion [34]. Thus, we speculate that islets of NZO-IF and ob/ob mice, which have lower NEFA plasma levels and less fat in the pancreas than NZO-AL mice, show a lower NEFA exposition and a lower insulin secretion.

In fact, we demonstrated the direct influence of adipocytes on islet function. The co-culture of islets and adipocytes has not been intensively used so far. We compared the insulin secretion of islets co-cultured either with pancreas-derived adipocytes or iWAT-derived adipocytes. Under both conditions, insulin secretion was markedly elevated, especially in NZO islets. An elevated insulin secretion and the lack of a potentiation of insulin secretion in response to a glucose challenge were also visible in islets of B6 mice which were either co-cultured with 3T3-L1 adipocytes or pancreatic adipocytes. This observation demonstrates the negative crosstalk of adipocytes to β-cells which appears to be mediated via NEFA.

Up to now we do not know the differences between adipocytes derived from pancreatic precursor cells and cells from iWAT, but our actual data show that pancreatic adipocytes release more NEFA than iWAT adipocytes. It is well known that NEFA increase insulin secretion [35].

The power of our study is that we clearly show the different impact of ectopic fat in liver and pancreas on glucose homeostasis. Our results are also unique because APCs from pancreas have never been isolated and analyzed before. The limitation is the lack of intensive comparison of pancreatic adipocytes with those of other depots. Further investigations will be necessary to deeply elucidate the specific role of pancreatic adipocytes on islet physiology and diabetes risk.

5. Conclusion

In conclusion, we found that diabetes-susceptible and diabetes-resistant mice differ markedly in their pancreas fat content, that adipocytes induce hypersecretion of insulin when co-cultured with islets and that intermittent fasting is an effective and non-invasive way to prevent fat accumulation in the pancreas and to improve glucose tolerance and insulin sensitivity.

Author contributions

C.Q., A.S., T.J.S., and C.B. conceived and designed the experiments. C.Q., N.D., A.G., M.S., F.G., and A.H. contributed to the acquisition and analysis of data. C.Q. and A.S. drafted and wrote the manuscript. M.S., F.G., and T.J.S. were responsible for discussions and critical revision of the manuscript. M.S. and A.S. edited the manuscript. All authors contributed to the interpretation of data and approved its final version for publication. A.S. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Declaration of Competing Interest

All authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.metabol.2019.05.005.

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