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Gastric bypass improves ß-cell function and increases β-cell mass in a porcine model

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Abstract

The most frequently used, and effective, treatment for morbid obesity is Roux-en-Y gastric bypass surgery (RYGB), which results in rapid remission of T2D in most cases. To what extent this is accounted for by weight loss or other factors remains elusive. To gain insight into these mechanisms, we investigated the effects of RYGB on ß-cell function and ß-cell mass in the pig, a species highly reminiscent of the human. RYGB was performed using linear staplers during open surgery. Sham-operated pigs were used as controls. Both groups were fed a low calorie diet for 3 weeks after surgery. Intravenous glucose-tolerance tests were performed 2 weeks after surgery. Body weight in RYGB-pigs and sham-operated, pair-fed control pigs developed similarly. RYGB-pigs displayed improved glycaemic control, which was attributed to increases in ß-cell mass, islet number and number of extra-islet ß-cells. Pancreatic expression of insulin and glucagon was elevated, and cells expressing the GLP-1-receptor were more abundant in RYGB-pigs. Our data from a pig model of RYGB emphasize the key role of improved ß-cell function and ß-cell mass to explain the improved glucose tolerance after RYGB as food intake and body weight remained identical.

Introduction

Roux-en-Y gastric bypass (RYGB) leads to remission of type 2 diabetes (T2D) in most patients within days after surgery (1). Importantly, this occurs long before any substantial weight loss has occurred (2). The reason for this remains a controversy, as studies have shown that the beneficial effects of GBP on T2D are weight loss-independent (e.g.(3)) and others suggest that they result from reduced food intake (4). Clinical studies have shown that RYGB has greater effect on remission of T2D than for example vertical sleeve gastrectomy, despite similar weight loss (5). This is in support of weight-independent factors underlying the resolution of T2D upon RYGB. One factor, accounting for the beneficial metabolic effects of GBP, may be changes in circulating levels of gut hormones and their effects on the islets. In particular, increased levels of the incretin hormone glucagon-like peptide 1 (GLP-1) have been implicated as a factor contributing to remission of T2D (2; 6). The effect of RYGB on glucose-dependent insulinotropic peptide (GIP) is less clear (6; 7). Other factors, including gut microbiota (8), intestinal glucose sensing (9) and bile acids (10), may also contribute. Nevertheless, it is of great clinical importance to resolve this issue, as it will have a strong impact on how treatment for a large group of patients is devised. In fact, if a specific mechanism were to be identified, it could be used as the basis for a new treatment modality.

One problem in the dissection of effects of RYGB on glucose metabolism is that studies on ß-cell mass in humans are lacking and it is extremely difficult to generalize from data in rodents (11) because of huge differences in pancreatic anatomy and physiology between the species. To circumvent these problems we developed a porcine model of RYGB, the results of which we present in the current paper.

Research Design and Methods

Animals. Castrated male pigs (Swedish Landrace x Yorkshire x Hampshire; Swedish Agricultural University, Lund, Sweden) (26±2.5 kg) were kept in individual pens with feeding trough, drinking nipple and heating lamp. Pens were cleaned daily. Pigs were habituated for one week prior to the study to avoid stress during tests.

Surgery.

a) *Roux-en*-Y *gastric bypass.* Pigs (n=4) were operated through an upper midline incision under general halothane anaesthesia. The gastric pouch (12-15 ml) was constructed using linear staplers (GIA80, blue cartridges, Covidien, Mansfield, MA). The stomach was divided horizontally, 3 cm from the gastro-esophageal transition (4 cm staple length). With a second stapler the stomach was vertically completely divided ending close to esophagus. The small intestine (total length approximately 500 cm) was followed from caecum and proximally to the duodeno-jejunal transition. Sixty centimeters from the duodeno-jejunal junction, the intestine was divided using a GIA-staple device as above and a hand-sewn side-to-side anastomosis using continuous 4-0 monofilament absorbable suture was made 150 cm further distally. The jejunal end of the Roux-limb (alimentary limb) was brought up and anastomosed to the lowest part of the gastric pouch by a linear stapler and completed by continuous 4-0 monofilament absorbable suture.

b) *Sham-operation*. Pigs (n=6) were operated through an upper midline incision under general halothane anaesthesia. The bowel was gently manipulated but not transected. The

pigs were kept under anaesthesia for the same time as the average RYGB-operation (70 minutes).

c) *Post-surgical management.* Pigs were closely monitored and treated prophylactically with ampicillin (Doktacillin, 15 mg*kg^{-1}) and buprenorphine (Temgesic 0.15 mg) during and three days after surgery. After surgery, all pigs were given three meals per day (at 0800, 1300 and 1800) of low-calorie diet (250 ml ModiFast, Stocksund, Sweden; 220 kcal; 25E% protein, 52E% carbohydrates and 21E% fat (6).

Intravenous glucose tolerance test (IVGTT). Prior to surgery jugular vein catheters were implanted under halothane anaesthesia. IVGTTs were performed two weeks post-operatively. After an overnight fast, basal blood samples were drawn at -10 and -5 minutes. Pigs were then given a glucose bolus (500 mg*ml^{-1}) at a dose of 1 g*kg^{-1} bodyweight through a jugular vein catheter. Blood was collected at indicated time points after glucose administration.

Blood sampling and storage. Blood was collected into chilled EDTA-tubes. Tubes were kept on ice until centrifugation (1500*g, 15 minutes, 4°C). Plasma was stored at -80°C until analysis.

Plasma analyses. Glucose was analyzed, using the Infinity Glucose Oxidase kit (ThermoScientific, Lexington, MA) and insulin, using a porcine ELISA (Mercodia, Uppsala, Sweden) according to the manufacturers' instructions.

Tissue handling, immunohistochemistry (IHC) and antibodies. Tissues were collected at sacrifice 20 days post-operatively and processed as previously described (12). Antibodies are presented in Table 1.

Morphometry. Immunofluorescence was examined in an epifluorescence microscope (Olympus BX60, Olympus, Tokyo, Japan). For ß-cell mass quantification, all islets in 9 sections from head, body and tail of the pancreas were analyzed. Insulin-stained area and section area were calculated using BioPix software (Gothenburg, Sweden). For each pig, 80±11 islets were analyzed. ß-cell mass was expressed as the ratio of insulin-stained area to section area. Extra-islet ß-cells density was expressed as cell number per section area. Sections were randomly selected and the identity of specimens was unknown to the observer. In addition, density of immunoreactive cells was quantified on coded slides in five randomly selected visual fields (0.63 mm^2 visual field) in each of three randomly selected sections.

Quantitative real-time PCR. Pancreatic RNA was extracted using Nucleo Spin RNA II (Macherey Nagel, Bethlehem, PA). cDNA was generated using RevertAid First Strand cDNA Synthesis kit (ThermoScientific, Waltham, MA). Real-time PCR was run on a Viia7 (Applied Biosystems, San Francisco, CA) using TaqMan® assays (insulin, Ss03386682_u1; glucagon, Ss03384069_u1; HPRT1, Ss03388275_g1). 25 ng cDNA was run under the following conditions: 1 cycle of 50°C for 2 minutes and 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. mRNA

expression was calculated using the $2^{-\Delta\Delta(Ct)}$-formula and expressed as arbitrary units in relation to the reference gene HPRT1.

Statistics. Data are presented as mean±SEM. Unpaired two-tailed Student´s t-test or two-way ANOVA was used to calculate statistical significant differences. $P<0.05$ was considered statistically significant.

Results

Body weight and food intake. RYGB and sham-surgeries were successfully conducted. Both groups displayed similar body weight development after surgery. A small reduction in body weight gain in RYGB-pigs was evident at one time-point, 14 days post surgery (3% loss versus 5% gain, in RYGB- and sham-pigs, respectively, $p<0.05$) (Suppl Fig 1).

Intravenous glucose tolerance test. IVGTT was performed to assess ß-cell function. RYGB-pigs responded with an attenuated rise (40%, $p<0.001$) in glucose levels (Figure 1A). Both basal and 2-h levels of glucose were lower in RYGB-pigs (Figure 1A). This was paralleled by a more sustained insulin response: RYGB-pigs had 2-fold higher peak values than the sham-pigs (Figure 1B). HOMA-ß index trended towards improved ß-cell function in RYGB-pigs ($p<0.0516$; Figure 1C). Acute insulin response (AIR) trended towards an increase in RYGB-pigs ($p=0.09$; Figure 1D). The $AUC_{glucose}$ was lower ($p<0.05$) in RYGB-pigs while the increase in $AUC_{insulin}$ did not reach statistical significance ($p=0.068$). However, the $AUC_{insulin}/AUC_{glucose}$-ratio, reflecting total insulin

secretion corrected for glucose during the IVGTT, was significantly higher in RYGB-pigs ($p<0.05$; Figure 1E).

Islet morphology. To examine whether increased ß-cell mass could explain enhanced insulin release in RYGB-pigs, we performed morphometric analyses of the pancreas. Indeed, this revealed a doubling of ß-cell mass in GBP-pigs ($p<0.05$; Figure 2A). There was a trend towards larger islet size ($p=0.07$; Figure 2B). Determination of islet number revealed that GBP-pigs possessed 1.9-fold more islets ($p<0.05$; Figure 2C). As an indication of increased islet neogenesis (13), RYGB-pigs displayed a higher frequency of extra-islet ß-cells ($p<0.05$; Figure 2D). In agreement with increased ß-cell mass, the number of insulin-immunoreactive cells per pancreas area was 1.8-fold ($p<0.05$; Figure 3A) greater in RYGB-pigs. The number of glucagon-immunoreactive cells per pancreas area was 1.5-fold ($p<0.05$; Figure 3B) greater in RYGB-pigs. These changes in cell density were reflected by trends towards increased expression of pancreatic insulin mRNA ($p=0.069$; Figure 3C) and glucagon mRNA ($p=0.063$; Figure 3D) in RYGB-pigs. Given the putative role of incretins in the metabolic effects of RYGB, we also assessed pancreatic expression of the receptors for GIP (GIP-R) and GLP-1 (GLP-1R). Indeed, the density of cells immunoreactive for GLP-1R was 3.8-fold higher ($p<0.01$) in RYGB-pigs (Figure 3F), while the number of GIP-R-immunoreactive cells was similar in both groups of pigs (Figure 3E).

Discussion

RYGB frequently results in improved glycaemia in T2D patients (14). In fact, most patients with T2D that undergo RYGB experience remission from the disease before significant weight loss occurs (2; 14). The mechanism underlying this rapid remission remains unknown. A body of evidence shows increased insulin secretion and levels of GLP-1 after RYGB (15). However, it is not known how ß-cell mass is affected by RYGB. Here, we show that RYGB provokes improved ß-cell function, as well as increased ß-cell mass in the pig.

Human studies report improved ß-cell function after RYGB in T2D patients and non-diabetic controls (15; 16). Studies aiming to uncover the mechanism have been performed in rodents (17). However, due to the pronounced differences in food composition, GI-tract and pancreas physiology, these results may not be easily translated into the human. Since pancreatic anatomy in pigs resembles the human more than the rodent (18), a porcine RYGB-model was developed. A potential limitation of the study was that although RYGB provokes massive weight loss in morbidly obese humans (1) and in obese rodent models (17), RYGB-pigs displayed similar body weight development as the sham-pigs. Whether this is related to the use of non-obese, non-diabetic pigs or if it is due to species differences remains to be elucidated, but weight loss has been reported to be a function of preoperative excess weight in humans (19). Although the physiology of glucose homeostasis is less well characterized in pigs compared to, e.g., rodent models, RYGB improved glycaemic control in the pigs even though they were neither glucose-intolerant nor diabetic. We found lower fasting and 2-h glucose levels, as well as increased insulin secretion during an IVGTT. This can most likely be attributed to enhanced ß-cell function. In line with these data, RYGB-pigs displayed an increased ratio

of $AUC_{insulin}/AUC_{glucose}$ and a trend towards higher HOMA-β. Thus, the present data are in line with previous reports on the effect of RYGB on glucose homeostasis and insulin secretion in humans (2; 3; 14), but also demonstrate that this improvement in glucose metabolism is associated with an increased functional ß-cell mass.

A key finding was that RYGB-pigs displayed greater ß-cell mass than sham-operated, pair-fed control pigs. This was due to larger islet size and increased number of islets. As an indication of increased islet neogenesis, RYGB-pigs had more extra-islet ß-cells than control-pigs. Occurrence of such cells are, at least in rodents, suggested to be associated with increased islet number and neogenesis (13). Increased ß-cell mass is a novel finding and in line with the long-term effects of duodenal-jejunal bypass (DJB) in GK-rats reported by Speck *et al.* (20). Although it has been reported that RYGB increases the incidence of nesidioblastosis (21), attempts to study the effects of bariatric surgery on ß-cell mass in humans are few and provide conflicting results. Inabnet *et al.* report that DJB and vertical sleeve gastrectomy result in increased number of ß-cells in humans 90 days after surgery, as assessed by PET-scanning and the vesicular monoamine transporter type 2 (VMAT)-index (22). In contrast, Meier *et al.* found ß-cell formation to be unaffected by RYGB (23).

Although regulation of gut hormones is less well studied in pigs vs. rodents, we found more GLP-1R-immunoreactive cells in the islets of RYGB-pigs, possibly resulting in increased incretin sensitivity in the islets after RYGB. Indeed, GLP-1, and GLP-1 receptor agonists exert anti-apoptotic effects in ß-cells (24; 25), a mechanism possibly underlying the beneficial effects of RYGB on ß-cell mass observed in the present study.

Overall, our data suggest that RYGB has beneficial effects on ß-cell function and ß-cell mass, an effect which cannot be explained by reduced food intake and weight loss. The pig appears to be an animal model well suited for mechanistic studies on the effects of RYGB.

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Author contribution: A.L. conducted the study, performed experiments and wrote the manuscript; P.S. co-wrote the manuscript; M.E. performed the surgeries and edited the manuscript; E.G.V. edited the manuscript; S.P. provided veterinarian advice; M.G. co-wrote and edited the manuscript; H.M. wrote the manuscript; J.H. performed the surgeries and edited the manuscript, L.G. wrote the manuscript; N.W. conceptualized the study and wrote the manuscript. All authors participated in finalizing the manuscript. Dr. Nils Wierup, is the guarantor of this manuscript and as such has had full access to all data

and takes responsibility for the integrity of the data and the accuracy of the data analysis.

The authors have nothing to disclose.

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Figure legends:

Figure 1. Glucose and insulin levels after RYGB and sham-surgery. RYGB-pigs had lower fasting and 2h glucose, and a 40% lower response in glucose, during an IVGTT compared to sham-pigs (**A**). In line with this, RYGB-pigs had higher insulin levels than sham-pigs (**B**). The HOMA-ß index trended towards improved ß-cell function (p=0.0516) (**C**). Also, the acute insulin response (AIR) trended towards an increase in the RYGB-pigs (p=0.09) (**D**). The AUC-ratio between insulin and glucose was higher in RYGB-pigs compared to sham-pigs (**E**). *, p<0.05; **, p<0.01; ***, p<0.001.

Figure 2. Pancreas morphology in RYGB-pigs and sham-pigs. RYGB-pigs possessed 2-fold higher ß-cell mass than sham-pigs (**A**). RYGB-pigs trended towards increased mean islet size (**B**). The number of islets per total area was higher in the RYGB-pigs (**C**) and RYGB-pigs had higher density of extra-islet ß-cells (**D**). *, p<0.05.

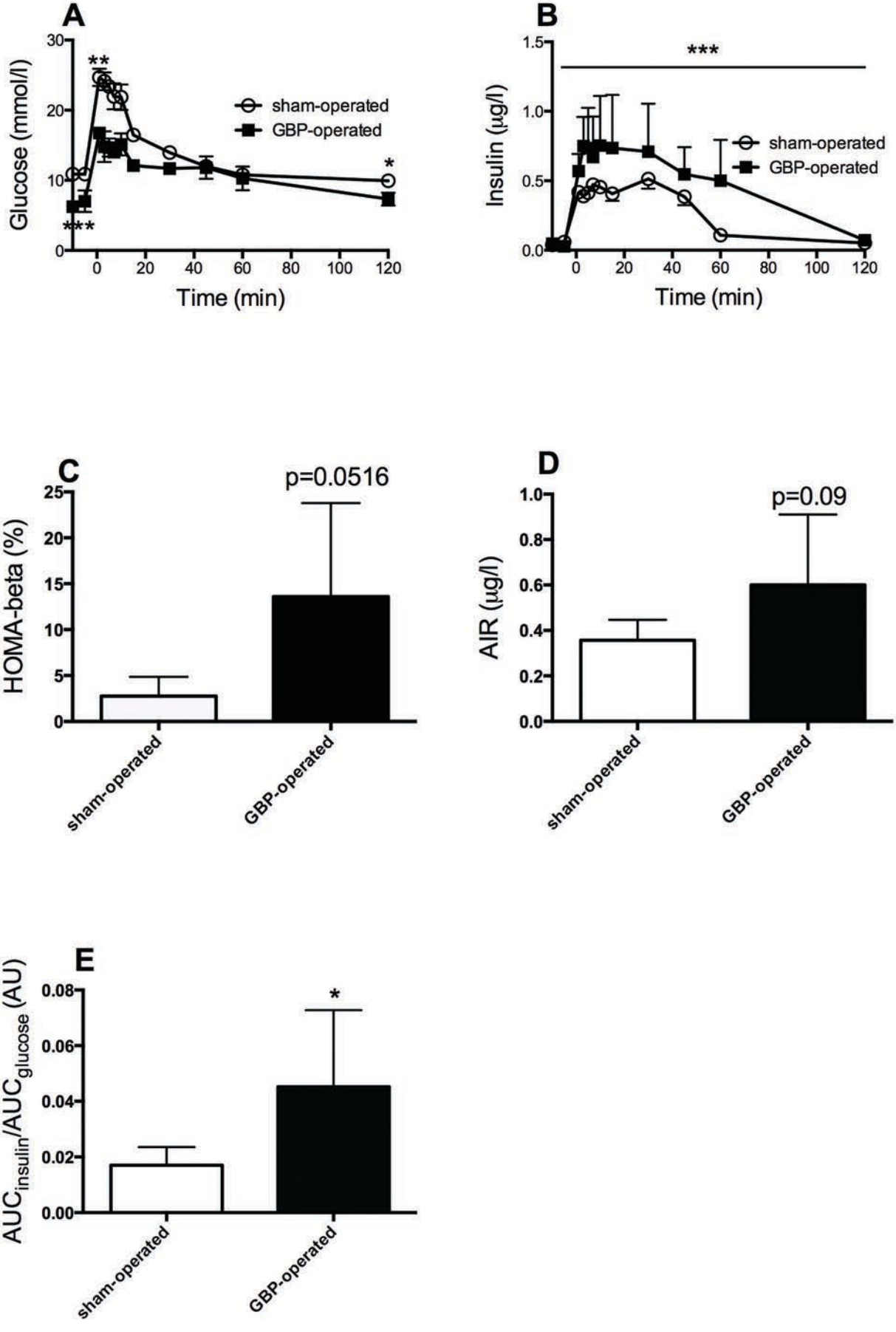
Figure 3. Protein and mRNA expression in RYGB-pigs and sham-pigs. The density of insulin-producing ß-cells **(A)** and glucagon-producing α-cells **(B)** was increased in RYGB-pigs compared to sham-pigs. Trends towards increased insulin mRNA expression (**C**; p=0.069) and glucagon mRNA expression (**D**; p=0.063) were observed in the RYGB-pigs. Density of GIP-1R **(E)** was unaltered whereas density of GLP-1R was increased **(F)** in RYGB-pigs compared to sham-pigs. *, p<0.05; **, p<0.01; ***, p<0.001.

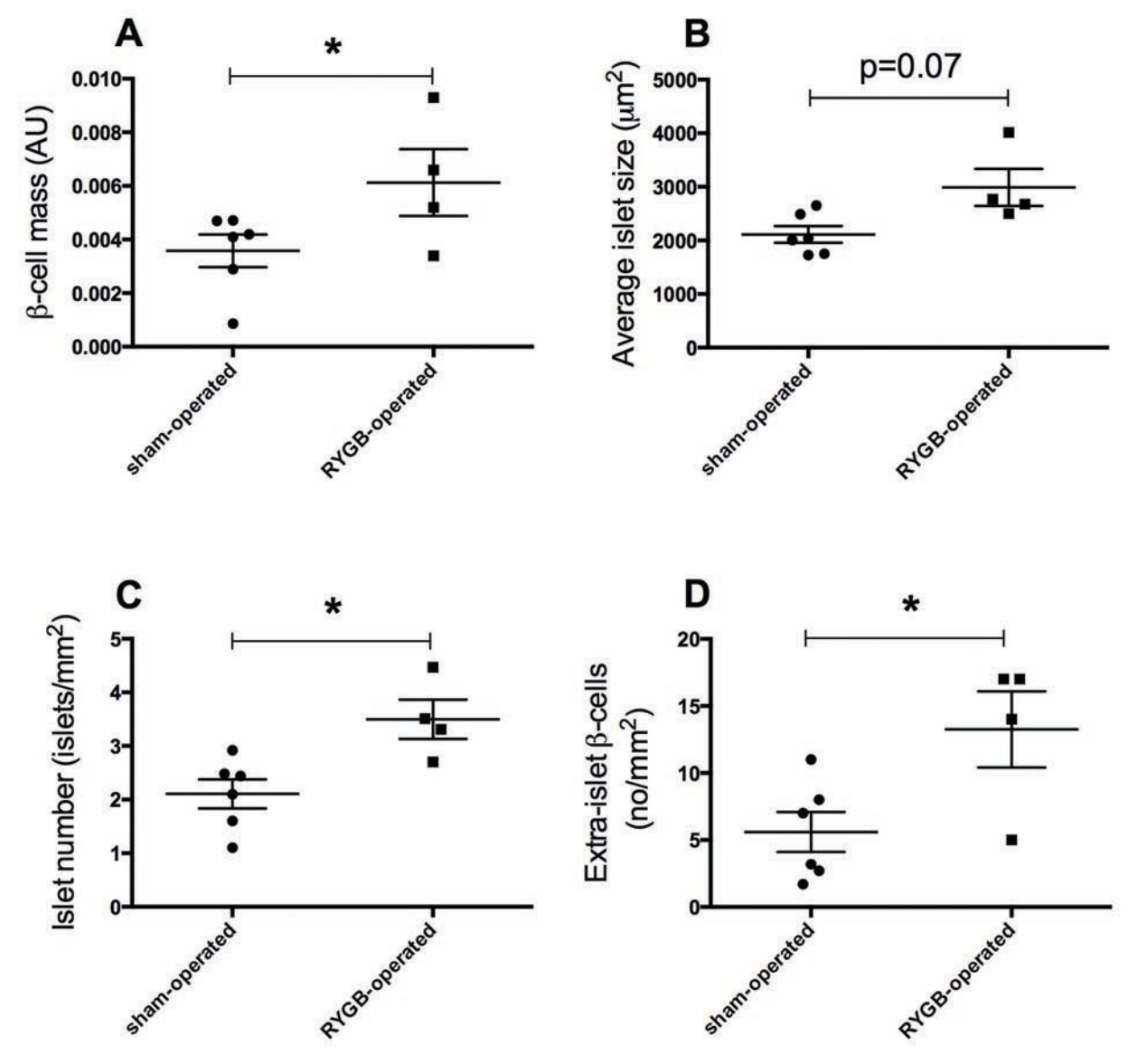
Supplementary Figure 1. Body weight development in the two groups of pigs remained identical except for one time point, where a small reduction in body weight gain was observed in RYGB-pigs compared to sham-pigs. *, $p<0.05$.

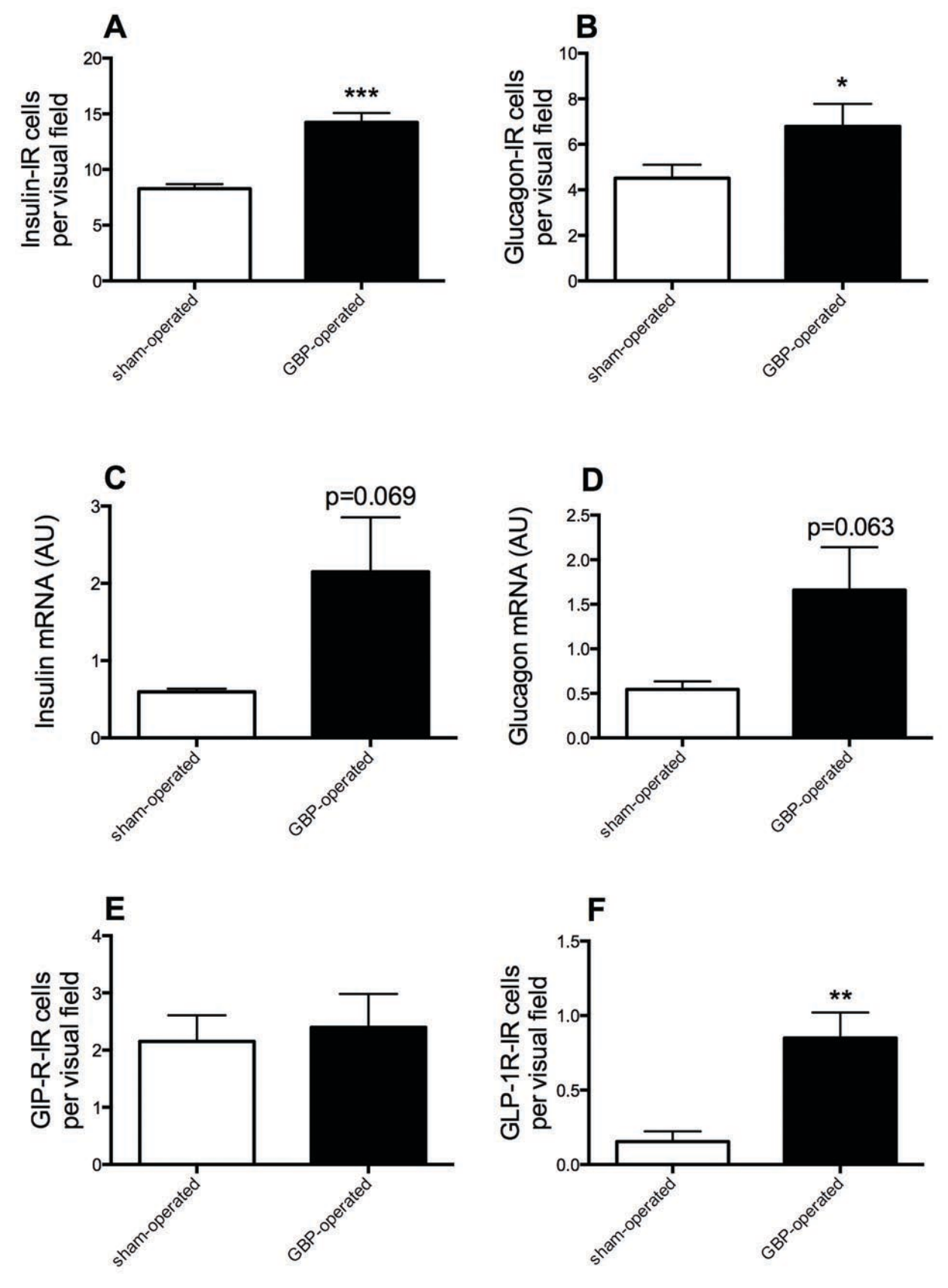
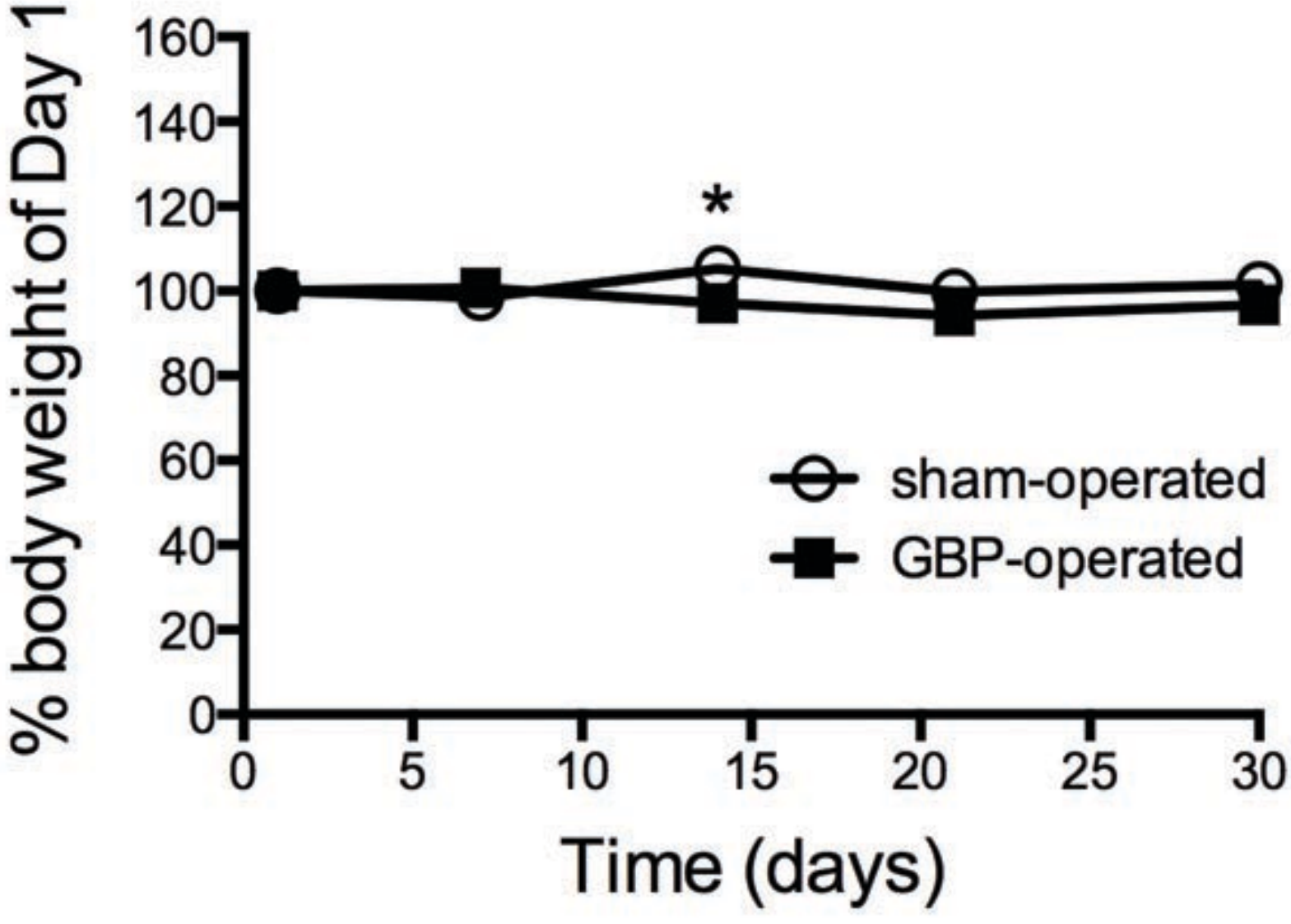
Table 1. Details of antisera used in the immunohistochemical examination of the pancreas.

Hormone	Code	Dilution	Source
GIP-R	RbαGIPr551#4	1:400	Kind gift from Dr. T Kieffer (Vancouver, Canada)
GLP-1R	156/30	1:200	Kind gift from Dr. S Mojsov (New York, NY; USA)
Glucagon	7811	1:10000	EuroDiagnostica (Malmö, Sweden)
Insulin	M9003	1:5000	EuroDiagnostica

Goat anti-rabbit Cy2 was used as secondary antibody for all antisera except for insulin for which donkey anti-guinea pig Cy2 was used.


A
Glucose (mmol/l)
Time (min)
sham-operated
GBP-operated
B
Insulin (µg/l)
Time (min)
sham-operated
GBP-operated
C
HOMA-beta (%)
p=0.0516
sham-operated
GBP-operated
D
AIR (µg/l)
p=0.09
sham-operated
GBP-operated
E
AUCinsulin/AUCglucose (AU)
sham-operated
GBP-operated


A
*
β-cell mass (AU)
0.010
0.008
0.006
0.004
0.002
0.000
sham-operated
RYGB-operated
B
p=0.07
Average islet size (μm²)
5000
4000
3000
2000
1000
0
sham-operated
RYGB-operated
C
*
Islet number (islets/mm²)
5
4
3
2
1
0
sham-operated
RYGB-operated
D
*
Extra-islet β-cells (no/mm²)
20
15
10
5
0
sham-operated
RYGB-operated


A
Insulin-IR cells per visual field
20
15
10
5
0
***
sham-operated
GBP-operated
B
Glucagon-IR cells per visual field
10
8
6
4
2
0
*
sham-operated
GBP-operated
C
Insulin mRNA (AU)
3
2
1
0
p=0.069
sham-operated
GBP-operated
D
Glucagon mRNA (AU)
2.5
2.0
1.5
1.0
0.5
0.0
p=0.063
sham-operated
GBP-operated
E
GIP-R-IR cells per visual field
4
3
2
1
0
sham-operated
GBP-operated
F
GLP-1R-IR cells per visual field
1.5
1.0
0.5
0.0
**
sham-operated
GBP-operated

Supplemental Figure 1