Intestinal adaptation and Reg gene expression induced by anti-diabetic duodenal-jejunal bypass surgery in Zucker fatty rats

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The anti-diabetic mechanism of bariatric surgery includes specific changes in the secretion of incretins. To identify additional players originating from the gut, we evaluated the effects of duodenal-jejunal bypass (DJB) in morbidly obese Zucker fatty rats. A fast relief of hyperglycemia and hyperinsulinemia was achieved even before a significant weight loss occurred. Fourteen days after DJB, we characterized the changes in intestinal histochemistry in the bypassed duodenum and shortcut jejunum that was re-anastomosed directly to the starting point of the duodenum, and compared to the corresponding regions of sham-operated rats. The bypassed duodenum exhibited mucosal atrophy and apoptosis, and decreased proliferative renewal. In shortcut jejunum, DJB resulted in 40% significantly enlarged intestinal circumference and increased epithelial proliferation, especially in putative transit-amplifying (TA) cells and the crypt. As Reg family proteins promote cell growth and survival, we explored their expression in the intestine. Using immunohistochemistry, Reg1, -3α and -3β were normally expressed in intestinal mucosa. After DJB, the level of Reg1 protein was reduced whereas Reg3α and -3β were not changed in bypassed duodenum. Downstream in shortcut jejunum, the levels of Reg1 and -3β were greatly induced, and especially concentrated in the putative TA cells. Our results revealed significant changes in the integrity and proliferation of the intestinal mucosa as a consequence of DJB, and in cell- and isoform-specific expression of Reg proteins within the replicating mucosal epithelium; and provide evidence indicating that the activation of Reg proteins may contribute to intestinal compensation against increased load and/or to improving insulin sensitivity.
Human and rodent studies have demonstrated that bariatric (metabolic) surgery is a very effective treatment for morbid obesity that causes steady weight loss and ameliorates type 2 diabetes (T2D) by improving insulin secretion and sensitivity (3, 28, 30). The anti-diabetic mechanism has been shown to be independent of weight loss at least in part and may include specific changes in the intestines such as increased secretion of gut hormones glucagon-like peptide-1 (GLP-1), gastric inhibitory peptide (GIP) and peptide YY (PYY) that boost the β-cell function and/or improve insulin actions (32, 33, 37, 45). More recently, jejunal glucose sensing has been shown to be essential for controlling glucose production via a gut-brain-liver neurocircuit (2). While factors such as incretins and PYY have been implicated in the glycemic control, little is known about other molecular players involved in the entero-islet responses to bariatric operations. Reg family proteins are normally expressed in the pancreas and gastrointestinal tract and have been proposed to promote cell proliferation and/or inhibit apoptosis. Reg1 promoted cell replication in the pancreatic islets (13, 41, 43) and gastric and intestinal mucosa; Reg1 deletion resulted in reduced epithelial repair and regeneration upon gastric and intestinal injuries (6, 9, 31, 34). We reported that forced expression of Reg3α accelerate the proliferation of insulin-producing cells (7). Reg3β played an important role in the development and regeneration of motor neurons (14) and stimulated liver regeneration after partial hepatectomy (1, 10, 35). In the pancreas and our recent report, β-cell specific overexpression of Reg3β induced pro-islet gene expression and protected the islet cells (46). Both Reg3α and -3β were also expressed in the gastrointestinal tract including in human (5, 22, 42); however, their functions in the regeneration of intestinal epithelium have not been explored.

In order to investigate intestinal adaptation to bariatric surgery and the involvement of
Reg proteins in the anti-diabetic mechanism, we have studied Zucker fatty rats, which carry mutant leptin receptors and display hyperphagia, obesity, diabetes and/or hypertension (19, 24, 39). In previous reports, these rats responded well to various forms of bariatric surgery, e.g. gastric banding decreased food intake and body weight (11); duodenal-jejunal bypass (DJB) improved glucose tolerance (32); and Roux-en-Y gastric bypass (RYGB) decreased food intake and body weight, improved insulin sensitivity and insulin production (24). We have surgically bypassed a segment of the small intestine, 15 cm, including the entire duodenum and proximal jejunum from food (but maintained normal flow of bibliopancreatic juice) in the DJB group. As a consequence, the bypassed intestinal segment displayed autolysis and atrophy whereas the shortcut jejunum exhibited increased proliferation and enlargement in response to increased food flux. In the meantime, Reg protein expression was significantly upregulated in the shortcut segment and was particularly high in the putative transit-amplifying (TA) cells, indicating that these proteins may function as growth factors to mediate intestinal mucosal proliferation after DJB.

Materials and Methods

DJB surgery on Zucker fatty rats: male Zucker fatty rats, purchased from Charles River, were individually housed and had free access to food and water unless specified otherwise. All animal handling procedures were approved by the Research Institute Animal Care Committee of McGill University Health Centre. Male rats of 3.5-month-old were randomized into sham or DJB groups by body weight, blood glucose and serum insulin levels. As shown in Fig. 1, for DJB at upper panel, rats were anesthetized with isoflurane. The abdominal cavity was exposed through a ventral midline incision, at 0.75 cm below the starting point of the duodenum.
pylori), a suture tightly tied the duodenum, and then an incision was made immediately upstream. Bowel continuity was interrupted at the level of the distal jejunum (15 or 10 cm from the pylori or ligament of Treitz respectively). The distal limb was directly connected to the duodenum incision (duodenal-jejunal anastomosis) and the proximal limb carrying the biliopancreatic juices was reconnected downward to the alimentary limb at a distance of the same length of bypassed duodenum and proximal jejunum from the duodenal-jejunal anastomosis. For sham operation, rats underwent a similar surgical procedure, however all resections were re-anastomosed to maintain the physiologic flow of food through the bowel as showed in the lower panel. Rats were returned to normal feeding 5 days after surgery.

**Post-operation observation of blood glucose, insulin and glucose tolerance**: rats were kept for two months and body weight, blood glucose and serum insulin levels were monitored continuously. Eleven days after DJB surgery, an intraperitoneal glucose tolerance test (IPGTT) was performed with D-glucose at 1 g/kg body weight after 16 h fasting, and blood glucose level was measured from tail vein at 0, 15, 30, 60 and 120 min. The difference between glucose tolerance was calculated by area under the curve. To explore the changes in Reg gene expression and islet function, rats were sacrificed 14 d and 60 d after the surgery, respectively, and segments of intestine and pancreas were removed for immunohistochemistry. The experiment was repeated three times: N=3 for 60 d; N=3 for 14 d; and N=5 for 14 d (3) or 60 d (2).

**Histology and immunohistochemistry**: intestinal segments and pancreas were fixed in 4% paraformaldehyde and embedded in paraffin. Pancreatic and intestinal cross sections were cut at 5 μm thickness. The intestinal circumference and wall thickness were measured based on hematoxylin-eosin (HE) stained histology. To detect apoptotic cells, the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL; Millipore, Billerica, MA) assay
was performed. For immunohistochemistry, the primary antibodies against Ki67 and insulin (Millipore), Reg1 (Dr. H Okamoto, Japan) (21), Reg3α and Reg3β (R&D Systems, Minneapolis, MN), or Reg3δ (INGAP; Dr. M Petropavlovskaya of McGill) (42) were incubated overnight at 4°C, followed by horse peroxidase-conjugated secondary antibodies. The signals were visualized by diaminobenzidine substrate (Vector Labs, Burlingame, CA). To quantify cell proliferation in Fig. 4C, 15 crypts from intestinal sections were randomly selected and the number of Ki67 positive vs. total cells was counted, the % Ki67 positive cells was calculated and averaged in each rat. For dual-labeled immunofluorescence, pancreatic sections were incubated with anti-glucagon (Santa Cruz Biotechnology, Santa Cruz, CA) and Alex594-conjugated secondary antibody (Invitrogen, Carlsbad, CA); followed by anti-insulin and Alexa488-conjugated secondary antibody (Invitrogen). To evaluate the extent of islet fibrosis occurred in obesity and diabetes, pancreatic sections were incubated with 0.1% Direct Red 80 dissolved in aqueous saturated picric acid (Sigma-Aldrich, St. Louis, MO) for 75 min followed by two washes with acidified water. Collagen was visualized as red filaments under light microscope (47). To quantify the difference, 10-15 imaging fields (400 x) containing islet clusters were randomly selected from each pancreas of 3 sham- and 3 DJB-operated rats, the thickness of collagen fibers and the extent of collagen deposition were scored from 1 to 4 with 1 as the least and 4 the worst fibrosis. The average score from each pancreas/rat was considered and tested by student’s t-test. The % area of insulin, glucagon and fibrosis was quantified using Northern Eclipse software as reported (20, 37).

**Data presentation and statistical analysis**: data were presented as mean ± SE and plotted using SigmaPlot (Version 11, Systat Software, Chicago, IL). Student’s t-test and one-way ANOVA followed by Tukey’s test were performed using SigmaPlot with the statistical
Results

DJB caused early relieves of hyperglycemia and hyperinsulinemia before weight loss

We studied bariatric intervention against obesity and diabetes in Zucker fatty rats. Starting from the same body weight before surgery, DJB rats show a tendency of less weight gain 7 d after surgery compared to sham-operated rats; the weight difference became significant from 21 d onward and reached 80 g after 40 d (P < 0.05 by t-test, Fig. 2A). The blood glucose level of DJB rats was decreased from 7 d and maintained at 20-40 mg/dL lower than sham control at each time point (P < 0.05 by ANOVA, Fig. 2B). Almost immediately after the surgery, from 3 d onward, serum insulin level at random fed state was dramatically decreased by 30-50% in DJB rats compared to sham-operated animals (P < 0.05 when the two curves were compared by ANOVA, Fig. 2C). As for glucose tolerance, there was no difference before the surgery (data not shown). Eleven days after the surgery, DJB rats exhibited significantly improved glucose tolerance compared to sham-operated rats (P < 0.01 by t-test on AUC, Fig. 2D); which was reconfirmed again on 47 d (data not shown). We thus have successfully established an anti-diabetic bariatric surgery in obese rats.

DJB caused mucosal atrophy and autolysis in the bypassed duodenum

To evaluate morphologic change in the small intestine affected by DJB, we performed histology 14 d after the surgery. As shown in the cross sections of sham-operated rats in Fig. 3, top-left panel, the dense and tall mucosal villi were mainly composed of enterocytes that were evenly stained by hematoxylin-eosin and include scattered mucus-secreting goblet cells (better
viewed in Fig. 4A left panel, scattered dots in higher magnification). After DJB, top-right panel, the duodenal villi were mostly broken and/or shortened, measured by a 31% significant decrease in intestinal wall thickness and a tendency of decreased intestinal circumference (Table 1).

To further evaluate changes in the rates of cell death and proliferation, we performed immunohistochemistry against TUNEL and Ki67, respectively. As shown in Fig. 4A, left panel, TUNEL positive cells were concentrated at the tips of the mucosal villi, representing active and normal turnover of epithelial cells in sham-operated rats. In DJB rats, right panel, ~1/3 of the mucosal layer was detached from the villi as a result of atrophy or autolysis, resulting in a loss of TUNEL-positive cells. The rate of cell proliferation was assessed by immunostaining of nuclear antigen Ki67 which is present during all active phases of the cell cycle and absent in quiescent state (17). As shown in Fig. 4B, left panel, continuous columns of enterocytes were labeled by Ki67, together with the TUNEL staining at the tip of mucosal villi (Fig 4A left panel), representing normal and active cellular renewal in sham-operated rats. In bypassed duodenum of DJB rats, Fig. 4B right panel, the columns of Ki67-positive cells (marked by red lines) were significantly shortened, with the mucosal villi disorganized. The bypassed duodenum exhibited more quiescent cells (arrow) and interrupted epithelial replication as a consequence of diminished requirement for digestion and absorption.

**DJB induced adaptive mucosal proliferation and enlargement of the shortcut jejunum**

The general morphological feature of the distal jejunum was rather similar to that of the duodenum in sham-operated rats (Fig. 3, left panels), in the tall and dense mucosal villi representing healthy enterocytes. In contrast to bypassed duodenum, however, there were two obvious changes in the shortcut jejunum after DJB, i.e. the intestinal circumference was
increased 40% and the villous folding became more complex, there were horizontal layers of enterocytes (Table 1 and Fig. 3, bottom-right panel). Together they would significantly increase the absorptive surface to accommodate the increased digestive load caused by DJB. TUNEL staining revealed scattered epithelial apoptosis in the mucosa to a similar extent in both DJB and sham-operated rats (data not shown). Virtually all mucosal enterocytes were stained positive for Ki67 in sham-operated rats, again indicating constant and normal cellular renewal, but the putative stem cell population in the crypt was mostly quiescent (arrow in Fig. 4C, left panels). After DJB, the Ki67 positive cells were expanded to include most of the crypt including the putative transit-amplifying (TA) and stem cells, right panels, indicating increased rate of cell replication compared to sham-operated rats. The double arrows pointed to expanded layers of mucosal (including putative stem) cells that were replicating, averaged at 88 ± 9% of total cells for DJB vs. 43 ± 4% for sham-operated (N=3, P<0.01 exemplified by Fig. 4C bottom panels). As a result, DJB rats exhibited higher density of the mucosal villi and enlargement of this portion of the intestine (Fig. 4C right panels and Fig 3 bottom-right panel). The opposite characteristics of bypassed and shortcut intestinal segments in DJB rats, consistent to early report of human jejunoileal bypass for obesity (8), clearly demonstrated an adaption and compensation to altered food flux caused by the surgery.

Decreased level of Reg1 immunostaining in the bypassed duodenum after DJB

Reg family proteins promote cell proliferation and regeneration in a number of tissues and could be involved in DJB-induced intestinal adaptation (7, 38). We next examined possible changes in Reg1, -3α, -3β, and -3δ expression by immunohistochemistry (Fig. 5). The staining of Reg1, -3α, and -3β was detected as brown pigmentation at various levels in the intestinal
mucosa. In the duodenum of sham-operated rats, Reg1 was normally expressed at high level across the mucosal villi, Reg3α protein was expressed at low level, while Reg3β expression was very rarely detected in patches of enterocytes (better viewed in higher magnification in Fig 5D). Whereas in DJB rats, the level of Reg1 expression was reduced, especially from the layer of putative TA cells (arrow in Fig 5A). In an attempt to quantify the decrease, Reg1 protein was barely detectable using Western blot and protein isolated from the paraffin blocks using Qproteome FFPE tissue kit (Qiagen; data not shown) (44). No obvious change in the patterns of either Reg3α or Reg3β staining was observed (Fig 5B to 5D). The reduced Reg1 level correlated with decreased proliferation and increased cell death in the bypassed duodenum (Fig 4), supporting a role of Reg1 as growth factor for intestinal epithelial cells. However, Reg3δ immunoreactivity was not detected in either the duodenum or jejunum, compared to its positive staining in the pancreatic islet α-cells and ductal cells (data not shown) (42). The only other two Reg protein isoforms in rats, Reg3γ and Reg4, were not studied here.

**Increased Reg1, Reg3α and Reg3β levels in the shortcut jejunum after DJB**

In contrast to mucosal atrophy of the bypassed duodenum, the shortcut jejunum displayed adaptive proliferation; opposite changes in the levels of Reg1, -3α and -3β proteins were expected in DJB vs. sham-operated rats. In sham-operated animals, Reg1 was widely expressed across the mucosal layers with relatively high level in the putative TA cells but only sporadically in the crypt (Fig 6A left panels). In DJB rats, Reg1 staining was greatly enhanced with predominant induction in the putative TA cells (arrow, Fig. 6A right panels). Reg3α was expressed in all mucosal cells and the level appeared much higher than in the proximal duodenum in sham-operated rats (Fig 6B vs. Fig 5B left panels). In DJB rats, although the
overall level of Reg3α expression in the shortcut segment did not seem to elevate from sham-operated rats, it was more concentrated in the putative TA cells (arrow in Fig 6B). Reg3β was normally dispersed in a few individual cells in the jejunum of sham-operated rats. In DJB rats, it was visibly induced in the putative TA cells (arrow, Fig. 6C right panels). All three Reg proteins studied exhibited specific induction in the putative TA cells in the shortcut jejunum after DJB. To quantify the overall increase and/or cell-specific induction, Reg1 and Reg3β proteins were barely detectable using Western blot, while the sample variability in total tissue Reg3α level did not support a meaningful conclusion either (data not shown).

Intestinal stem cells are proposed to reside at the base of the crypts of Lieberkuhn; from the bottom to the top, they give rise to the TA cells, which further divide and differentiate into absorptive enterocytes, mucus-secreting goblet and enteroendocrine cells in sham-operated rats (23, 40). Normally, the putative stem cells were not very active in cell cycle progression (largely negative for Ki67 labeling, Fig 4B and 4C left panels by arrow) and did not exhibit Reg protein expression (Figs 5 and 6, below the layers marked by arrows). In the shortcut jejunum after DJB, these cells exhibited clearly increased proliferation by Ki67 staining and sign of expansion (Fig. 4C, right panels by double arrows). Above the stem cell layer, the putative TA cells exhibited increased expression of Reg1, -3α, and -3β genes, shown by the arrows in Fig 6 right panels, indicating possible involvement of these proteins in mucosal proliferation.

**Decreased intra-islet fibrosis in the pancreas after DJB**

As part of the diabetic mechanism, significant islet hyperplasia and fibrosis have been reported in Zucker fatty rats and other T2D models (4, 27, 47). The anti-diabetic gastric bypass and DJB have been proposed to improve pancreatic islet function directly, or as a result of food...
restriction, weight loss and/or improvement of insulin sensitivity (26, 29, 36). We thus evaluated pancreatic islet morphology 2 months after DJB or sham operation (Fig. 7, top panels). Dual-labeled immunofluorescence of insulin and glucagon confirmed islet hyperplasia; however, there was no significant difference in the islet density and the relative ratio of insulin and glucagon staining in DJB vs. sham-operated animals. Islet fibrosis was evident in both sham-operated and DJB rats (arrows in middle panels). Normally collagen was mainly present in the islet capsule as visualized by Sirius Red staining (47). In sham-operated Zucker fatty rats, thick collagen fibers in red were dispersed within the hyperplastic islet clusters, demonstrating intra-islet fibrosis (Fig. 7, bottom-left, marked by arrows). In DJB rats, the extent and thickness of the intra-islet collagen filaments were significantly diminished. In Direct Red 80-stained sections, the area of fibrosis was decreased from $15.3 \pm 0.5\%$ to $7.1 \pm 0.3\%$ ($P<0.02$), average collagen deposition score from $3.0 \pm 0.4$ to $2.1 \pm 0.2$ ($P=0.047$; N=3), confirming improved islet fibrosis (Fig. 7, bottom-right), which was consistent with the reported finding in the non-obese diabetic Goto-Kakizaki (GK) rats 1 yr after the surgery (37). Thus, although there was no obvious change in islet cell mass caused by DJB, diminished fibrosis was evident after 2 mo.

Discussion

In this study we demonstrated significant anti-diabetic effect of DJB in correcting hyperglycemia and hyperinsulinemia in Zucker fatty rats which is in agreement with previously reported findings (11, 24, 32). In response to the surgical rerouting, bypassed duodenum exhibited clear mucosal atrophy associated with decreased proliferation and increased cell death. On the contrary, the shortcut jejunum displayed the opposite changes, e.g. enlarged intestinal circumference, increased mucosal proliferation, expanded proliferating zone and more folded
mucosal villi in compensation to expedited food load. In order to explore putative proliferative roles of the Reg proteins to either intestinal mucosa or pancreatic islets, we detected decreased Reg1 expression in the bypassed duodenal mucosa but significantly increased Reg1, -3α and -3β expression in the shortcut jejunal mucosa, especially in the putative layer of TA cells. The close association of isoform- and segment-specific changes of Reg protein expression with the intestinal adaptation suggests a role of Reg proteins in this process and/or indirectly in the anti-diabetic effects. Our data further indicate that not only Reg1 but also Reg3α and -3β may function as growth factors to promote the proliferation of intestinal mucosa.

DJB diverted food flux from the stomach directly to the distal jejunum and caused adaptive changes in the small intestines. The abolished demand in the segment of duodenum and proximal jejunum for digestion and absorption led to increased apoptosis, decreased proliferation and mucosal atrophy. Morphologically, this segment displayed a thinner intestinal wall and a diminished circumference. In contrast, with increased load of less digested food flux the re-anastomosed distal jejunum increased the ability for digestion and absorption through compensatory hyperplasia in the mucosa. Morphologically, the shortcut jejunum displayed increased villus density, a thicker wall and circumference enlargement. To our knowledge, these morphological characterizations after DJB, supported by specific markers of cellular proliferation and apoptosis have not been reported.

Bariatric surgery is a very effective treatment for morbid obesity that causes steady weight loss and ameliorates T2D by improving insulin secretion and sensitivity (3, 28, 30). At least part of the anti-diabetic effect has been shown to be independent of weight loss and may have to do with increased secretion of GLP-1 and GIP (32, 33, 37, 45). According to the hindgut hypothesis, the expedited delivery of nutrient chime to the distal intestines, and the earlier
presentation of undigested food trigger greater production of GLP-1 and PYY, enhancing physiological signals that improves glucose metabolism (12, 15). Gastric bypass was also found to activate gluconeogenesis in distal small intestine and release increased glucose into the portal vein. A proposed detection system in the portal vein transmits the glucose signal to the brain via afferent nervous system (40), which boosts satiety and an efferent signal to cause decreased glucose production in the liver and improved insulin sensitivity (25, 41). Recent report further established an essential role of jejunal glucose sensing in the control of hepatic glucose production (2). Additional considerations were given to impaired ghrelin secretion, increased serum bile acids level and alternations in additional gut factors, such as the numerous bioactive peptides normally produced in the intestines (16, 18).

The DJB procedure has been indicated to improve glucose homeostasis independent of weight loss and/or food restriction in various animal models. In diabetic Zucker fatty rats, DJB greatly normalized blood glucose level to the level of the nondiabetic controls (33). Even in non-obese diabetic GK rats, DJB ameliorated hyperglycemia, increased post-prandial GLP-1 secretion, and increased the enteroendocrine cells coexpressing GLP-1 and GIP in the distal jejunum shortcut to the stomach. In the long term, DJB increased $\beta$-cell mass and decreased islet fibrosis in GK rats and contributed to a persistent improvement of glucose homeostasis (32, 37).

In our study, DJB-caused improvement in glycemic parameters was consistent with a previous report using similar model (33). At 60 d after DJB, there was no change in the relative ratio of $\alpha$- and $\beta$-cells in the islets of our rats, similar to the findings of a mouse study (45). Nevertheless, there seemed to be decreased collagen deposition in between the hyperplasic islet clusters thus relieved intra-islet fibrosis in the pancreas of DJB rats (Fig 7, bottom panels). It is established that the $\beta$-cell area is inversely correlated with the extent of islet fibrosis, as reported in GK rats.
(37). Similarly in Zucker fatty rats, DJB exerted its beneficial effects on the pancreas by attenuating islet fibrosis, which may aid the preservation of islet mass in the long run beyond 2 months.

Reg proteins have been proposed as growth factors to promote cell replication and regeneration in various tissues including pancreas, liver, motor neurons and gastrointestinal tracts. This study explored whether their expression is affected by the adaptive changes of the small intestines, and the induction of Reg proteins in the shortcut intestines may promote mucosal replication and adaptation. Using immunohistochemistry, Reg1, -3α and -3β were localized to mucosal epithelium at different levels whereas Reg3δ was not detected. Reg1 acted as a growth factor to stimulate islet β-cell regeneration through PI3K/ATF-2/cyclin D1 signaling pathway and prevented the development of diabetes after 90% pancreatectomy in rats (13, 38). In the gastrointestinal tract, Reg1 promoted gastric mucosal proliferation and regeneration after injury (9, 31). Further, data from knockout mice suggested that Reg1 is required for normal cell proliferation and intestinal turnover, and the treatment of recombinant Reg1 inhibited indomethacin-induced injury in the small intestine (6, 34). In non-GI systems, Reg3β was proposed a growth factor mediating ciliary neurotrophic factor (CNTF)-mediated survival signaling in motor neurons (14); transgenic overexpression in the liver accelerated hepatocyte regeneration after partial hepatectomy and protected drug-induced hepatitis; and Reg3β gene deletion delayed liver regeneration and enhanced liver sensitivity to oxidative stress (1, 10, 35).

For another member of the family, Reg3α was reported by us to increase insulinoma cell growth and cellular CDK4/cyclin D1 level (7). In the small intestine, Reg3α and Reg3β were expressed in an age-dependent fashion (5, 22, 42); however, their role in normal intestinal development and the adaptive response to DJB were elusive. Here we showed increased Reg1 and Reg3β
expression in the distal jejunum, which was positively correlated with increased mucosal proliferation. Although the overall level of Reg3α was not increased, its expression seemed to be more inducible in the replicating and putative TA cells after DJB, supporting a functional involvement. The mucosal stem cells are normally located at the crypt base and actively divide to maintain rapid turnover of the epithelial enterocytes. In the shortcut intestine, we provided evidence consistent to increased stem cell replication and differentiation into the epithelium in adaption to increased food load after DJB. The parallel upregualtion of Reg1, -3β, and -3α genes in the putative TA cells suggests that these proteins may play a common role of promoting intestinal mucosal replication and adaptation to DJB-induced changes.

In summary, we demonstrate that anti-diabetic DJB caused distinct changes in the bypassed and re-anastomosed intestinal segments of Zucker fatty rats. The duodenum and proximal jejunum being bypassed underwent autolysis and atrophy; whereas the shortcut distal jejunum exhibited greatly increased mucosal cell proliferation. Several isoforms of Reg proteins were normally expressed by the mucosal cells in both segments but differentially affected by the operation. In the bypassed segment, Reg1 expression was decreased with reduced mucosal replication. In the re-anastomosed intestinal segment, Reg gene expression was markedly enhanced especially in the putative TA cells with concurrent increase in the rate of epithelial proliferation. Reg1 has been proposed to be a growth factor for intestinal cells; based on our observation, Reg3α and -3β showed similar changes consistent for them to be involved in the generation and/or maintenance of intestinal mucosa too. However, whether the induced Reg proteins in the intestinal cells affected islet function as endocrine factors, or intestinal adaptation only, remained to be determined.
Acknowledgements

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Table 1. Changes in intestinal wall thickness and circumference in the bypassed duodenum (A) and shortcut jejunum (B) 14 d after DJB. Mean ± S.E. N=3, NS: not significant. P values were derived from unpaired t-tests.

<table>
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<th>Parameter (mm)</th>
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<th>DJB</th>
<th>P value</th>
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<td>Wall thickness</td>
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<td>0.8 ± 0.1</td>
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<td></td>
<td>B: Jejunum</td>
<td>1.2 ± 0.1</td>
<td>1.3 ± 0.2</td>
<td>NS</td>
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<tr>
<td>Circumference</td>
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<td>B: Jejunum</td>
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Figure legends

Figure 1. Diagram of the duodenal-jejunal bypass (DJB) surgery and the segments of intestines used for histology. The proximal duodenum was tied at 0.75 cm and an incision was made immediately upstream, to allow anastomosis of proximal jejunum (15 cm down the pylori). The distal duodenum/jejunum end was reconnected to a downstream jejunal incision at 30 cm down the pylori. For sham operation, rats underwent a similar surgical procedure, however all resections were re-anastomosed to maintain the physiologic flow of food through the bowel. Histological analysis was made in cross sections of segments 1 (A, duodenum) and 3 (B, jejunum) and presented in Figs 3 to 6.

Figure 2. DJB caused early relieves of hyperglycemia and hyperinsulinemia, followed by weight loss and improved glucose tolerance. A. Change in body weight. *P < 0.05 from 21 d vs. sham-operated rats using t-test. B. Change in blood glucose level measured in fed status. *P < 0.05 vs. sham-operated rats tested by one-way ANOVA. C. Change in serum insulin level measured in fed status. *P < 0.05 vs. sham-operated rats by ANOVA. D. Results of i.p. glucose tolerance test 11 d after DJB surgery. **P < 0.01 vs. sham-operated rats by t-test and area under the curve. Panels A to C were based on two experiments using total 5 sham-operated and 5 DJB rats; panel D was based on two experiments using total 6 sham-operated and 6 DJB rats.

Figure 3. Histological changes of the small intestines after DJB, 14 d after surgery. Hematoxylin-eosin staining showed autolysis and atrophy of bypassed duodenum (segment A, up-right panel) and intestinal distension of shortcut jejunum (segment B, bottom-right) in DJB rats. Each panel was a representative of more than ten images taken from 3 rats. The experiments were done twice. The result of dimensional quantification was summarized in Table 1. The Scale bar was 1 mm.
Figure 4. DJB-induced changes in cellular apoptosis and proliferation in the intestinal mucosa. A. In bypassed duodenum (segment 1 or A), TUNEL staining using diaminobenzidine substrate (brown) was localized at the tip of villi in sham-operated rats but more widely distributed in the broken villi in DJB rats. B. Changes in the pattern of enterocyte proliferation marked by nuclear staining of Ki67. Decreased mucosal proliferation in bypassed duodenum (segment 1 or A) in DJB rats vs. sham-operated, marked by red lines representing the average heights of Ki67-positive columns and especially in putative TA cells marked by arrows. C. Increased mucosal proliferation marked by Ki67 staining especially in the putative TA cells in shortcut jejunum (segment 3 or B), illustrated in 100X (top) and 400X (bottom) magnifications. Double arrows highlight expanded layers of Ki67-positive putative TA and stem cells. Based on two experiments using 3 sham- and 3 DJB-operated rats each time; representative images of more than five in each group were illustrated. The Scale bar was 250 or 60 microns as marked.

Figure 5. Immunohistochemical changes of Reg1, -3α, and -3β in bypassed duodenum 14 d after DJB. A. The level of Reg1 staining was decreased in the bypassed segment, especially in the putative TA cells (arrow) of DJB rats. B. Reg3α staining was weak and not altered in DJB rats. C. Reg3β staining was barely detectable in both DJB and sham-operated rats, in 100X. D. Reg3β staining in 400X showing brown pigmentation in the mucosal cells of both DJB and sham-operated rats. Based on two experiments using 3 sham- and 3 DJB-operated rats each time; representative images of more than five in each group were illustrated. The Scale bar was 250 or 60 microns as marked.

Figure 6. Changes in the immunohistochemistry of Reg1, -3α, and -3β in shortcut jejunum 14 d after DJB. A. The level of Reg1 staining was increased in mucosal epithelium and especially the putative TA cells (arrow) in DJB rats, captured at 100X (top) and 400X.
(bottom) magnifications. **B.** The overall Reg3α staining was not altered in DJB rats, except an enriched expression in the putative TA cells (arrow). **C.** Reg3β staining was barely detectable in sham-operated rats but increased in DJB rats, especially in the putative TA cells marked by arrows. Note the particularly higher expression of all three Reg proteins in putative TA cells, consistent with increased Ki67 expression and cellular proliferation as shown in Fig. 5. Based on two experiments using 3 sham- and 3 DJB-operated rats each time; representative images of more than five in each group were illustrated. The Scale bar was 250 or 60 microns as marked.

**Figure 7. Improved intra-islet fibrosis in the pancreas of DJB rats 2 months after surgery.** Top panels, double immunofluorescence of insulin (green) and glucagon (red) confirmed islet hyperplasia (vs. lean mice, data not shown); however, there was no difference in the relative ratio of insulin and glucagon staining in DJB rats vs. sham-operated rats. Middle panels, immunostaining of insulin using diaminobenzidine substrate (brown) showing evidence of islet fibrosis by arrows. Bottom panels, collagen fibers were visualized by Sirius Red staining (arrow), intra-islet fibrosis was lower in DJB vs. sham-operated rats. The experiments were done twice on total 5 sham-operated and 5 DJB rats. Representative images of more than ten in each group were illustrated. The Scale bars from top down were 125, 125 and 60 microns.
References


Fig. 1
DJB

Stomach
Liver
Pancreas
Duodenum & Proximal Jejunum (15 cm)
Jejunum (15 cm)

Sham

A=1, B=3

Stomach
Liver
Duodenum & Jejunum (30 cm)
Jejunum
Pancreas

Legend:
- biliopancreatic juice
- gastric contents
- anastomosis
Fig. 2A, B

A

Body weight (g)

Sham
DJB

Days after surgery

B

Blood glucose level (mg/dL)

Sham
DJB

Days after surgery

* * *
Fed

Days after surgery

Serum insulin level (ng/ml)

Sham
DJB

GTT 11d

Minutes after glucose injection

Blood glucose level (mg/dL)

Sham
DJB

Fig. 2C, D
Fig. 3 (HE)
Fig. 4A, B (Bypassed duodenum)
C. Ki67

Fig. 4C (Shortcut jejunum)
Fig 5A, B (Bypassed duodenum)
Fig 5C, D (Bypassed duodenum)
A. Reg1

Sham

DJB

Fig 6A (Shortcut jejunum)
B. Reg3α

Fig 6B (Shortcut jejunum)
C. Reg3β

Fig 6C (Shortcut jejunum)
Fig 7 (Pancreas 200, 200, 400X)