

Pancreatic islet regeneration through PDX-1/Notch-1/Ngn3 signaling after gastric bypass surgery in db/db mice

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Abstract. In view of the compelling anti-diabetic effects of gastric bypass surgery (GBS) in the treatment of morbid obesity, it is important to clarify its enhancing effect on pancreatic islets, which is closely linked with diabetes remission in obese patients, as well as the underlying mechanisms. The present study evaluated the effects of GBS on glycemic control and other pancreatic changes in db/db mice. The db/db mice were divided into Control, Sham and GBS group. A significant improvement in fasting plasma glucose levels and glucose intolerance were observed post-surgery. At 4 weeks after surgery, further noteworthy changes were observed in the GBS group, including improved islet structure (revealed by immunohistochemical analysis), enhanced insulin secretion, pancreatic hyperplasia and a marked increase in the ratio of β -cells to non- β endocrine cells. Furthermore, notable changes in the levels of Notch-1, pancreatic and duodenal homeobox 1 (PDX-1) and neurogenin 3 (Ngn3) were observed in the GBS group, indicating a potential role of Notch signaling in pancreatic islet regeneration after surgery. In addition, results obtained in PDX-1 knockout (KO), Notch-1 KO and Ngn3 KO mouse models with GBS suggested that elevated PDX-1 resulted in the inhibition of Notch-1, further facilitated Ngn3 and thus promoted pancreatic β -cell regeneration after GBS. The present findings demonstrated that GBS in db/db mice resulted in pancreatic islet regeneration through the PDX-1/Notch-1/Ngn3 signaling pathway, which also reflected the important role of the gastrointestinal system in metabolism control.

Introduction

Type 2 diabetes mellitus (T2DM) is rapidly becoming an epidemic worldwide and patients with T2DM are increasingly diagnosed as being overweight or obese (1,2). Indeed,

the increase in the prevalence of obesity is frequently accompanied by an elevation in the number of cases of T2DM. Hyperglycemia is a characteristic included in the clinical manifestation of T2DM. Bariatric surgery is an effective approach to achieve sustained and persistent weight loss for the treatment of morbid obesity (3,4). Given the limitation of drug therapy, novel treatment strategies focusing on surgical treatment are most promising for the control of T2DM. Earlier in the 1980s, a notable improvement in T2DM has been reported after Roux-en-Y gastric bypass (5), and subsequent studies confirmed the marked therapeutic effect of gastric bypass on T2DM (6-8). Animal experiments show that gastric bypass improves the function of pancreatic islets and reduces blood glucose in diabetic mice (9-11). One possibility of achieving substantial decrements in blood glucose after gastric bypass in patients with T2DM is regeneration of pancreatic islets (12), but the specific mechanism underlying the hypoglycemic effect of islet regeneration following gastric bypass treatment has remained to be fully elucidated.

Current knowledge on pancreatic development suggests that Notch signaling is required for early embryonic development and the regulation of cell-fate decisions during organogenesis (13-15). Regeneration of the pancreas has been confirmed to occur through activation of Notch signaling in human chronic pancreatitis (16), as well as during acute pancreatitis in the adult murine pancreas (17). Furthermore, during pancreatic carcinogenesis, the tumor-initiating effect of transforming growth factor- α appears to be mediated via Notch signaling through enlarging a cluster of undifferentiated precursor cells (18). However, to the best of our knowledge, no previous study has assessed the mechanism by which Notch signaling mediates islet regeneration following gastric bypass surgery in patients with T2DM. The db/db mouse model of T2DM exhibits the characteristics of obesity, hyperglycemia and insulin resistance associated with human T2DM and was therefore used in the present study (19-21). To elucidate the possible mechanism underlying diabetes remission after gastric bypass surgery in T2DM patients, the present study analyzed the proteins involved in the Notch signaling pathway as well as regeneration of pancreatic islets following gastric bypass surgery in db/db mice.

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Materials and methods

Animals. Twelve-week-old male C57BL/KsJ db/db mice (n=45) and the gene knockout (PDX-1 KO, Notch-1 KO and

Ngn3 KO) C57BL/KsJ db/db mice (n=35) (each weighing 40–50 g) were purchased from the National Rodent Laboratory Animal Center (Shanghai, China). Animals were housed in a well-ventilated holding room at a controlled temperature (24°C) and humidity (55%) under a 12-h light/dark cycle with free access to water and food. The protocol of the present study was approved by the Institutional Research Ethics Committee of Shanghai Eighth People's Hospital (Shanghai, China). Ninety db/db mice were randomly divided into three groups: Control group, Sham group (undergoing sham operation) and GBS group (undergoing gastric bypass surgery).

Surgical procedures. Surgeries were performed according to previous protocols (22). In brief, gastric bypass surgery was performed on overnight fasted mice under continuous isoflurane anesthesia. The body of the stomach in mice has a white line indicating the border between the forestomach and glandular stomach. The stomach was carefully divided into two segments by suture along the white line. Special care was taken at the small curve of the stomach to avoid damaging the left and right gastric arteries and vagus nerve, and to ensure that the upper pouch (white pouch) was continuous with the esophagus. A biliopancreatic limb extending 16 cm from the ligament of Treitz was transected. The distal segment was anastomosed to the gastric remnant and the proximal segment was drained into 12 cm of the Roux limb by side-to-side anastomosis. For Sham-operated animals, the incision was made at a spot 16 cm from the ligament of Treitz. The intestine was then reconnected by side-to-side anastomosis, without intestinal rearrangement, and the incision was closed.

Oral glucose tolerance test (OGTT) and measurement of plasma glucose and insulin. For fasting plasma glucose (FPG), blood was collected through cutting the tail tip in conscious animals after fasting overnight. Samples were centrifuged and plasma glucose was analyzed using the glucose oxidase method (Roche/Hitachi 917; Roche Diagnostics, Mannheim, Germany).

For the OGTT, after 12–14 h of fasting, a glucose load (2 g/kg body weight) was orally administered. Glucose levels were measured in blood obtained by tail bleeding with a glucometer (Roche; Milpitas, CA, USA) at 0, 10, 20, 30, 40, 60, 80, 100 and 120 min after glucose administration. For the measurement of insulin, plasma was evaluated using a radioimmunoassay kit (Diagnostics Products Corp., Los Angeles, CA, USA).

Pancreas weight changes and morphological and immunohistochemical analyses. The mice were euthanized at post-operative 4 weeks. Pancreases were carefully excised, weighed, fixed in 4% paraformaldehyde for 4 h at room temperature, washed, transferred to cassettes and positioned in the same manner. The fixed tissues were dehydrated in ethanol, cleared in xylene and then embedded in paraffin blocks. Sections (5 μ m) were cut sequentially, mounted on polylysine-coated slides, rehydrated and subjected to insulin immunohistochemical staining to observe the histopathological changes. The slides were microwaved for antigen retrieval, and treated for blocking the endogenous peroxidase activity

as described previously (23). The slides were incubated with insulin antibody (12018; 1:1,000 dilution; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) overnight at 4°C, then incubated with FITC-conjugated anti-mouse IgG (F9137; 1:100 dilution; Sigma-Aldrich; Merck KGaA) secondary antibody for 1.5 h at room temperature. The slides were covered with DAPI mounting medium or an aqueous mounting solution.

For determination of the β -cell/non- β endocrine cell ratio, 10 sections (at least 200 μ m apart) were immunostained for insulin and counterstained with DAPI. Sections were examined using a confocal microscope (A1; Nikon, Tokyo, Japan) connected to a computer with NIS elements v4.3 software (Nikon). Images (magnification, x4; spanning the entire tissue for each slide) were acquired using an X-Y motorized microscope with resolution sufficient to identify single β -cells, and 'stitched together' using the MetaMorph software program (v.7; Molecular Devices, Sunnyvale, CA, USA). Insulin staining in each section was calculated by MetaMorph software and then checked manually to remove irrelevant spots or to add β -cells that stained weakly. MetaMorph-quantified total tissue area (based on measurement of DAPI-stained area) and insulin-positive area (based on measurement of glucagon/somatostatin/c-peptide cocktail-positive area) to generate β -cell/non- β endocrine cell ratio.

Western blot analysis. Western blotting was performed as described previously (24), with certain modifications. Total protein was extracted from pancreatic tissues and measured with the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Protein (20 μ g) was separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Merck KGaA, Darmstadt, Germany). After blocking for 1 h with 5% non-fat dry milk in PBS, membranes were incubated with specific primary antibodies including Notch1 (sc-71719; 1:1,000), pancreatic and duodenal homeobox 1 (PDX-1) (sc-390792; 1:800), neurogenin 3 (Ngn3) (sc-13793; 1:100) (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) overnight at 4°C, then incubated with goat anti-rabbit horseradish peroxidase (HRP)-coupled secondary (sc-2030; 1:1,000; Santa Cruz Biotechnology, Inc.) for 1.5 h at room temperature and detected by chemiluminescence (Thermo Fisher Scientific, Inc., Waltham, MA, USA). β -actin was used as a protein loading control.

RNA isolation and reverse-transcription quantitative polymerase chain reaction (RT-qPCR). RT-qPCR was performed as previously described (25), with certain alterations. In brief, total RNA was extracted from cells with TRIzol reagent (Thermo Fisher Scientific, Inc.) and refined using an RNeasy Mini kit (Qiagen, Valencia, CA, USA) in accordance with the manufacturer's protocol. Samples (1 μ g RNA) were reverse-transcribed to cDNA in a first-strand cDNA synthesis reaction with PrimeScript RT-PCR kit (Takara Biotechnology Co., Ltd., Dalian, China), at 37°C for 25 min, then incubated at 85°C for 5 sec in 20 μ l of reaction volume. Synthesized cDNA was amplified by real-time PCR in a Chromo 4 instrument (Bio-Rad Laboratories, Inc.) using the SsoFast™ EvaGreen Supermix (Bio-Rad Laboratories, Inc.) and then analyzed with Opticon Monitor Analysis 2.0 Software (Bio-Rad Laboratories, Inc.). Specificity was determined by electrophoretic analysis

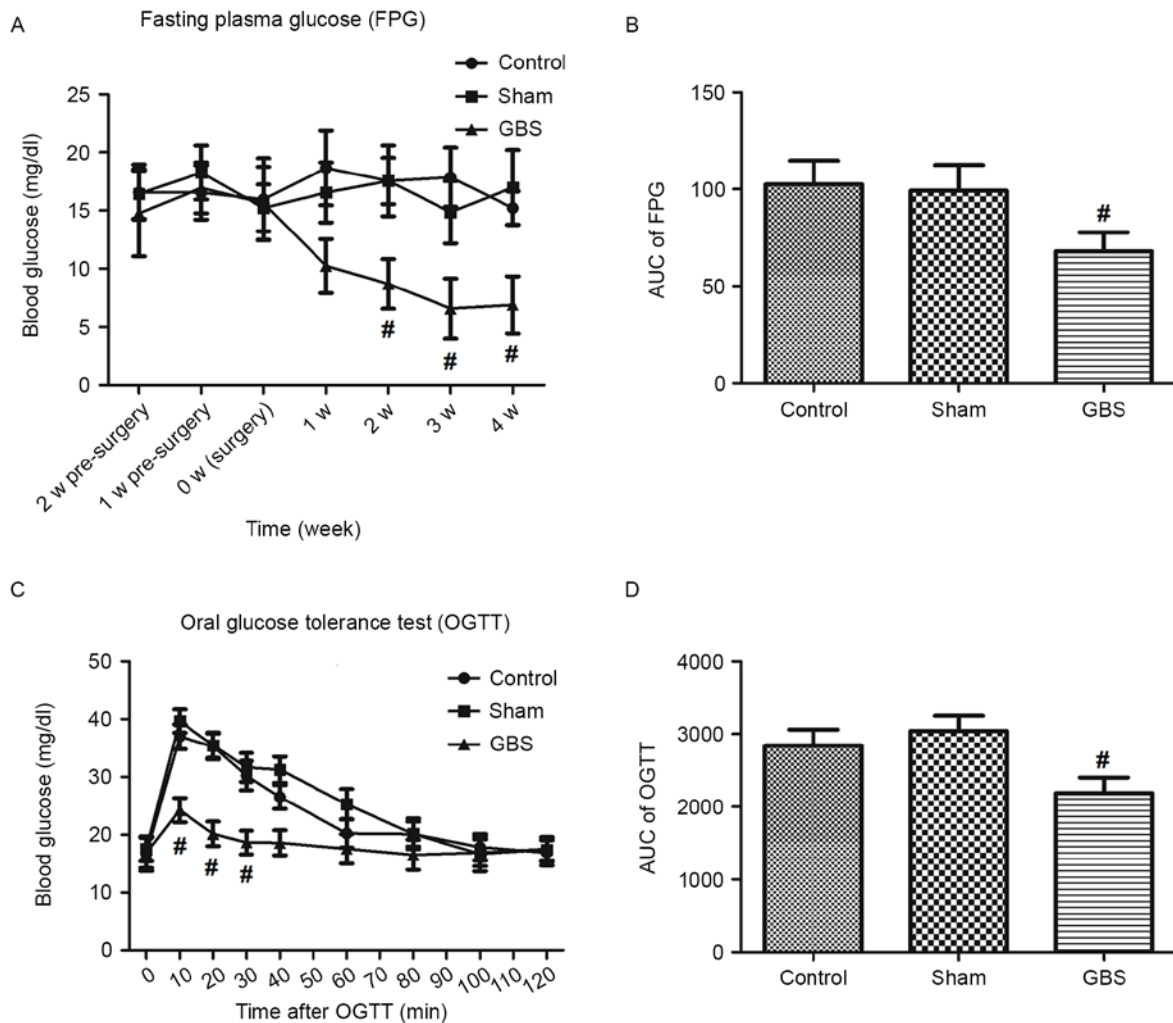


Figure 1. Effects of GPS on glycemic control in db/db mice. (A) FPG in db/db mice was evaluated weekly for two weeks prior to surgery as well as four weeks after surgery. Mice were fasted overnight and blood glucose levels were measured. (B) AUC of FPG. (C) The first week after GPS, OGTT were performed among Control, Sham and GBS groups. Mice were fasted overnight and then orally administered glucose (2 g/kg body weight). Blood glucose levels were measured at the indicated time-points. (D) AUC of OGTT. * $P < 0.05$ vs. control group. FPG, fasting plasma glucose; AUC, area under curve; GBS, gastric bypass surgery; OGTT, oral glucose tolerance tests.

of the reaction products. GAPDH was used as an internal standard. The PCR primers used were as follows: Notch-1, sense GCTACAAGTGCCTGTGTGTC and antisense GTTGGTGTCGAGTTGGAGC; Notch-2, sense CACCTTGAACTGCAGACAT and antisense GGATGACCAAGTCTGTGATG; Notch-3, sense ACTGGACCTCGCTGTGAGAC and antisense GCAGCTGAAGCCATTGACTC; Notch-4, sense GCTATGTGTCTCAGTGGTCA and antisense AAGCTTGGCCTGGCATCTCT; PDX-1, sense GGATGAAGTCTACAAAGCTCACGC and antisense CCAGATCTTGATGTGTCTCTCGGTC; Ngn3, sense CAATCGAATGCACAACTCA and antisense GGGAGACTGGGAGTAGAGG; GAPDH, sense CACTGGCATGGCCTTCCGT and antisense, CTTACTCCTTGGAGGCCAT.

Statistical analysis. Values are expressed as the mean \pm standard error. Statistical analysis was performed with SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). Statistical analysis was performed using analysis of variance when appropriate, followed by an unpaired Student's *t*-test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effects of gastric bypass surgery on metabolic control in db/db mice. The status of FPG in db/db mice was evaluated weekly for two weeks prior to surgery as well as four weeks after surgery. The first two weeks prior to operation, FPG levels did not differ significantly between Control, Sham and GBS group, but FPG levels and the area under the curve (AUC) of FPG in the GBS group was significantly lower than that in the Control and Sham group at the first week after operation (Fig. 1A and B). The first week after operation, OGTTs were performed in the three groups. The blood glucose levels at time 0 did not differ significantly between Control, Sham and GBS groups; however, subsequent to surgery, the blood glucose levels in the GBS group were significantly lower than those in the Control and Sham group at different time-points, but elevated glucose levels in the GBS group at 10 min after the oral glucose gavage were gradually restored to original glucose levels at 120 min (Fig. 1C), indicating glucose intolerance remission after gastric bypass surgery. Simultaneously, in the GBS group, the AUC of glucose according to the OGTT was significantly declined

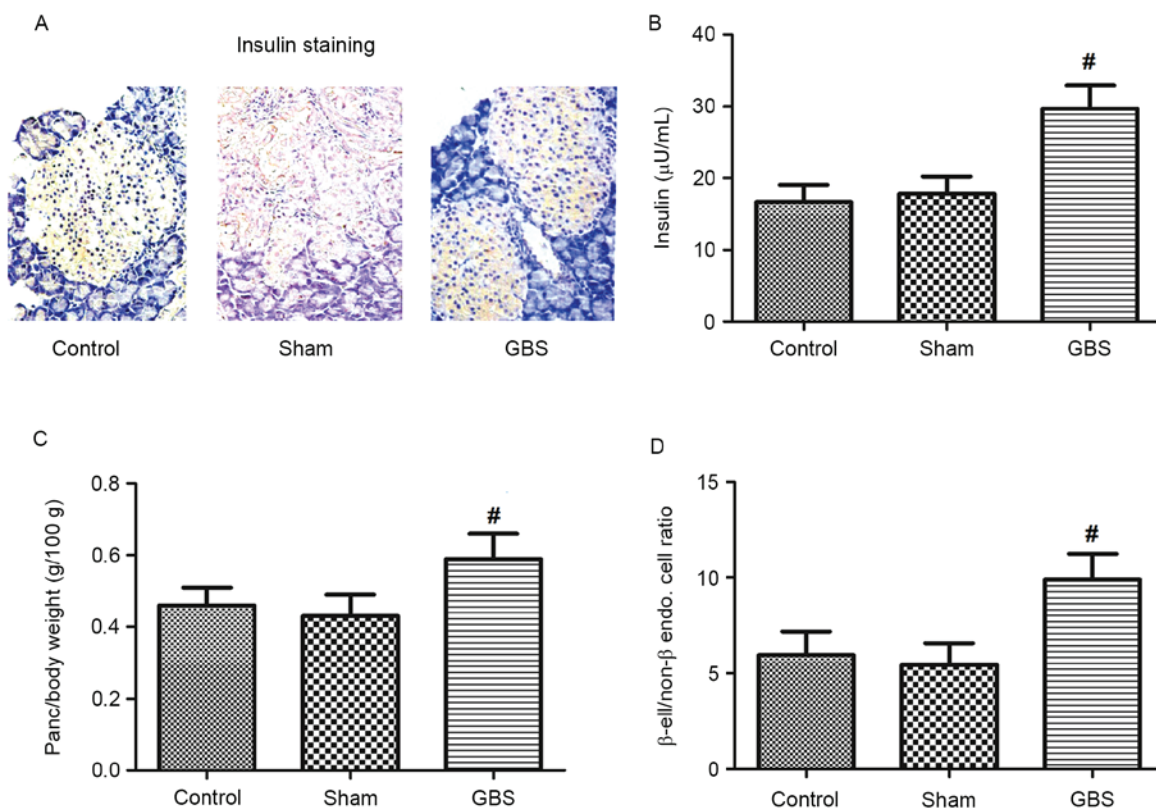


Figure 2. Pancreatic hyperplasia after gastric bypass surgery in db/db mice. (A) Representative microscopy/scanned immunohistochemistry images of islets in three groups stained with insulin (red brown, magnification x400). (B) Insulin secretion. (C) Pancreas weight expressed as g per 100 g body weight. (D) Ratio of insulin-positive β -cells to non- β endocrine cells. [#] $P < 0.05$ vs. control group. GBS, gastric bypass surgery; endo., endocrine.

compared with that in the Control and Sham group (Fig. 1D). These findings indicated that hyperglycemia and impaired glucose tolerance were improved in db/db mice after gastric bypass surgery.

Effects of gastric bypass surgery on islet morphology and β -cell mass. Animals were sacrificed 4 weeks post-surgery. Pancreases were carefully excised, weighed and subjected to insulin immunohistochemical staining to observe any morphological changes. As shown in Fig. 2A, the islet structure in the Control and Sham groups was similar, as was islet cell histopathology: Reduced islets and increased interstitial fibrosis of islets, discrete distribution of β -cell mass due to the destruction of agglomerates formed, swelling deformation of a large amount of β -cells and damage on the formation of vacuoles were observed. In the GBS group, however, islet structure and histopathology were obviously improved: Abundant islets and reduced interstitial fibrosis of islets, a tight cell arrangement with massive β -cells in the central core as well as regeneration and hypertrophy of the β -cell mass were observed. In addition, Control and Sham group exhibited heterogeneous insulin staining relative to that in the GBS group, which was also weak. Meanwhile, insulin secretion in GBS group was significantly higher than that in the Control and Sham groups (Fig. 2B). Pancreas weight expressed as g per 100 g body weight was similar between Control and Sham groups, but was significantly higher in the GBS group (Fig. 2C). Furthermore, the ratio of β -cells to non- β endocrine cells did not differ significantly between Control and Sham

groups, but was notably higher in the GBS group (Fig. 2D). These results suggested that gastric bypass surgery may promote pancreatic islet regeneration.

Effects of gastric bypass surgery on proteins involved in Notch signaling. Based on the known role of Notch signaling in regulating epithelial differentiation in pancreatic development, the levels of proteins involved in Notch signaling were detected among the three groups. As shown in Fig. 3A, compared with the Control and Sham groups, western blot analyses showed that the level of Notch-1 in the GBS group was conspicuously lower, but the levels of Notch-2, Notch-3 and Notch-4 did not obviously differ among the three groups. However, a marked increase in PDX-1 levels and a decrease in Ngn3 levels was observed in the GBS group. The RT-qPCR results further consolidated the results of the western blot analysis (Fig. 3B-G). All of these results indicated a potential role of Notch signaling in regulating pancreatic islet regeneration after gastric bypass surgery in db/db mice.

Mechanism involved in pancreatic islet regeneration after gastric bypass surgery. To confirm the specific mechanism underlying the association between Notch signaling and pancreatic islet regeneration after gastric bypass surgery, the GBS group was divided into four groups: Control group, PDX-1 KO group, Notch-1 KO group and Ngn3 KO group. As shown in Fig. 4, Notch-1 or Ngn3 gene-deficient db/db mice did not exhibit any significant changes in the level of PDX-1. Notch-1 was significantly higher in the PDX-1 KO group, but was not

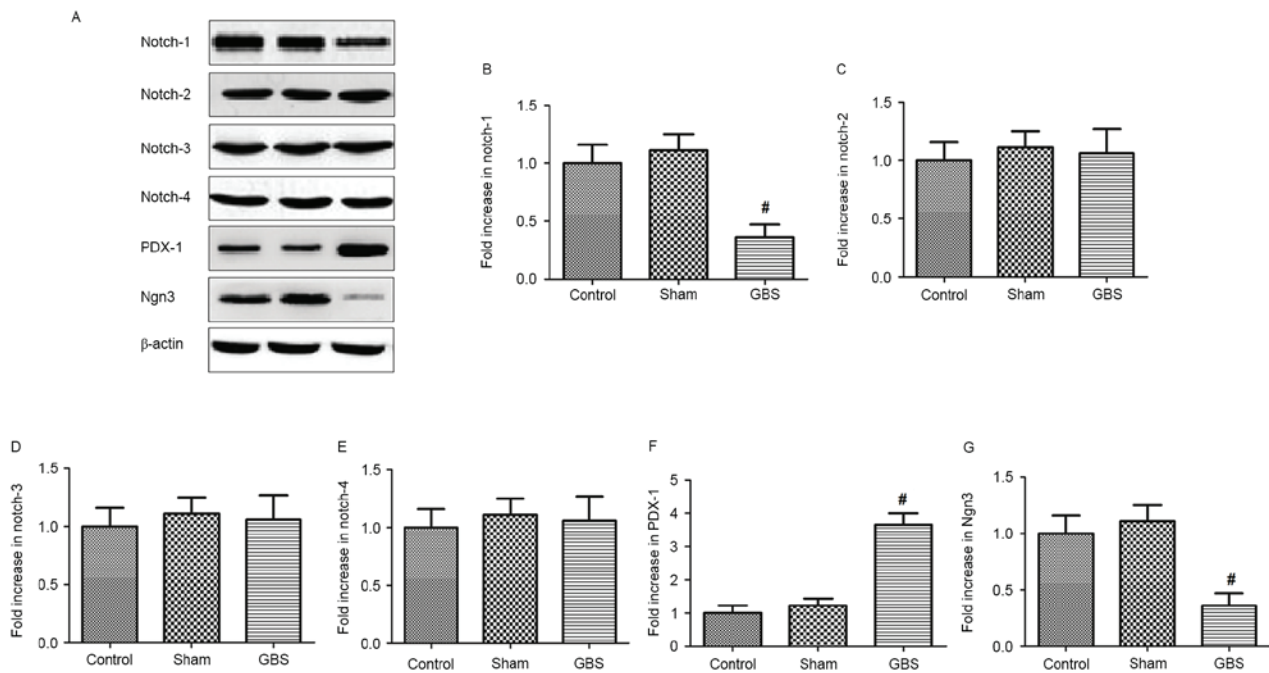


Figure 3. Levels of proteins involved in Notch signaling after gastric bypass surgery in db/db mice. (A) Levels of Notch1-4, PDX-1 and Ngn3 measured through western blotting in three groups. β -actin was used as a protein loading control. (B-G) Fold increase in (B) Notch-1, (C) Notch-2, (D) Notch-3, (E) Notch-4, (F) PDX-1 and (G) Ngn3 detected in the three groups through reverse-transcription quantitative polymerase chain reaction analysis. [#] $P < 0.05$ vs. control group. PDX-1, pancreatic and duodenal homeobox 1; Ngn3, neurogenin 3; GBS, gastric bypass surgery.

notably affected in the Ngn3 KO group. The PDX-1 KO group exhibited a significant decline in Ngn3, while it was obviously increased in the Notch-1 KO group. Considering that PDX-1 was significantly increased after gastric bypass surgery, it was hypothesized that elevated PDX-1 caused an inhibition of Notch-1 and further facilitated Ngn3, thus stimulating pancreatic β -cell regeneration after gastric bypass surgery. Therefore, the present study further investigated the influence of PDX-1/Notch-1/Ngn3 on pancreatic islets in the GBS group. As shown in Fig. 5A, insulin secretion was obviously decreased in the PDX-1 KO and Ngn3 KO groups, while being significantly elevated in the Notch-1 KO group. Similarly, a significant decrease in the β -cell to non- β endocrine cell ratio was observed in the PDX-1 KO and Ngn3 KO groups, while the Notch-1 KO group showed a tremendous increase in the ratio of β -cell to non- β endocrine cells (Fig. 5B). All of these results indicated that PDX-1/Notch-1/Ngn3 signaling had a crucial role in regulating pancreatic islet regeneration in db/db mice after gastric bypass surgery.

Discussion

T2DM frequently occurs in insulin-resistant subjects with hyperglycemia. A major challenge in reversing the deteriorative glycemic control in T2DM is to improve the function of pancreatic islets and increase the β -cell mass to meet the requirement for insulin. Gastric bypass surgery has been reported to be effective in reversing T2DM or preventing disease progression, resulting in a marked amelioration of diabetes in numerous morbidly obese patients (26,27). Therefore, in the present study, FPG, glucose tolerance, insulin secretion, morphological changes in pancreatic islets as well as the underlying mechanisms were assessed in

db/db mice after gastric bypass surgery. The results revealed that gastric bypass surgery led to significant improvements in hyperglycemia and impaired glucose tolerance in db/db mice, which was consistent with the results of earlier studies (28,29), indicating the recovery of degenerative glycemic control capacity after surgery. In addition, a significant decrease in the AUC of FPG and OGTT results were observed in the present study.

Previous studies have reported the associations between intestine and pancreas. Haegel *et al* (30) observed exocrine pancreas hyperplasia in experimental rats following massive resection of the small intestine. Numerous studies have addressed the beneficial effect of gastrointestinal hormones on glycemic control after gastric bypass surgery in diabetic patients (31-33), particularly glucagon-like peptide-1 (GLP-1). Elevated GLP-1 markedly enhanced insulin secretion and also improved the function of pancreatic β -cells, thus ameliorating hyperglycemia after gastric bypass surgery in diabetic subjects (34,35). GLP-1 is presumably associated with pancreatic β -cell neogenesis (36). As expected, in the present study, elevated insulin levels were observed in db/db mice following gastric bypass surgery, which may have resulted from an increased β -cell mass and significant improvement in the structure of islets observed through insulin immunohistochemical staining. An increased β -cell area has been reported in rat models following a different type of bariatric surgery (37), as well as increased β -cell mass in porcine models (38). In the present study, the ratio of β -cells to non- β endocrine cells was elevated after gastric bypass surgery, suggesting an increase in β -cells after surgery. In addition, pancreatic hyperplasia was observed in parallel with increased pancreas weight following surgery.

Further elucidation of the specific mechanism responsible for gastric bypass surgery-induced islet regeneration will

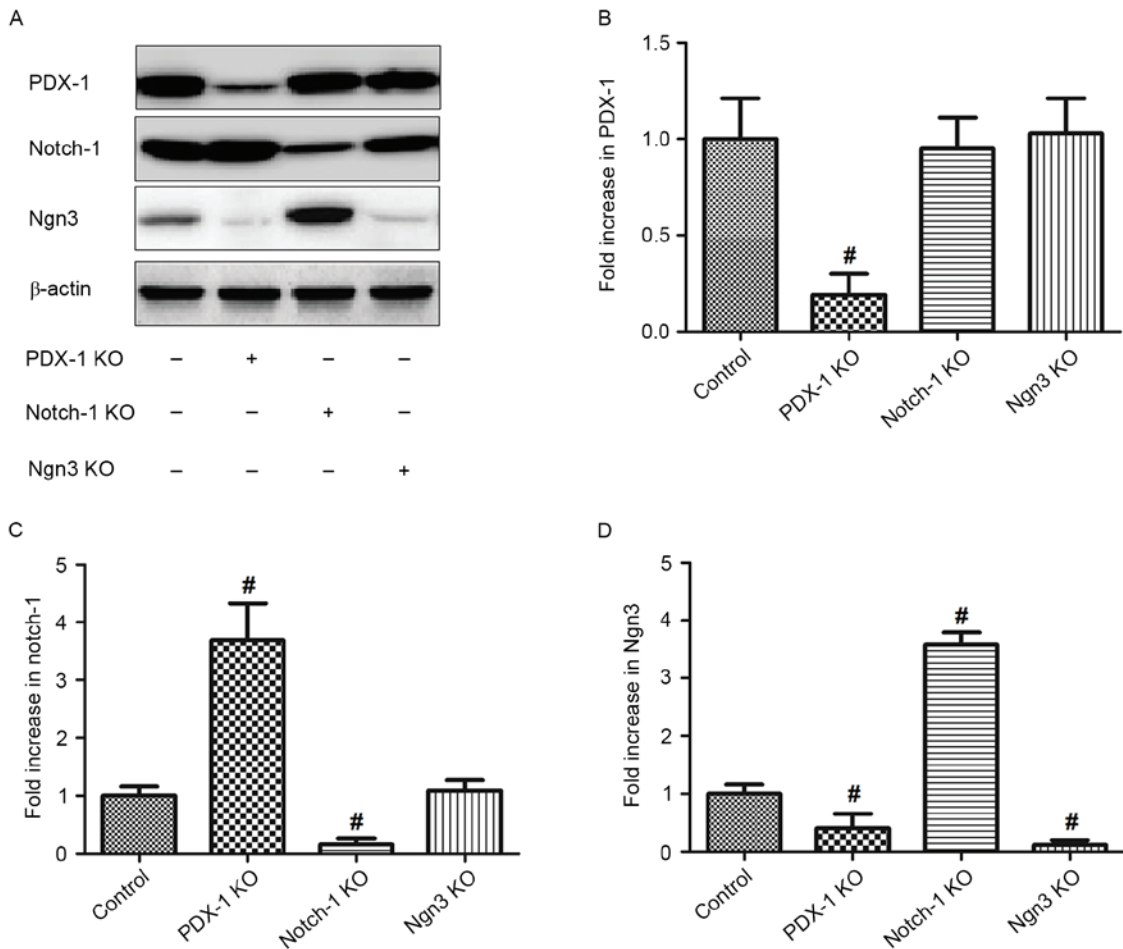


Figure 4. Potential mechanism involved in pancreatic islet regeneration after gastric bypass surgery in db/db mice. The GBS group was sub-divided into four groups: Control group, PDX-1 KO group, Notch-1 KO group and Ngn3 KO group. (A) Levels of Notch-1, PDX-1 and Ngn3 in the four groups. The levels of PDX-1, Notch-1, and Ngn3 were measured by western blot analysis. β-actin was used as a protein loading control. (B-D) Fold increase in (B) PDX-1, (C) Notch-1 and (D) Ngn3 measured in the four groups using reverse-transcription quantitative polymerase chain reaction analysis. #P<0.05 vs. control group. PDX-1, pancreatic and duodenal homeobox 1; Ngn3, neurogenin 3; GBS, gastric bypass surgery; KO, knockout.

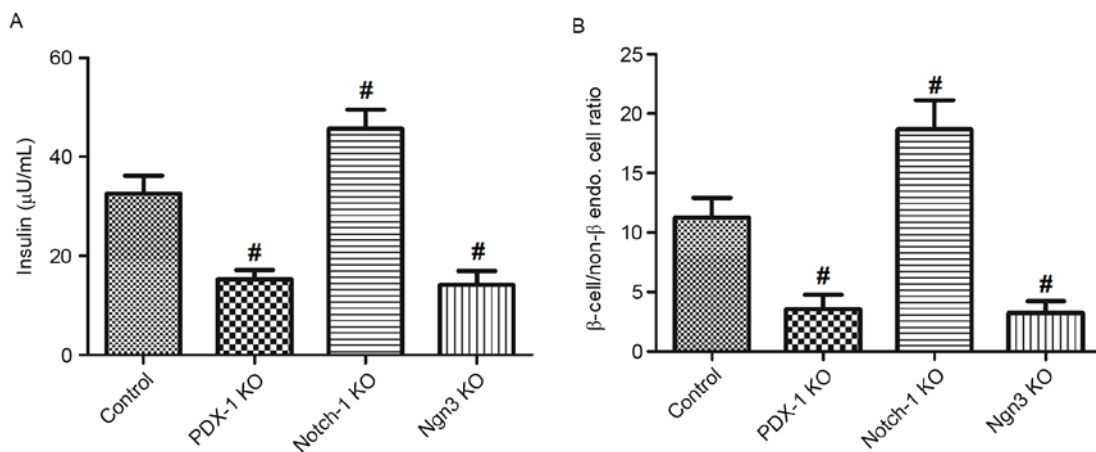


Figure 5. Influence of PDX-1/Notch-1/Ngn3 signaling on pancreatic islets in the GBS group. The GBS group was divided into four groups: Control group; PDX-1 KO group, Notch-1 KO group and Ngn3 KO group. (A) Insulin secretion in the four groups. (B) The ratio of β-cell to non-β endocrine cell in the four groups. #P<0.05 control group. PDX-1, pancreatic and duodenal homeobox 1; Ngn3, neurogenin 3; GBS, gastric bypass surgery; KO, knockout.

largely enhance the current understanding of T2DM pathogenesis and facilitate the development of T2DM therapy. The current knowledge on pancreatic development suggests a

potential role of Notch signaling in pancreatic hyperplasia. Notch was first reported in 1917, and subsequently, Notch-1, -2, -3 and -4 were found in vertebrates (39), among which

Notch-1 exerted a pre-dominant effect on pancreatic embryonic development (40). In addition, pancreatic development may be regulated by PDX-1, which is expressed in pancreatic progenitor cells (41). It has been shown that the activation of Notch-1 prevented exocrine and endocrine differentiation of pancreatic progenitor cells, leaving a large amount of pancreatic epithelial cells in the neonatal stage, an undifferentiated state with PDX-1 being expressed (17). Ngn3 is recognized as a master regulator of pancreatic endocrine formation and Notch signaling as an important negative regulator of Ngn3 gene expression (42). Studies also suggested a link between Ngn3 and Notch signaling in pancreatic embryonic development. In a study by Qu *et al* (43), elevated Notch/Hes1 signaling was reported to inhibit Ngn3, subsequently negatively affecting endocrine differentiation. The Notch signaling target Hes1 appears to have a crucial role in this process, as it directly binds and represses Ngn3. In the present study, notable changes in the levels of PDX-1, Notch-1 and Ngn3 were observed in db/db mice following gastric bypass surgery. Furthermore, the findings obtained with PDX-1 KO, Notch-1 KO and Ngn3 KO db/db mouse models with gastric bypass surgery suggested that the PDX-1/Notch-1/Ngn3 axis is involved in pancreatic islet regeneration after surgery, as elevated PDX-1 due to gastric bypass surgery produced the inhibition of Notch-1 and further facilitated Ngn3. These findings demonstrated that gastric bypass surgery in db/db mice produced pancreatic islet regeneration through PDX-1/Notch-1/Ngn3 signaling. The present study provided evidence for a novel mechanism by which diabetes remission is induced by pancreatic islet cell regeneration after gastric bypass surgery in db/db mice.

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