

Gastrin Treatment Stimulates β -Cell Regeneration and Improves Glucose Tolerance in 95% Pancreatectomized Rats

Noèlia Téllez, Géraldine Joanny, Jéssica Escoriza, Marina Vilaseca, and Eduard Montanya

Laboratory of Diabetes and Experimental Endocrinology (N.T., G.J., J.E., M.V., E.M.), Department of Clinical Sciences, University of Barcelona; Institut d'Investigacions Biomèdiques de Bellvitge, (IDIBELL) (N.T., G.J., J.E., M.V., E.M.), CIBER de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM) (N.T., G.J., J.E., M.V., E.M.), Endocrine Unit (J.E., E.M.), Hospital Universitari de Bellvitge, L'Hospitalet de Llobregat, Barcelona, Spain

β -Cell mass reduction is a central aspect in the development of type 1 and type 2 diabetes, and substitution or regeneration of the lost β -cells is a potentially curative treatment of diabetes. To study the effects of gastrin on β -cell mass in rats with 95% pancreatectomy (95%-Px), a model of pancreatic regeneration, rats underwent 95% Px or sham Px and were treated with [15 leu] gastrin-17 (Px+G and S+G) or vehicle (Px+V and S+V) for 15 d. In 95% Px rats, gastrin treatment reduced hyperglycemia (280 ± 52 mg vs. 436 ± 51 mg/dl, $P < 0.05$), and increased β -cell mass (1.15 ± 0.15 mg) compared with vehicle-treated rats (0.67 ± 0.15 mg, $P < 0.05$). Gastrin treatment induced β -cell regeneration by enhancing β -cell neogenesis (increased number of extraislet β -cells in Px+G: 0.42 ± 0.05 cells/mm² vs. Px+V: 0.27 ± 0.07 cells/mm², $P < 0.05$, and pancreatic and duodenal homeobox 1 expression in ductal cells of Px+G: $1.21 \pm 0.38\%$ vs. Px+V: $0.23 \pm 0.10\%$, $P < 0.05$) and replication (Px+G: $1.65 \pm 0.26\%$ vs. S+V: $0.64 \pm 0.14\%$; $P < 0.05$). In addition, reduced β -cell apoptosis contributed to the increased β -cell mass in gastrin-treated rats (Px+G: $0.07 \pm 0.02\%$, Px+V: $0.23 \pm 0.05\%$; $P < 0.05$). Gastrin action on β -cell regeneration and survival increased β -cell mass and improved glucose tolerance in 95% Px rats, supporting a potential role of gastrin in the treatment of diabetes. (*Endocrinology* 152: 2580–2588, 2011)

β -Cell mass reduction is a central aspect in the development of type 1 and type 2 diabetes (1, 2), and substitution or regeneration of the lost β -cells is a potentially curative treatment of diabetes. Islet transplantation is an emerging treatment for diabetes, but the scarcity of human islets precludes a wide use of islet transplantation unless alternative sources of β -cells are found. *Ex vivo* generation of new β -cells, either by replication or by differentiation from stem cells, could provide enough insulin-producing cells to make this treatment widely available (3). Alternatively, regeneration of the endogenous β -cells by replication of preexisting β -cells or by neogenesis from adult progenitor cells could increase the pancreatic β -cell mass

and restore normoglycemia. Thus, the search of molecules that could induce or stimulate β -cell regeneration is the subject of intensive research.

Gastrin is an enterogastric hormone synthesized by the G cells located in gastric pits in the antrum of the stomach. In fetal life, gastrin is expressed in the pancreas, both in islets and scattered among endocrine cells (4). Endogenous gastrin expression is activated in the developing pancreas during the secondary transition phase, a period of rapid pancreatic development when proto-differentiated ducts proliferate and differentiate into exocrine and endocrine pancreas (4, 5). In this period, islet precursors in the fetal duct divide actively and bud from the ductal epithelium to

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

Copyright © 2011 by The Endocrine Society

doi: 10.1210/en.2011-0066 Received January 20, 2011. Accepted April 18, 2011.

First Published Online May 10, 2011

Abbreviations: BrdU, 5-Bromo-2'-deoxyuridine; CCK, cholecystokinin; Ct, cycle threshold; IPGTT, intraperitoneal glucose tolerance test; PDX-1, pancreatic and duodenal homeobox 1; Px, pancreatectomy.

form new islets, in a process called islet neogenesis (6). In the postnatal period, gastrin expression rapidly disappears from the pancreas, and the administration of gastrin alone has shown no effects on β -cell mass in different experimental conditions (7–10). However, in combination with epidermal growth factor, gastrin has increased β -cell mass and/or improved glucose homeostasis in several rodent models of diabetes (8, 11), and increased β -cell mass in cultured adult human pancreatic cells (9). In this study we determined the effect of gastrin administration alone on islet regeneration in rats that underwent 95% pancreatectomy (95%-Px), a well-established model of β -cell regeneration. Gastrin administration increased β -cell neogenesis and replication, and reduced β -cell apoptosis, resulting in increased β -cell mass, and improved glucose homeostasis.

Materials and Methods

Animals

Experimental procedures were reviewed and approved by the Ethical Committee of the University of Barcelona. Male Sprague Dawley rats (Harlan Interfauna Ibérica, Spain), 4–5 wk of age and weighing approximately 100 g underwent a 95% Px. Four groups were studied: 95%-Px rats treated with gastrin (Px+G), 95%-Px rats treated with vehicle (Px+V), sham-Px rats treated with gastrin (S+G), and sham-Px rats treated with vehicle (S+V). Treatment with [15 leu] gastrin-17 (150 μ g/kg, Transition Therapeutics Inc., Toronto, Canada) (12) or vehicle (PBS) was started immediately before surgery. All animals were injected sc every 12 h for 15 d.

Metabolic control

Fed morning plasma glucose levels were monitored on d 3, d 7, d 10, d 14, and d 15 from the snipped tail with a portable meter. Blood samples were taken approximately 12 h after the previous gastrin injection and just before the next injection. Body weight was simultaneously determined.

Intraperitoneal glucose tolerance test (IPGTT) was performed on d 14 after 95%-Px or sham-Px. Glucose (2 g/kg of body weight) was injected after 2 h of fasting, and blood glucose was determined 0, 30, 60, 90, and 120 min after glucose injection. GraphPad Prism 4 software (GraphPad Software, Inc., San Diego, CA) was used to calculate the area under the curve.

Px and pancreas remnant harvesting

95%-Px was performed as previously described (13). Briefly, animals were anesthetized with 5% isoflurane (Forane; Abbott Laboratories, Madrid, Spain), and maintained anesthetized with an isoflurane-(1.5%) air mixture. Ninety five percent of the pancreas was removed carefully by gentle abrasion with cotton applicators in order to leave major blood vessels intact; the pancreatic remnant was the tissue between the common bile duct and the first loop of the duodenum. Particular attention was placed to removing the small flap of pancreas attached to the pylorus. Sham Px was accomplished by breaking splenic and duodenal mesenteric connections and gently handling the pancreas with

the fingertips. Immediately after surgery, animals received one dose (2.5 mg/kg) of meloxicam (Metacam; Boehringer Ingelheim, Ingelheim, Germany) that was subsequently administered for the next 3 d after surgery for analgesia. Animals were anesthetized 15 d after Px with ketamine (70 mg/kg), diazepam (5.6 mg/kg), and atropine (0.5 mg/kg), and pancreatic remnants were harvested. In sham animals, the equivalent of the remaining 5% tissue left after 95%-Px was harvested 15 d after sham surgery. The excised pancreatic remnants were cleared of fat and lymph nodes, weighed, fixed in 4% PBS-buffered paraformaldehyde, and processed for paraffin embedding.

Morphometry

β -Cell mass, endocrine non- β -cell mass, ductal cell mass, and acinar cell mass was determined by point-counting morphometry on immunoperoxidase-stained sections, using a 48-point grid to obtain the number of intercepts over β -cells, endocrine non- β - β cells, ductal cells, acinar cells, and over other tissue (14). Tissue sections were stained for β -cells with a rabbit antihuman insulin antibody (1:50; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), for the endocrine non- β -cells of the islets with a cocktail of antibodies: rabbit antiswine glucagon (1:500, DAKO Corp., Carpinteria, CA), rabbit antihuman somatostatin (1:500, DAKO) and rabbit antihuman pancreatic polypeptide (1:2000, Chemicon International, Inc., Temecula, CA), and for cytokeratins with rabbit anticow PanCK antiserum (1:3000) (DAKO) and visualized with LSAB+^{HRP} system (DAKO).

A nomogram relating number of points counted to volume density and expected relative SE in percentage of mean (<10%) was used to determine the number of intercepts needed for a representative sampling (15). Approximately 20,000 points were counted in each pancreas, and at least two to three sections (150 μ m apart) were included for each animal. All measurements were performed by a blinded observer. The relative volume of each cell type was calculated by dividing the number of points over that particular cell type by the total number of points over pancreatic tissue. For each pancreatic cell type of interest, cell mass was obtained by multiplying its relative volume by the pancreas weight.

β -Cell neogenesis

β -cell neogenesis was determined by three indirect methods (14).

Islets budding from ducts

Islet cells were considered to be budding from ducts when they were in contact or in near contact (less than five cells apart) with ductal cells. Insulin-stained sections were visualized, and the number of islets that were less than five cells apart from ducts (periductal), and those islets that were more than five cells distant from ducts (extraductal) were independently counted. Results were expressed as percentage of periductal or extraductal islets. Two to three sections (150 μ m apart) from each pancreas were analyzed to get a representative result.

Extraiislet β -cell clusters (groups of less than five cells)

The number of small clusters (less than five cells) of extraiislet insulin-positive cells was counted in insulin-stained pancreatic sections (14). To standardize counting, the total pancreatic tissue area was measured with an image-analytical software (AnalySIS 3.0, Soft Imaging System, Münster, Germany), and results were

expressed as number of extraislet β -cell clusters per square millimeter of pancreatic tissue. To get a representative result, three sections were counted from each pancreatic remnant.

Pancreatic and duodenal homeobox 1 (PDX-1) expression in ductal cells

Ductal epithelium has been proposed as a source of pancreatic progenitors (16). We counted the cells in the ductal epithelium that expressed PDX-1, a major regulator of β -cell identity and function (17) that has been found to be up-regulated in a number of β -cell regeneration models (18–20). Ductal cells were identified by its typical columnar cell morphology. Slides were treated with DNase (10 μ g/ml; Sigma, St. Louis, MO) for 1 h at 37 C. Immunofluorescence for PDX-1 (1/250; Chemicon International) was performed using as secondary antibody an Alexa fluor 546-labeled antirabbit IgG (1:400, Molecular Probes, Eugene, OR), and nuclei were stained with DRAQ5 (Biostatus Ltd., Leicestershire, UK). Sections were visualized under a confocal microscope (TCS-SL Spectral Confocal Microscope; Leica, Wetzlar, Germany). A minimum of 2500 epithelial ductal cells were counted per pancreas.

Cell replication

Rats were injected with the thymidine analog 5-bromo-2'-deoxyuridine (BrdU; Sigma), 100 mg/kg ip 6 h before the pancreas remnant was harvested. To assess β -cell replication, sections were double stained with immunoperoxidase for BrdU using a Cell Proliferation Kit (Amersham, Amersham, UK), and for insulin using a rabbit antihuman insulin antibody (1:50; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) (14, 21).

Ductal and acinar cell replication was determined by double immunofluorescence. For BrdU detection the rat anti-BrdU antibody (1:100) (Abcam, Cambridge, UK) combined with the Alexafluor 488-labeled antirat IgG (1:400) was used. Rabbit antihuman amylase antibody (1:50; Sigma) and rabbit anticow cytokeratin antiserum (1:3000; DAKO) were used for acinar and ductal cell identification, respectively. Alexafluor 555-labeled antirabbit IgG (1:400) was used as secondary antibody. β -Cell, acinar, and ductal replication was expressed as percentage of BrdU-positive β -cells, acinar cells, or ductal cells respectively. For β -cell replication, at least 1200 cells per pancreas were counted. For ductal and acinar replication, more than 2500 cells were counted per pancreas in a minimum of 10 different fields distributed across the whole pancreatic section.

β -Cell apoptosis

Sections were double stained by immunoperoxidase for apoptotic nuclei (black) with the terminal deoxynucleotide transferase-mediated dUTP nick end labeling technique (In Situ Cell Death Detection Kit, ApopTag; Intergene, Oxford, UK) and for insulin (*brown*) using a rabbit antihuman insulin antibody (1:50) (Santa Cruz Biotechnology) (14). A minimum of 1200 cells were counted per pancreas.

Individual β -cell area

The mean cross-sectional area of individual β -cells, a measure of β -cell size, was determined on the immunoperoxidase-stained sections for insulin with the image-analytical software (AnalySIS 3.0, Soft Imaging System) (21). The perimeter of insulin-positive area in each islet of the section was carefully traced on the com-

puter's monitor to exclude any other tissue, and β -cell nuclei of each islet were counted. To calculate the area of the individual β -cells, the total β -cell area in the section was divided by the number of β -cell nuclei.

RNA isolation, quantification, and retrotranscription

RNA was isolated from the pancreas and isolated islets of Px+V (n = 10) and S+V (n = 10) groups; in each group, five animals were used for gene expression analysis in pancreas, and five animals were used for gene expression analysis in isolated islets. Sham-pancreas and pancreatic remnants were rapidly harvested, immersed in RNAlater solution (Ambion, Warrington, UK) and chopped in small pieces (<5 mm) and kept overnight at 4 C. RNAlater was then removed and pancreatic tissue was resuspended with 1ml TRIzol Reagent (Sigma) for homogenization (Ultraturrax; Janke & Kunkel, Stauffen, Germany). Total RNA was purified according to the manufacturer's instructions (PureLink Micro-to Midi System; Invitrogen, Carlsbad, CA). Islets were isolated by collagenase (Collagenase P; Roche, Penzberg, Germany) digestion and hand picked to obtain a population of pure islets (22). RNA was extracted with RNeasy Mini Kit (QIAGEN, Crawley, UK), eluted with diethylpyrocarbonate-treated water and quantified using NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA). RNA quality was assessed with the Bioanalyzer 2100 (Agilent Technologies, Inc., Palo Alto, CA). The RNA Integrity Number score ranged from 6.9 to 8.3 for pancreases and pancreatic remnants and was higher than 8 for islet samples. cDNA synthesis was performed from 5 μ g or 1 μ g of total RNA for pancreas or isolated islets respectively using the Superscript III First-strand cDNA synthesis system (Invitrogen).

Quantitative PCR

PCR was run in a 7900HT Fast Real-Time PCR system (Applied Biosystems) with a 384-well plate allowing all samples to be amplified in the same run for each gene. Reactions were performed using TaqMan Gene Expression Assays and TaqMan Gene Expression Master Mix (Applied Biosystems) following the manufacturer protocol in a final volume of 20 μ l. In each reaction 250 ng or 25 ng of pancreas cDNA or isolated islets cDNA, respectively, were loaded. The assays used in this study were: cholecystokinin A receptor (*cckar*): Rn00562164_m1, cholecystokinin B receptor (*cckbr*): Rn00565867_m1, and eukaryotic 18S rRNA (used as endogenous control). Gene expression of *cckar* and *cckbr* was expressed as Δ Ct, the difference between cycle threshold (Ct) of each target gene and Ct of the endogenous control. Ct is the cycle at which sample crosses the point of detection.

Statistical analysis

Results were expressed as means \pm SE. Statistics were performed using GraphPad Prism 4 software (GraphPad Software, San Diego, CA), and differences among means were evaluated using Student's *t* test, the one-way ANOVA combined with Tukey's test for *post hoc* analysis or the two-way ANOVA combined with Bonferroni's test for *post hoc* analysis as appropriate. *P* < 0.05 was considered significant.

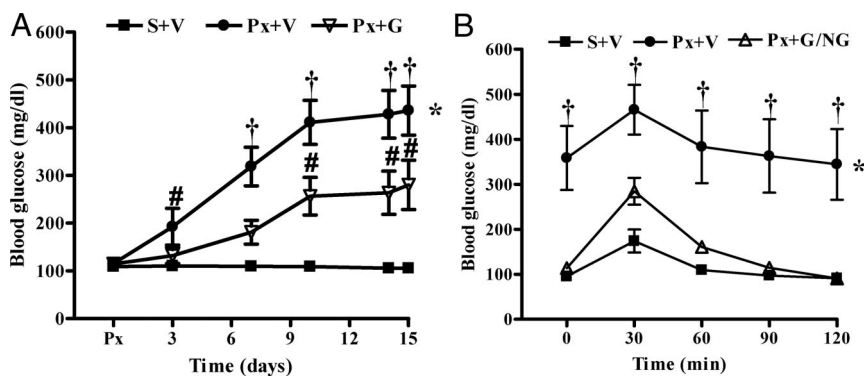


FIG. 1. Effects of gastrin treatment on metabolic evolution of 95%-Px rats. A, Blood glucose of 95%-Px rats treated with vehicle (Px+V; circles, $n = 9$), with gastrin (Px+G; triangles, $n = 12$) and sham-operated rats treated with vehicle (S+V; squares, $n = 10$). B, Intraperitoneal glucose tolerance tests of 95%-Px rats treated with vehicle (Px+V; circles, $n = 8$), gastrin-treated Px rats that remained normoglycemic (Px+G/NG; triangles, $n = 7$), and sham-operated rats (S+V; squares, $n = 9$). Values are means \pm SE. *, $P < 0.05$ vs. all other groups for ANOVA repeated measures. ANOVA: $P < 0.05$; #, $P < 0.05$ vs. S+V for Tukey's test, †, $P < 0.05$ vs. all other groups for Tukey's test.

Results

Gastrin treatment improved glucose homeostasis in 95%-Px rats

All Px rats treated with vehicle developed hyperglycemia that was already detected on d 3 (193 ± 39 mg/dl vs. S+V: 110 ± 2 mg/dl, $P < 0.05$), and that was severe from d 7 (318 ± 40 mg/dl) until the end of the study (d 15: 436 ± 51 mg/dl) (Fig. 1A). Gastrin treatment prevented and delayed the development of hyperglycemia: 58% (seven of

12) of Px+G rats maintained normoglycemia throughout the study, and the appearance of hyperglycemia was delayed in the remaining animals (d 3: 169 ± 34 mg/dl; d 7: 265 ± 25 mg/dl, $P = \text{ns}$ vs. S+V group). The IPGTT showed similar blood glucose levels in normoglycemic Px+G (Px+G/NG) and sham-operated animals (area under the curve of Px+G/NG: 19404 ± 1077 ; S+V: 14607 ± 1048), and lower than in Px+V groups (Px+V: $46,892 \pm 3450$, $P < 0.01$) (Fig. 1B). Pancreatectomized rats treated with vehicle and gastrin showed similar weight gain (Supplemental Appendix 1 published on The Endocrine Society's Journals Online web site at <http://endo.endojournals.org>), indicating that the improved glucose homeostasis found in Px+G rats was not due to an anorectic action of gastrin.

12) of Px+G rats maintained normoglycemia throughout the study, and the appearance of hyperglycemia was delayed in the remaining animals (d 3: 169 ± 34 mg/dl; d 7: 265 ± 25 mg/dl, $P = \text{ns}$ vs. S+V group). The IPGTT showed similar blood glucose levels in normoglycemic Px+G (Px+G/NG) and sham-operated animals (area under the curve of Px+G/NG: 19404 ± 1077 ; S+V: 14607 ± 1048), and lower than in Px+V groups (Px+V: $46,892 \pm 3450$, $P < 0.01$) (Fig. 1B). Pancreatectomized rats treated with vehicle and gastrin showed similar weight gain (Supplemental Appendix 1 published on The Endocrine Society's Journals Online web site at <http://endo.endojournals.org>), indicating that the improved glucose homeostasis found in Px+G rats was not due to an anorectic action of gastrin.

Gastrin treatment increased β -cell mass in Px rats

β -Cell mass was higher in the pancreatic remnant of gastrin-treated rats (Px+G: 1.15 ± 0.15 mg) than in Px+V (0.67 ± 0.15 mg, $P < 0.05$) and sham-operated rats (S+V: 0.31 ± 0.07 mg; $P < 0.001$) (Fig. 2A). In Px+V rats β -cell mass was doubled compared with S+V animals, but differences did not reach statistical significance ($P = 0.083$). Gastrin treatment had no effect on S+G animals that showed similar blood glucose, body weight, IPGTT, and β -cell mass (0.27 ± 0.06 mg) than S+V group (Supplemental Appendix 2). Thus, no additional studies were performed in S+G group.

The endocrine non- β -cell mass (α , δ , and pancreatic polypeptide cells) was approximately 2 times higher in Px+G group than in Px+V rats (Fig. 2B). Ductal and acinar cell masses were similar in pancreatic remnants from both Px groups (Fig. 2, C and D).

Gastrin treatment increased β -cell neogenesis and replication and reduced β -cell apoptosis

β -Cell neogenesis

β -Cell neogenesis was determined by three indirect methods: quantification of ductal cells expressing PDX-1, of islets budding from ducts, and of extraislet β -cells (groups of < 5 cells).

The percentage of ductal cells expressing the transcription factor PDX-1 was increased in gastrin-treated rats (Px+G: $1.21 \pm 0.38\%$) compared with Px+V ($0.23 \pm 0.10\%$, $P = 0.03$), and with S+V rats ($0.28 \pm 0.21\%$, $P < 0.05$) (Fig. 3, A–D). The percentage of periductal islets was

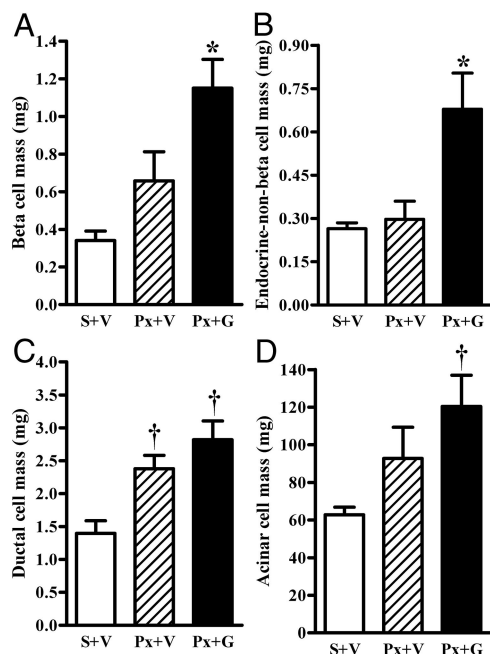


FIG. 2. Effects of gastrin treatment on β -cell mass (A), endocrine non- β -cell mass (B), ductal cell mass (C), and acinar cell mass (D) of pancreatic remnants of sham-operated rats (S+V, $n = 10$), vehicle-treated 95%-Px rats (Px+V, $n = 9$), and gastrin-treated 95%-Px rats (Px+G, $n = 12$). Values are means \pm SE. ANOVA: $P < 0.05$, *, $P < 0.05$ vs. all other groups; †, $P < 0.05$ vs. S+V group for Tukey's test.

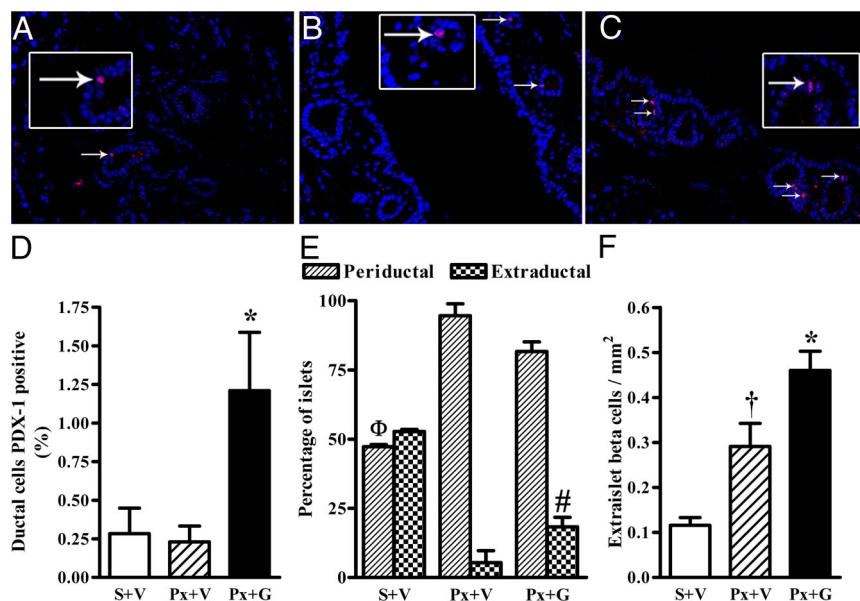


FIG. 3. Effects of gastrin treatment on β -cell neogenesis. Confocal images of PDX-1 immunofluorescence in pancreatic remnants of sham-operated rats (S+V, $n = 6$) (A), vehicle-treated 95%-Px rats (Px+V, $n = 6$) (B), and gastrin-treated 95%-Px rats (Px+G, $n = 6$) (C). Insets show the nuclear labeling of PDX-1 in epithelial ductal cells. D, Quantification of PDX-1 expression in ductal cells. E, Percentage of islets within the ducts or less than five cells apart from ducts (dashed bars) and percentage of islets more than five cells apart from ducts (squared bars). F, Number of β -cell clusters with less than five β -cells. Values are means \pm SE. ANOVA: $P < 0.05$. *, $P < 0.05$ vs. all other groups; Φ , $P < 0.05$ vs. periductal islets of Px groups; #, $P < 0.05$ vs. extraductal islets of Px+V group and †, $P < 0.05$ vs. S+V for Tukey's test.

higher in pancreatectomized rats than in sham-operated rats (Px+V: $87.1 \pm 6.5\%$; S+V: $44.7 \pm 0.3\%$; $P < 0.05$). Gastrin treatment did not further increase the percentage of periductal islets (Px+G = $79.4 \pm 4.1\%$) (Fig. 3E). The number of extraislet β -cells was doubled in vehicle-treated Px rats compared with sham-operated rats (Px+V: 0.29 ± 0.05 cells/mm², S+V: 0.11 ± 0.02 cells/mm²; $P < 0.05$) and was further increased in gastrin-treated Px rats (Px+G: 0.46 ± 0.04 cells/mm²; $P < 0.05$ vs. both Px+V and S+V groups) (Fig. 3F). Overall, these results indicate that gastrin treatment enhanced β -cell neogenesis.

β -Cell replication

β -Cell replication was doubled in gastrin-treated Px rats compared with sham-operated rats (Px+G: $1.65 \pm 0.26\%$; S+V: $0.64 \pm 0.14\%$; $P < 0.05$) (Fig. 4A). β -Cell replication in Px+V rats ($1.07 \pm 0.15\%$) was not significantly different from Px+G ($P = 0.07$) or S+V ($P = 0.083$) groups.

Apoptosis

β -Cell apoptosis was increased in 95%-Px rats treated with vehicle compared with sham-operated animals (Px+V: $0.23 \pm 0.05\%$; S+V: 0.08 ± 0.02 ; $P < 0.05$) (Fig. 4B). Gastrin treatment protected β -cells from the in-

creased apoptosis found in Px+V rats (Px+G: $0.07 \pm 0.02\%$, $P < 0.05$ vs. Px+V).

Cross-sectional individual β -cell size and islet size

The area of individual β -cells was similarly increased in 95%-Px rats treated with gastrin or with vehicle (Px+G: 244 ± 13 μ m², Px+V: 269 ± 20 μ m²) compared with sham-operated rats (S+V: 197 ± 16 μ m², $P < 0.05$), indicating that gastrin treatment did not induce further β -cell hypertrophy (Fig. 4C).

Gastrin-treatment increased ductal and acinar cell replication in pancreatic remnants

Ductal cell proliferation was higher in 95%-Px rats treated with gastrin than in Px+V and S+V groups (Px+G: $1.49 \pm 0.19\%$; Px+V: $0.89 \pm 0.14\%$; S+V: $0.35 \pm 0.15\%$, $P < 0.05$) (Fig. 5C). Acinar cell replication was also increased in Px+G group ($1.38 \pm 0.28\%$) compared with Px+V ($0.48 \pm 0.12\%$, $P < 0.05$) and S+V ($0.46 \pm 0.07\%$, $P < 0.05$) groups. (Fig. 5F).

Similar effects of gastrin on replication and apoptosis in 95%-Px normoglycemic and hyperglycemic rats

Because chronic hyperglycemia impairs β -cell proliferation and induces β -cell apoptosis (23), we analyzed the effects of gastrin treatment in the animals that remained normoglycemic and that developed hyperglycemia. β -Cell mass, neogenesis, replication, and apoptosis were similar in Px+G rats that remained normoglycemic and in those that developed hyperglycemia after Px (Supplemental Appendix 3), indicating that the effects of gastrin on β -cells were not due to the improved metabolic control of gastrin-treated animals, and suggesting a direct effect of gastrin on β -cells.

Expression of gastrin receptors genes *cckar* and *cckbr* in pancreas and islets

The enhanced β -cell replication and reduced β -cell apoptosis found in Px+G rats suggested a direct action of gastrin on pancreatic β -cells. However, the expression of the specific gastrin receptor (cholecystikinin B receptor; CCKBR) in islets is not well established. Thus, we determined the expression of *cckbr* and *cckar* genes in the pan-

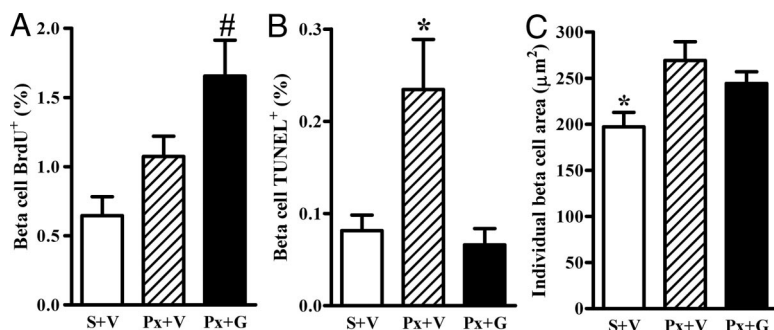


FIG. 4. Effects of gastrin treatment on β -cell replication (A), apoptosis (B), and β -cell size (C) in sham-operated rats (S+V, $n = 10$), vehicle-treated 95%-Px rats (Px+V, $n = 9$) and gastrin-treated 95%-Px rats (Px+G, $n = 12$). Values are means \pm SE. ANOVA: $P < 0.05$; #, $P < 0.05$ vs. S+V group and *, $P < 0.05$ vs. all other groups for Tukey's test.

creas and in isolated islets of Px+V and S+V animals. *cckar* gene was similarly expressed in pancreas and isolated islets from Px+V and S+V groups. In contrast, *cckbr* was exclusively expressed in the pancreas of Px+V rats and was detected neither in the pancreas of S+V rats nor in isolated islets of Px or S+V rats (Table 1).

Discussion

In this study we have shown that gastrin treatment reduced hyperglycemia in 95%-Px rats and increased pancreatic β -cell mass by enhancing β -cell neogenesis and replication and reducing β -cell apoptosis.

Partial Px is an established model of β -cell regeneration that shows very active cell regeneration immediately after surgery that gradually decreases and by d 14 has returned to basal levels (24–26). In 95%-Px rats, the regenerative response is insufficient to overcome the reduction in β -cell mass, and severe hyperglycemia develops soon after Px, as shown by the metabolic evolution of vehicle-treated rats. In this study, gastrin treatment reduced the severity of hyperglycemia in 95%-Px rats, and in more than half of the animals completely prevented the development of hyperglycemia, and even of glucose intolerance, as shown by the normal glucose levels in the IPGTT. Gastrin has no known effects on insulin secretion, and the beneficial effects on glucose control can be attributed to the increased β -cell mass.

Gastrin treatment enhanced β -cell neogenesis as indicated by the higher percentage of extraislet β -cells and the increased expression of the β -cell transcription factor PDX-1 in ductal cells of 95%-Px rats treated with gastrin. A role for gastrin on β -cell neogenesis has been suggested in the fetal pancreas when gastrin and gastrin receptors are transiently and specifically expressed during the secondary transition phase (4, 5, 27, 28). Indirect evidence for a role of gastrin in islet neogenesis was obtained in patients

with hypergastrinemia caused by gastrin-expressing islet tumors and atrophic gastritis that showed nesidioblastosis, an abnormal persistence of active islet neogenesis in the postnatal pancreas characterized by islets budding from pancreatic ducts (29). In animal models the combination of gastrin with an epidermal growth factor family ligand (8, 10) or a glucagons-like peptide 1 analog induced β -cell neogenesis (7, 12), and in the INGAS/TGF- α double transgenic mice an increased number of β -cells was present within the metaplastic ducts (30). However, in the normal pancreas gastrin alone showed no effects on β -cell neogenesis or mass. We have now found that treatment with gastrin alone increased β -cell neogenesis and mass in pancreatectomized rats but had no effect on sham animals. Overall, the results suggest that gastrin exerts no neogenic effect on the normal adult rat pancreas, but it may become an important factor when the tissue undergoes remodeling, an interpretation supported by the neogenic effect of gastrin in rats with pancreatic duct ligation (11).

Pancreatic ducts are considered to harbor adult progenitor cells capable to differentiate into endocrine and exocrine pancreas after partial Px (26), in a process that would recapitulate the embryonic development by reactivating latent acinar/islet precursors in pancreatic ducts (26). The ductal origin of new exocrine and endocrine cell types has been recently shown in postnatal and adult mice using the lineage tracing method (16), although other studies yielded negative results (31). Our finding of an increased expression of the β -cell transcription factor PDX-1 in ductal cells of gastrin-treated animals, and the higher percentage of periductal islets in gastrin and vehicle-treated animals after 95% Px, is consistent with the proposed role of ductal cells in β -cell neogenesis. The transient increment in PDX-1 expression in ductal cells after partial Px has been also associated with ductal cell proliferation (21). Supporting this association, we found that PDX-1 expression in ductal cells and ductal cell replication were both increased in gastrin-treated rats 15 d after Px, but they were not increased in pancreatectomized animals treated with vehicle.

β -Cell proliferation was modestly enhanced by gastrin treatment in pancreatectomized rats. The mitogenic effect of gastrin was found both in the animals that remained normoglycemic and in those that became hyperglycemic after Px, indicating that the gastrin-increased β -cell proliferation was not dependent on blood glucose. However, in rats with pancreatic duct ligation, the acute administration of gastrin had not increased β -cell replication (11).

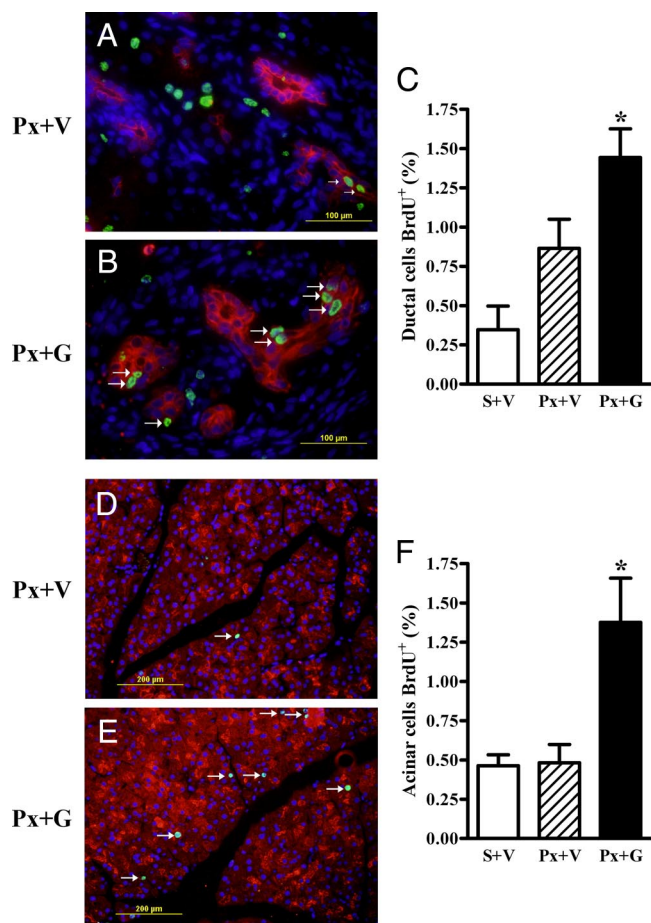


FIG. 5. Ductal and acinar cell replication. Micrographs show BrdU (green) and PanCK (red) double stained sections of pancreatic remnants from vehicle-treated 95%-Px rats (Px+V) (A) and gastrin-treated 95%-Px rats (Px+G) (B), and BrdU (green) and amylase (red) double stained sections of pancreatic remnants from Px+V rats (D) and Px+G rats (E). Arrows show ductal (A and B) or acinar (C and D) cells with immunoreactivity for BrdU in their nuclei. Quantification of ductal cell proliferation (C) and of acinar cell proliferation (F). (Px+V, n = 7; Px+G, n = 8). Values are means \pm SE. ANOVA: $P < 0.05$; *, $P < 0.05$ vs. all other groups for Tukey's test.

Differences between these two experimental models, dose and duration of gastrin treatment, and day of pancreas analysis could account for the different results. In patients with hypergastrinemia caused by gastrin-secreting tumors the size of islets is increased (29, 32, 33) and, more re-

TABLE 1. Gene expression of *ccka* and *cckb* receptors in pancreas and isolated islets

	Pancreas		Islets	
	S+V	Px+V	S+V	Px+V
<i>cckar</i>	6.59 \pm 1.67	5.65 \pm 0.59	6.53 \pm 0.15	6.17 \pm 0.45
<i>cckbr</i>	ND	15.28 \pm 0.69	ND	ND

Real-time quantitative PCR from sham pancreas (S+V) (n = 5), pancreatic remnants of 95%-Px rats (Px+V) (n = 5), islets isolated from S+V animals (n = 5), and islets isolated from pancreatic remnants of Px+V animals (n = 5) was performed to identify *cckar* and *cckbr* gene expression. Values are means \pm SE of Δ Ct (target gene Ct - endogenous control Ct). ND, Nondetectable.

cently, increased β -cell replication, fractional β -cell area, and mean islet size have been identified in islets adjacent to gastrinomas, suggesting a paracrine effect of gastrin on β -cell replication (34). Partial Px is a well-established model of β -cell regeneration in young rats. Because recent studies have described a decline in β -cell replication with age (35–37), it remains to be known whether gastrin would increase β -cell proliferation in old pancreatectomized rats.

A potential additional factor contributing to increased β -cell mass is β -cell hypertrophy. The study of the individual β -cell size showed that although 95%-Px induced β -cell hypertrophy, gastrin did not further increase it. The result is in agreement with the lack of effect of gastrin infusion on β -cell size in duct-ligated rats (11).

Gastrin treatment reduced β -cell apoptosis in 95%-Px rats. The increased β -cell apoptosis in 95%-Px rats treated with vehicle was probably driven by chronic exposure to hyperglycemia (23, 38). β -Cell apoptosis was normal in the subgroup of gastrin-treated rats that developed hyperglycemia, indicating that the effect of gastrin cannot be attributed to the reduced hyperglycemia. The antiapoptotic properties of gastrin have been shown in other cell types such as gastrointestinal and AR42J cells (39, 40), but we are unaware of previous data showing an antiapoptotic action of gastrin in pancreatic β -cells. In acinar cells, the antiapoptotic action of gastrin is mediated by the phosphorylation of insulin receptor substrate-1, stimulation of phosphoinositide 3-kinase, AKT, ERK, and MAPK (40).

The amidated peptides of the gastrin-cholecystokinin (CCK) family exert their actions mainly via two G protein-coupled receptors, CCK-A and CCK-B receptors. The CCK-A receptor (also known as CCK-1 receptor) has an affinity for CCK that is 100- to 500-fold greater than for gastrin, and the CCK-B receptor (also known as CCK-2 receptor) has equivalent high affinities for both hormones (41). In the rat, CCK-A receptors are expressed in the normal exocrine pancreas, but CCK-B receptors are not (16, 42–45). There is general agreement that CCK-B receptor is not expressed in adult β -cells (46–48), and the expression in α - and δ -cells is controversial, with some studies reporting its presence in rat and human α -cells (46), but not in δ cells (48), and other studies showing colocalization with somatostatin but not with glucagon positive cells (47, 49). In this study, we did not detect the expression of *cckbr* gene in the pancreas or in islets of S+V animals. In pancreatectomized rats, gene expression of *cckbr* remained negative in islets isolated from the pancreatic remnant but was found in the pancreatic remnant. The expression of the CCK-B receptor has been reported in other experimental models of pancreatic damage, such as partial duct ligation (45) and azaserine-induced pan-

creatic carcinoma in rats (50). Although the expression of CCK-B in newly formed extraislet β -cells cannot be excluded, the results suggest that in partially pancreatectomized rats, gastrin could enhance islet neogenesis acting on the newly expressed CCK-B receptor in the exocrine pancreas.

We detected the expression of *cckar* gene in pancreas as well as in isolated islets from both sham and 95%-Px rats. The expression in islets is in agreement with the previously reported colocalization of insulin and CCK-A receptor (47). The involvement of CCK-A receptor in pancreatic growth has been previously suggested (51). Thus, although gastrin is a poor activator of CCK-A receptor at physiological concentrations (41), the dose of gastrin used in our study may have allowed the interaction with CCK-A receptor, providing a mechanistic basis for the effects of gastrin on β -cell replication and apoptosis.

In summary, we have found that gastrin administration to 95%-Px rats enhanced β -cell neogenesis, increased β -cell replication, and reduced β -cell apoptosis. Gastrin action on β -cell regeneration and survival increased β -cell mass and improved glucose tolerance, supporting a potential role of gastrin in the treatment of diabetes.

Acknowledgments

We thank the Biology Unit of the Scientific-Technical Services (Serveis Científicotècnics), University of Barcelona, for their assistance. We thank Kelly Roche for the excellent technical assistance.

Address all correspondence and requests for reprints to: Eduard Montanya, Endocrine Unit (13-2), Hospital Universitari Bellvitge, Feixa Llarga, s/n, 08907 L'Hospitalet de Llobregat, Barcelona, Spain. E-mail: montanya@ub.edu or Noèlia Téllez, Laboratory of Diabetes and Experimental Endocrinology 4136, School of Medicine (Campus de Bellvitge), Pavelló de Govern Fourth floor, Feixa Llarga s/n, 08907-L'Hospitalet de Llobregat, Barcelona, Spain, E-mail: ntellez@ub.edu.

This work was supported by grants PI 06/0891 (to E.M.) and PI 10/00636 (to E.M.) from Instituto de Salud Carlos III (ISCIII), Promt-0901 from Center of Regenerative Medicine in Barcelona (CMRB), and Novo Nordisk. CIBERDEM is a project of ISCIII. G. J. was supported by a grant from Fundació IDIBELL.

Disclosure Summary: The authors have nothing to disclose.

References

- Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA, Butler PC 2003 β -Cell deficit and increased β -cell apoptosis in humans with type 2 diabetes. *Diabetes* 52:102–110
- Devendra D, Liu E, Eisenbarth GS 2004 Type 1 diabetes: recent developments. *BMJ* 328:750–754
- Montanya E 2004 Islet- and stem-cell-based tissue engineering in diabetes. *Curr Opin Biotechnol* 15:435–440
- Larsson LI, Rehfeld JF, Sundler F, Håkanson R 1976 Pancreatic gastrin in foetal and neonatal rats. *Nature* 262:609–610
- Hadjivanova C, Dufresne M, Poirat S, Sozzani P, Vaysse N, Moroder L, Fourmy D 1992 Pharmacological and biochemical characterization of cholecystokinin/gastrin receptors in developing rat pancreas. Age-related expression of distinct receptor glycoforms. *Eur J Biochem* 204:273–279
- Bouwens L, Klöppel G 1996 Islet cell neogenesis in the pancreas. *Virchows Arch* 427:553–560
- Suarez-Pinzon WL, Power RF, Yan Y, Wasserfall C, Atkinson M, Rabinovitch A 2008 Combination therapy with glucagon-like peptide-1 and gastrin restores normoglycemia in diabetic NOD mice. *Diabetes* 57:3281–3288
- Suarez-Pinzon WL, Yan Y, Power R, Brand SJ, Rabinovitch A 2005 Combination therapy with epidermal growth factor and gastrin increases β -cell mass and reverses hyperglycemia in diabetic NOD mice. *Diabetes* 54:2596–2601
- Suarez-Pinzon WL, Lakey JR, Brand SJ, Rabinovitch A 2005 Combination therapy with epidermal growth factor and gastrin induces neogenesis of human islet β -cells from pancreatic duct cells and an increase in functional β -cell mass. *J Clin Endocrinol Metab* 90:3401–3409
- Rooman I, Bouwens L 2004 Combined gastrin and epidermal growth factor treatment induces islet regeneration and restores normoglycaemia in C57Bl6/J mice treated with alloxan. *Diabetologia* 47:259–265
- Rooman I, Lardon J, Bouwens L 2002 Gastrin stimulates β -cell neogenesis and increases islet mass from transdifferentiated but not from normal exocrine pancreas tissue. *Diabetes* 51:686–690
- Suarez-Pinzon WL, Lakey JR, Rabinovitch A 2008 Combination therapy with glucagon-like peptide-1 and gastrin induces β -cell neogenesis from pancreatic duct cells in human islets transplanted in immunodeficient diabetic mice. *Cell Transplant* 17:631–640
- Montaña E, Bonner-Weir S, Weir GC 1994 Transplanted β -cell response to increased metabolic demand. Changes in β -cell replication and mass. *J Clin Invest* 93:1577–1582
- Montanya E, Téllez N 2009 Pancreatic remodeling: β -cell apoptosis, proliferation and neogenesis, and the measurement of β -cell mass and of individual β -cell size. *Methods Mol Biol* 560:137–158
- Weibel ER 1979 Point counting methods. *Stereological methods*. New York: Academic Press; 101–161
- Inada A, Nienaber C, Katsuta H, Fujitani Y, Levine J, Morita R, Sharma A, Bonner-Weir S 2008 Carbonic anhydrase II-positive pancreatic cells are progenitors for both endocrine and exocrine pancreas after birth. *Proc Natl Acad Sci USA* 105:19915–19919
- McKinnon CM, Docherty K 2001 Pancreatic duodenal homeobox-1, PDX-1, a major regulator of β cell identity and function. *Diabetologia* 44:1203–1214
- Tiemann K, Panienska R, Klöppel G 2007 Expression of transcription factors and precursor cell markers during regeneration of β cells in pancreata of rats treated with streptozotocin. *Virchows Arch* 450:261–266
- Rooman I, Heremans Y, Heimberg H, Bouwens L 2000 Modulation of rat pancreatic acinoductal transdifferentiation and expression of PDX-1 in vitro. *Diabetologia* 43:907–914
- Sharma A, Zangen DH, Reitz P, Taneja M, Lissauer ME, Miller CP, Weir GC, Habener JF, Bonner-Weir S 1999 The homeodomain protein IDX-1 increases after an early burst of proliferation during pancreatic regeneration. *Diabetes* 48:507–513
- Téllez N, Montolio M, Estil-lés E, Escoriza J, Soler J, Montanya E 2007 Adenoviral overproduction of interleukin-1 receptor antagonist increases β -cell replication and mass in syngeneically transplanted islets, and improves metabolic outcome. *Diabetologia* 50:602–611
- Nácher V, Rurell M, Merino JF, Aranda O, Soler J, Montana E 1996 β -Cell growth and mass are preserved in long-term syngeneic

- islet transplantation in streptozocin-induced diabetic Lewis rats. *Diabetes* 45:1541–1546
23. Biarnés M, Montolio M, Nacher V, Raurell M, Soler J, Montanya E 2002 β -Cell death and mass in syngeneically transplanted islets exposed to short- and long-term hyperglycemia. *Diabetes* 51:66–72
 24. Hayashi KY, Tamaki H, Handa K, Takahashi T, Kakita A, Yamashina S 2003 Differentiation and proliferation of endocrine cells in the regenerating rat pancreas after 90% pancreatectomy. *Arch Histol Cytol* 66:163–174
 25. Bonner-Weir S, Trent DF, Weir GC 1983 Partial pancreatectomy in the rat and subsequent defect in glucose-induced insulin release. *J Clin Invest* 71:1544–1553
 26. Bonner-Weir S, Baxter LA, Schuppin GT, Smith FE 1993 A second pathway for regeneration of adult exocrine and endocrine pancreas. A possible recapitulation of embryonic development. *Diabetes* 42:1715–1720
 27. Brand SJ, Fuller PJ 1988 Differential gastrin gene expression in rat gastrointestinal tract and pancreas during neonatal development. *J Biol Chem* 263:5341–5347
 28. Bardram L, Hilsted L, Rehfeld JF 1990 Progastrin expression in mammalian pancreas. *Proc Natl Acad Sci USA* 87:298–302
 29. Larsson LI, Ljungberg O, Sundler F, Håkanson R, Svensson SO, Rehfeld J, Stadil R, Holst J 1973 Antor-pyloric gastrinoma associated with pancreatic nesidioblastosis and proliferation of islets. *Virchows Arch A Pathol Anat* 360:305–314
 30. Wang TC, Bonner-Weir S, Oates PS, Chulak M, Simon B, Merlino GT, Schmidt EV, Brand SJ 1993 Pancreatic gastrin stimulates islet differentiation of transforming growth factor α -induced ductular precursor cells. *J Clin Invest* 92:1349–1356
 31. Solar M, Cardalda C, Houbracken I, Martín M, Maestro MA, De Medts N, Xu X, Grau V, Heimberg H, Bouwens L, Ferrer J 2009 Pancreatic exocrine duct cells give rise to insulin-producing β cells during embryogenesis but not after birth. *Dev Cell* 17:849–860
 32. Zollinger RM, Ellison EH 1955 Primary peptic ulcerations of the jejunum associated with islet cell tumors of the pancreas. *Ann Surg* 142:709–723; discussion, 724–728
 33. Creutzfeldt W, Arnold R, Creutzfeldt C, Track NS 1975 Pathomorphologic, biochemical, and diagnostic aspects of gastrinomas (Zollinger-Ellison syndrome). *Hum Pathol* 6:47–76
 34. Meier JJ, Butler AE, Galasso R, Rizza RA, Butler PC 2006 Increased islet β cell replication adjacent to intrapancreatic gastrinomas in humans. *Diabetologia* 49:2689–2696
 35. Tschén SI, Dhawan S, Gurlo T, Bhushan A 2009 Age-dependent decline in β -cell proliferation restricts the capacity of β -cell regeneration in mice. *Diabetes* 58:1312–1320
 36. Montanya E, Nacher V, Biarnés M, Soler J 2000 Linear correlation between β -cell mass and body weight throughout the lifespan in Lewis rats: role of β -cell hyperplasia and hypertrophy. *Diabetes* 49:1341–1346
 37. Rankin MM, Kushner JA 2009 Adaptive β -cell proliferation is severely restricted with advanced age. *Diabetes* 58:1365–1372
 38. Laybutt DR, Sharma A, Sgroi DC, Gaudet J, Bonner-Weir S, Weir GC 2002 Genetic regulation of metabolic pathways in β -cells disrupted by hyperglycemia. *J Biol Chem* 277:10912–10921
 39. Stepan V, Ramamoorthy S, Pausawadi N, Logsdon CD, Askari FK, Todisco A 2004 Role of small GTP binding proteins in the growth-promoting and antiapoptotic actions of gastrin. *Am J Physiol Gastrointest Liver Physiol* 287:G715–G725
 40. Ramamoorthy S, Stepan V, Todisco A 2004 Intracellular mechanisms mediating the anti-apoptotic action of gastrin. *Biochem Biophys Res Commun* 323:44–48
 41. Dufresne M, Seva C, Fourmy D 2006 Cholecystokinin and gastrin receptors. *Physiol Rev* 86:805–847
 42. Ramiya VK, Maraist M, Arfors KE, Schatz DA, Peck AB, Cornelius JG 2000 Reversal of insulin-dependent diabetes using islets generated in vitro from pancreatic stem cells. *Nat Med* 6:278–282
 43. Yatoh S, Dodge R, Akashi T, Omer A, Sharma A, Weir GC, Bonner-Weir S 2007 Differentiation of affinity-purified human pancreatic duct cells to β -cells. *Diabetes* 56:1802–1809
 44. Bouwens L, Pipeleers DG 1998 Extra-insular β cells associated with ductules are frequent in adult human pancreas. *Diabetologia* 41:629–633
 45. Rooman I, Lardon J, Flamez D, Schuit F, Bouwens L 2001 Mitogenic effect of gastrin and expression of gastrin receptors in duct-like cells of rat pancreas. *Gastroenterology* 121:940–949
 46. Saillan-Barreau C, Dufresne M, Clerc P, Sanchez D, Corominola H, Moriscot C, Guy-Crotte O, Escricuet C, Vaysse N, Gomis R, Tarasova N, Fourmy D 1999 Evidence for a functional role of the cholecystokinin-B/gastrin receptor in the human fetal and adult pancreas. *Diabetes* 48:2015–2021
 47. Morisset J, Julien S, Lainé J 2003 Localization of cholecystokinin receptor subtypes in the endocrine pancreas. *J Histochem Cytochem* 51:1501–1513
 48. Brand SJ, Tagerud S, Lambert P, Magil SG, Tatarkiewicz K, Doiron K, Yan Y 2002 Pharmacological treatment of chronic diabetes by stimulating pancreatic β -cell regeneration with systemic co-administration of EGF and gastrin. *Pharmacol Toxicol* 91:414–420
 49. Morisset J, Wong H, Walsh JH, Lainé J, Bourassa J 2000 Pancreatic CCK(B) receptors: their potential roles in somatostatin release and δ -cell proliferation. *Am J Physiol Gastrointest Liver Physiol* 279:G148–G156
 50. Zhou W, Pivoski SP, Longnecker DS, Bell Jr RH 1992 Novel expression of gastrin (cholecystokinin-B) receptors in azaserine-induced rat pancreatic carcinoma: receptor determination and characterization. *Cancer Res* 52:6905–6911
 51. Pivoski SP, Zhou W, Longnecker DS, Jensen RT, Mantey SA, Bell Jr RH 1994 Stimulation of in vivo pancreatic growth in the rat is mediated specifically by way of cholecystokinin-A receptors. *Gastroenterology* 107:1135–1146