

Gastrin induces ductal cell dedifferentiation and β -cell neogenesis after 90% pancreatectomy

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Abstract

Induction of β -cell mass regeneration is a potentially curative treatment for diabetes. We have recently found that long-term gastrin treatment results in improved metabolic control and β -cell mass expansion in 95% pancreatectomised (Px) rats. In this study, we investigated the underlying mechanisms of gastrin-induced β -cell mass expansion after Px. After 90%-Px, rats were treated with gastrin (Px+G) or vehicle (Px+V), pancreatic remnants were harvested on days 1, 3, 5, 7, and 14 and used for gene expression, protein immunolocalisation and morphometric analyses. Gastrin- and vehicle-treated Px rats showed similar blood glucose levels throughout the study. Initially, after Px, focal areas of regeneration, showing mesenchymal cells surrounding ductal structures that expressed the cholecystokinin B receptor, were identified. These focal areas of regeneration were similar in size and cell composition in the Px+G and Px+V groups. However, in the Px+G group, the ductal structures showed lower levels of keratin 20 and β -catenin (indicative of duct dedifferentiation) and higher levels of expression of neurogenin 3 and NKX6-1 (indicative of endocrine progenitor phenotype), as compared with Px+V rats. In Px+G rats, β -cell mass and the number of scattered β -cells were significantly increased compared with Px+V rats, whereas β -cell replication and apoptosis were similar in the two groups. These results indicate that gastrin treatment-enhanced dedifferentiation and reprogramming of regenerative ductal cells in Px rats, increased β -cell neogenesis and fostered β -cell mass expansion.

Key Words

- ▶ β cell mass
- ▶ dedifferentiation
- ▶ duct
- ▶ gastrin
- ▶ neogenesis
- ▶ diabetes

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Introduction

β -cell mass reduction is a central event in the development of types 1 and 2 diabetes, and β -cell regeneration is a potentially curative treatment for the disease. Strategies aiming to regenerate β -cell mass include stimulation of replication and survival of β -cells, generation of new β -cells from progenitor cells and reprogramming of other cell types

to functional β -cells. In the adult pancreas, new β -cells have been generated from different non- β -cell sources (i.e. acinar, ductal, and non- β islet-cells) through transdifferentiation and transdetermination processes (Minami *et al.* 2005, Inada *et al.* 2008, Zhou *et al.* 2008, Collombat *et al.* 2009, Thorel *et al.* 2010). The identification of molecules

that could induce or stimulate β -cell regeneration in diabetic patients is the subject of intensive research.

The major endocrine β -cell expansion takes place during the development of embryonic pancreas. Development of the pancreas occurs by the sequential differentiation of multipotent pancreatic progenitors that give rise to both, exocrine and endocrine lineages (Fishman & Melton 2002, Gu *et al.* 2002). A subpopulation of endocrine progenitor cells expressing the pro-endocrine factor neurogenin 3 (NEUROG3) appears within the multipotent pancreatic progenitor pool (Gu *et al.* 2002) and subsequent hierarchical expression of specific transcription factors results in progressive differentiation and maturation into different hormone-producing islet cells (Collombat *et al.* 2006, Murtaugh 2007, Rieck *et al.* 2012). NEUROG3 expression peaks during the major endocrine cell differentiation phase of the embryonic pancreas, known as 'secondary transition'. During this period gastrin and its high-affinity receptor cholecystokinin B receptor (CCKBR) are expressed within the endocrine domain of the developing pancreas (Larsson *et al.* 1976, Suissa *et al.* 2013). In post-natal life, pancreatic gastrin and CCKBR expression declines along with β -cell neogenesis (Rooman *et al.* 2001, Suissa *et al.* 2013). Ectopic expression of gastrin and transforming growth factor alpha in the pancreas or administration of gastrin in combination with epidermal growth factor or glucagon-like peptide 1 analogues has been shown to increase the β -cell mass and/or to improve glucose tolerance (Wang *et al.* 1993, Rooman *et al.* 2002, Suarez-Pinzon *et al.* 2005, 2008a,b), supporting a potential role of gastrin in the treatment of diabetes. In a recent study, we have found that long-term gastrin treatment improved glucose tolerance by increasing the functional β -cell mass in 95% pancreatectomised (Px) rats (Téllez *et al.* 2011).

Subtotal Px results in pancreatic regeneration with increased exocrine and endocrine cell mass expansion (Bonner-Weir *et al.* 1983, 1993, Téllez *et al.* 2011) and has been extensively used as a model for the study of pancreatic endocrine regeneration in the adult (Bonner-Weir *et al.* 1993, Xu *et al.* 1999, Fernández *et al.* 2006, Figeac *et al.* 2012). Increased β -cell replication and neogenesis have been demonstrated to play a role in β -cell regeneration after partial Px, but genetic cell lineage tracing studies have shown discrepant results with respect to the importance of neogenesis during normal growth and after pancreatic injury (Inada *et al.* 2008, Xu *et al.* 2008, Solar *et al.* 2009, Kopinke *et al.* 2011, Kopp *et al.* 2011, Al-Hasani *et al.* 2013, Courtney *et al.* 2013, Pfeifer *et al.* 2013, Xiao *et al.* 2013). After partial Px, regenerative ducts branch from the

common pancreatic duct (CPD) and acquire a progenitor-like phenotype, based on the expression of a cadre of transcription factors expressed in the developing pancreas (i.e. *Pdx1*, *Neurog3*, *Sox9*, *Hnf1b*, *Ptf1a* and *Nkx6.1*), and form focal areas of regeneration (Li *et al.* 2010). New β -cells are formed within these areas, even though their contribution to the β -cell mass expansion after Px is unclear.

In this study, we investigated the mechanisms underlying gastrin-induced β -cell expansion in 90% Px rats. Gastrin treatment enhanced the dedifferentiation and reprogramming of regenerative ductal cells, and increased β -cell neogenesis and β -cell mass regeneration.

Material 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, Sant Feliu de Codines, Spain), 4- to 5-weeks-old and weighing approximately 100 g were subjected to a 90% Px. Three groups were studied: 90%-Px rats treated with gastrin (Px+G), 90%-Px rats treated with vehicle (Px+V) and sham-Px rats treated with vehicle (S+V). Treatment with (15 leu) gastrin-17 (150 μ g/kg, Transition Therapeutics, Toronto, Ontario, Canada) (Téllez *et al.* 2011) or vehicle (PBS) was started immediately before surgery. All animals received s.c. injections every 12 h from the day of surgery until harvesting of pancreatic remnants. Fed morning plasma glucose levels were monitored daily from the snipped tail with a portable meter. The blood samples were taken approximately 12 h after the previous gastrin injection and just before the next injection.

Px and pancreas remnant harvesting

Pancreatectomy 90%-Px was performed as described previously (Bonner-Weir *et al.* 1983). Briefly, animals were anaesthetised with 5% isoflurane (Forane, Abbott) and anaesthesia was maintained with an isoflurane (1.5%)–air mixture. Ninety percent of the pancreas was removed by gentle abrasion with cotton applicators, being careful to leave major blood vessels intact; the pancreatic remnant was the tissue between the common bile duct and the first loop of the duodenum, and corresponded to $8.5 \pm 0.8\%$ of the total pancreas. Sham Px was accomplished by breaking splenic and duodenal mesenteric connections and by gently handling the pancreas with the fingertips. Immediately after surgery, the animals received one

dose (2.5 mg/kg) of meloxicam (Metacam, Boehringer Ingelheim, Germany) that was subsequently administered for the next 3 days after surgery for analgesia.

Tissue harvesting For sham-operated animals, the equivalent of the remaining 10% tissue left after 90%-Px (remnant-equivalent) was collected on day 1 ($n=3$), day 3 ($n=3$), day 5 ($n=3$), day 7 ($n=3$), and day 14 ($n=5$) after sham surgery. Tissue weight was similar among remnants from 1 to 7 days after surgery, thus pancreatic remnants were pooled into one single group (S+V, $n=12$). For Px rats, pancreatic remnants were harvested on day 1 ($n=10$), day 3 ($n=10$), day 5 ($n=10$), day 7 ($n=10$), and day 14 ($n=10$) after surgery. The excised pancreatic remnants were weighed and fixed in 4% PBS-buffered paraformaldehyde and processed for paraffin embedding. For the gene expression analysis, remnant-equivalents were harvested on days 1, 3, 5, and 7 after sham surgery and were pooled in one single group (S+V, $n=6$), and pancreatic remnants were harvested on day 1 ($n=10$), day 3 ($n=10$), day 5 ($n=8$), and day 7 ($n=8$), after 90%-Px. The tissues were rapidly immersed in RNAlater solution (Ambion, Applied Biosystems), chopped into small pieces (<5 mm) and kept overnight at 4 °C until total RNA extraction was performed.

Morphometry

β -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 and over other pancreatic tissue (Montanya & Tellez 2009). The tissue sections were stained for insulin

with a rabbit anti-human insulin antibody (antibody description given in Table 1) and visualised with the LSAB+^{HRP} system (DAKO, Carpinteria, CA, USA). A nomogram relating number of points counted to volume density and expected relative standard error in percentage of mean (<10%) was used to determine the number of intercepts needed for a representative sampling (Weibel 1979). Approximately, 20 000 points were counted in each pancreas, and at least two sections (150 μ m apart) were included for each animal.

Ductal cell mass was determined on keratin 20 (KRT20)-labelled samples using the above described procedure. At least two sections (150 μ m apart) were included for each animal. All measurements were performed by a blinded observer. The β -cell and ductal-cell relative volume was calculated by dividing the number of points over β -cells or ductal-cells by the total number of points over pancreatic tissue. Mass of β -cell and ductal cell mass was obtained by multiplying its relative volume by the pancreas weight.

The focal areas of regeneration were identified based on mesenchymal cell infiltration and observation of ductal structures with KRT20-positive cells (Li *et al.* 2010). The relative volume was determined on slides stained for KRT20/vimentin (Table 1) using the Leica Application Suite (LAS) Software (Leica Microsystems, Heerbrugg, Switzerland).

Immunostaining and image analysis

The primary antibodies are listed in Table 1. For NKX6.1 immunofluorescence, the biotin–streptavidin amplification

Table 1 Primary antibodies used for immunostaining

| Name | Source species | Dilution | Antigen retrieval | Vendor | Ref no. |
|----------------------|----------------|----------|------------------------------------|---|----------|
| Amylase | Rabbit | 1/50 | Trypsin | Sigma | A-8273 |
| B-catenin H-102 | Rabbit | 1/200 | Citrate buffer pH 6 + MW | Santa Cruz | SC-7199 |
| BrdU (KIT) | Mouse | 1/100 | HCl + trypsin | GE Health Care | RPN20 |
| CCKBR | Goat | 1/100 | Tris–EDTA buffer pH 9 + MW | Everest Biotech Ltd. (Upper Heyford, Oxfordshire, UK) | EB-06767 |
| Insulin | Guinea pig | 1/200 | Citrate buffer pH 6 + MW | Abcam (Cambridge, UK) | Ab-7842 |
| Insulin | Rabbit | 1/50 | Citrate buffer pH 6 + MW | Santa Cruz | SC-9168 |
| Krt20 | Mouse | 1/15 | Citrate buffer pH 6 + MW + trypsin | DAKO | M-7019 |
| Neurog3 ^a | Mouse | 1/1000 | DNase | DSHB (Developmental Studies Hybridoma Bank, Iowa city, IA, USA) | F25A1B3 |
| NKX6.1 ^b | Mouse | 1/100 | DNase | DSHB | F55A10 |
| PanCK | Rabbit | 1/500 | Citrate buffer pH 6 + MW + trypsin | DAKO | Z-0622 |
| Vimentin | Mouse | 1/100 | Citrate buffer pH 6 + MW + Trypsin | DAKO | M-0725 |

TSA, tyramide signal amplification; MW, microwave.

^aTSA fluorescein system was used.

^bBiotin–streptavidin-conjugated Alexa Fluor 555 amplification was used.

system was used (Invitrogen) and for NEUROG3 visualisation TSA amplification was required (TSA Plus Cyanine3/Fluorescein System, Perkin Elmer LAS, Inc., Boston, MA, USA).

For β -catenin, images were acquired with an Olympus DP70 camera-associated software under equal exposure time and brightness/contrast settings. For KRT20, images were acquired from a confocal microscope (TCS-SL Spectral Confocal Microscope, Leica, Wetzlar, Germany) using identical settings among samples. For co-expression analysis, 488 and 647 Alexa Fluor-labelled secondary antibodies were used in order to rule out false double-positive cells. Fluorescence intensity was determined by using the open-source image processing package based on ImageJ, FIJI Software (National Institutes of Health, Bethesda, MD, USA).

Cell replication

The rats were received injections of thymidine analogue 5-bromo-2'-deoxyuridine (BrdU; Sigma), 100 mg/kg, i.p. 1 h before the pancreas remnant was harvested. To assess β -cell replication, the sections were double stained with immunoperoxidase for BrdU using a Cell Proliferation Kit (GE Health Care, Amersham, Buckinghamshire, UK), and for insulin using a rabbit anti-human insulin antibody.

Ductal cell replication was determined by double immunofluorescence. For BrdU detection, the mouse anti-BrdU (1:100; Cell Proliferation Kit, GE Health Care) combined with the Alexa Fluor 488-labelled anti-mouse IgG (1:400) was used. Rabbit anti-cow cytokeratin antiserum (1:3000; DAKO) was used for ductal cell identification and Alexa Fluor 555-labelled anti-rabbit IgG (1:400) was used as secondary antibody. β -cell and ductal cell replication was expressed as percentage of BrdU-positive β - or ductal cells respectively. At least 1200 cells/pancreas were counted.

β -cell apoptosis

The sections were double stained with immunoperoxidase for apoptotic nuclei (black) with the TUNEL technique (*In Situ* Cell Death Detection Kit, ApopTag, Intergene, Oxford, UK) and for insulin (brown) using a rabbit anti-human insulin antibody (Montanya & Tellez 2009). A minimum of 1200 cells/pancreas were counted.

RNA isolation, quantification, and retrotranscription

Pancreatic tissue was resuspended with 1 ml TRIzol reagent (Sigma) for homogenisation (Ultraturrax, Janke

& Kunkel, Stauffen, Germany). Total RNA was purified according to the manufacturer's instructions (PureLink Micro-to Midi System, Invitrogen). RNA quality was assessed with the Agilent Bioanalyzer 2100 (Agilent Technologies, Inc., Santa Clara, CA, USA) and the RNA integrity number (RIN) score ranged from 6.7 to 8.3. cDNA synthesis was carried out from 5 μ g of total RNA using the Superscript III First-strand cDNA synthesis system (Invitrogen).

QPCR

PCR was run in a 7900HT Fast Real-Time PCR system (Applied Biosystems) with 384-well optical plates 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's protocol in a final volume of 20 μ l with 250 ng of cDNA in each reaction. A full listing of assays (Applied Biosystems), gene names and assay identification numbers are given in Table 2. Relative quantities (RQs) were calculated using ABI SDS 2.2.2 RQ for 7900HT Software (Applied Biosystems) and the $2^{-\Delta\Delta C_t}$ analysis method with eukariotic 18S rRNA as the endogenous control. Gene expression was expressed with a RQ value resulting from the calculations performed in the SDS2.2.2. RQ values were normalised to give a mean of 100 for control rats to aid in comparison across genes with varying basal abundance. The cholecystokinin receptor B (*Cckbr*) mRNA was undetectable in control pancreases. Thus, for analysis of *Cckbr* gene expression, a Px+V sample was used as a reference sample.

Table 2 Gene expression assays used for real-time qPCR

| Gene name | Gene symbol | Assay ID |
|---|-------------------------------|---------------|
| Carbonic anhydrase 2 | <i>Car2</i> | Rn01462065_m1 |
| Catenin (cadherin-associated protein), beta 1 | <i>Ctnnb1</i> | Rn00584431_g1 |
| Cholecystokinin B receptor | <i>Cckbr</i> | Rn00565867_m1 |
| Gastrin | <i>Gast</i> | Rn01420745_m1 |
| hnf1 homeobox B | <i>Hnf1β</i> | Rn00447453_m1 |
| Keratin 20 | <i>Krt20</i> | Rn00597548_m1 |
| Neurogenic differentiation 1 | <i>Neurod1</i> | Rn00824571_s1 |
| Neurogenin 3 | <i>Neurog3</i> | Rn00572583_s1 |
| nk6 homeobox 1 | <i>Nkx6-1</i> | Rn00581973_m1 |
| One cut homeobox 1 | <i>Onecut1/Hnf6</i> | Rn00575362_m1 |
| Pancreatic and duodenal homeobox 1 | <i>Pdx1</i> | Rn00755591_m1 |

Statistical analyses

Results were expressed as means \pm s.e.m. Analyses were performed using GraphPad Prism 4 Software (GraphPad Software Inc, La Jolla, CA, USA), and differences among means were evaluated using the Student's *t*-test or the one-way ANOVA combined with Tukey's test for *post hoc* analysis as appropriate. A *P* value of <0.05 was considered significant.

Results

Metabolic evolution

Blood glucose levels were similar between S+V, Px+V, and Px+G rats throughout the study (day 3; S+V: 101 ± 6 mg/dl; Px+V: 119 ± 5 mg/dl; Px+G: 111 ± 6 mg/dl and day 14; S+V: 111 ± 7 mg/dl; Px+V: 124 ± 6 mg/dl; Px+G: 109 ± 4 mg/dl; Fig. 1).

Upregulation of gastrin and CCKBR in pancreatic remnants early after Px

Gastrin and its high-affinity receptor CCKBR were transcriptionally upregulated on days 1 and 3 after Px in both Px+V and Px+G groups (Fig. 2A and B). CCKBR immunostaining was positive in a few acinar cells in the sham and Px groups, whereas β -cells were largely negative (Supplementary Fig. 1, see section on supplementary data given at the end of this article). The highest positivity was found in regenerative ductal cells from the focal areas of regeneration (Fig. 2C). The number of CCKBR-positive cells per unit pancreatic area was higher in pancreatic remnants from Px+V rats than in S+V rats (day 3; S+V:

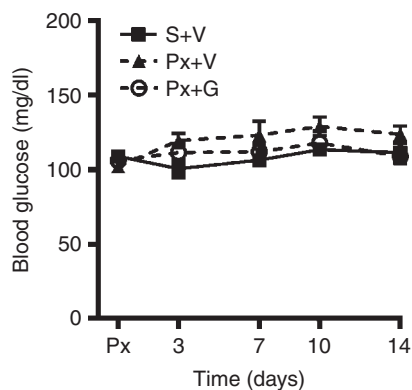


Figure 1

Metabolic evolution of sham-operated and Px rats. Blood glucose concentrations of sham (S+V) and Px rats treated with vehicle (Px+V) or with gastrin (Px+G). Values are means \pm s.e.m. $n \geq 5$.

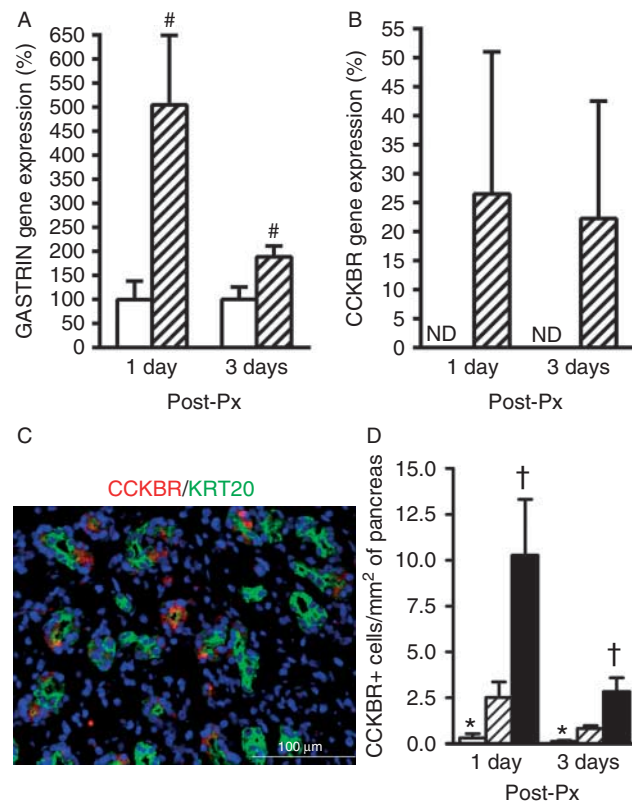


Figure 2

Gastrin and CCKBR expression following 90%-Px. Gene expression of (A) gastrin and (B) CCKBR in pancreatic remnant-equivalents from sham-Px rats (S+V, white bars) and pancreatic remnants from 90%-Px rats (Px+V, hatched bars), 1 and 3 days after surgery. (C) Representative image of CCKBR (red) and KRT20 (green) double immunofluorescence of pancreatic remnants from Px+V rats. An area of regeneration is depicted. (D) Quantification of CCKBR expressing cells per mm² of pancreas in sham (S+V, white bars) and Px rats-treated with vehicle (Px+V, hatched bars) or with gastrin (Px+G, black bars). Values are means \pm s.e.m. #*P* < 0.05 vs Px+V for Student's *t*-test. ANOVA, *P* < 0.05; **P* < 0.05 vs all other groups; and †*P* < 0.05 vs Px+V for Tukey's test. ND, not detected.

0.12 ± 0.05 cells/mm² of pancreas, Px+V: 0.83 ± 0.15 cells/mm² of pancreas; *P* = 0.004) and was further increased by gastrin treatment (Px+G: 2.84 ± 0.75 cells/mm² of pancreas; *P* = 0.03) (Fig. 2D).

Similar ductal cell expansion in gastrin- and vehicle-treated rats

On days 1 and 3 after 90% Px, areas of regeneration with ductal structures surrounded by mesenchymal tissue can be easily identified and distinguished from the rest of the pancreatic remnant. The appearance of islet endocrine cells within these areas of regeneration is considered indicative of islet neogenesis (Bonner-Weir *et al.* 1993,

Li *et al.* 2010). On day 1 after Px, the epithelial ductal cells of the CPD showed strong BrdU incorporation; on day 3, BrdU was mainly detected within the focal areas of regeneration. These focal areas of regeneration contained KRT20-positive ductal cells (regenerative ducts) surrounded by mesenchymal cells (Fig. 3A), and increased in size from days 1 to 3 after Px (Fig. 3B). Pancreatic remnant weight was similar in both Px groups (day 3; Px+V: 107.2 ± 7.64 mg; Px+G: 99.2 ± 5.7 mg, $P=0.42$) and the KRT20-positive cell relative area was similarly increased in both Px groups compared with sham-operated rats (Fig. 3C), indicating that gastrin did not further increase the ductal cell mass and areas of regeneration after Px.

Enhanced ductal cell dedifferentiation in gastrin-treated rats

Gene expression was analysed in the entire pancreatic remnants of Px+V and Px+G rats and in the whole remnant-equivalents of S+V rats. Initially after Px, gastrin-treated pancreatic remnants showed reduced gene expression levels of ductal- and epithelial-cell markers compared with Px+V remnants (Fig. 4A) despite the similar increment in the relative area of ductal cell found in both Px groups (Fig. 3C).

In pancreases of sham-operated rats, KRT20 was exclusively expressed in epithelial ductal cells and centroacinar/terminal ductal cells, with similar immunofluorescence

intensity among different types of ducts (CPD, interlobular and intralobular ducts, data not shown). In Px animals, KRT20 was expressed in the ductal cells of both the focal areas of regeneration and of the rest of the remnant, although the former exhibited lower KRT20 immunofluorescence intensity (Supplementary Fig. 2A and B, see section on supplementary data given at the end of this article). In gastrin-treated animals, KRT20 fluorescence intensity was even lower than in Px+V remnants (Fig. 4B and C), paralleling the lower gene expression of *Krt20* in this group.

β -catenin immunoreactivity was found in the plasma membranes of ductal, acinar and islet cells in pancreases of sham-operated animals, with higher immunofluorescence intensity in ductal than in acinar cells (Supplementary Fig. 2C and D). In pancreatic remnants, ductal cells in the focal areas of regeneration showed lower immunoreactivity for β -catenin than that for native ducts (Supplementary Fig. 2E and F), and it was further reduced in Px+G remnants (Fig. 4D and E).

The ductal cells in focal areas of regeneration of Px+G remnants showed higher cell replication than those of the Px+V group (Px+V, $2.02 \pm 0.47\%$ and Px+G, $4.23 \pm 0.90\%$; $P<0.05$; Fig. 4F). Overall, these results indicate that the presence of ductal-cell dedifferentiation after Px was enhanced by gastrin treatment.

Increased expression of transcription factors involved in endocrine specification by gastrin treatment

After Px, differentiation of the focal areas of regeneration into an endocrine phenotype involves the expression of pancreatic and endocrine progenitor markers in regenerative ducts, recapitulating embryonic pancreas development (Li *et al.* 2010).

Gene expression of *Neurog3*, *Neurod1* and *Pdx1* was similar in S+V and Px+V remnants at all time points (Fig. 5A). *Nkx6.1* gene expression was doubled in Px+V remnants compared with S+V on day 3 after Px, but differences were not statistically significant (Fig. 5A). In contrast, in Px+G remnants gene expression of *Neurog3* and *Neurod1* on day 1 and of *Pdx1* and *Nkx6-1* on day 3 after Px were significantly increased compared with sham and Px+V groups (Fig. 5A).

NEUROG3 and NKX6-1 proteins were detected in the ductal cells from the focal areas of regeneration (Fig. 5B and C). On day 3 after Px, the expression of NKX6.1 in KRT20-positive cells was significantly increased in gastrin-treated animals (Px+G, $57.6 \pm 3.9\%$ and Px+V, $41.7 \pm 0.7\%$; $P=0.028$; Fig. 5D).

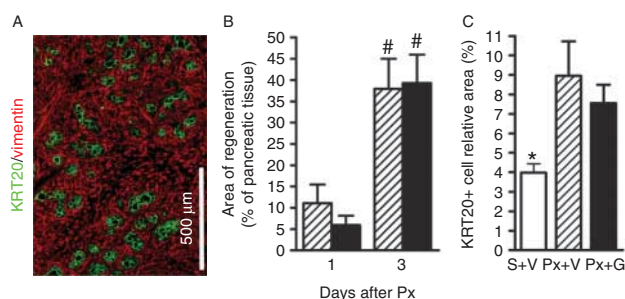
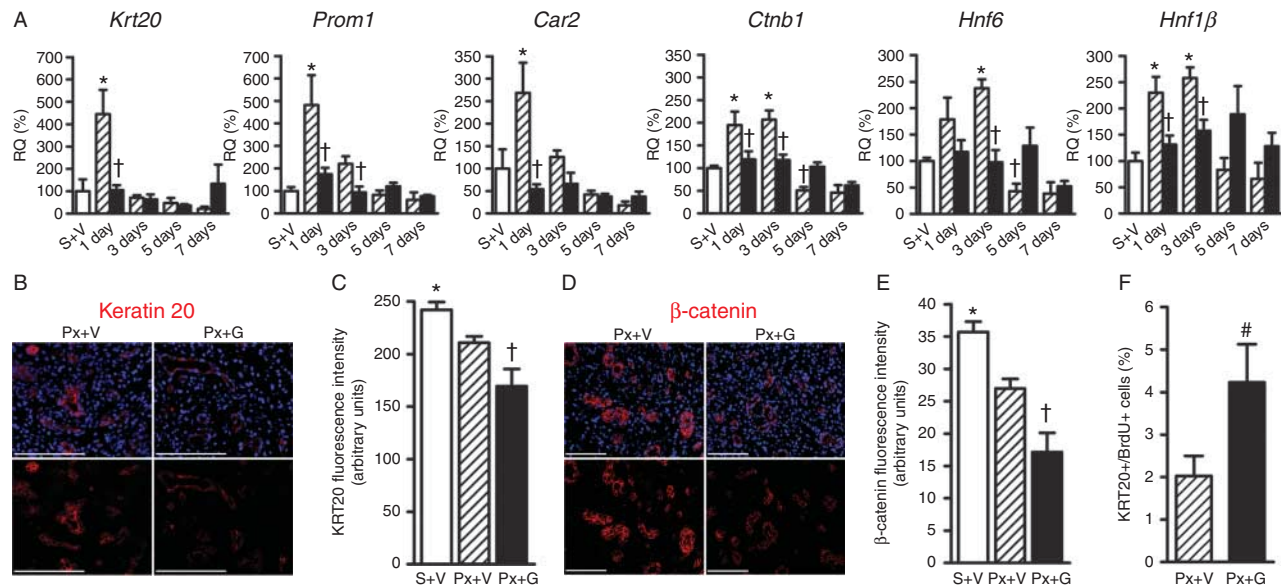


Figure 3 Focal areas of regeneration and ductal-cell relative volume after pancreatectomy (Px). (A) Representative fluorescence microscopy image of keratin 20 (KRT20) (Alexa Fluor 488, green) and vimentin (Alexa Fluor 555, red) in focal areas of regeneration in pancreatic remnants of Px rats, 3 days after Px. (B) Relative volume of the focal areas of regeneration in pancreatic remnants from Px+V (hatched bars) and Px+G (black bars) rats, 1 and 3 days after Px. (C) KRT20+ cell relative volume in pancreatic remnant-equivalents from sham-Px (S+V, white bars), and pancreatic remnants from Px+V (hatched bars) and Px+G (black bars) rats, 3 days after surgery. Values are means \pm s.e.m. from five independent experiments. # $P<0.05$ vs 1 day after Px for Student's *t*-test. ANOVA, $P<0.05$; * $P<0.05$ vs other groups for Tukey's test.

**Figure 4**

Ductal and epithelial cell markers expression after Px and gastrin treatment. (A) Gene expression profile of ductal markers in remnant-equivalents from sham-Px rats (S+V, white bars) and pancreatic remnants from Px+V (hatched bars) and Px+G (black bars) rats on days 1, 3, 5, and 7 after surgery. (B) Confocal microscopy images of keratin 20 (KRT20) immunofluorescence (Alexa Fluor 555, red) in areas of regeneration from pancreatic remnants of Px+V and Px+G rats, 3 days after Px. (C) KRT20 immunofluorescence intensity quantification. (D) Fluorescence microscopy

images of β-catenin immunofluorescence (Alexa Fluor 555, red) in areas of regeneration from pancreatic remnants of Px+V and Px+G rats, 3 days after Px. (E) β-catenin immunofluorescence intensity quantification. (F) Ductal cell replication in the focal areas of regeneration of pancreatic remnants from Px+V and Px+G rats 3 days after Px. Px, pancreatectomy; RQ, relative quantity. Values are means ± s.e.m. from five independent experiments. ANOVA, $P < 0.05$; * $P < 0.05$ vs Px+V and † $P < 0.05$ vs Px+V for Tukey's test. # $P < 0.05$ for Student's *t*-test.

Increased β-cell neogenesis and mass in gastrin-treated rats

On day 3 after surgery, single β-cells or small β-cell clusters were found within the areas of regeneration continuous to or in close contact with ducts (Fig. 6A), at a density of 3 ± 0.33 and 8 ± 1.21 insulin-positive cells/mm² in Px+V and Px+G pancreases respectively ($P = 0.004$). The number of small β-cell clusters (less than five insulin-positive cells) within the regenerative pancreas was significantly increased in Px+G remnants (Fig. 6B).

On day 3 after surgery, β-cell apoptosis was similar in both Px groups and 2.5 times higher compared with sham-operated rats, but differences did not reach statistical significance (Fig. 6C). β-cell replication was increased similarly in Px groups compared with sham-operated rats (Fig. 6D). Replication was increased in β-cells within the focal areas of regeneration compared with those in the non-regenerating pancreas in Px+V and Px+G rats (Fig. 6E).

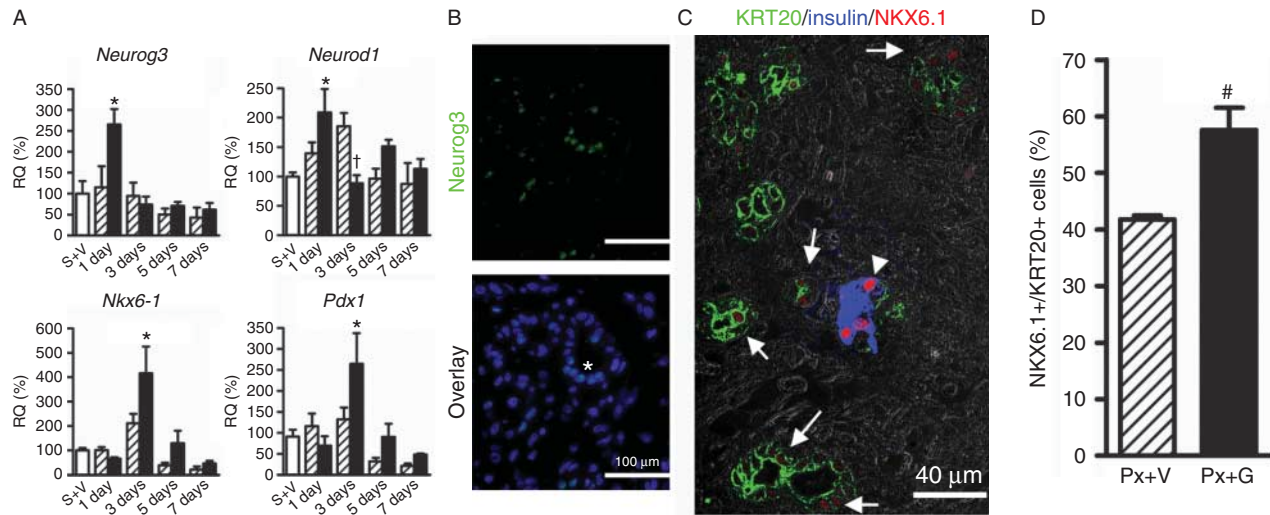
β-cell relative area was similar between Px groups on day 1 after Px. On day 3, it was significantly increased in Px+G, compared with Px+V rats, and remained higher

on day 14 (Fig. 6F). Accordingly, β-cell mass was increased in Px+G rats on days 3 and 14 after Px compared with Px+V and S+V rats. As previously reported, β-cell mass was increased in Px+V rats compared with S+V, on day 14 (Fig. 6G).

Discussion

In this study, we showed that the expansion of the ductal cell compartment in the regenerative pancreas was paralleled by downregulation of KRT20 and β-catenin and by NKX6-1 expression in ductal cells. Gastrin treatment enhanced Px-induced ductal cell dedifferentiation, further stimulated ductal cell NKX6-1 expression in the regenerative pancreas, increased the number of newly formed β-cells and fostered a rapid β-cell mass expansion.

Gastrin and its high-affinity receptor, CCKBR, were upregulated after 90% Px, providing a mechanistic explanation for the effects of gastrin treatment. In addition, this expression of gastrin and CCKBR has similarities with their expression during the secondary transition in embryonic pancreas development (Larsson

**Figure 5**

Effects of gastrin treatment on the expression of transcription factors involved in endocrine specification. (A) Gene expression profile of transcription factors involved in endocrine specification in pancreatic remnant equivalents from sham-Px rats (S+V; white bars) and pancreatic remnants from Px+V (hatched bars) and Px+G (black bars) rats. (B) Fluorescence microscopy images of neurogenin 3 (NEUROG3) immunofluorescence (fluorescein, green) in the focal areas of regeneration from pancreatic remnants on day 1 after Px. NEUROG3 positivity is found in epithelial ductal cells (asterisk) and in the surrounding mesenchymal cells. (C) Confocal microscopy images of keratin 20 (KRT20; Alexa Fluor 488,

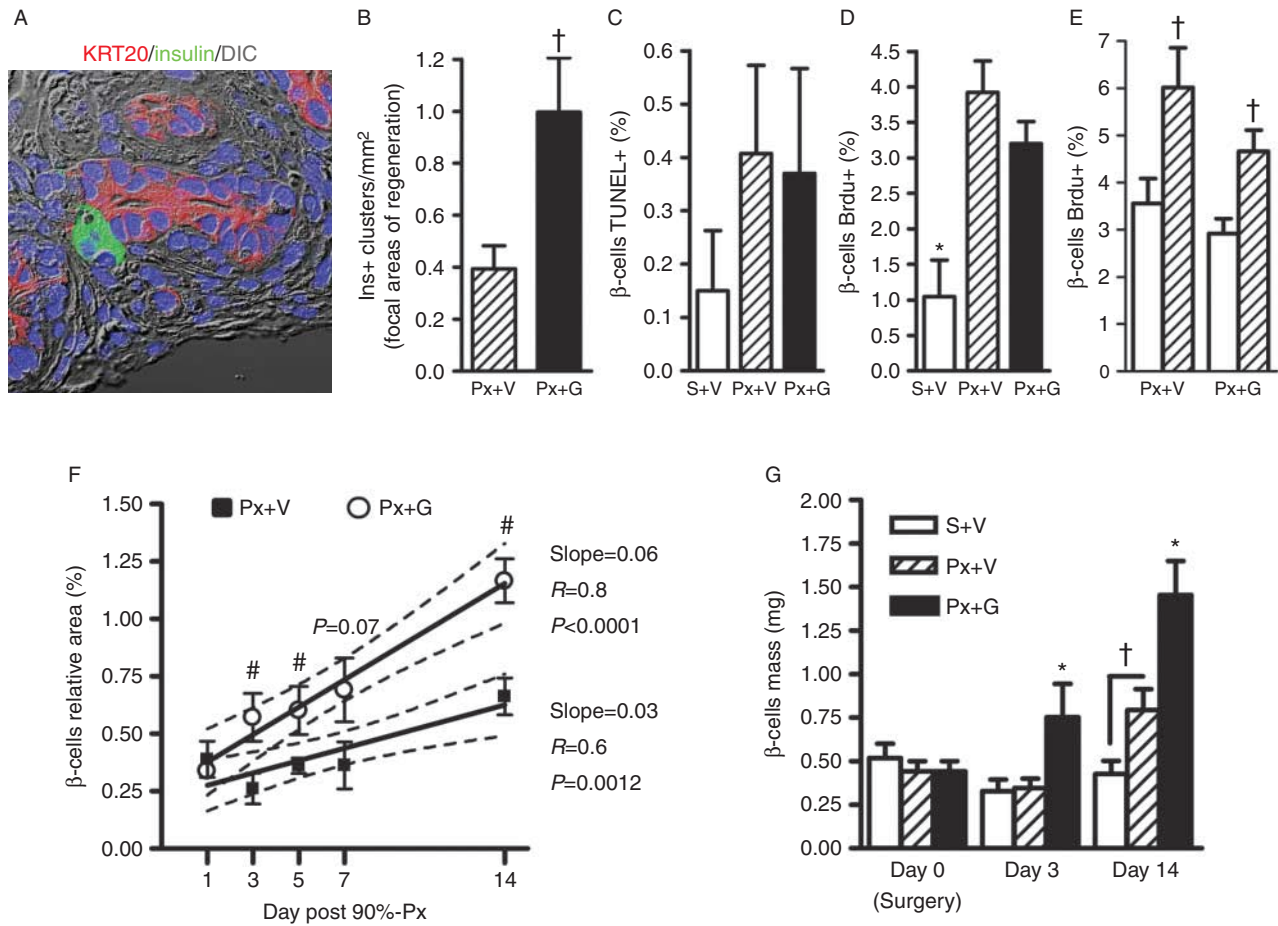
green), insulin (Alexa Fluor 647, blue) and NKX6.1 (Alexa Fluor 555, red) immunofluorescence in the focal areas of regeneration from pancreatic remnants 3 days following Px. Ductal structures with low expression of NKX6.1 in the nuclei (arrows) and insulin clusters with high expression of NKX6.1 in the nuclei (arrow head) are shown. (D) Quantification of KRT20 positive cells, within the focal areas of regeneration, expressing low levels of NKX6.1. Px, pancreatectomy. Values are means \pm s.e.m. ANOVA, $P < 0.05$; * $P < 0.05$ vs all other groups and $^{\dagger}P < 0.05$ vs Px+V for Tukey's test. $^{\#}P < 0.05$ for Student's *t*-test.

et al. 1976, Suissa *et al.* 2013), when a burst of endocrine β -cell differentiation takes place.

Gastrin treatment did not modify ductal cell mass expansion after Px, but enhanced the dedifferentiation of ductal cells in the regenerative pancreas. Ductal cell mass was similar in gastrin- and vehicle-treated Px rats as shown by the similarly increased ductal cell relative volume and focal areas of regeneration in pancreatic remnants in the two groups. In contrast, gene expression of ductal cell markers (*Krt20*, *Car2*, *Prom1*, *Hnf1b* and *Hnf6*) in the pancreatic remnants of gastrin-treated rats was lower compared with vehicle-treated rats. Ductal cells in the focal areas of regeneration of pancreatic remnants from vehicle-treated Px rats showed reduced expression of KRT20 and β -catenin, supporting the previously reported acquisition of a dedifferentiated phenotype in regenerative ductal cells (Li *et al.* 2010). Gastrin treatment resulted in further dedifferentiation of ductal cells in the regenerative pancreas, as indicated by the even lower expression of KRT20 and β -catenin that was concordant with the lower gene expression of ductal cell markers in this group compared with vehicle-treated Px rats. Mature ductal cells are characterised by strong and polarised KRT20 expression

(Bouwens *et al.* 1995), while multipotent embryonic ductal cells show absent or minor unpolarised expression of the intermediate filament (Di Bella *et al.* 2009). In addition, KRT20 expression has been found to be downregulated in the *in vitro* human ductal-to- β -cell differentiation process (Dodge *et al.* 2009, Téllez *et al.* 2013), supporting differential KRT20 expression in ductal cells depending on the maturation status. On the other hand, the reduced cytoplasmic accumulation of β -catenin in newly formed regenerative ducts could facilitate delamination of endocrine precursors from ductal structures as found in embryonic pancreas development (Kesavan *et al.* 2009, Gouzi *et al.* 2011). It has been recently reported that loss of E-cadherin accumulation in the plasma membrane by sustained expression of *Neurog3* in the mouse endoderm is sufficient to trigger cell delamination from the endodermal epithelium (Gouzi *et al.* 2011). Thus, the lower gene expression of ductal cell markers and KRT20 and β -catenin immunoreactivity indicates enhanced ductal dedifferentiation in gastrin-treated rats after Px.

Pancreatic remnants of gastrin-treated rats showed transiently increased gene expression of the endocrine progenitor markers *Neurog3*, *Neurod1*, *Pdx1*, and *Nkx6-1*

**Figure 6**

Increased β -cell neogenesis and mass in gastrin-treated rats. (A) Representative confocal microscopy image of keratin 20 immunofluorescence (Alexa Fluor 647, red) and insulin (Alexa Fluor 488, green) of areas of regeneration from pancreatic remnants, 3 days after pancreatectomy (Px). (B) Number of small β -cell clusters per mm^2 of regenerative pancreas. (C) β -cell apoptosis and (D) β -cell replication in pancreatic remnant equivalents of sham-operated pancreases (S+V, white bars) and pancreatic remnants from Px+V (hatched bars) and Px+G (black bars) rats, 3 days after surgery. (E) Cell replication of β -cells located within the non-

regenerative (white bars) or regenerative (hatched bars) pancreas, 3 days after pancreatectomy. (F) Evolution of the β -cell relative area after 90%-Px in pancreatic remnants from Px+V (black squares) and Px+G (open circles). (G) β -cell mass of pancreatic remnant equivalents of sham pancreas (S+V, white bars) and pancreatic remnants from Px+V (hatched bars) and Px+G (black bars) rats, the day of surgery, 3 and 14 days after Px. Values are means \pm s.e.m. Student's *t*-test, $^{\dagger}P < 0.05$. ANOVA, $P < 0.05$; $\#P < 0.05$ vs Px+V group for Student's *t*-test; $^*P < 0.05$ vs all other groups for Tukey's test.

initially after surgery, indicating that gastrin treatment enhanced Px-induced cell progenitor competence of ductal cells in the regenerative pancreas. *Neurog3* is required for pancreatic endocrine cell differentiation during embryonic development (Apelqvist *et al.* 1999, Gradwohl *et al.* 2000, Schwitzgebel *et al.* 2000, Grapin-Botton *et al.* 2001). In the post-natal pancreas, *Neurog3* expression is clearly downregulated and it is only very modestly expressed in mature islet cells (Gu *et al.* 2002, Kodama *et al.* 2005, Dror *et al.* 2007, Joglekar *et al.* 2007, Wang *et al.* 2009). In rodent models of pancreatic endocrine regeneration, *Neurog3* gene expression is significantly upregulated (Xu *et al.* 2008, Solar *et al.* 2009,

Kopp *et al.* 2011) and it has been found in the pre-existing β -cells (Kopp *et al.* 2011, Xiao *et al.* 2013), within ducts (Xu *et al.* 2008) and/or in mesenchymal cells next to ducts (Al-Hasani *et al.* 2013, Courtney *et al.* 2013, Pfeifer *et al.* 2013). In this study, we have found *Neurog3* expression in regenerative ducts after Px, in accordance with previous data (Li *et al.* 2010). We have identified the presence of mesenchymal cells positive for *Neurog3* surrounding the regenerative ducts. A similar finding has been recently reported in a mouse model of induced β -cell neogenesis by *Pax4* misexpression in α -cells, where *Neurog3*-induced epithelial-to-mesenchymal transition (EMT) was identified in duct-lining cells (Al-Hasani

et al. 2013). It has recently been reported that sustained expression of *Neurog3* in the mouse endoderm is sufficient to trigger EMT and cell delamination from the endodermal epithelium (Gouzi *et al.* 2011). Therefore, the expression of *Neurog3* in duct cells and in mesenchymal cells may indicate that EMT occurred in the ductal epithelium of focal areas of regeneration.

Nuclear NKX6-1 expression was found in some regenerative ductal cells in Px+V rats and it was significantly increased in pancreatic remnants of gastrin-treated rats. NKX6-1 is required for the development of endocrine α and β cells in the foetal pancreas (Henseleit *et al.* 2005) and drives pancreatic progenitor cells into the endocrine/ductal cell lineage. Specifically, continuous expression of NKX6-1 in the entire pancreatic progenitor pool significantly represses *Ptf1a* expression, preventing acinar cell differentiation, and favours an endocrine over a ductal cell fate choice (Schaffer *et al.* 2010). In the post-natal pancreas, nuclear NKX6-1 expression is restricted to endocrine β -cells (Sander *et al.* 2000), and it has been shown that induction of NKX6-1 expression in PDX1-expressing liver cells promotes endocrine cell reprogramming (Gefen-Halevi *et al.* 2010). The presence of NEUROG3 and NKX6-1 expression in a subpopulation of ductal cells indicates that these cells have an endocrine progenitor-like phenotype. Their increased number in the gastrin-treated group provides a potential mechanism for the increased neogenesis in these animals.

The increased β -cell relative area and mass found in Px+G group compared with Px+V group pancreatic remnants initially after Px was most probably due to differences in β -cell neogenesis, because β -cell replication, apoptosis and pancreas weight were similar for the two Px groups. Differences in β -cell relative area and mass between the Px+V and Px+G groups persisted 2 weeks after Px, indicating that early expansion of the β -cell compartment determined the long-term evolution of β -cell mass.

This study supports the concept that after partial Px, in addition to β -cell replication, new β -cells are also formed by neogenesis, that neogenesis contributes to β -cell mass expansion and that the process is enhanced by gastrin treatment. The lower expression of KRT20 and β -catenin in the regenerating ducts of gastrin-treated Px rats and the increased number of duct cells expressing NKX6-1 indicate that gastrin enhanced dedifferentiation and progenitor competence of the ductal compartment of the pancreas. We propose that this would lead to an enrichment of the endocrine progenitor pool, which could represent a source of new endocrine cells.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-14-0222>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

N T researched data, contributed to the discussion, wrote, reviewed, and edited the manuscript. E M contributed to the discussion and reviewed the manuscript.

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