Rapid Publication

Regeneration of Pancreatic Islets After Partial Pancreatectomy in Mice Does Not Involve the Reactivation of Neurogenin-3

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Understanding the factors and mechanisms involved in β-cell regeneration will guide therapeutic efforts to augment β-cell mass in patients with diabetes. Neurogenin-3 (Ngn3) is a bHLH transcription factor that responds to Notch signaling and whose expression marks endocrine progenitors. During fetal development, all endocrine cells are derived from Ngn3$^+$ precursors. Although expression of Ngn3 in the adult pancreas has not been reported, it has been suggested that islet regeneration in adult organisms recapitulates embryonic developmental pathways. Here, we investigated whether β-cell regeneration in adult mice recapitulates the embryonic pathway involving Ngn3 activation. Despite full recovery of β-cell mass after 50% partial pancreatectomy (Ppx) in BALB/c mice, no pancreatic Ngn3 immunoreactivity was detected, even when the β-cell trophic glucagon-like peptide-1 receptor agonist exendin-4 was administered after the procedure. Even when we used the stable expression of enhanced green fluorescent protein (EGFP) in *Ngn3*$^{EGFP/+}$ mice to trace Ngn3 expression after Ppx, no pancreatic Ngn3 expression was detected. Although ectopic expression of Ngn3 can promote an endocrine transcriptional program in adult cells and may thus have therapeutic potential in the development of surrogate β-cells, our studies indicate that a reactivation of endogenous Ngn3 expression is not required for adult β-cell regeneration in vivo. *Diabetes* 55: 269–272, 2006

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Received for publication 5 October 2005 and accepted in revised form 5 December 2005.

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Posted on the World Wide Web at http://diabetes.diabetesjournals.org on 16 December 2005.

EGFP, enhanced green fluorescent protein; Ngn3, neurogenin-3; Ppx, partial pancreatectomy.

A failure of β-cell mass is now recognized to play a pathogenetic role in both type 1 and type 2 diabetes. Whereas transplantation with donor islets (and potentially surrogate sources of β-cells) has achieved some success in clinical trials, an alternative approach is the stimulation of endogenous β-cell regeneration in diabetic patients. Thus, an understanding of the factors and mechanisms involved in β-cell formation and regeneration will inform therapeutic efforts to augment β-cell mass in patients with diabetes.

During pancreas development, the endocrine compartment in the islets of Langerhans differentiates progressively into five main endocrine cell types, the α-, β-, δ-, ε-, and PP-cells, which express glucagon, insulin, somatostatin, ghrelin, and pancreatic polypeptide, respectively, as their principal hormone products (1,2). Cell fate is determined by a tightly regulated hierarchy of transcription factors, of which neurogenin-3 (Ngn3) is the earliest factor that specifically regulates the development of the endocrine compartment (rev. in 2). *Ngn3*$^{-/-}$ mice have absolutely no endocrine islet development (3), and transgenic overexpression of Ngn3 results in the activation of an islet differentiation program in vivo and in cultured pancreatic ductal cell lines (4–7).

Ngn3 mRNA is first detected in the dorsal pancreatic epithelium at embryonic day 9 (E9.0) in the mouse. As the pancreas develops, the number of *Ngn3*$^+$ cells increases and peaks at ~E15.5. Expression then gradually declines but is still detectable in the neonatal period (4,7). Interestingly, both *Ngn3* mRNA and protein are undetectable in insulin- and glucagon-producing cells, suggesting that the expression of *Ngn3* is extinguished before the final differentiation of the hormone-producing cells (4,7). This transient expression activation of Ngn3 may result from its ability to negatively autoregulate its own expression (8). Taken together, these data indicate that Ngn3 expression marks an islet progenitor pool, a concept that is supported by recent lineage-tracing studies (9,10).

Although expression of Ngn3 in the adult pancreas has not been reported, it has been suggested that islet regeneration in adult organisms recapitulates embryonic developmental pathways (11). However, recent evidence indicates that replication of existing β-cells may be the primary mechanism of renewal in adult organisms (12,13). Yet, others have suggested the existence of intraislet progenitor pools, which may result in neogenesis via pathways distinct from those activated during embryonic development (14,15). Here, we sought to determine

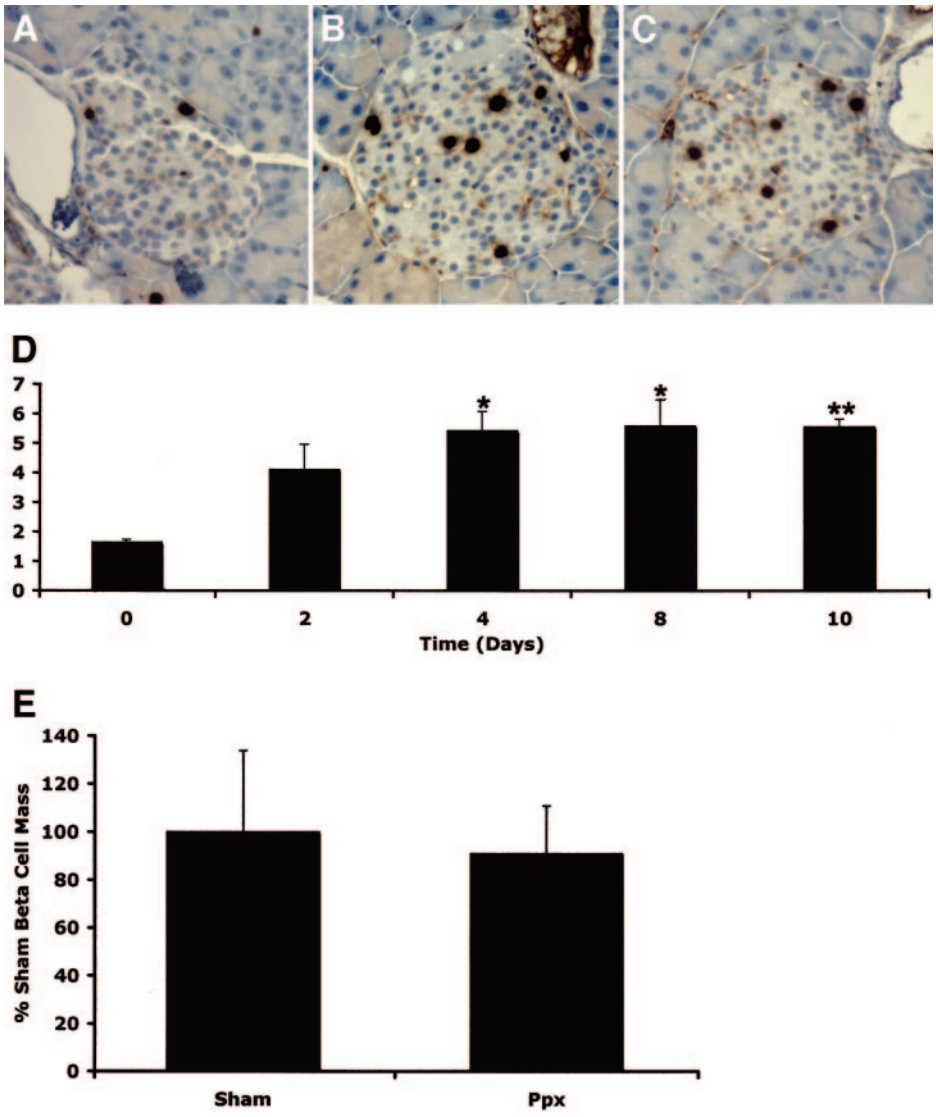

FIG. 1. β-Cell mass fully regenerates after 50% Ppx in BALB/c mice. BrdU incorporation into islet cells of control (*A*) and Ppx mice at 2 (*B*) and 10 (*C*) days after surgery. *D*: Quantitation of percentage of $BrdU^+$ islet cells on days 0–10 after Ppx (n = 2–3/time point; *$P < 0.05$, **$P < 0.01$ vs. day 0). *E*: Full recovery of β-cell mass 4 weeks after 50% Ppx (n = 3/group; P = NS).

whether β-cell regeneration in the adult organism recapitulates the embryonic pathway involving Ngn3 activation.

RESEARCH DESIGN AND METHODS

Eight- to 10-week-old male BALB/c mice were purchased from Charles River Laboratories and housed under standard conditions. Female *Ngn3*$^{EGFP/+}$ mice (age 8 weeks) were generated as previously described (16) and maintained on a mixed Sv129/C57Bl6 genetic background. All procedures were approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

Surgical model. Mice were anesthetized by administration of Nembutal Sodium solution (50 mg/kg i.p.). The abdomen was opened through a left lateral incision. The entire splenic portion of the pancreas was surgically removed, resulting in an ~50% pancreatectomy, confirmed by weighing the removed and remnant portions during a pilot study. Sham operation was performed by opening the abdomen while leaving the pancreas intact. The incision was closed using 4-0 silk for the peritoneum and clips for the skin.

Islet replication and β-cell mass. For islet replication measurements, BrdU (200 mg/kg) was administered 6 h before sacrifice. BrdU incorporation was assessed using a BrdU-specific mouse monoclonal antiserum (Sigma, St. Louis, MO). At least 1,200 islet endocrine cell nuclei were counted per pancreas. The rate of proliferation is expressed as the percentage of islet cell nuclei that are also BrdU positive. BrdU incorporation was determined at 0, 2, 4, 8, and 10 days after pancreatectomy.

β-Cell mass was determined 5 weeks after partial pancreatectomy by point-counting morphometry, as previously described (17). A section through the maximal footprint region of the pancreas was stained with guinea pig anti-human insulin (1:5,000; Linco Research, St. Charles, MO). The secondary antiserum was biotinylated donkey anti–guinea pig (1:200; Vector Laboratories, Burlingame, CA), and color development was with the ABC kit and diamino benzidine (Vector Laboratories, Burlingame, CA). Sections were evaluated using a Cool Snap digital camera (Photometrics, Huntington Beach, CA) connected to an Eclipse E600 microscope (Nikon). Using a 9 × 10 grid of points, the percentage of points overlying β-cells was determined and then multiplied by the weight of the pancreas to determine the mass of β-cells. At least 200 random fields were assessed for each pancreas.

Embryo dissection. E15.5 embryos were dissected from timed pregnancies. Digestive tracts, including liver, stomach, pancreas, and intestine, were removed and placed in 1× PBS. Enhanced green fluorescent protein (EGFP) expression was visualized and images were captured using a Leica dissecting microscope (Leica MZF III).

Whole-mount immunofluorescence. After vibratome sectioning, tissue slices were placed in 1× PBS in 24 wells and rinsed three times with 1× PBS. Tissues were washed in 1× PBS/0.1% TritonX for 40 min at room temperature and then blocked in 1× PBS/0.1% TritonX/5% BSA on a rotator at room temperature for 1 h. Guinea pig anti-insulin antibody (Linco) was diluted with 1× PBS/0.1% TritonX/5% BSA at 1:1,000 and incubated with the sections overnight at 4°C. Tissues were washed three times in 1× PBS/0.1% TritonX/1% BSA for 1.5 h, and anti–guinea pig antibody conjugated to rhodamine was added at 1:50 in 1× PBS/0.1% TritonX/5% BSA for 2 h at room temperature. Tissues were washed three times in 1× PBS/0.1% TritonX/1% BSA for 1.5 h, rinsed once in 1× PBS, mounted in 50% glycerol/PBS, and examined using confocal microscopy.

Ngn3 expression. Ngn3 immunostaining was carried out as we described previously (18). Ngn3-EGFP expression was assessed in pancreatic and intestinal tissues harvested 4 days, 2 days, and 2 h postpancreatectomy. Tissues were fixed in 4% paraformaldehyde for 40 min, rinsed three times in 1× PBS, and then embedded in 4% agarose/PBS for vibratome sectioning.

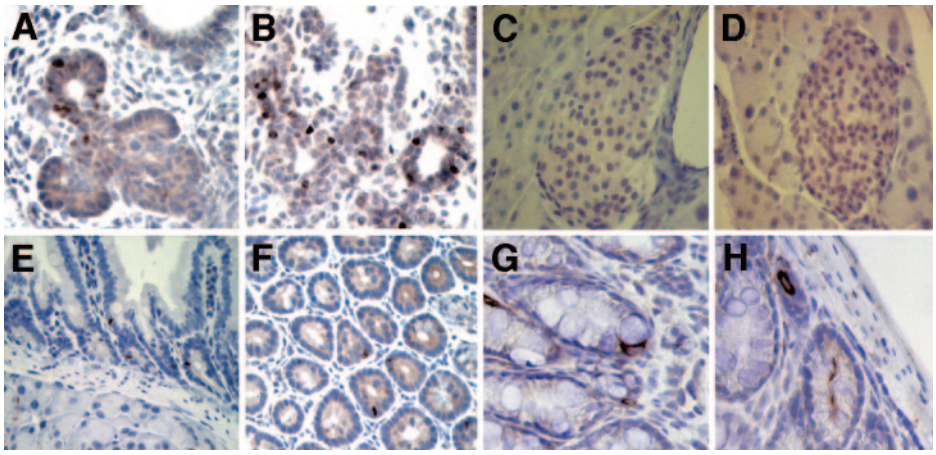

FIG. 2. Ngn3 protein is undetectable after 50% Ppx in BALB/c mice. Expression of Ngn3 in E13.5 (*A*) and E15.5 (*B*) embryonic pancreatic epithelium but not adult islets of sham-operated (*C*) or 50% Ppx (*D*) mice 24 h after surgery. Ngn3 is detected in the nucleus (*E* and *F*) and cytoplasm (*G* and *H*) of a subset of small intestinal epithelial cells in adult sham and Ppx mice.

Tissue sections (150 μm) were cut, placed on microscope slides, and mounted in 50% glycerol/PBS. Slides were examined using confocal microscopy (Leica).

RESULTS AND DISCUSSION

We sought to determine whether β-cell regeneration in adult mice recapitulates the embryonic pathway involving the proendocrine transcription factor Ngn3. The 50% partial pancreatectomy (Ppx) model was chosen because β-cell mass fully regenerates in this paradigm. Islet replication after 50% Ppx in BALB/c mice increases from a basal rate of 1.7% to ~5–6% by day 4 after surgery and remains elevated until at least day 10 (Fig. 1*A–D*). This correlates well with the nearly complete recovery of β-cell mass by 4 weeks after Ppx (Fig. 1*E*). In contrast, pancreatic organ weight only partially recovers to 65 ± 0.98% of sham-operated pancreas weight ($P = 0.003$), suggesting that the endocrine compartment has greater regenerative capacity than the exocrine compartment in this surgical model and genetic background.

To determine whether β-cell regeneration involves a reactivation of Ngn3 expression, we first examined Ngn3 immunoreactivity at 0, 0.5, 1, 2, 3, 4, 6, 8, and 10 days after Ppx. Although our conditions permitted detection of Ngn3 during fetal development (Fig. 2*A* and *B*), we failed to detect specific Ngn3 staining in the pancreas at any post-Ppx time points (Fig. 2*C* and *D*). In contrast, strong nuclear and cytoplasmic Ngn3 staining of a subset of intestinal crypt epithelial cells was detected in both sham and Ppx mice (Fig. 2*E–H*), confirming the efficacy of the staining protocol in adults.

Administration of the glucagon-like peptide-1 receptor agonist exendin-4 stimulates pancreatic Pdx-1 expression in mice (19) and augments the regeneration of β-cell mass after 90% Ppx in rats and mice (20 and D.D.L., D.A.S., unpublished observations). Therefore, we next examined Ngn3 expression in mice that received exendin-4 (1 nmol/kg i.p. once daily) for 10 days after surgery. Tissues were harvested at the same time points as described above (0–10 days). Once again, we detected no pancreatic Ngn3 immunoreactivity at any time point (data not shown).

We considered the possibility that Ngn3 reactivation in this model occurs during a narrow temporal window that escaped detection due to the short half-life of Ngn3 protein and the spacing of the time points chosen for analysis. To address this issue, we used *Ngn3*$^{EGFP/+}$ mice in which one Ngn3 allele is replaced by EGFP (16). Due to the long half-life of EGFP in vivo, even transient activation of the Ngn3 locus would result in EGFP expression that is easily

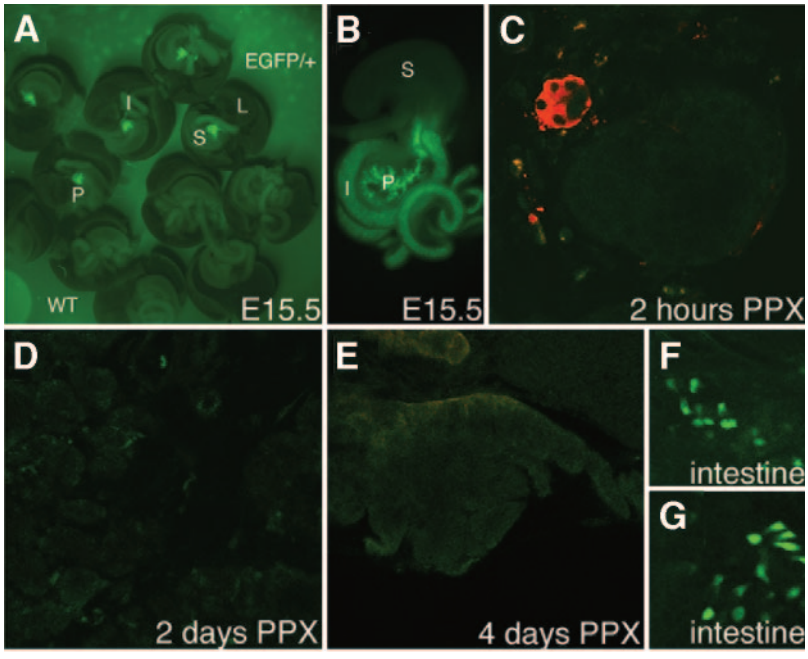

FIG. 3. The Ngn3 locus fails to be reactivated after 50% Ppx. *A*: Ngn3-EGFP expression was detected in the gastrointestinal organs of E15.5 heterozygous embryos but not in wild-type embryos. *B*: Enlarged image of the digestive tract of a E15.5 heterozygous embryo. Ngn3-EGFP expression was not induced in 8-week-old heterozygous animals 2 h (*C*), 2 days (*D*), and 4 days (*E*) after 50% Ppx. In *C*, pancreatic β-cells were labeled by insulin staining (red). *F–G*: Ngn3-EGFP expression was detected in the intestinal crypts of 8-week-old *Ngn3*$^{EGFP/+}$ mice. I, intestine; L, liver; P, pancreas; S, stomach.

detectable for at least 48 h after gene activation. Thus, we can trace the fate of Ngn3-expressing cells regardless of the duration of Ngn3 gene transcription. Examination for EGFP expression both early (2 h) and later (2 and 4 days) after Ppx failed to detect EGFP signal in the pancreas (Fig. 3*C–E*), despite obvious expression during embryonic development (Fig. 3*A* and *B*) and in the intestine of adult Ppx animals (Fig. 3*F* and *G*).

Taken together, these data indicate that Ngn3 is not expressed in adult mouse pancreatic tissue and that its expression is not reactivated during islet regeneration after partial pancreatic resection. These findings complement recent lineage-tracing studies implicating replication from existing β-cells as the primary mechanism of β-cell renewal in adult mice (12). Although ectopic expression of Ngn3 can promote an endocrine transcriptional program in adult cells and may thus have therapeutic potential in the development of a surrogate β-cell source for transplantation, our studies indicate that a reactivation of endogenous Ngn3 expression is not required for adult β-cell regeneration in vivo.

ACKNOWLEDGMENTS

This work was supported by a type 1 diabetes pilot grant from the Penn Diabetes Center (P30-DK-19525 to D.A.S. and K.H.K.), NIDDK (R01 DK55342 to K.H.K.), an American Diabetes Association Career Development Award (7-01-CD-15 to D.A.S.), and the Morphology Core of the Center for Molecular Studies of Liver and Digestive Diseases (P30 DK50306).

The authors acknowledge Biva M. Desai and Cyrus Farzad for outstanding technical assistance.

REFERENCES

1. Prado CL, Pugh-Bernard AE, Elghazi L, Sosa-Pineda B, Sussel L: Ghrelin cells replace insulin-producing beta cells in two mouse models of pancreas development. *Proc Natl Acad Sci U S A* 101:2924–2929, 2004
2. Habener JF, Kemp DM, Thomas MK: Minireview: transcriptional regulation in pancreatic development. *Endocrinology* 146:1025–1034, 2005
3. Gradwohl G, Dierich A, LeMeur M, Guillemot F: Neurogenin3 is required for the development of the four endocrine cell lineages of the pancreas. *Proc Natl Acad Sci U S A* 97:1607–1611, 2000
4. Schwitzgebel VM, Scheel DW, Conners JR, Kalamaras J, Lee JE, Anderson DJ, Sussel L, Johnson JD, German MS: Expression of neurogenin3 reveals an islet cell precursor population in the pancreas. *Development* 127:3533–3542, 2000
5. Heremans Y, Van De Casteele M, in't Veld P, Gradwohl G, Serup P, Madsen O, Pipeleers D, Heimberg H: Recapitulation of embryonic neuroendocrine differentiation in adult human pancreatic duct cells expressing neurogenin 3. *J Cell Biol* 159:303–312, 2002
6. Gasa R, Mrejen C, Leachman N, Otten M, Barnes M, Wang J, Chakrabarti S, Mirmira R, German M: Proendocrine genes coordinate the pancreatic islet differentiation program in vitro. *Proc Natl Acad Sci U S A* 101:13245–13250, 2004
7. Apelqvist A, Li H, Sommer L, Beatus P, Anderson DJ, Honjo T, Hrabe de Angelis M, Lendahl U, Edlund H: Notch signalling controls pancreatic cell differentiation. *Nature* 400:877–881, 1999
8. Smith SB, Watada H, German MS: Neurogenin3 activates the islet differentiation program while repressing its own expression. *Mol Endocrinol* 18:142–149, 2004
9. Gu G, Dubauskaite J, Melton DA: Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors. *Development* 129:2447–2457, 2002
10. Schonhoff SE, Giel-Moloney M, Leiter AB: Neurogenin 3-expressing progenitor cells in the gastrointestinal tract differentiate into both endocrine and non-endocrine cell types. *Dev Biol* 270:443–454, 2004
11. Bonner-Weir S, Baxter LA, Schuppin GT, Smith FE: A second pathway for regeneration of adult exocrine and endocrine pancreas: a possible recapitulation of embryonic development. *Diabetes* 42:1715–1720, 1993
12. Dor Y, Brown J, Martinez OI, Melton DA: Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. *Nature* 429:41–46, 2004
13. Gershengorn MC, Hardikar AA, Wei C, Geras-Raaka E, Marcus-Samuels B, Raaka BM: Epithelial-to-mesenchymal transition generates proliferative human islet precursor cells. *Science* 306:2261–2264, 2004
14. Zulewski H, Abraham EJ, Gerlach MJ, Daniel PB, Moritz W, Muller B, Vallejo M, Thomas MK, Habener JF: Multipotential nestin-positive stem cells isolated from adult pancreatic islets differentiate ex vivo into pancreatic endocrine, exocrine, and hepatic phenotypes. *Diabetes* 50:521–533, 2001
15. Seaberg RM, Smukler SR, Kieffer TJ, Enikolopov G, Asghar Z, Wheeler MB, Korbutt G, van der Kooy D: Clonal identification of multipotent precursors from adult mouse pancreas that generate neural and pancreatic lineages. *Nat Biotechnol* 22:1115–1124, 2004
16. Lee CS, Perreault N, Brestelli JE, Kaestner KH: Neurogenin 3 is essential for the proper specification of gastric enteroendocrine cells and the maintenance of gastric epithelial cell identity. *Genes Dev* 16:1488–1497, 2002
17. De Leon DD, Deng SP, Madani R, Ahima RS, Drucker DJ, Stoffers DA: Role of endogenous glucagon-like peptide-1 in islet regeneration after partial pancreatectomy. *Diabetes* 52:365–371, 2003
18. Esni F, Stoffers DA, Takeuchi T, Leach SD: Origin of exocrine pancreatic cells from nestin-positive precursors in developing mouse pancreas. *Mech Dev* 121:15–25, 2004
19. Stoffers DA, Kieffer TJ, Hussain MA, Drucker DJ, Bonner-Weir S, Habener JF, Egan JM: Insulinotropic glucagon-like peptide 1 agonists stimulate expression of homeodomain protein IDX-1 and increase islet size in mouse pancreas. *Diabetes* 49:741–748, 2000
20. Xu G, Stoffers DA, Habener JF, Bonner-Weir S: Exendin-4 stimulates both β-cell replication and neogenesis, resulting in increased β-cell mass and improved glucose tolerance in diabetic rats. *Diabetes* 48:2270–2276, 1999