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β Cells Can Be Generated from Endogenous Progenitors in Injured Adult Mouse Pancreas

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SUMMARY

Novel strategies in diabetes therapy would obviously benefit from the use of beta (β) cell stem/progenitor cells. However, whether or not adult β cell progenitors exist is one of the most controversial issues in today's diabetes research. Guided by the expression of Neurogenin 3 (Ngn3), the earliest islet cell-specific transcription factor in embryonic development, we show that β cell progenitors can be activated in injured adult mouse pancreas and are located in the ductal lining. Differentiation of the adult progenitors is Ngn3 dependent and gives rise to all islet cell types, including glucose responsive β cells that subsequently proliferate, both in situ and when cultured in embryonic pancreas explants. Multipotent progenitor cells thus exist in the pancreas of adult mice and can be activated cell autonomously to increase the functional β cell mass by differentiation and proliferation rather than by self-duplication of pre-existing β cells only.

INTRODUCTION

Numerous mechanisms that control differentiation of endocrine progenitor cells in the embryonic pancreas have been disclosed (Jensen, 2004) but our knowledge on the existence of precursors and generation of islet cells in the postnatal pancreas depends merely on descriptive data and indirect proof (Bonner-Weir and Weir, 2005; Bouwens and Rooman, 2005). Long-term culture of heterogeneous populations of pancreas cells favors enrichment of beta (β) cell-like phenotypes (Bonner-Weir et al., 2000; Seaberg et al., 2004; Suzuki et al., 2004) that under certain conditions were able to reverse hyperglycemia when transplanted in diabetic mice (Hao et al., 2006; Ramiya et al., 2000). None of these studies, however, was conclusive in demonstrating the existence and origin of a bona fide β cell progenitor in postnatal pancreas. The elusiveness of this cell type reached a summit when genetic lineage tracing provided evidence that pre-existing β cells, rather than stem/progenitor cells, are the major source of new β cells in adult mice both under normal physiological conditions and after 70% or 50% pancreatectomy (Dor et al., 2004; Teta et al., 2007).

Two major problems are at the basis of this ambiguous scenario: the slow turnover of adult β cells and the lack of specific markers to trace their origin. We overcame these hurdles by (1) forcing the generation of new β cells through partial duct ligation (PDL) in the pancreas of adult mice and (2) using transgenic reporter mice that allow tracing of the promoter activity of *Ngn3* as a marker of adult progenitor cell recruitment. PDL stimulates doubling of the β cell mass in rats (Wang et al., 1995), and Ngn3 is an essential master switch for differentiation of embryonic islet cell progenitors (Apelqvist et al., 1999; Gradwohl et al., 2000; Gu et al., 2002; Schwitzgebel et al., 2000) and extremely rare in normal postnatal pancreas (Gu et al., 2002).

RESULTS

Activation of *Ngn3* Gene Expression Induces β Cell Hyperplasia in Adult Mice

Pancreatic β cells have a slow turnover under normal physiological conditions but expand rapidly under certain experimental conditions like PDL (Wang et al., 1995). In Balb/c mice, the duct leading to the pancreatic tail was closed while the organ's head located adjacent to the stomach and duodenum remained unaffected. Within 1 week far most of the acinar exocrine cells underwent apoptosis (Figure S1A) and likely were scavenged by $CD45^+$ cells recruited to the ligated tail part of pancreas (Figure S1C) (Scoggins et al., 2000). Moreover, duct cell-cycle activity was strongly elevated (Figures S1B and S2E), and consequently, the density of duct structures significantly increased (Figures 1A, S1A, and S1B). The weight of the pancreatic tail decreased (Figure S2A), while body weight and glycemia remained unaffected (Figures S2B and S2C). The total $insulin^+$ cell mass in

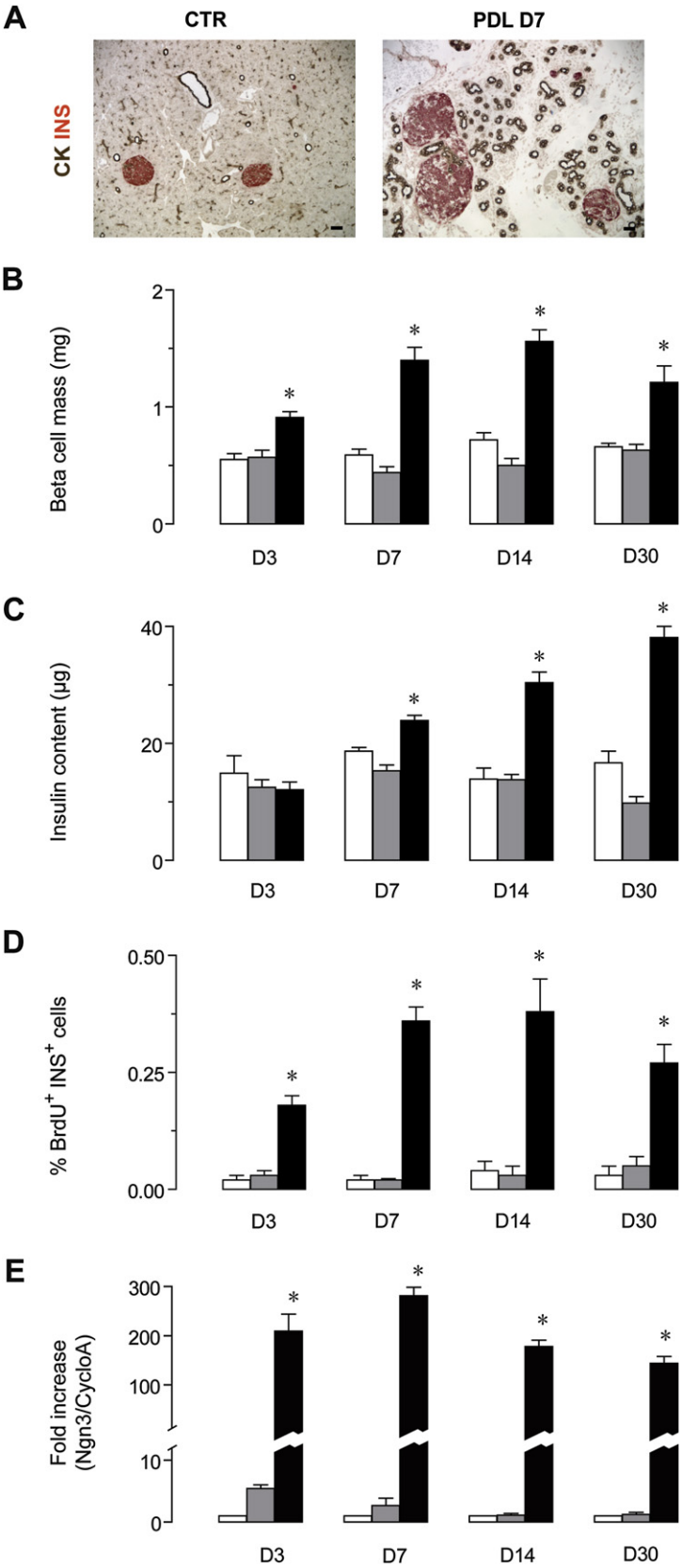

Figure 1. PDL Activates *Ngn3* Gene Expression and Increases β Cell Mass in Adult Pancreas

(A–C) In 8-week-old BALB/C mice, the duct that connects pancreatic tail and duodenum was ligated, and the ligated tail of PDL pancreas at day 7 (PDL D7) was compared to the tail of sham-treated pancreas (CTR) by immunohistochemistry for cytokeratin$^+$ (CK) ductular complexes and insulin$^+$ cells (A). Magnification bars are 100 µm. Several parameters were measured (see Experimental Procedures) at 3, 7, 14, and 30 days following ligation. PDL increased the insulin$^+$ cell mass (mg) (B) and the insulin content (µg) (C) of the tail part of pancreas more than 2-fold (black bars) as compared to the unligated head of the same pancreas (gray bars) and the tail of a sham-operated pancreas (white bars).

(D) At 3, 7, 14, and 30 days after PDL and 1 hr before sacrifice, the nucleotide analog BrdU (50 mg/kg) was injected intraperitoneum. The number of insulin$^+$ BrdU$^+$ cells on pancreatic tissue sections was 10-fold higher in the ligated tail versus control tail or head of pancreas. A similar relative increase of BrdU$^+$ β cells was seen when BrdU had been applied 16 hr before sacrifice (4.60% ± 0.51% in ligated tail of pancreas at day 7 following PDL versus 0.66% ± 0.15% in unligated tail).

(E) A more than 50-fold increase of Ngn3 transcripts was observed in ligated versus unligated part of pancreas by real time RT-PCR using a mouse Ngn3-specific TaqMan probe. All results shown are representative of three or more independent experiments. *: $p < 0.001$ ligated versus unligated pancreas tail.

the ligated part of pancreas increased more than 2-fold within 1 week following surgery (Figures 1B and S1D), and the absolute amount of immunoreactive insulin had doubled 1 week later, a lag period likely needed for β cell maturation (Figure 1C). The individual β cell size remained similar under all conditions tested (Figure S2D), meaning that the treatment induced an increase in cell number rather than in cell size. During this period, the number of β cells in active cell cycle increased 10-fold as established by incorporation of the nucleotide analog BrdU (Figures 1D and S1E). As PDL robustly induced generation of new β cells, we investigated the importance of progenitor cell activation and β cell proliferation in doubling of the β cell mass. A strong activation of expression of *Ngn3*, a well-established marker for embryonic islet cell progenitors, was observed specifically in the ligated part of adult mouse pancreas within 3 days following injury. Maximal levels of Ngn3 transcript were reached within 1 week and subsequently decreased slowly (Figure 1E). Besides neo-formation of β cells, the number of apoptotic β cells increased significantly (Figure S1F). Apoptotic β cells were not preferentially in active cell cycle (Figure S1G).

To investigate the causal relationship between the doubling of β cell mass and the activation of *Ngn3* gene expression, the latter was knocked down during PDL-induced formation of endocrine pancreas. Recombinant lentiviruses that encode 2 Ngn3-specific short hairpin (sh) interfering RNA molecules (Le-sh1Ngn3 and Le-sh2Ngn3), or a control, scrambled sequence (Le-scr) (Baeyens et al., 2006) were injected into the pancreatic duct via the papilla Vateri, followed by ligation of the tail duct. The viruses constitutively express the reporter protein eGFP that allowed us to evaluate the efficiency and specificity of infection in the whole organ and in tissue sections. Injection of reporter virus in sham-treated pancreas transduced 62% of total cells (Figure S3). When virus injection was combined with duct ligation, 18% ± 10% of total cells expressed detectable levels of eGFP 1 week after surgery (Figure 2A). Following infection with lentivirus expressing shRNA, acinar cells disappeared similarly as in control PDL pancreas, and no off-target effects on differentiation and proliferation of duct cells were observed (Figures S4A–S4C). In Le-shNgn3-injected pancreas the Ngn3 transcript abundance was 70% ± 11% (sh1) and 49% ± 4% (sh2) lower than in sham- and Le-scr-injected PDL pancreas at day 7 (Figures 2B, upper and S5). PDL-induced increase of the β cell mass was prevented by 66% ± 17% (sh1) and 26% ± 10% (sh2) following infection with Le-shNgn3 (Figure 2B, middle). These data strongly suggest that β cell formation following PDL depends at least partly on Ngn3 activity. The induced BrdU labeling index of insulin$^+$ cells decreased with 77% ± 10% (sh1) and 32% ± 12% (sh2) by Le-shNgn3 versus Le-scr injection (Figure 2B, lower), indicating that an important fraction of BrdU$^+$ β cells (Figure 1D) were derived from differentiated Ngn3$^+$ cells.

Ngn3$^+$ Cells in Adult Pancreas Originate from Hormone$^-$ Progenitors Near Ducts and Become Islet Cells

Given the activated *Ngn3* expression in injured pancreas of adult mouse, we attempted to track these islet progenitor cells in transgenic Ngn3-nLacZ mice, expressing a nuclear β-galactosidase (β-gal) reporter protein under control of a 6.9 kb genomic sequence that includes the *Ngn3* promoter and faithfully recapitulates the spatial expression of *Ngn3* in the embryonic as well as in the adult pancreas (G.M. and G.G., unpublished data). Histochemistry for β-gal activity revealed blue nuclei in adult mouse duodenum (data not shown), known to constitutively express *Ngn3* in enteroendocrine progenitor cells (Jenny et al., 2002; Schonhoff et al., 2004). The Ngn3 reporter was also detected in the ligated tail of PDL pancreas but not in the unligated head or in the pancreatic tail of sham-operated mice. The localization of 785 β-gal$^+$ cells was examined in six mice, 7 days following PDL (Figure 3A). Of all β-gal$^+$ cells 15% ± 1% were immunoreactive for duct cell-specific cytokeratins (CK) (Figure 3B) and half of the β-gal$^+$CK$^+$ cells were lining the duct lumen as shown by confocal scanning microscopy (Figure 3C). Furthermore, *Ngn3* was expressed in duct-lining cells that activated Pdx1 expression following PDL (Figure 3I). No marker typical for any pancreatic cell type was expressed on 51% ± 2% β-gal$^+$ cells (Figure 3D), one-third of which were still in contact with CK$^+$ duct cells (Figure 3B). Immunohistochemical staining with a Ngn3-specific antibody showed that the Ngn3$^+$ cells in duct-ligated pancreas were devoid of islet cell-specific hormones (Figures 3E–3F). On the other hand, the long half-life of the reporter protein β-gal (Gonda et al., 1989) allowed tracing the fate of Ngn3$^+$ cells to their endocrine descendants. Indeed, 34% ± 4% of β-gal$^+$ cells contained transcripts encoding islet hormones at day 7 following PDL (Figures 3G and 3H). Half the number of hormone-expressing βGal$^+$ cells was still in contact with duct cells but none of these were part of the luminal lining. No β-gal$^+$ cells costained for amylase (not shown). While these lineage-tracing data strongly suggest that the observed Ngn3$^+$ cells originate from islet hormone$^-$ cells among the lining of ducts and migrate to become hormone$^+$ cells within the islet structures in adult mice, they do not fully exclude the alternative possibility that endocrine cells dedifferentiated to Ngn3$^+$ cells. Therefore, we traced permanently labeled β cells from INS-Cre/R26R mice with PDL and found the label absent from Ngn3$^+$ cells (Figure S6B) supporting differentiation of Ngn3$^+$-to-islet cells.

Ngn3$^+$ Progenitor Cells Can Be Purified from Adult Mouse Pancreas

Based on the number of β-gal$^+$ cells in 30 tissue sections from the PDL pancreas of three mice, approximately 5000 β-gal$^+$ cells were present in the ligated tail, a sufficiently high number to endeavor their isolation. By flow cytometry, Ngn3$^+$ cells were isolated from the PDL pancreas of reporter mice that express eGFP under control of the same 6.9 kb *Ngn3* promoter fragment as used in the Ngn3-nlacZ mice. As for β-gal, the half-life of eGFP (Corish and Tyler-Smith, 1999) exceeds that of Ngn3 (Lee et al., 2001), and consequently the reporter protein was still present in a fraction of the hormone-positive descendants of the preendocrine cells (data not shown). These GFP$^+$ cells with hormone-containing vesicles were excluded, and only nongranulated GFP$^+$ cells were considered as endocrine progenitors. Seven days following partial duct ligation, PI$^-$/GFP$^+$/TSQ$^-$/LowSSC cells (termed GFP/LSSC cells from hereon) that were viable, green fluorescent, and contained only few granules could be isolated from PDL pancreas of Ngn3-eGFP mice (Figure 4A). The transcript encoding Ngn3 was 200-fold enriched, and those encoding insulin and glucagon were very rare in the progenitor

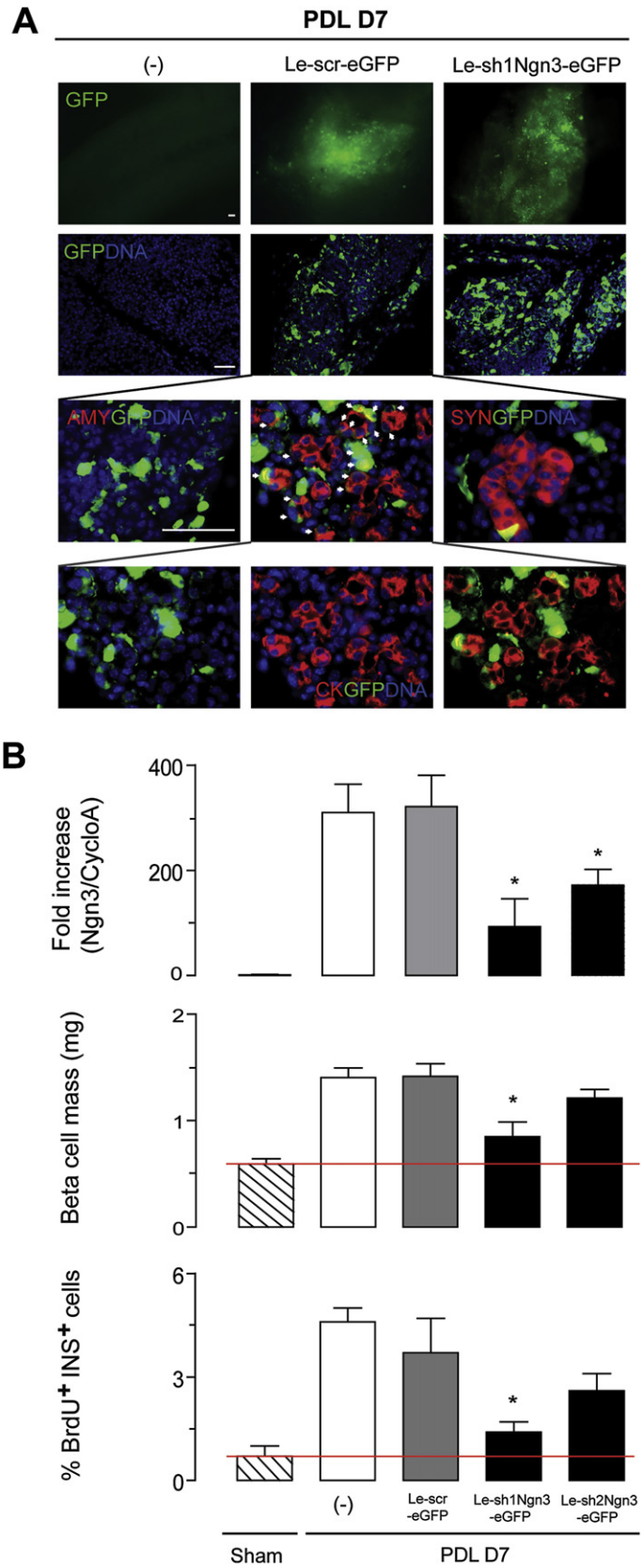

Figure 2. Knockdown of Ngn3 Impairs PDL-Induced β Cell Generation

(A) The pancreatic duct of adult BALB/C mice was injected with recombinant lentiviruses encoding reporter eGFP and two different shRNAs for specific interference with Ngn3 transcript (Baeyens et al., 2006) (Le-sh1Ngn3-eGFP and Le-sh2Ngn3-eGFP) or control sequence (Le-scr-eGFP), immediately followed by PDL. The efficiency of infection was determined 7 days following sham- or virus injection and PDL by direct fluorescence of whole pancreas tail (upper row) and by immunostaining for the reporter on pancreas sections (middle row). The specificity was defined by the fraction of GFP^+ cells that immunostained positive for duct cell-specific cytokeratins (47% ± 9%) or the islet cell marker synaptophysin (5% ± 2%). As most acinar cells had disappeared at day 7 following PDL, no GFP^+ cells were $amylase^+$ (lower rows).

(B) As a result of the Ngn3-specific knockdown by Le-shNgn3-eGFP in day 7 PDL pancreas (black bar), the fold activation of *Ngn3* was decreased by 70% ± 11% (sh1) and 49% ± 4% (sh2) as compared to the effect of Le-scr-eGFP infection (gray bar, upper). The β cell mass more than doubled in the tail of Le-scr-infected PDL pancreas compared to sham-operated control (hatched bar), but this effect was inhibited by 66% (sh1) and 26% (sh2) following infection with Le-shNgn3 (middle). Ngn3 knockdown also reduced the increase in the number of $insulin^+$ cells that incorporated BrdU following Le-shNgn3 injection (lower). Abundance of transcripts was quantified by real-time RT-PCR using TaqMan probes. β cell mass and the fraction of $BrdU^+$ $insulin^+$ cells were determined as in Figure 1, except that BrdU was supplied 16 and 2 hr before sacrifice. All results shown are representative of three independent experiments. *: $p < 0.05$ Le-shNgn3-eGFP versus Le-scr-eGFP infected PDL pancreas. Magnification bars are 100 μm.

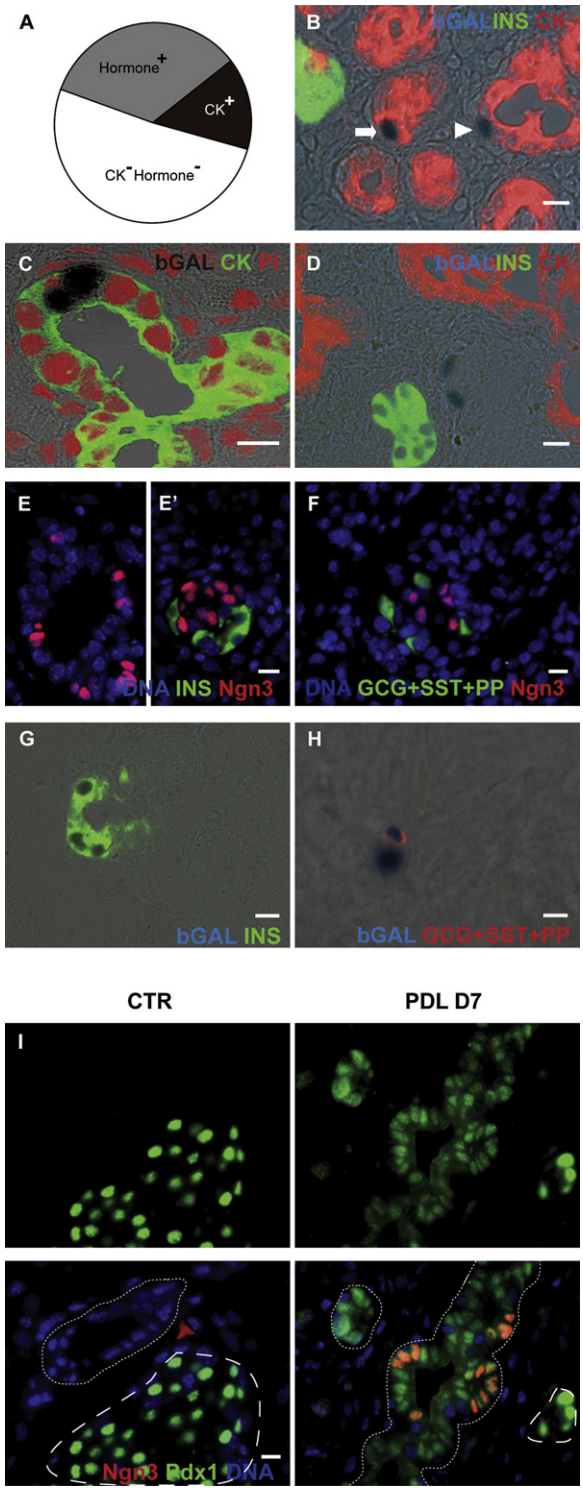

Figure 3. New Islet Cells Derive from Hormone$^-$ Progenitors among the Lining of Ducts in PDL Pancreas

(A–I) Duct ligation induced *Ngn3* promoter activation in the pancreatic tail of the outbred CD1 x C57BL/6 strain of Ngn3-nlacZ reporter mice (data not shown), similar as in Balb/c mice. Expression of lacZ, coding for the long-living reporter β-gal under the control of *Ngn3* promoter sequences, allowed tracking of the *Ngn3*-expressing cells and their descendants. Ngn3$^+$ cells were detected by histochemical staining of Ngn3-reporter activity (B–D, G, and H). The identity of the β-gal-expressing cells was determined by combined immunohistochemical detection of ductal cytokeratins and/or islet hormones (B–D, G, and H). An overview of the distribution of coexpressing cells was based on the examination of 785 β-gal$^+$ cells in the ligated pancreas of 6 mice (A). 15% ± 1% of all β-gal$^+$ cells expressed duct cell-specific cytokeratins (CK) but no insulin (INS) (B, arrow) or other islet cell hormones (not shown). Half of the β-gal$^+$ CK$^+$ hormone$^-$ cells were in direct contact with the duct lumen (C). Half of the β-gal$^+$ cells did not express CK or islets markers (here INS) (D), one-third of which were in contact with CK$^+$ cells (B, arrowhead). While immunostaining for endogenous Ngn3 showed no coexpression with islet hormones, insulin (E, E′), glucagon (GCG), somatostatin (SST), or pancreatic polypeptide (PP) (F), one-third of all β-gal$^+$ cells were hormone$^+$ indicating that Ngn3$^+$ cells are the source of endocrine islet cells (G and H). DAPI (blue) (E and F) and PI (red) (C) stain the nuclei. All sampling was done 1 week following PDL. No Ngn3 or β-gal signal was detected in tail of unligated or head of ligated pancreas. Sham-treated and PDL D7 pancreas were stained simultaneously for Pdx1 and Ngn3 showing coexpression in cells lining the duct of PDL pancreas (I). Magnification bars are 10 μm.

population as compared to nonsorted PDL pancreas cells (Figure 4B). A similar cell population was isolated from the pancreas of Ngn3$^{eYFP/+}$ knock add-on mice (Mellitzer et al., 2004), corroborating faithful recapitulation of Ngn3 expression in the Ngn3-eGFP mice (Figure 4D) (data not shown). The GFP/LSSC cell population represented 0.04% of the total number of sorted pancreatic cells. Of this sorted population, 93% ± 4% immunostained GFP$^+$, 90.1% ± 5% Ngn3$^+$, and none were insulin$^+$ (Figure 4E). As expected, the fraction of insulin$^+$ cells was high in the GFP$^+$/TSQ$^+$/HSSC (termed GFP/HSSC from hereon) cell population (85% ± 10%) and in total pancreas (3.0% ± 0.8%).

GFP/LSSC cells, sorted from GCG-Cre/R26R/Ngn3-eGFP mice pancreas (Figure S6C) 7 days following PDL, lacked β-gal (800 cells counted), while the reporter was expressed in 5% of islet cells (Figure S6D). In addition to the differentiation of Ngn3$^+$-to-β cells (Figure S6B) and the similar amount of Ngn3 transcripts in islets isolated from the tail of ligated and sham-treated pancreas (lower than in total PDL pancreas) (Figure 4C), these data provide strong evidence against islet-to-Ngn3$^+$ cell dedifferentiation.

Due to the controversial aspect of progenitor cells in adult pancreas, we compared these GFP/LSSC cells with Ngn3$^+$ cells isolated from E13.5 embryonic pancreas and from adult duodenal crypt region, as these Ngn3$^+$ cell types are generally accepted to be genuine endocrine progenitors (Gu et al., 2002; Jenny et al., 2002; Schonhoff et al., 2004). While 99.8% of the sorted cells from embryonic pancreas and adult duodenum were GFP-positive, none immunostained positive for insulin. The abundance of Ngn3 transcripts in these cells was more than 100-fold higher than in the nonsorted cell populations (data not shown). Electron micrographs of GFP/LSSC cells isolated from embryonic and adult PDL pancreas showed rounded cells that were 3-fold smaller than adult mouse β cells (985 ± 112 μm^3 versus 3052 ± 178 μm^3) and had relatively large nuclei (695 ± 98 μm^3 versus 565 ± 76 μm^3) with a remarkable amount of heterochromatin in the periphery of the nuclei (Figure 4F). The ultrastructural features of Ngn3$^+$ cells isolated from adult duodenum were similar to those of the pancreatic GFP/LSSC cells (data not shown). In contrast to the many dark secretory granules containing mature insulin that are present in all differentiated β cells,

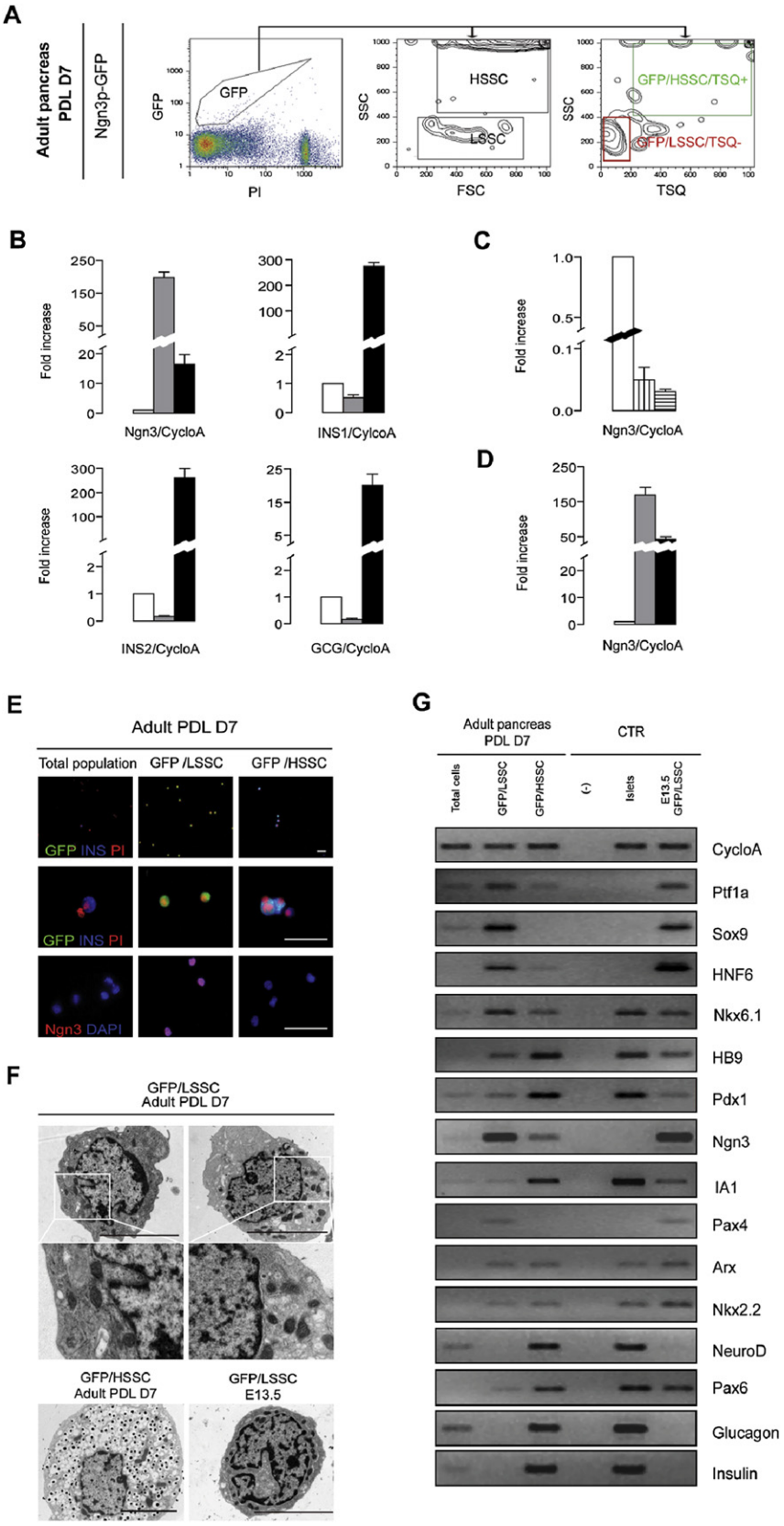

Figure 4. Ngn3$^+$ Cells Isolated from Adult Pancreas Have an Embryonic Islet Cell Progenitor Phenotype

(A) GFP$^+$ cells were isolated by flow cytometry from adult PDL pancreas (day 7) of Ngn3 reporter mice, based on GFP expression and low degree of granulation. First, viable Ngn3$^+$ cells were isolated based on their GFP fluorescence and capacity to exclude propidium iodide. Then, they were separated from the hormone$^+$ cells according to their low degree of cellular granulation that was evaluated in two ways, namely by binding of the Zn^{2+}-chelator 6-methoxy-8-p-toluene sulfonamide quinoline (TSQ) to hormone peptides in secretory vesicles and cellular sideward scattering (SSC) properties. The resulting cell population is PI$^-$/GFP$^+$/low SSC/TSQ$^-$, in brief GFP/LSSC (red window), while granulated GFP$^+$ cells are PI$^-$/GFP$^+$/high SSC/TSQ$^+$ or GFP/HSSC (green window). GFP/LSSC cells were not detected in wild-type littermates.

(B–D) Quantification of transcript levels by RT-QPCR (see Table S1 and Experimental Procedures). RNA was extracted from the total population of nonsorted pancreas cells (white bars), GFP/LSSC cells (gray bars), GFP/HSSC cells (black bars), and islets from sham-treated (vertical lines) or PDL (horizontal lines) pancreas from transgenic mice with random Ngn3-eGFP insertion (B and C) or eYFP added on the Ngn3 locus (D). All RT-PCR results shown are representative of three independent experiments.

(E) Immunodetection of insulin$^+$ and Ngn3$^+$ cells on cytospins of nonsorted and sorted cells from PDL D7 pancreas. Enrichment of GFP$^+$ and Ngn3$^+$ cells and depletion of insulin$^+$ cells in the GFP/LSSC fraction (0 insulin$^+$ on 3000 GFP/LSSC cells, a fraction of the GFP/LSSC cells from 48 PDL mice) was confirmed by immunocytochemistry.

(F) Ultrathin sections of GFP/LSSC (upper panels) and GFP/HSSC cells (lower left panel) from adult PDL (day 7) and of GFP/LSSC cells from E13.5 pancreas (lower right panel) were analyzed on transmission electron micrographs. All cells were isolated from Ngn3-eGFP transgenic mice. Magnification bars are 100 μm in (E) and 10 μm in (F).

(G) Compared to nonsorted pancreas cells (Total cells) or GFP/HSSC cells, the expression of progenitor marker Ngn3 and of developmental transcription factors Ptf1a, Sox9, HNF6, and Nkx6.1, located upstream of Ngn3 during embryogenesis, was high in GFP/LSSC cells while that of its direct targets and differentiation markers was low or absent. The presence of transcripts was determined by conventional RT-PCR amplification with specific primers (Experimental Procedures). cDNA from adult mouse islet cells and from GFP$^+$ cells isolated from E13.5 pancreas of Ngn3-GFP reporter mice served as control (CTR). The negative control (-) contained no cDNA.

most GFP/LSSC cells from embryonic or adult pancreas were nongranulated and few granules were found in only 5% ± 1% of them. The latter had inclusions of low electron density, without a halo (Figure 4F), typical for cells with unprocessed hormone (Orci et al., 1985) and another indication of the immature cell state. Ngn3 cells isolated from adult regenerating pancreas thus strongly resemble progenitors of endocrine cells in embryonic pancreas and adult duodenum.

More extensive gene-expression profiling revealed that transcription factors expressed upstream of Ngn3 in early pancreas epithelium (Ptf1a, Sox9, HNF6, and Nkx6.1) were also enriched in GFP/LSSC cells, while the ones that continue to be expressed in mature islet cells (Hlxb9 and Pdx1) were higher in GFP/HSSC than LSSC cells. Transcription factors acting downstream of Ngn3 (IA1, Pax4, Arx, Nkx2.2, NeuroD1, Pax6) were overall low in GFP/LSSC cells, also illustrating their early endocrine differentiation status (Figure 4G).

Ngn3$^+$ Cells from Adult Pancreas Differentiate to Functional Islet Cells In Vitro

When cultured in 1% or 10% serum, either in suspension, as monolayer, or in 3D collagen gel, over 90% of the GFP/LSSC cells died after 1 day (data not shown). All factors required for endogenous Ngn3$^+$ cells to survive and differentiate into islet cells should, however, be present in the embryonic pancreas in situ but also in embryonic organ culture (Miralles et al., 1998) (Figures S7A–S7C). We therefore considered the ex vivo cultured embryonic mouse pancreas as an appropriate microenvironment to investigate the capacity of the Ngn3$^+$ cells isolated from adult mouse pancreas to differentiate into mature islet cells (Figure 5A). In wild-type (WT) E12.5 pancreatic explants, the differentiating endocrine cells derived from endogenous *Ngn3*-expressing cells, since no islet hormone$^+$ cells appeared in explants from *Ngn3* homozygous null mutant embryos (Figure 5B). To exclude interference with these endogenous embryonic Ngn3$^+$ cells, the isolated GFP/LSSC cells from normal embryonic and ligated adult pancreas of Ngn3-eGFP mice were microinjected in embryonic Ngn3$^{-/-}$ pancreas. No insulin or glucagon peptide or transcript could be detected in the engrafted Ngn3$^{-/-}$ explants following 1 day of culture (Figure 5B,C). After 7 days of culture, WT explants as well as engrafted—but not sham-injected—Ngn3$^{-/-}$ explants contained transcripts encoding the four islet hormones as well as their corresponding peptides (Figures 5B and 5C). No cell expressed more than one hormone simultaneously (data not shown). We further examined whether the observed endocrine differentiation was cell autonomous or whether fusion or signaling between injected adult Ngn3$^+$ cells and explanted embryonic pancreas was involved. First, when GFP/LSSC cells were preincubated with CellTracker Orange (CMTMR) and injected in explanted pancreas of Ngn3$^{-/-}$ embryonic mice, the injected GFP/LSSC cells differentiated since some of them expressed insulin already at day 4 of culture (Figure S8A). Second, when mouse GFP/LSSC cells were cultured in explants of rat embryonic pancreas and differentiating β cells were immunostained by species-specific antibodies directed against insulin C peptide (Blume et al., 1990), both mouse and rat cells independently differentiated to C peptide$^+$ cells (Figure S8B). Finally, when GFP/LSSC cells isolated from Ngn3-eGFP mice that constitutively express β-gal were cultured in pancreas explants from embryonic *Ngn3*$^{-/-}$ mice, all differentiated, hormone$^+$ cells were β-gal$^+$ (Figure S8C). Consequently, the endocrine cells originate directly from the injected GFP/LSSC cells without cell fusion.

When explants were labeled with BrdU during the last 16 hr of culture, the injected GFP/LSSC cells did not enter the cell cycle after 1 day, while 22% ± 6.2% of the newly differentiated insulin$^+$ cells were active in S phase at day 7 (Figure 5D).

WT explants contained 137 ± 37 ng of insulin following 7 days of culture (versus 1.2 ± 0.8 ng at day 1) and *Ngn3*$^{-/-}$ explants supplemented with adult GFP/LSSC cells had 35 ± 7 ng insulin (versus 0.2 ± 0.2 at day 1). To evaluate the degree of differentiation of the GFP/LSSC cells, we measured glucose responsiveness of the insulin release. Glucose induced a 1.5-fold increase of insulin secretion from explanted E12.5 pancreas of WT mice at day 7 of culture (Figure 5E). Embryonic pancreas from *Ngn3*$^{-/-}$ mice acquired glucose responsiveness when injected with GFP/LSSC cells from adult Ngn3-eGFP mice (PDL D7), since their insulin release increased 2.6-fold when stimulated with 20 mmol/L glucose (Figure 5E).

DISCUSSION

Our study demonstrates convincingly that the adult mouse pancreas contains islet cell progenitors and that expansion of the β cell mass following injury induced by ligation of the pancreatic duct depends at least partly on the activation of *Ngn3* gene expression and the ensuing differentiation of endogenous progenitor cells in a cell-autonomous, fusion-independent manner. Partial duct ligation induces a strong inflammatory response and a loss of acinar cells. Both processes may be important in signaling for increase of the β cell mass under these conditions of injury, but it is unclear at this moment whether they play a role in the normal physiology of a healthy pancreas where the importance of self-duplication rather than stem cell differentiation is well documented (Dor et al., 2004; Teta et al., 2007). Activation of *Ngn3* and doubling of the β cell mass could be prevented up to 66% by Ngn3-specific RNA interference, suggesting a considerable contribution of progenitor cells to the observed β cell hyperplasia. In nonligated pancreas 67% of the cells were transduced compared to only 18% in ligated pancreas. This difference is due to the disappearance of acinar cells, the most abundant cell type of the infected pancreas and to a massive recruitment of uninfected immune response cells to the pancreas affected by inflammation following PDL. The efficient Ngn3 knockdown can be explained by (1) the infection of 67% of pancreas cells before PDL is carried out, (2) the specific location of an important fraction of *Ngn3*-expressing cells, targets of the interfering RNA, among or in contact with duct cells that line the site of injection and therefore are exposed directly to the virus, and (3) a near 100% knockdown of Ngn3 expression by Le-sh1Ngn3 (Baeyens et al., 2006). The remaining increase in β cell mass in spite of Ngn3 knockdown likely is due to cycling of (1) pre-existing β cells and/or (2) progenitor cells that were uninfected or that had differentiated beyond the Ngn3$^+$ stage before being infected.

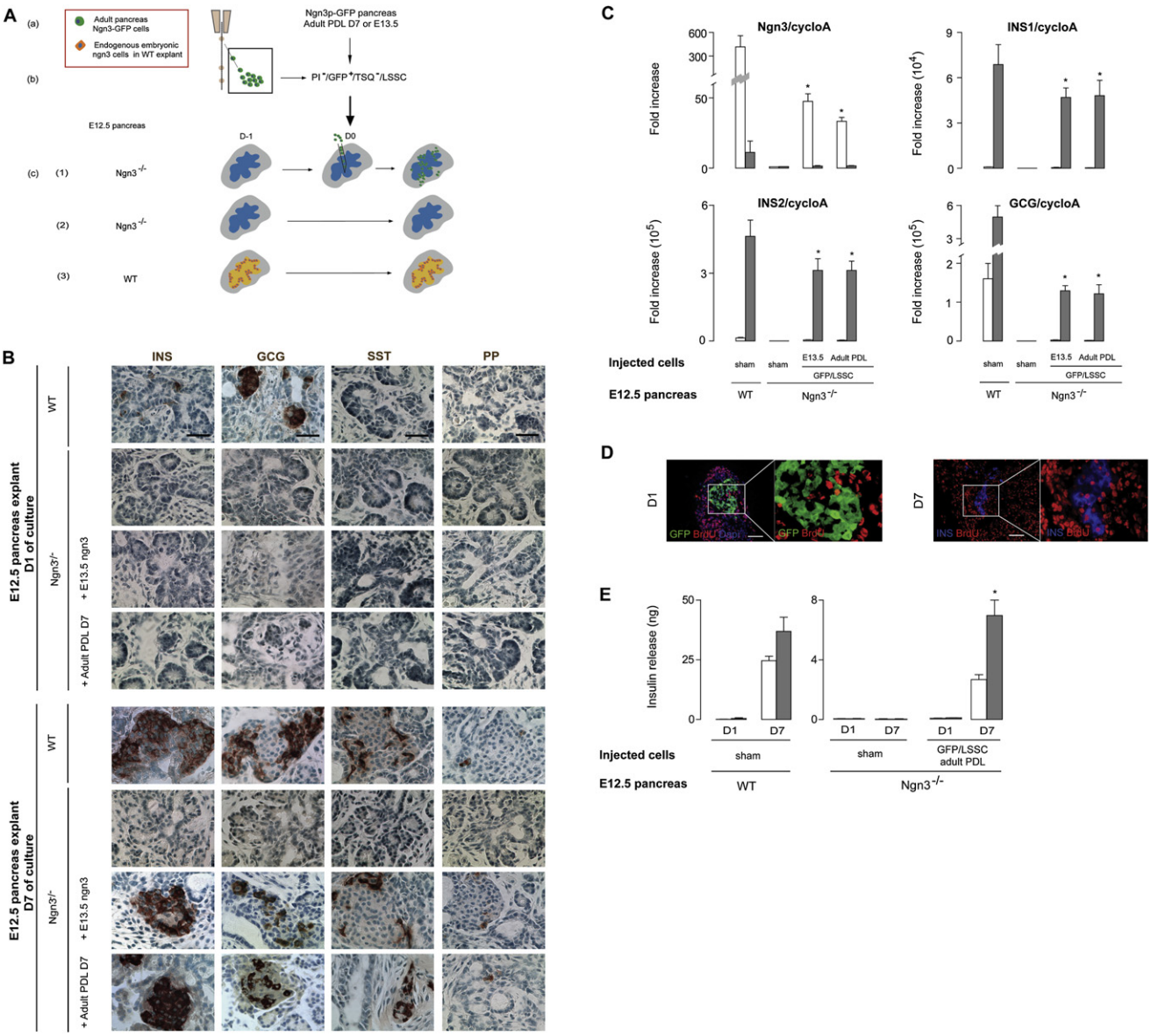

Figure 5. Ngn3⁺ Cells from Adult Pancreas Differentiate In Vitro into Functional β Cells

(A) The following is a schematic overview of the experiment: GFP/LSSC cells were isolated by flow cytometry from adult (PDL D7) or embryonic (E13.5) pancreas. Embryonic pancreas was explanted from homozygous *Ngn3* null mutant mice or their WT littermates at E12.5 (D-1). One day later (D0), 500 GFP/LSSC cells were microinjected into the embryonic pancreas and kept in culture for 1 or 7 days.

(B) Following 1 day in culture, WT embryonic explants immunostained positive for insulin and glucagon but *Ngn3*$^{-/-}$ embryonic explants did not, even when injected with GFP/LSSC cells from adult PDL. After 1 week of culture, WT explants expressed insulin and glucagon, somatostatin, and pancreatic polypeptide but *Ngn3*$^{-/-}$ explants did not. However, when engrafted with GFP/LSSC cells from E13.5 or adult PDL pancreas, the four islet hormones were detected in *Ngn3*$^{-/-}$ explants. Magnification bar is 100 μm.

(C) RNA was extracted from the explants described in (B), cultured for 1 (white bar) or 7 (gray bar) days and transcript levels encoding Ngn3, insulin 1 and 2, and glucagon were determined by quantitative RT-PCR (see Experimental Procedures). The negative control contained no cDNA.

(D) While cell-cycle activity was high in the explant cultured for 1 day, the engrafted GFP/LSSC cells from adult PDL pancreas were out of cycle. After their differentiation to insulin⁺ cells, however, the injected cells reinitiated cell cycle. Explants were labeled with BrdU during the last 16 hr of culture.

(E) The glucose-responsive insulin release by embryonic pancreas from *Ngn3*$^{-/-}$ mice engrafted with GFP/LSSC cells was determined at day 1 and day 7 of culture, following incubation in 6 mmol/L (white bars) or 20 mmol/L (gray bars) glucose for 24 hr. Explants from embryonic pancreas of WT mice and of nonengrafted *Ngn3*$^{-/-}$ mice were taken as positive and negative control, respectively. All results shown are representative of three independent experiments. *: $p < 0.001$ insulin release at 6 mmol/L versus 20 mmol/L glucose.

While under normal physiological conditions the slow course of β cell proliferation is sufficient to compensate for their low turnover and expansion (Dor et al., 2004; Teta et al., 2007), our data in injured tissue demonstrate a rapid course of hyperplasia that depends on progenitor cell recruitment. This pathway may not be active after 50%–70% partial pancreatectomy (PPx) (Dor et al., 2004; Teta et al., 2007), a less robust injury model in which Ngn3-expressing cells remain absent (Lee et al., 2006) and the β cell mass indeed increases much slower than following duct ligation (Bouwens and Rooman, 2005).

The Ngn3$^+$ islet cell progenitors coexpress cytokeratins when located among the cells that line the pancreatic ducts and were activated by PDL as shown by expression of Pdx1. In PDL pancreas, the Ngn3$^+$ cells near and within islets did not express any of the islet cell hormones, nor did permanently labeled islet cells express Ngn3. Finally, islets isolated from PDL pancreas contained less Ngn3 mRNA than total PDL pancreas, excluding dedifferentiation of pre-existing islet cells as the basis of the phenomena we describe. The detection of β-gal in the progeny of Ngn3$^+$ cells that already expressed the hormones, some of which were in islets, suggests a migration from duct to islet by the progenitor cells. The ultrastructure of Ngn3$^+$ cells from adult pancreas revealed an immature phenotype, but when injected in an embryonic microenvironment that supports islet progenitor differentiation, the GFP/LSSC cells became functional endocrine islet cells among which were β cells with glucose responsive insulin release. We confirmed that the 6 kb promoter recapitulates the endogenous Ngn3 expression by performing PDL on the pancreas of Ngn3$^{eYFP/+}$ knock-add-on mice (Mellitzer et al., 2004) and showing that YFP/LSSC cells are similar to the GFP/LSSC cells found in PDL pancreas from Ngn3-GFP mice. The endogenous progenitor cell type we isolated from adult mouse pancreas is different from the atypical ones isolated from neonatal (Suzuki et al., 2004) or adult (Seaberg et al., 2004) mouse pancreas that expressed *Ngn3* but had a high proliferation capacity and gave rise to pancreatic (Seaberg et al., 2004; Suzuki et al., 2004) and neuronal (Seaberg et al., 2004) cell types in vitro. None of these expanded colonies formed islet cells with significant glucose responsive insulin release. Recently, the nonendocrine fraction of the human pancreas, containing undifferentiated epithelial cells that expressed markers of pancreatic duct cells, was used to generate new insulin-producing cells when grafted together with cells of fetal pancreas under the kidney capsule of mice (Hao et al., 2006). An important similarity with our study is the requirement of an embryonic microenvironment able to produce essential growth and differentiation factors. Cytokeratin 19, the marker used by Hao et al. (2006) is ambiguous, though, since it is expressed in islet cells undergoing dedifferentiation (Gao et al., 2005). Ngn3, however, is the only unambiguous marker known for islet progenitors in the embryonic (Gu et al., 2002) and in the adult pancreas (present study). Our data provide the first direct evidence for the existence of endogenous endocrine islet cell progenitors in adult mouse pancreas. This cell population is similar to the one that gives rise to the islets during embryonic development and represents an obvious target for therapeutic regeneration of β cells in diabetes. Indeed, our findings reveal the significance of investigating the feasibility of (1) isolating facultative β cell progenitors and newly formed β cells from human pancreas in order to expand and differentiate them in vitro and transplant them in diabetic patients and (2) composing a mix of factors able to activate β cell progenitors to expand and differentiate in situ in patients with an absolute or relative deficiency in insulin.

EXPERIMENTAL PROCEDURES

Mouse Manipulations

All mice experiments were performed in accordance with our institutional "Ethical Committee for Animal Experiments" and national guidelines and regulations. The pancreatic duct of 8 weeks old mice (Balb/C, C57BL/6 x CD1 Ngn3-nLacZ, Ngn3-eGFP, Ngn3$^{eYFP/+}$ [Mellitzer et al., 2004], Ngn3-eGFP/ROSA26-lacZ, Ngn3-eGFP/GCG-Cre/R26R, INS-Cre/R26R) was ligated as described in rats (Wang et al., 1995) with some minor modifications. GCG-Cre and INS-Cre were kindly provided by Pedro Herrera (University of Geneva), and ROSA26-lacZ and R26R were from Philippe Soriano (Fred Hutchinson Cancer Research Center). Following clamping of the distal bile duct, 60 μl containing 2 × 10^7 TU of recombinant lentiviruses that express short hairpin RNA molecules directed against Ngn3 (Le-sh1Ngn3 5′-GTGCTCAGTTCCAATTCCA-3′ and Le-sh2Ngn3 5′-GACCCTGCGCTTCGCCCAC-3′) or a random control shRNA (Le-scr 5′-GAGCATGCGAGCCATGCAC-3′) (Baeyens et al., 2006) were slowly injected in the pancreatic duct (Taniguchi et al., 2003) or in explant of embryonic pancreas. We minimized possible off-target effects by careful selection of the RNAi sequences using the si*DESIGN* Center (http://www.dharmacon.com) (Reynolds et al., 2004). Candidate target shRNA sequences were blasted against mouse transcript and genomic databases. Their sequence similarity with genes other than Ngn3 (100% identity) was "not significant." The highest similarity (74%–79% identity) was between synuclein alpha and Sh1 and between Ngn2 or gastric inhibitory polypeptide receptor and Sh2. From E12.5 or E13.5 embryos of WT or *Ngn3*$^{-/-}$ mice, the dorsal lobes of pancreas were isolated as described (Duvillie et al., 2003; Miralles et al., 1998), cultured in RPMI1640 + 10% fetal calf serum (Hyclone), and microinjected (Eppendorf TransferMan NK) with 500 GFP/LSSC cells that were collected in a micropipette with 20 μm diameter.

Isolation and Labeling of Ngn3-eGFP Cells

GFP/LSSC cells were obtained from embryonic (E13.5) and adult (PDL D7) pancreas of Ngn3-eGFP mice following dissociation to single cells (collagenase, 0.3 mg/ml and trypsin, 10 μg/ml, Sigma), filtration (30 μm), incubation with PI (2μg/ml, Sigma), and TSQ (2 μg/ml, Molecular Probes) for 15 min and sorting on a FACSAria (Becton Dickinson). GFP/LSSC cells were labeled by incubation for 10 min in presence of 5 μM CellTracker Orange CMTMR (Invitrogen, Molecular Probes).

RNA and Protein Analysis

Total RNA was isolated from tissue (RNeasy, QIAGEN) or cells (Picopure, Arcturus). Only RNA with RNA Integrity Number ≥ 7 (2100 BioAnalyzer, Agilent) was further analyzed. cDNA synthesis and RT-PCR were done as described (Mellitzer et al., 2006) using specific primers (Table S1). Quantitative PCR was performed using mouse-specific primers and probes recognizing insulin 1 (Mm01259683), insulin 2 (Mm00731595), glucagon (Mm00801712), CD45 (Mm00448463_m1), F4/80 (Mm00802530_m1), and cyclophilin A (Mm02342429) with TaqMan Universal PCR master mix on an ABI Prism 7700 Sequence Detector, and data were analyzed using the Sequence Detection Systems Software, Version 1.9.1 (all Applied Biosystems). The following sequences were used for analysis of Ngn3: 5′-GTCGGGAGAACTAGGATGGC-3′ go (forward primer)p, 5′-GGAGCAGTCCCTAGGTATG-3′ (reverse primer), and 5′-CCGGAGCCTCGGACCACGAA-3′ go(probe). The abundance of Ngn3, insulin 1, insulin 2, glucagon, CD45, and F4/80 transcripts was normalized versus the abundance of the transcript encoding the housekeeping protein cyclophilin A.

Samples for immunohistochemistry (IHC) were fixed in 4% formaldehyde (FA) for 4 hr respectively at RT following embedding in paraffin or at 4°C followed by overnight in 20% sucrose and freezing. Samples for immunocytochemistry (ICC) were fixed in 4% FA for 10 min. Paraffin sections (4–5 μm)

were incubated with antisera specific for insulin (1/5000, guinea pig), glucagon (1/3000, rabbit) and somatostatin (1/5000, rabbit) (generated at the Diabetes Research Center, Brussels), pancreatic polypeptide (1/5000, rabbit, gift from Lilly), synaptophysin (1/50, rabbit, Zymed), pan-keratin (1/1000, rabbit, Dako Cytomation), amylase (1/500, rabbit, Sigma), PHH3 (1/400, rabbit, Upstate Biotechnology), BrdU (1/10, mouse, Cappel), GFP (1/100, rabbit or goat, Abcam), activated caspase 3 (1/200, rabbit, Cell Signaling), CD45 (1/50, rat, BD-PharMingen), F4/80 (1/10, rat, Serotec), Ngn3 (1/2000, mouse, Ole Madsen, Hagedorn Research Institute, Gentofte) (Zahn et al., 2004), and Pdx1 (1/1000, rabbit), mouse-specific C peptide (1/6000, rabbit), and rat-specific C peptide (1/6000, rabbit) (Beta Cell Biology Consortium, Antibody Core). The primary rat-specific anti-C peptide was labeled using a fluorophore-labeled Fab fragment directed against its Fc portion (Zenon, Molecular Probes). Antigen retrieval was required for recognition of synaptophysin, PHH3 and Ngn3 (microwave), BrdU, and pankeratin (proteinase K). Secondary antibodies for detection of guinea pig, rabbit, goat, or mouse antibodies were labeled by fluorescence (Cy3, Cy2, Cy5, or AMCA) (Jackson ImmunoResearch Labs) or by ABC/DAB (DakoCytomation/Becton Dickinson). Signals of Ngn3 were amplified using the TSA-Cy3 System (Perkin Elmer Life). Nuclei were labeled by Hoechst 33342 (4 μg/ml, Sigma) or Sytox green (5 μM, Invitrogen). Images were viewed using normal (Zeiss Axioplan 2) or confocal scanning (Leica DMIRE) microscopy and morphometrically analyzed using NIH ImageJ (versus1.3.1). For electron microscopy samples were prepared as in Heremans et al. (2002).

Quantitative analysis of the β cell mass (calculated on the basis of at least 9 sections, 150 μm apart from each other, per pancreas tail or head) and the number of $BrdU^+$ $insulin^+$ cells was done as described by Bogdani et al. (2003) (Figure S9).

Insulin content of adult and embryonic pancreas and medium insulin were determined by radioimmunoassay using mouse insulin RIA kit (Linco Research Inc.).

Glucose response of adult and embryonic GFP/LSSC cells, cultured in $Ngn3^{-/-}$ explants for 1 or 7 day(s), was assayed for insulin release in the medium following incubation with 6 or 20 mmol/L glucose during the last 24 hr. Positive and negative controls were sham-injected embryonic pancreas explants from WT and $Ngn3^{-/-}$ mice, respectively.

Data Analysis

All values are depicted as mean ± standard error of the mean (SEM) from at least three independent experiments and considered significant if $p < 0.05$. All data were statistically analyzed by multivariate comparison (two-way ANOVA) with Bonferroni correction or one-way ANOVA with Newman-Keuls correction.

Supplemental Data

Supplemental Data include seven figures and one table and can be found with this article online at http://www.cell.com/cgi/content/full/132/2/197/DC1/.

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