BETA 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 cell stem/progenitor cells. However, whether or not adult beta 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 beta 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 beta 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 beta cell mass by differentiation and proliferation rather than by self-duplication of pre-existing beta cells only.

Key words: endocrine pancreas; stem cell; neurogenin 3; cell differentiation; cell proliferation
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 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 beta cell progenitor in postnatal pancreas. The elusiveness of this cell type reached a summit when genetic lineage tracing provided evidence that pre-existing beta cells, rather than stem/progenitor cells, are the major source of new beta 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 beta cells and the lack of specific markers to trace their origin. We overcame these hurdles by (i) forcing the generation of new beta cells through partial duct ligation (PDL) in the pancreas of adult mice and (ii) 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 beta 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 post-natal pancreas (Gu et al., 2002).
RESULTS

Activation of Ngn3 Gene Expression Induces Beta Cell Hyperplasia in Adult Mice

Pancreatic beta cells have a slow turnover under normal physiological conditions but expand rapidly under certain experimental conditions like partial duct ligation (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 one 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 (Figure S1B,S2E) and consequently, the density of duct structures significantly increased (Figure 1A,S1A,B). The weight of the pancreatic tail decreased, (Figure S2A) while body weight and glycemia remained unaffected (Figure S2B,C). The total insulin+ cell mass in the ligated part of pancreas increased more than 2-fold within one week following surgery (Figure 1B,S1D) and the absolute amount of immunoreactive insulin had doubled one week later, a lag period likely needed for beta cell maturation (Figure 1C). The individual beta 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 beta cells in active cell cycle increased 10-fold as established by incorporation of the nucleotide analogue BrdU (Figure 1D,S1E). As PDL robustly induced generation of new beta cells, we investigated the importance of progenitor cell activation and beta cell proliferation in doubling of the beta 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 one week and subsequently decreased slowly (Figure 1E). Besides neo-formation of beta cells, the number of apoptotic beta cells increased significantly (Figure S1F). Apoptotic beta cells were not preferentially in active cell cycle (Figure S1G).

To investigate the causal relationship between the doubling of beta 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 one week after surgery (Figure 2A). Following infection with lentivirus expressing short hairpin RNA, acinar cells disappeared similarly as in control PDL pancreas and no off-target effects on differentiation and proliferation of duct cells were observed (Figure S4A-C). 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 (Figure 2B, upper and S5). PDL-induced increase of the beta cell mass was prevented by 66 ± 17% (sh1) and 26 ± 10% (sh2) following infection with Le-shNgn3 (Figure 2B, middle). These data directly demonstrate that beta 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 vs Le-scr injection (Figure 2B, lower), indicating that an important fraction of BrdU\(^+\) beta 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 \textit{Ngn3} expression in injured pancreas of adult mouse we attempted to track these islet progenitor cells in transgenic Ngn3-nLacZ mice, expressing a nuclear \(\beta\)-galactosidase (\(\beta\)Gal) reporter protein under control of a 6.9 kb genomic sequence that includes the \textit{Ngn3} promoter and faithfully recapitulates the spatial expression of Ngn3 in the embryonic as well as in the adult pancreas (GM and GG, unpublished data). Histochemistry for \(\beta\)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 \(\beta\)Gal\(^+\) cells was examined in 6 mice, 7 days following PDL (Figure 3A). Of all \(\beta\)Gal\(^+\) cells 15 ± 1% were immunoreactive for duct cell-specific cytokeratins (CK) (Figure 3B) and half of the \(\beta\)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% \(\beta\)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 (Figure 3E-F). On the other hand, the long half-life of the reporter protein \( \beta \)Gal (Gonda et al., 1989) allowed tracing the fate of Ngn3\(^+\) cells to their endocrine descendants. Indeed, 34 ± 4% of \( \beta \)Gal\(^+\) cells contained transcripts encoding islet hormones at day 7 following PDL (Figure 3G,H). Half the number of hormone expressing \( \beta \)Gal\(^+\) cells was still in contact with duct cells but none of these were part of the luminal lining. No \( \beta \)Gal\(^+\) cells co-stained 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 beta 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 \( \beta \)Gal\(^+\) cells in 30 tissue sections from the PDL pancreas of 3 mice, approximately 5000 \( \beta \)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 \( \beta \)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 pre-endocrine cells (data not shown). These GFP\(^+\) cells with
hormone-containing vesicles were excluded and only non-granulated 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 population as compared to non-sorted PDL pancreas cells (Figure 4B). A similar cell population was isolated from the pancreas of Ngn3eYFP/+ knock add-on mice (Mellitzer et al., 2004), corroborating faithful recapitulation of Ngn3 expression in the Ngn3-eGFP mice (Figure 4D and 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 βGal (800 cells counted) while the reporter was expressed in 5% of islet cells (Figure S6D). In addition to the differentiation of Ngn3+-to-beta 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). 99.8% of the sorted cells from embryonic pancreas and adult duodenum were GFP-positive while none immunostained positive for insulin. The abundance of Ngn3 transcripts in these cells was more than 100-fold higher than in the non-sorted 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 beta cells (985 ± 112 µm³ vs. 3052 ± 178 µm³) and had relatively large nuclei (695 ± 98 µm³ vs. 565 ± 76 µm³) with a remarkable amount of heterochromatin in the periphery of the nuclei (Figure 4F). The ultra-structural 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 beta cells, most GFP/LSSC cells from embryonic or adult pancreas were non-granulated 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 DIFFERENITATE 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 one 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) (Figure S7A-C). 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 micro-injected 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 4 islet hormones as well as their corresponding peptides (Figure 5B,C). 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. Firstly,
when GFP/LSSC cells were pre-incubated 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). Secondly, when mouse GFP/LSSC cells were cultured in explants of rat embryonic pancreas and differentiating beta 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 βGal were cultured in pancreas explants from embryonic Ngn3−/− mice, all differentiated, hormone+ cells were βGal+ (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 h 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 (vs 1.2 ± 0.8 ng at day 1) and Ngn3−/− explants supplemented with adult GFP/LSSC cells had 35 ± 7 ng insulin (vs 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 beta 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 beta 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 beta cell mass could be prevented up to 66% by Ngn3-specific RNA interference, suggesting an important contribution of progenitor cells to the observed beta cell hyperplasia. In non-ligated pancreas 67% of the cells were transduced compared to only 18% in ligated pancreas, due to the disappearance of acinar cells, the most abundant cell type of the infected pancreas and a massive recruitment of uninfected immune response cells to the pancreas affected by inflammation following PDL. That Le-shNgn3 transduction of a relatively small fraction of cells in the ligated pancreas resulted in an efficient Ngn3 knockdown can be explained by (i) 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 (ii) a near 100% knockdown of Ngn3 expression by Le-sh1Ngn3 (Baeyens et al., 2006). The remaining increase in beta cell mass in spite of Ngn3 knockdown likely is due to cycling
of (i) pre-existing beta cells and/or (ii) progenitor cells that were uninfected or that had differentiated beyond the Ngn3+ stage before being infected.

While under normal physiological conditions the slow course of beta cell proliferation is sufficient to compensate for their low turn over 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 beta cell mass indeed increases much slower than following duct ligation (Bouwens and Rooman, 2005).

The Ngn3+ islet cell progenitors co-express 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 βGal 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 beta cells with glucose responsive insulin release. We confirmed that the 6kb promoter recapitulates the endogenous Ngn3 expression by
performing PDL on the pancreas of Ngn3\textsuperscript{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 \textit{in vitro}. None of these expanded colonies formed islet cells with significant glucose responsive insulin release. Recently, the non-endocrine 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. (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 beta cells in diabetes. Indeed, our findings reveal the significance to investigate the feasibility of (i) isolating facultative beta cell progenitors and newly formed beta cells from human pancreas in order to expand and differentiate them \textit{in vitro} and transplant them in diabetic patients and (ii) composing a
mix of factors able to activate beta 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\textsuperscript{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 of 5 x 10\textsuperscript{6} 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 siDESIGN® Center (www.Dharmacon.com) (Reynolds et al., 2004). Candidate target short hairpin RNA 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\textsuperscript{-/-} 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 micro-injected (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 (2 µg/ml, Sigma) and TSQ (2 µg/ml, Molecular Probes) for 15 min and sorting on a FACSArria (Becton Dickinson). GFP/LSSC cells were labeled by incubation for 10 minutes in presence of 5µM 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). For analysis of Ngn3: forward primer 5’-GTCGGGAGAACTAGGATGGC-3’, reverse primer 5’-GGAGCAGTCCCTAGGTATG-3’ and probe 5’-CCGGAGCCTCGGACCACGAA-3’). 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 4h resp. at RT following embedding in paraffin or at 4°C followed by ON in 20% sucrose and freezing. Samples for immunocytochemistry (ICC) were fixed in 4% FA for 10 minutes. 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 (4µg/ml, Sigma) or
Sytox green (5µM, Invitrogen). Images were viewed using normal (Zeiss Axiosplan 2) or confocal scanning (Leica DMIRE) microscopy and morphometrically analyzed using NIH ImageJ (vs.1.3.1). For electron microscopy samples were prepared as in Heremans et al. (Heremans et al., 2002).

Quantitative analysis of the beta 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. (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 days, was assayed for insulin release in the medium following incubation with 6 or 20 mmol/L glucose during the last 24 h. 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 (s.e.m.) from ≥ 3 independent experiments and considered significant if p < 0.05. All data were statistically analyzed by multi-variate comparison (2-way ANOVA) with Bonferroni correction or 1-way ANOVA with Newman-Keuls correction.
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REFERENCES


**FIGURE LEGENDS**

**Figure 1:** PDL activates Ngn3 gene expression and increases beta cell mass in adult pancreas. In 8 weeks 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 (C) (µg) of the tail part of pancreas more than 2-fold (black bars) as compared to the unligated head of the same pancreas (grey bars) and the tail of a sham-operated pancreas (white bars). (D) At 3, 7, 14 and 30 days after PDL and 1 hour before sacrifice, the nucleotide analogue BrdU (50 mg/kg) was injected intra peritoneum. The number of insulin+ BrdU+ cells on pancreatic tissue sections was 10-fold higher in the ligated tail vs control tail or head of pancreas. A similar relative increase of BrdU+ beta cells was seen when BrdU had been applied 16 hours before sacrifice (4.60 ± 0.51% in ligated tail of pancreas at day 7 following PDL vs. 0.66 ± 0.15% in unligated tail). (E) A more than 50-fold increase of Ngn3 transcripts was observed in ligated vs unligated part of pancreas by real time RT-PCR using a mouse Ngn3-specific TaqMan probe. All results shown are representative of 3 or more independent experiments. *: p<0.001 ligated vs unligated pancreas tail.

**Figure 2:** Knock-down of Ngn3 impairs PDL-induced beta cell generation. (A) The pancreatic duct of adult BALB/C mice was injected with recombinant lentiviruses encoding reporter eGFP and 2 different short hairpin RNAs 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 row). (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 (grey bar, upper). The beta 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. Beta cell mass and the fraction of BrdU⁺ insulin⁺ cells were determined as in Figure 1, except that BrdU was supplied 16 and 2h before sacrifice. All results shown are representative of 3 independent experiments. *: p<0.05 Le-shNgn3-eGFP vs 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. 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 βGal 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,H). The identity of the βGal-expressing cells was determined by combined immunohistochemical detection of ductal cytokeratins and/or islet hormones (B-D,G,H). An overview of the distribution of co-expressing cells was based on the examination of 785 βGal+ cells in the ligated pancreas of 6 mice (A). 15 ± 1% of all βGal+ cells expressed duct cell-specific cytokeratins (CK) but no insulin (INS) (B, arrow) or other islet cell hormones (not shown). Half of the βGal+ CK+ hormone− cells were in direct contact with the duct lumen (C). Half of the βGal+ 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 co-expression with islet hormones, insulin (E, E’), glucagon (GCG), somatostatin (SST) or pancreatic polypeptide (PP) (F), one third of all βGal+ cells were hormone+ indicating that Ngn3+ cells are the source of endocrine islet cells (G,H). DAPI (blue) (E-F) and PI (red) (C) stain the nuclei. All sampling was done one week following PDL. No Ngn3 or βGal 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 co-expression in cells lining the duct of PDL pancreas (I). Magnification bars are 10 µm.

**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 Zn²⁺-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 non-sorted pancreas cells (white bars), GFP/LSSC cells (grey 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,C) or eYFP added on the Ngn3 locus (D). All RT-PCR results shown are representative of 3 independent experiments. (E) Immunodetection of insulin+ cells on cytospins of non-sorted 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) Ultra-thin 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 non-sorted 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.

**FIGURE 5: Ngn3+ cells from adult pancreas differentiate in vitro into functional beta cells.** (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 micro-injected 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 one 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 4 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 (grey 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 h 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 (grey bars) glucose for 24 hours. Explants from embryonic pancreas of WT mice and of non-engrafted Ngn3-/- mice were taken as positive and negative control, respectively. All results shown are representative of 3 independent experiments. *: p<0.001 insulin release at 6 mmol/L vs 20 mmol/L glucose.
Figure 1

A

B

C

D

E

Fold increase (Ngn3/CycloA)
Figure 2

A

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B

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Figure 3

A. Diagram showing the relationship between hormones and CK.

B. Image showing INS, DNA, GCG+SST+PP, and Ngn3.

C. Image showing bGAL and INS.

D. Image showing GCG+SST+PP and Ngn3.

E and E'. Images showing DNA, INS, Ngn3, GCG+SST+PP, and Ngn3.

F. Image showing CK and Hormone.

G. Image showing bGAL and INS.

H. Image showing bGAL and Ngn3.

I. Images showing CTR and PDL D7.
**Figure 4**

**A**

<table>
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<th>Adult pancreas</th>
<th>GFP/LSSC</th>
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**B**

- **Fold increase**
  - Ngn3/CycloA
  - INS1/CycloA
  - INS2/CycloA
  - GCG/CycloA

**C**

- **Fold increase**
  - Ngn3/CycloA

**D**

- **Fold increase**
  - Ngn3/CycloA

**E**

**Adult PDL D7**

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<th>Total population</th>
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**F**

- **GFP/LSSC**
  - Adult PDL D7
  - E13.5

**G**

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- **Proteins**
  - CycloA
  - Ptf1a
  - Sox9
  - HNF6
  - Nkx6.1
  - HB9
  - Pdx1
  - Ngn3
  - IA1
  - Pax4
  - Arx
  - Nkx2.2
  - NeuroD
  - Pax6
  - Glucagon
  - Insulin
Figure 5

(a) Adult pancreas
Ngn3-GFP cells
Endogenous embryonic
ngn3 cells in WT explant

(b) E12.5 pancreas
D-1
D0

(c) (1) Ngn3-/-
(2) Ngn3-/-
(3) WT

B

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| E12.5 pancreas explant D1 of culture
Ngn3-/- + Adult PDL D7 | ![Image](image5) | ![Image](image6) | ![Image](image7) | ![Image](image8) |
| + E13.5 ngn3   | ![Image](image9) | ![Image](image10) | ![Image](image11) | ![Image](image12) |

C

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D

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Injected cells
sham sham E13.5 Adult PDL GFP/LSSC
WT Ngn3-/-

E12.5 pancreas
Injected cells
sham sham E13.5 Adult PDL
WT Ngn3-/-

Fold increase (10^6)
0 3 6
0 3 6
0 3 6
0 3 6
0 3 6
0 3 6
0 3 6
0 3 6
0 3 6
0 3 6