Regeneration relies mainly on the plasticity of the differentiated cells within the pancreas. This mechanism of embryonic pancreas differentiation in response to injury, and contribute to regeneration of the pancreas. This mechanism of replication of pre-existing differentiated cells, hypertrophy of both hepatic stem cells and oval cells are recognized as tissue-specific stem cells, regeneration occurs mainly by replication of existing differentiated hepatocytes (Fausto and Campbell, 2003). Pancreas tissues also turn over slowly but have substantial regenerative capacity in response to injury. Following partial pancreatectomy (Px), pancreatic-duct ligation and cerulein-induced pancreatitis, pancreatic endocrine, acinar and/or duct tissues regrow (Bonner-Weir et al., 1993; Gu and Sarvetnick, 1993; Jensen et al., 2005; Wang et al., 1995). The identification of pancreatic stem cells has been elusive but they might exist.

Following a 90% Px in the adult rat (Bonner-Weir et al., 1993; Bonner-Weir et al., 1983) regeneration is extensive. After 4 weeks, the endocrine and exocrine pancreas increased eightfold and sixfold, respectively. The regenerative mechanisms include enhanced proliferation and differentiation into all pancreatic cell types, forming new lobes of pancreas (Bonner-Weir et al., 1993). We have hypothesized that mature pancreatic ducts could act as facultative stem cells or a pool of potential progenitors both in vivo and in vitro (Bonner-Weir et al., 2004; Yatoh et al., 2007). This process could be accomplished through dedifferentiation to a progenitor-like phenotype and then redifferentiation through a pathway such as used during normal embryonic development. Indeed, in the cerulein-induced pancreatitis model, in which only acinar cells are lost, the ensuing acinar regeneration occurs predominantly through acinar cell dedifferentiation into cells that resemble embryonic pancreatic precursors that proliferate; their subsequent redifferentiation to acinar cells parallels that of embryonic pancreas development (Jensen et al., 2005).

Development of the mammalian pancreas occurs by the sequential differentiation of multipotent pancreatic progenitors located at foregut endoderm (Gittes, 2009; Slack, 1995). These pancreatic progenitors can be defined by the cadre of transcription factors, including several hepatocyte nuclear factors (Tcf1/2, FoxA2, Hnf4 and Hnf6/Onecut-1) as well as Pdx1, Hb9, Ptf1a-p48 and Sox9 (Collombat et al., 2006; Murtough, 2007). The endocrine progenitor cells then appear within this progenitor pool and are recognized by the expression of the pro-endocrine factor Neurogenin3 (Ngn3). Nkx2.2, Pax4, Pax6, MafA, MafB and Arx are expressed later and result in sequential differentiation and maturation of different lineages of hormone-producing islet cells (Collombat et al., 2006; Murtough, 2007; Nishimura et al., 2006). A more recent study showed that one of the acinar-associated enzymes carboxypeptidase a1 (Cpa1) is expressed in Pdx1+ Ptf1a+ cells located at distal ‘tip’ domains in developing pancreatic tubules,
Ducts as progenitors during regeneration

Journal of Cell Science

marking a distinct compartment of multipotent cells in the embryonic pancreas (Zhou et al., 2007). Although much progress has been made in understanding the expression and function of factors regulating organogenesis and differentiation in embryonic pancreas, less is known about their role in the expansion of pancreatic, and specifically β-cell, mass after birth or during regeneration.

In the Px model, in which all pancreatic cell types are regenerated, the molecular changes during regeneration are still unknown. In the current study, we define stages of the regeneration after Px and examine the expression of factors related to pancreas development. We show that: (1) rapid loss of differentiated phenotype in mature ducts is a very early event in pancreatic regeneration after Px; (2) the regenerating foci originate from and are continuous with the common pancreatic duct (CPD); (3) the proliferating ducts of these foci possess a molecular profile that is similar to pancreatic progenitors; and (4) the differentiating endocrine β-cells derived from these cells follow a maturation pathway similar to that in embryonic development. In summary, we provide strong evidence that mature pancreatic duct cells can regress and recapitulate the embryonic differentiation program during regeneration. Here, we map the molecular events mediating this process of dedifferentiation and redifferentiation to the different pancreatic cell types. Clues from regeneration models might provide new approaches to induce the formation of new β-cells in vivo.

Results

Early loss of Hnf6 and downstream duct-related transcription factors occurs before proliferation during regeneration

The expression of the transcription factor Hnf6 is an important marker of the duct epithelial phenotype. During development, Hnf6 is expressed throughout the undifferentiated ductal epithelium and becomes restricted to the ducts in the adult pancreas; the absence of Hnf6 expression results in perturbed ductal morphogenesis (Pierreux et al., 2006). A recent study using conditional gene inactivation showed that Hnf6 has an important role both at early and late pancreatic development stages and is essential for maintenance of adult pancreatic duct morphology (Zhang et al., 2009). Therefore, we examined the expression of Hnf6 as a marker of differentiated ductal epithelial cells in the pancreas following Px.

We found a rapid loss of Hnf6, which precedes decreased expression of other ductal markers and proliferation of the duct cells. By Px+4 hour Hnf6 mRNA was significantly (P<0.01) decreased whereas mRNA for Sox9 and Tcf2 (Hnf1β) were not significantly decreased until 16 hours (Fig. 1A). In our previous study, increase in proliferating (BrdU+) cells in the CPD was detected only from Px +20 hours onwards, after proliferation, duct cells transiently (48-72 hours) expressed Pdx1 protein without a change in levels of Pdx1 mRNA (Sharma et al., 1999). Consistent with those findings, mRNA expression of another replication marker PcnA was increased approximately 2.5-fold (P<0.01) in isolated Px +16 hour CPD compared with CPD from time-matched sham-operated rats (Fig. 1A) by real-time RT-PCR. Previously, we suggested that the ductal phenotype was lost with replication but had not examined the timing (Sharma et al., 1999). Here, the expression of Hnf6 was already decreasing (~50%) by Px +4 hours, reaching 20% of the level observed in the sham-operated control by 16 hours (Fig. 1A). This decrease in Hnf6 mRNA precedes that of two other transcription factors Sox9 and Tcf2 (Fig. 1A).

Decreased Hnf6 protein also preceded proliferation. In adult rat pancreas, Hnf6 protein was expressed in ductal cells of the CPD (Fig. 1B), many small pancreatic ducts and some centroacinar cells (data not shown). At Px +1 day and +2 days, both the immunostaining intensity and number of positive cells for Hnf6 decreased in the CPD (Fig. 1B), indicating that duct cells change their molecular profile within 48 hours of Px surgery. At Px +3-4 days, both the number of Hnf6-expressing cells and their intensity greatly increased (Fig. 1B), but returned to near-sham level by Px +5-7 days (Fig. 1B). A similar expression pattern was observed for Sox9 (Fig. 1C). It is noted that, for unknown reasons, Hnf6 and Sox9 protein expression increased, whereas levels of their RNA did not (Fig. 1A). This pattern was similar to what we previously reported for Pdx1 expression in Px rats (Sharma et al., 1999). In addition to duct-associated transcription factors, mRNA expression of an epithelial marker, e-cadherin (Cdh1), and a functional duct marker, cystic fibrosis transmembrane conductance regulator (Cftr), decreased by Px +1 day, the period when ducts in the CPD begin to proliferate (Fig. 1D). Thus, the reduction of Hnf6 might initiate the loss of the differentiated phenotype and the dedifferentiation of ducts to a progenitor-cell-like phenotype.

In our previous studies using either 12 hour or 6 hour incorporation of BrdU before sacrifice, labeled cells were increased compared with the control at Px +24-36 hours (Bonner-Weir et al., 1993; Sharma et al., 1999); importantly there was no increase in BrdU-labeled cells at either 12 or 18 hours after Px. To further examine the relationship between loss of Hnf6 and proliferation, we co-stained for Hnf6 and the proliferation marker Ki67 in the CPD from sham-operated, Px +10 hours and Px +1 day rats (Fig. 1E,F). As with PCNA, Ki67 expression was induced during early G1 and was maintained until mitosis, during which it is degraded (Gerdes et al., 1984). In sham-operated rats (n=5) most epithelial cells in the CPD were Hnf6+ and 15.3±1.3% were Ki67+. At Px +10 hours (n=3), a greater proportion of the cells had decreased intensity of Hnf6 staining (more Hnf6low) than in the sham-operated control, and yet the percentage of Ki67+ cells was unchanged (12.4±6.6%). At Px +1 day (n=4), there was no further change in Hnf6 staining, but now 60.3±13.8% of the ductal epithelial cells were positive for Ki67. These data indicate that duct proliferation only occurs after the loss of Hnf6 protein. The dynamic expression pattern of Hnf6 protein in the CPD after Px might reflect its requirement in both maintaining duct differentiation (Px +1-2 days) and regulating growth of pancreatic progenitors (Px +3-4 days) during regeneration.

Extensive branching morphogenesis of the CPD forms foci of regeneration

Following rapid expansion of the ductal cells of the CPD at Px +1-2 days, areas of regenerating ductules are transiently seen starting at approximately 60 hours after Px (Bonner-Weir et al., 1993; Sharma et al., 1999). These areas are well-defined structures consisting of abundant stromal cells, increasingly branched ductules, and differentiating acinar and islet cells. Their appearance follows that of the proliferation of the CPD and their disappearance coincides with the growth of the pancreatic remnant (Bonner-Weir et al., 1993). The lack of a cell-lineage-tracing system in rats precludes the use of genetic-lineage tracing to show that the regenerating foci are derived from the CPD. Instead we examined the continuity of the ducts with the CPD in three ways.

In the first, injection of eosin-gelatin solution into the pancreatic ductal tree allowed the visualization of its three-dimensional
structure (for experimental details, see the Materials and Methods). There were consistently more branches along the CPD from Px +4 day rats \((n=4)\) than from sham-operated rats \((n=3)\) (Fig. 2A), suggesting that the CPD is probably the origin of the regenerating foci. To further characterize these branching tissues, we dissected them from the CPD and performed Pdx1 immunostaining to determine whether their histology resembled regenerating foci (Sharma et al., 1999). These CPD-derived tissues were comprised of stroma and Pdx1+ ducts (Fig. 2B,C). Then, on serial sections (Fig. 2C), we observed the smaller-branching Pdx1+ ducts and the open lumen structures seen in the foci (Sharma et al., 1999). Additionally, sequential transverse sections of isolated CPDs from Px rats (illustrated in supplementary material Fig. S1A) showed the continuity of the duct epithelium of foci and the CPD (Fig. 2D,E and supplementary material Fig. S1B). These data show that the foci tissues, with enriched duct and stroma profiles, are indeed
Fig. 2. The regenerating foci develop from the CPD. (A) In ductal casts made by injection of eosin and gelatin through the CPD, more numerous branches (arrows) from the CPD are seen at Px +4 days compared with sham-operated animals. The image on the far right is at higher magnification. (B,C) Pdx1 immunostaining of the branched tissues cut from Px +4 day CPD (n=7). Areas enriched in Pdx1+ duct profiles and stroma tissues resemble the young regenerating foci (detailed below) observed in pancreatic sections. Images from serial sections (C) show the branching of Pdx1+ ducts into open-lumen ductal structures as in foci. Red-dotted lines highlight gradual expansion from a small duct profile into more branching Pdx1+ ducts (red asterisks). The sections were collected every 10 μm. (D) In these consecutive sections transversely cut along an isolated CPD, the continuity of the ductal epithelium of the CPD and the branching ductules of an early-forming focal area (yellow lines) is shown. (E) High-magnification image of the red square in D; the arrow highlights the direct connection between CPD and a duct in foci. Scale bars: 50 μm.

Finally, images of whole-mount immunostaining for the epithelial marker E-cadherin and progenitor marker Pdx1 in isolated CPDs from Px rats were taken as Z-stacks of optical sections and viewed either as reconstructed three-dimensional structures or as single frames. At Px +2 days, a number of E-cadherin+ structures began to protrude from the CPD (Fig. 3A,B); these structures extended to form elongated structures by Px +3-4 days (Fig. 3C-F). The structures expressed Pdx1 protein in branching networks with higher levels of Pdx1 expression at the tips of these structures (supplementary material Fig. S2). The Pdx1 expression was similar to that of the young regenerating foci in sectioned tissues from Px +3-4 day rats (see below, Fig. 6A). Together, these results show the continuity of the CPD and the emerging branching regenerating foci, thus suggesting that the regenerating foci originate from mature ducts, i.e. the CPD.

Regeneration following 90% Px is an asynchronous process and several stages of regenerating foci are observed within a single pancreas

Large numbers of foci can be detected within a single pancreas after Px (Fig. 4A); the number peaks at Px +3-4 days (Fig. 4B). The foci have well-defined boundaries and increasingly complex cellular organization; we suggest they are new lobes in formation. Based on their similarity with branching and differentiation seen in the developing rat pancreas (supplementary material Fig. S3A), we classified them by cellular composition and morphology: a young stage, which is predominantly stromal with a few duct tubules and an occasional hormone-positive cell; an intermediate stage, with more ductal profiles that result from further branching, but cells are mostly cuboidal without much specialization; and a complex, mature stage, which has densely packed ductal profiles and differentiating acinar cells and islets (Fig. 4C). Indeed, immunostaining of these foci showed enrichment of E-cadherin+ duct epithelium in young foci and amylase+ cells in more mature foci (Fig. 4D), implying duct-to-acinar differentiation, as seen in development of the rat embryonic pancreas (supplementary material Fig. S3B). These observations suggest that the foci reflect different stages of the regeneration process within the same pancreas, prompting us to examine the dynamics of gene expression within the stages.

Embryonic progenitor markers are expressed in ducts of young foci and are decreased in differentiating foci

Since we hypothesized that differentiation of regenerating foci recapitulates pancreatic development, we examined the expression of markers of pancreatic or endocrine progenitors during development (Collombat et al., 2006; Murtaugh, 2007) in newly formed foci. The use of laser-capture microdissection (LCM) allowed the selective isolation of the newly formed ductal epithelium of young regenerating foci for examination of the gene expression profile (Fig. 5A). The enrichment of duct epithelium in LCM samples was confirmed by increased expression of the epithelial markers Cdh1 (3.6-fold, n=8 isolated ducts from young foci from Px +3-4 day rats) and Ck20 (cytokeratin 20; 7.2-fold) and decreased the mesenchymal gene Vim (vimentin; 50% lower) in LCM-captured ducts compared with whole foci. By semiquantitative RT-PCR, expression of numerous pancreatic progenitor markers, including Hnf6, Nkx6.1, Ptf1a and Pdx1, was enhanced in LCM-captured ducts from foci compared with mature CPD ducts of control (both unoperated and sham-operated) rats (Fig. 5B). The ducts of foci had 10- to 30-fold more Pdx1, Hnf6, Sox9 and Tcf2 mRNA than CPDs from sham-operated rats by real-time PCR analysis (Fig. 5C).

Furthermore, the progenitor marker proteins were strongly expressed in focal regions, as seen with immunostaining (Fig. 6). In young foci, the Pdx1+ ductal epithelium co-expressed Sox9 and Tcf2 (Fig. 6A,B), which are only observed in early pancreatic progenitor tubules (Maestro et al., 2003; Seymour et al., 2007). Similarly, other proteins expressed in insulin-negative pancreatic progenitors, including FoxA2 (Fig. 6C), Nkx6.1 (Fig. 6D) and Glut2 (Fig. 6E) (Lee et al., 2005; Oster et al., 1998; Pang et al., 1994), were found in young regenerating foci. The initial strong
expression of Hnf6 and Sox9 protein in ducts of young foci waned as the foci matured (Fig. 7A,B), with a similar pattern for Tcf2 protein (data now shown). This decrease in progenitor marker expression as the foci mature suggests a dynamic differentiation process in regenerating foci after Px.

The endocrine progenitor marker Neurogenin 3 is activated during regeneration

For pancreatic epithelial cells to enter the endocrine lineage, they must first activate expression of the pro-endocrine transcription factor Ngn3 (Apelqvist et al., 1999; Gradwohl et al., 2000). In the developing mouse pancreas, Ngn3 expression peaks at E14.5 and then slowly decreases to undetectable levels after birth (Wang et al., 2009; White et al., 2008); its expression in individual cells is brief. By immunostaining, Ngn3 protein was detected only in the ducts of intermediate foci (Fig. 8D), scattered cells in the CPD (Fig. 8B) and some centroacinar cells in the remnant (Fig. 8D); Ngn3 expression was not detected in sham-operated pancreas (Fig. 8A), in very young foci (Fig. 8C) or in mature foci (data not shown). This finding suggested that Ngn3 is activated transiently after Px, mimicking its developmental expression in a narrow window of time.
Similar differentiation programs are seen in both developing and regenerating endocrine pancreas

Both insulin\(^+\) and glucagon\(^+\) cells form within the regenerating foci (Bonner-Weir et al., 1993) and the total number of endocrine cells increase as the foci mature (Hayashi et al., 2003). To assess the percentage of endocrine cells that were \(\beta\)-cells in the foci, sections double-stained for insulin (\(\beta\)-cell) and synaptophysin (pan-endocrine) cells were analyzed. In young foci, the endocrine mass consisted of small clusters or single cells with low proportion of insulin\(^+\) synaptophysin\(^+\) cells; the proportion of \(\beta\)-cells significantly increased as the foci matured (Fig. 9A).

We further examined the development of \(\beta\)-cells in regenerating foci by immunostaining for known pancreatic progenitor and \(\beta\)-cell markers. As insulin\(^+\) cells ‘bud’ from ductules, expression of Sox9, a crucial transcription factor expressed in pancreatic progenitors (Lynn et al., 2007), diminished (supplementary material Fig. S4A). Similarly, in patterns reminiscent of embryonic pancreas development, Nkx2.2 (supplementary material Fig. S4B), Nkx6.1 (Fig. 6D) and Glut2 (Fig. 6E) were expressed in insulin-negative ducts in younger foci, but were restricted to insulin\(^+\) cells as the foci matured. Maf factors are considered to be essential for endocrine differentiation (Artner et al., 2007; Nishimura et al., 2008), with the transition from MafB\(^+\) insulin\(^+\) to MafA\(^+\) insulin\(^+\) cells, which is a late step in development, even occurring after birth (Nishimura et al., 2006). Although available MafB antibodies do not react with rat tissue, the percentage of total MafA\(^+\) total insulin\(^+\) cells was lower in young regenerating foci than in mature foci (Fig. 9B,C), suggesting that these \(\beta\)-cells are immature. Interestingly, even in mature foci, the proportion of MafA\(^+\) insulin\(^+\) cells still had not reached that of islets of the remnant, suggesting that the transition from an immature to a mature \(\beta\)-cell was incomplete at 1 week after Px.

Discussion

Here, we examined the molecular mechanisms underlying the regeneration seen in 90% Px rats. The main mechanisms for restoring tissue mass during pancreas regeneration are replication of pre-existing differentiated cells, hypertrophy of individual \(\beta\)-

![Fig. 5. Progenitor marker mRNAs are enriched in LCM-excised duct epithelium of young regenerating foci compared with mature ducts (CPDs). (A) LCM enables specific excision of duct epithelium from young regenerating foci (yellow-dotted lines); from more mature foci, the RNA quality is compromised by the exocrine enzymes. (B) By semi-quantitative RT-PCR, pancreatic progenitor markers show greater expression in LCM-excised ducts from regenerating foci than in mature control ducts (CPD) from unoperated and sham-operated rats. Sample numbers given in parentheses. Ribosomal proteins L32 (Rpl32) and 18S were used as internal control. (C) Fold change of Pdx1, Sox9, Tcf2 and Hnf6 mRNA in LCM-excised ducts was compared with mature (sham-operated CPD) ducts using real time RT-PCR analysis, with Rpl32 as reference gene. n=3 samples of pooled LCM-excised ducts (each from three to four Px +3-4 day rats). Data presented as mean ± s.e.m.](image-url)

![Fig. 6. A set of shared progenitor markers is detected in ducts of young regenerating foci at Px +3-4 days. Immunostaining showed that ducts in young regenerating foci express proteins, mainly transcription factors, normally expressed in embryonic ductal progenitors. (A) Multiple progenitor proteins Sox9 (red), Tcf2 (green) and Pdx1 (blue) are expressed in the same ductules, as seen in embryonic duct epithelium. (B) Sox9 and Tcf2 co-localize in PanCk\(^+\) (blue) ductal epithelium in young foci, but not in acini of remnant tissues. (C) FoxA2 protein (green) is induced in E-cadherin\(^+\) (red) ductal epithelium. (D) Nkx6.1 (green) is expressed in ductal epithelium of foci (PanCk in red). (E) Glut-2 (green) is also detected in insulin (blue)-negative ductules (PanCk, red). Scale bars: 50 \(\mu\)m.](image-url)
cells and differentiation from stem or progenitor cells (Bonner-Weir et al., 1993; Bonner-Weir et al., 2004; Bouwens and Rooman, 2005; Xu et al., 1999). Using a cellular-lineage-tracing system, it was suggested that self-replication of pre-existing β-cells is the main mechanism that contributes to increased β-cell mass, even in 50% Px mice (Dor et al., 2004); however, the contributions from stem or progenitor cells cannot be ruled out owing to the low labeling efficiency. More recently, several studies provided further evidence that the stem or progenitor cells located in different pancreatic pools can be activated for the repair process under various physiological challenges and injuries. The in vivo cerulein-induced pancreatitis model supported the concept that pancreatic acini dedifferentiated and subsequently redifferentiated following a genetic program resembling that of embryonic pancreatic precursors, but the authors clearly stated there were no duct-like intermediates (Jensen et al., 2005). Xu and co-workers showed activation of Ngn3+ cells either located within or next to ducts during regeneration after duct ligation in mice (Xu et al., 2008). Lineage-tracing studies from our laboratory showed that ducts expressing carbonic anhydrase II give rise to both endocrine and exocrine cells after birth and after pancreatic ductal ligation (Inada et al., 2008). Our current data in support of the notion that ducts contribute to the pancreatic regeneration show that the regeneration follows a dedifferentiation-redifferentiation paradigm, in which mature ducts dedifferentiate or regress to a progenitor-like state and then recapitulate the normal development program to form all differentiated pancreatic cell types. It is still not clear whether all the cells can equally form all different pancreatic cell types. Our interpretation (Kushner et al., 2010) of a recent paper is that duct cells are heterogeneous in their potential to act as pancreatic progenitors. This analysis is consistent with the results from Solar et al. (Solar et al., 2009) showing that cells expressing enough Hnf1β to have effective Cre-recombination-mediated excision do not give rise to endocrine cells. Further work is needed to define the population(s) of duct cells that act as endocrine progenitors.

During development, Hnf6 is initially expressed throughout the pancreatic epithelium, but becomes restricted to the ducts, where it has been described as the master regulator for maintenance of the differentiated ductal phenotype (Clotman et al., 2002; Pierreux et al., 2006; Zhang et al., 2009). Thus, changes in Hnf6 might be necessary to trigger the dedifferentiation (CPDs in Px +1-2 day rats) to potential progenitors required for regeneration. The rebound expression of Hnf6 protein in the CPD and mRNA and protein in foci could be interpreted either as ducts simply redifferentiating back to their mature phenotype or their gaining the phenotype of embryonic pancreatic progenitors (Maestro et al., 2003). The transcriptional profile of LCM-isolated ducts from young-intermediate foci of Px +3-4 day rats (Fig. 5B) supports the latter interpretation because Ptf1a and Nkx6.1 are expressed in embryonic pancreatic progenitors and not in mature ducts (Fig. 5B) (Burlison et al., 2008; Jensen et al., 1996). Similar rapid falls of Hnf6 mRNA and protein were seen during bile-duct obstruction (Holterman et al., 2002; Tan et al., 2006). If these drops after bile-duct ligation are blunted by adenoviral overexpression of Hnf6, the wave of proliferation necessary for repair does not occur (Holterman et al., 2002), suggesting a complex role for Hnf6 during regeneration in ducts, both in liver and pancreas.

A striking finding in our study was the detection of a continuum of regenerating foci within an individual pancreas. This continuum makes it possible to examine dynamic molecular changes at different stages of regeneration without the confounding influence of intra-animal bias. Indeed, the decreasing expression of progenitor proteins (Fig. 7) and increasing maturity of islets (Fig. 9) in more mature foci provide direct evidence of progressive differentiation. The appearance of a wide range of foci facilitated the detection of
Ngn3<sup>+</sup> endocrine progenitors during regeneration. In a previous study using Ngn3-GFP mice, no Ngn3<sup>+</sup> cells were found during regeneration after 50% Px (Lee et al., 2006). However, in the duct-ligation model, Ngn3<sup>+</sup> cells were seen within or next to ducts, although their origin was unknown (Xu et al., 2008). This discrepancy might be due to: (1) differences in the severity of injury as stimulus for regeneration and/or (2) weak GFP signal in 50% Px mice. Ngn3 immunostaining was detected in the remnants of Px +3-4 day rats (Fig. 8) in a few cells of the CPD and an occasional centroacinar cell; this latter finding supports the suggestion that centroacinar cells could also be precursors during regeneration (Stanger et al., 2005). Indeed, previous studies showed progenitor markers ubiquitin carboxyl-terminal esterase L1 (Uchl1, or pgp9.5), Pdx1 and Epiplakin1 expressed in centroacinar cells (Hosotani et al., 2004; Suzuki et al., 2003; Yokoyama-Hayashi et al., 2002; Yoshida et al., 2008).

The regenerating foci we have described are seen not only after partial pancreatectomy in rodents, but also in other physiological or experimental conditions under which pancreatic remodeling occurs, such as pancreatitis (Bockman et al., 1982), chemical-induced carcinogenesis (Jimenez et al., 1999; Rao and Reddy, 1980) and some diabetic animals (Lipsett and Finegood, 2002; Wang et al., 2005). The term ‘tubular complexes’ has been used to describe both degenerative structures from injured acini or proliferative structures as we described; both structures are sometimes seen in the same pancreas. The degenerative structures partially resemble our regenerating foci, but they have residual amylase expression (which we do not see in the early foci, see Fig. 4D), an irregular luminal surface (also not seen in our ductal profiles) and less epithelial proliferation than in our foci. More recently, similar structures containing proliferating ductules were observed in sitagliptin-treated human islet amyloid polypeptide transgenic (HIP) rats (Matveyenko et al., 2009). The regenerating foci in some of these other experimental systems also express pancreatic progenitor markers, such as Pdx1, Ngn3, Nestin and Uchl1 proteins (Wang et al., 2005), suggesting that the induction of tubular complexes or regenerating foci could be a common initiating step in pancreatic regeneration. These observations underscore the importance of duct cells functioning as progenitors in regulating pancreatic mass (Inada et al., 2008; Trivedi et al., 2001).

Clues for the regulatory factors that promote differentiation of these progenitors into mature pancreatic cells might come from the knowledge of pancreatic organogenesis. During early development, distinct signals released from adjacent structures including notochord (Tgfβ), aorta (Vegf) and cardiac mesoderm (Fgfs and Bmps) are thought to be essential for the patterning of pancreatic endoderm (Deutsch et al., 2001; Kim et al., 1997; Lammert et al., 2001). In regenerating foci of Px rats, these local signaling molecules might arise from the mesenchymal (stromal) tissue surrounding the proliferating ductules. Examination of molecular ‘crosstalk’ between ductules and stroma in foci to explore triggers for differentiation of duct progenitors in regenerating foci will be important.

The dedifferentiation-redifferentiation paradigm seen in regenerating pancreas after Px can be observed in other tissues of different organisms. The clearest model might be the regenerating limbs and tails of amphibians (Slack, 1983; Slack et al., 2008).
has been shown by tracking dextran-injected myoblasts, that muscle cells could acquire plasticity during limb regeneration to form cartilage in the salamander *Ambystoma mexicanum* (axolotl) (Echeverri and Tanaka, 2002). Similar mechanisms might apply to the regeneration process in higher vertebrates (Odellberg, 2005). For example, after the resection of ventricular apex in adult zebrafish, cardiac cells close to the surgery site undergo epithelial-to-mesenchymal transition (EMT), a process that occurs in developing heart and is considered to be ‘dedifferentiation’, to regenerate the endothelial cells and smooth muscle that form new vessel tissues (Lepilina et al., 2006). However, in the mammalian heart, regeneration is mainly through the resident stem cells rather than through heart-derived dedifferentiated cells (Asoni and Sartore, 2009). Using a genetic lineage tracing system, it was shown that, through a proliferation-dedifferentiation process, differentiated cells [lung Clara cells (Rawlins et al., 2009) and renal tubular epithelium (Humphreys et al., 2008)] were the main sources of multiple cell types in normal growth and repair after tissue injury. These data suggest that cell plasticity is a more general mechanism for repair and regeneration and that the early step of dedifferentiation often accompanies an increasing proliferation index and activation of factors essential for wound healing and tissue remodeling (Carlson, 2005).

In conclusion, our data demonstrate the important role pancreatic ducts have in regeneration after injury. Although we have previously shown that self-duplication of both acinar and endocrine cells contribute to regeneration (Brockenbrough et al., 1988), the present data support the additional contribution of the dedifferentiation or regression of differentiated duct epithelial cells to less-differentiated progenitors, which expand and then redifferentiate into differentiated endocrine and exocrine cells following the embryonic developmental program. This mechanism of regeneration does not rely on self-duplication or on stem cells, but rather relies on the plasticity of the differentiation of cells within an organ.

**Materials and Methods**

**Partial pancreatectomy (Px) in adult rats**

90% Px was performed as previously described (Bonner-Weir et al., 1983) in young adult male Sprague-Dawley (SD) rats, weighing 95-110 g (TACONIC Laboratories). Morning-fed blood glucose values and body weights were measured over the first week after surgery. Animals were sacrificed by overdose of anesthesia at Px +4 days, +10 hours, +16 hours and +1-7 days. Consistent with our previous observations (Bonner-Weir et al., 1983), body weight gain was blunted for the first several days but by Px +7 days, the weight gain was similar for Px and sham control (data not shown); blood glucose levels only slightly differed starting at day 4 (104±19.2% of day 0 value; n=52) and were stably maintained until day 7. All animal procedures were approved by the Joslin Institutional Animal Care and Use Committee.

**Isolation of rat islets and CPDs**

Rat islets were isolated using a collagenase digestion protocol described before (Gotot et al., 1985). Using a modification of this procedure, CPDs were isolated (Sharma et al., 2009). Briefly, 1 ml M199 medium containing 1.5 mg/ml collagenase (Boehringer Mannheim, Indianapolis, IN) was injected into the parenchyma of the pancreas (Sharma et al., 1999). Using a modification of this procedure, CPDs were isolated (Ahnfelt-Ronne et al., 2007; Jorgensen et al., 2007). In brief, the isolated CPDs (three sham-operated, three Px +2 days, two Px +3 days and two Px +4 days) were hand dissected to remove excess acinar tissue, fixed in 4% PFA, equilibrated in cold absolute methanol, and then incubated in Dent’s bleach (methanol:DMSO:H2O2, 4:1:1) at room temperature for 2 hours. The bleaching step is crucial because it facilitates antibody penetration and reduces autofluorescent background (Alametal., 2007). Tissues were then rehydrated in PBS, blocked in 0.5% TBN buffer [supplied with Tyrannide Signal Amplification (TSA) fluorescein or Cy5 system, Perkin Elmer] and incubated in primary antibody. TSA amplification followed the manufacturer’s instructions. Stained tissues were equilibrated in absolute methanol and immersed in BABB (a 1:2 mixture of benzyl alcohol to benzyl benzoate; both from Sigma). Finally, the tissues were mounted in BABB in concavity slides (Fisher Scientific) with coverslips. The slides can be stored at 4°C without loss of fluorescence intensity for months. All images of whole-mount CPD immunostaining were taken using the Zeiss LSM 710 Live Confocal microscope in the Joslin DERC Advanced Microscopy Core as optical Z-sections using Zeiss LSM software. Raw files were analyzed and representative images were exported using Zen 2009 Light Edition.

**Laser-capture microdissection (LCM) on ducts in regenerating foci**

LCM was performed on an Arcturus PixCell IIE using manufacturer’s protocols as previously modified for pancreas (Ahn et al., 2007; Laybutt et al., 2003; Laybutt et al., 2002). Pancreas was embedded in Tissue Tek OCT medium and immediately frozen by immersion in chilled 2-methylbutane. Frozen blocks were stored at −80°C for future use (8 μm). Sections adjacent to those used for LCM were stained with H&E to determine the landmarks of ‘young’ foci of regeneration. For LCM, frozen sections were rapidly incubated sequentially in 70% ethanol (fixation), in DEPC-treated water (rehydration), H&E, and dehydrated in 70, 95 and 100% ethanol with a final step in xylene. Once air-dried, selective cells [duct or combined (duct+stroma) of whole foci] of early regenerating foci were captured under appropriate amplitude and pulse duration adjusted to allow complete tissue capture by 7.5 μm laser beam. To obtain good quality RNA, the sampling was done within 15 minutes of bringing the sections to room temperature. As a result of the digestive enzymes in the acini, only young foci yielded good quality RNA.

**Qualitative and real-time RT-PCR analysis**

Total RNA for CPDs isolated at Px +4 hours, +16 hours and +1-7 days was extracted using TRIzol (Invitrogen). RNA from LCM-captured tissues was extracted using Arcturus PicoPure RNA Isolation Kit following the manufacturer’s instructions. Purified RNA concentration was measured by NanoDrop TM 1000 Spectrophotometer (Thermo Scientific); reverse transcription was carried out using SuperScript III First-Strand Synthesis System (Invitrogen). Qualitative PCR reactions containing the mixture of cDNA, ReddyMixTM PCR Master Mix (ABgene) and sense and antisense primers (supplementary material Table S1) were processed in a thermal cycler (Bio-Rad) for indicated cycles. Samples were separated in 1.2% agarose gel. Real-time PCR was performed using ABI Prism® SDS 7900 PCR machine (Applied Biosystems). The SYBR-based detection (Applied Biosystems) method was validated and the SYBR Green detection analysis was used to determine the relative expression of target genes between different samples. Ribosomal protein L32 and S25 genes were used as the reference genes, and quantification was performed using the ΔΔCT method (Livak and Schmittgen, 2001).
Immunohistochemistry and image processing

For immunostaining, pancreas was excised, and either fixed in 4% PFA, embedded in paraffin and sectioned (5 μm) or embedded in Tissue Tek OCT medium and immediately frozen by immersion in chilled 2-methylbutane. Frozen blocks were stored at −80°C until sectioning (8 μm). Primary antibodies are listed in supplementary Table S2. All biotinylated and fluorescent-conjugated (Texas Red, Cy2, Cy3, Cy5 or AMCA) antibodies were obtained from Jackson Immunoresearch. streptavidin-conjugated Alexa Fluor 488 antibody was obtained from Invitrogen. Antigen retrieval was performed by either microwave treatment in 10 mM citrate acid buffer or in a PickCell 2100 antigen retriever in Retrievagen A solution (BD Biosciences). Peroxidase-based immunohistochemistry (ABC kit, Vector Labs) used chromagen 3,3'-diaminobenzidine (DAB, Sigma) and counterstaining of Harris’s Hematoxylin (Sigma). For immunofluorescence, either TSA system (Perkin Elmer) or biotin-streptavidin method was used. DAPI was used for nuclear staining. Stained sections were examined on Olympus BH-2 microscope or in confocal mode on a Zeiss LSM 410 microscope. Final images were compiled using Adobe Photoshop. All immunostaining results were reproducibly examined in at least five individual animals unless otherwise stated. For the percentage of MafA+ insulin+ in each stage were examined on Olympus BH-2 microscope or in confocal mode on a Zeiss LSM 410 microscope. Final images were compiled using Adobe Photoshop. All immunostaining results were reproducibly examined in at least five individual animals unless otherwise stated. For the percentage of MafA+ insulin+ in each stage.

Statistical analysis

Using GraphPad Prism 4, one-way ANOVA analyses followed by Dunnett’s comparison test were done to compare mRNA changes (Fig. 1A,D), percentage of Ki67+ cells in total CPD ducts and Hnf6 expression in total Ki67+ CPD ducts (Fig. 1E). The percentages of insulin+ synthaphysin+ and MafA+ insulin+ cells among individual cell populations (Fig. 9) were compared using two-tailed unpaired Student’s t-test.

This study was supported by grants from the National Institutes of Health R01 DK 66056 (S.B.-W.), P30 DK63686 (Joslin Diabetes and Endocrinology Research Center (DERC) Advanced Microscopy Core) as well as the Diabetes Research and Wellness Foundation and an important group of private donors. W.-C.L. was supported by a JDRF postdoctoral fellowship (3-2008-72). J.M.R. by funds from the NIH/NIDDK (K01 DK076791); and W.N. by the Mary K. Iaccoca Fellowship. Mouse monoclonal anti-ngn3 and anti-nkx6.1 antibodies (both generated by Ole D. Madsen) and mouse anti-nkx2.2 (generated by Thomas M. Jessell) were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa, IA 52242. We thank Christopher J. Cahill for superb technical support and Dr Loeken (Joslin Diabetes Center) for the use of fluorescent dissecting microscope. Deposited in PMC for release after 12 months.

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/123/16/2792/DC1

References


Table S1. Rat primers used for (semi-)quantitative RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Access number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hnf6</td>
<td>GAAAATAAAGCGTCCGTCCA</td>
<td>ACTCCTCCTTTCTCGCATTCA</td>
<td>NM_022671</td>
</tr>
<tr>
<td>Tcf1</td>
<td>ATGAGCCGTCGTCTCCAG</td>
<td>GTTGGATGGCACAGCAGGTG</td>
<td>X53297</td>
</tr>
<tr>
<td>Tcf2</td>
<td>TGCGGTGACTCAGCTACAGA</td>
<td>ATCTGTGACCACCATTGCAG</td>
<td>NM_013103</td>
</tr>
<tr>
<td>Pdx1</td>
<td>CGGACATCTCCCATACG</td>
<td>AAAGGGAGATGAAGCAGG</td>
<td>NM_022852</td>
</tr>
<tr>
<td>Sox9</td>
<td>TTCAATGAAGATGACAGCA</td>
<td>GTCCAGTCGTAGCCCTTATC</td>
<td>XM_001081628</td>
</tr>
<tr>
<td>Pcn9</td>
<td>TCACAAAGCCACTCCACTG</td>
<td>CGATCTTGGGAGCCAAATAA</td>
<td>NM_022381</td>
</tr>
<tr>
<td>Glut-2</td>
<td>TGGGTCTTCCAGTTATC</td>
<td>AGGCATCTGGTGTCTGTATG</td>
<td>NM_012879</td>
</tr>
<tr>
<td>Nkx2.2</td>
<td>ATCTGGTCTCAAAACCATCG</td>
<td>TGTACTGGGCCTTGATTTGC</td>
<td>XM_345446</td>
</tr>
<tr>
<td>Nkx6.1</td>
<td>ACTTGGCAGGACCAGAGAGA</td>
<td>GGGCTTGTTTGATCGTGTCAG</td>
<td>NM_031737</td>
</tr>
<tr>
<td>Ptf1a-p48</td>
<td>GGACTCCTCCCTCTCCTCTC</td>
<td>CTCCTGGGCTCCTACCTTTA</td>
<td>NM_053964</td>
</tr>
<tr>
<td>Uchl1 (Pgp9.5)</td>
<td>GAGAAGAAGCAGGAGCGTTCA</td>
<td>ATCTGCAGCAGAGGTCTTCTC</td>
<td>NM_017237</td>
</tr>
<tr>
<td>Cytokeratin 20</td>
<td>TACGAAACCATGCGCCAGC</td>
<td>GGAGATCGGCTCTCCACAG</td>
<td>NM_173128</td>
</tr>
<tr>
<td>Vimentin</td>
<td>ACATCCACCGCACCCTAC</td>
<td>CAACCTCCCTCATCCTCCTCTC</td>
<td>NM_031140</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>ATCTCCCCTGAGCAGGATTC</td>
<td>GCTCTTGGACACGCTTTTCTC</td>
<td>NM_033334</td>
</tr>
<tr>
<td>Cfr</td>
<td>TAAGCCATGGTCACAAGCAG</td>
<td>ACCAGCGAAGGCTTGTCTTTA</td>
<td>NM_031506</td>
</tr>
<tr>
<td>Rpl32</td>
<td>CAATGGTGTCTCTAAGACCA</td>
<td>CCTGGGCTTGGGAGGGTTG</td>
<td>NM_013226</td>
</tr>
<tr>
<td>18S ribosomal RNA</td>
<td>GCAATTATCCCTGTGAACG</td>
<td>GGCCTCAGTAAACCACATCC</td>
<td>M11188</td>
</tr>
</tbody>
</table>
Table S2. Primary antibodies used for immunostaining

<table>
<thead>
<tr>
<th>Name</th>
<th>Derived species</th>
<th>Vendor</th>
<th>Dilution (Method)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pdx-1</td>
<td>Rabbit</td>
<td>Gift from Jonathan Slack, University of Minnesota, Minneapolis, MA</td>
<td>1:6000 (Pa, TSA)</td>
</tr>
<tr>
<td>Idx-1</td>
<td>Rabbit</td>
<td>Our own</td>
<td>1:1000 (Pa, Bio-SA)</td>
</tr>
<tr>
<td>Hnf6</td>
<td>Rabbit</td>
<td>Santa Cruz Biotechnology</td>
<td>1:100 (Pa, ABC)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1:3000 (C, ABC)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1:150 (C, Bio-SA)</td>
</tr>
<tr>
<td>Sox9</td>
<td>Rabbit</td>
<td>Chemicon International</td>
<td>1:500 (Pa, ABC)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1:3000 (Pa, TSA)</td>
</tr>
<tr>
<td>FoxA2</td>
<td>Goat</td>
<td>Santa Cruz Biotechnology</td>
<td>1:100 (C, Bio-SA)</td>
</tr>
<tr>
<td>Glut2</td>
<td>Rabbit</td>
<td>Chemicon International</td>
<td>1:400 (C, Bio-SA)</td>
</tr>
<tr>
<td>MafA</td>
<td>Rabbit</td>
<td>Our own</td>
<td>1:400 (Pa, Bio-SA)</td>
</tr>
<tr>
<td>Nkx2.2</td>
<td>Mouse</td>
<td>Developmental Studies Hybridoma Bank</td>
<td>1:75 (C, Bio-SA)</td>
</tr>
<tr>
<td>Nkx6.1</td>
<td>Mouse</td>
<td>Developmental Studies Hybridoma Bank</td>
<td>1:500 (C, Bio-SA)</td>
</tr>
<tr>
<td>Ngn3</td>
<td>Mouse</td>
<td>Developmental Studies Hybridoma Bank</td>
<td>1:100 (C, Bio-SA)</td>
</tr>
<tr>
<td>Synaptophysin</td>
<td>Rabbit</td>
<td>DAKO</td>
<td>1:100 (Pa, Bio-SA)</td>
</tr>
<tr>
<td>Tcf2</td>
<td>Goat</td>
<td>Santa Cruz Biotechnology</td>
<td>1:100\textsuperscript{a} (Pa)</td>
</tr>
<tr>
<td>Insulin</td>
<td>Guinea pig</td>
<td>LINCO Research</td>
<td>1:300 \textsuperscript{a} (Pa)</td>
</tr>
<tr>
<td>Amylase</td>
<td>Goat</td>
<td>Santa Cruz Biotechnology</td>
<td>1:100 \textsuperscript{a} (Pa)</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>Mouse</td>
<td>BD Transduction Labs</td>
<td>1:100 \textsuperscript{a} (Pa)</td>
</tr>
<tr>
<td>Cytokeratin 20</td>
<td>Mouse</td>
<td>DAKO</td>
<td>1:400 \textsuperscript{a} (Pa)</td>
</tr>
<tr>
<td>β-catenin</td>
<td>Rabbit</td>
<td>Cell Signaling Technology</td>
<td>1:200 \textsuperscript{a} (Pa)</td>
</tr>
<tr>
<td>Pan-Cytokeratin</td>
<td>Rabbit</td>
<td>DAKO</td>
<td>1:100 \textsuperscript{a} (Pa)</td>
</tr>
</tbody>
</table>

\textsuperscript{a} The direct fluorescein-conjugated secondary antibody was applied for these primary antibodies

Abbreviations: Pa, paraffin-embedded section; C, cryosection; ABC, peroxidase-based immunohistochemistry (Elite ABC kit, Vector Labs); Bio-SA, biotin-streptavidin-conjugated fluorescein amplification; TSA, tyramide signal amplification (Perkin Elmer)