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Transdifferentiation of pancreatic ductal cells to endocrine β-cells

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Abstract

The regenerative process in the pancreas is of particular interest, since diabetes, whether Type 1 or Type 2, results from an inadequate amount of insulin-producing β-cells. Islet neogenesis, or the formation of new islets, seen as budding of hormone-positive cells from the ductal epithelium, has long been considered to be one of the mechanisms of normal islet growth after birth and in regeneration, and suggested the presence of pancreatic stem cells. Results from the rat regeneration model of partial pancreatectomy led us to hypothesize that differentiated pancreatic ductal cells were the pancreatic progenitors after birth, and that with replication they regressed to a less differentiated phenotype and then could differentiate to form new acini and islets. There are numerous supportive results for this hypothesis of neogenesis, including the ability of purified primary human ducts to form insulin-positive cells budding from ducts. However, to rigorously test this hypothesis, we took a direct approach of genetically marking ductal cells using CAII (carbonic anhydrase II) as a duct-cell-specific promoter to drive Cre recombinase in lineage-tracing experiments using the Cre-Lox system. We show that CAII-expressing pancreatic cells act as progenitors that give rise to both new islets and acini after birth and after injury (ductal ligation). This identification of a differentiated pancreatic cell type as an *in vivo* progenitor for all differentiated pancreatic cell types has implications for a potential expandable source for new islets for replenishment therapy for diabetes either *in vivo* or *ex vivo*.

Introduction

A goal of regenerative medicine is the therapeutic use of adult stem cells to treat many diseases. The regenerative process in the pancreas is of particular interest, since insulin-producing β-cells are lost in both Type 1 and Type 2 diabetes. The promise of embryonic stem cells is great, but, over the last few years, the task of controlling their differentiation to functional β-cells has been daunting and far slower than originally expected. Alternatives are the identification, expansion and differentiation of adult stem cells/progenitors or the expansion of pre-existing β-cells. Our efforts have been mainly on the former approach, as delineated below.

Mechanisms of growth of the endocrine pancreas

The continued and substantial growth (20-fold) of islet tissue after birth in rodents and humans (with additional rapid compensatory growth in response to increased demand) suggests the existence of adult progenitors [1]. Additionally, in mice and rats, there is clear evidence of pancreatic regeneration after some types of injury [2–4]. Two major mechanisms account for this increase in β-cells: replication of pre-existing β-cells and differentiation of new β-cells from progenitor/stem cells that were not β-cells. The latter, neogenesis, is seen as islet hormone-positive cells budding from ducts. There is no biological reason that there must be only one mechanism for replenishment of the islet cells; replication and neogenesis are not mutually exclusive.

Even with the large replicative capacity of β-cells in rodents, we have found in rats evidence of two waves of islets budding from ducts during the neonatal period: one immediately after birth and the second around weaning. Using data from our longitudinal study of the β-cell mass and its determinants [5], we estimate that over 30% of the new β-cells seen at day 31 were not from replication of pre-existing β-cells, but from differentiation from non-endocrine cells [6]. Additionally, increased neogenesis is reported in adult rodents given exendin-4 [7] or betacellulin [8], with transgenic overexpression of interferon γ [9] or TGF-α (transforming growth factor α) [10], and after 90% pancreatectomy in the adult rat [11]. The origin of these new cells is clearly from non-endocrine cells that reside in the pancreatic ducts. Whether adult stem cells, which may or may not be contained within the ductal structures, also contribute to this growth is still unclear, since there had been no direct proof of their existence. Even though there is a large body of correlative evidence of neogenesis, rigorous lineage-tracing studies to show the contribution of neogenesis have been lacking until now.

Key words: β-cell, diabetes neogenesis, pancreatic ductal cell, pancreatic progenitor, pancreatic regeneration.

Abbreviations used: CAII, carbonic anhydrase II; E, embryonic day; GFP, green fluorescent protein; MIP, mouse insulin I promoter; N-CAM, neural cell adhesion molecule; PDX-1, pancreatic duodenal homeobox-1; RT, reverse transcription.

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In vitro differentiation from non-endocrine cells

In vitro, there is considerable evidence of the induction of islet phenotype/genes from non-islet pancreatic cells. A number of pancreatic ductal cell lines have been induced *in vitro* to express islet hormones and other markers in response to GLP-1 (glucagon-like peptide 1)/exendin-4 [12–14], activin A and either HGF (hepatocyte growth factor) or betacellulin [15,16], the addition of PDX-1 (pancreatic duodenal homeobox-1) protein [17] and aggregation of tissue in serum-free medium [18]. Even more importantly, primary human pancreatic-duct-enriched (and islet-depleted) tissue, remaining after islet isolation, could be manipulated *in vitro* to generate islet-like structures that are glucose-responsive and have mature islet phenotypes [19]; our data were confirmed and extended by Otonkoski's group [20]. The evidence supports that these new islets were generated from the ductal epithelial cells. However, Gao et al. [21] reported that if they removed N-CAM (neural cell adhesion molecule)-positive cells, which they assumed to be the β-cells, from the starting material, they could no longer generate insulin-positive cells *in vitro*. However, we find that, in human pancreas, N-CAM is also expressed in much of the ductal tree, so more than β-cells were removed. In contrast, Zhao et al. [22] reported that, after removing insulin-producing cells by streptozotocin treatment *in vitro*, they could generate new insulin-producing cells *in vitro* from human pancreatic tissue. Additionally Hao et al. [23] labelled human pancreatic cells with a lentivirus after depleting dithizone-stained clusters containing β-cells, and showed that co-transplantation of these cells with human fetal pancreas resulted in islets differentiated from lentiviral-labelled adult tissue. As we reported previously [24], purified CA19-9$^+$ ductal cells from digested human pancreas depleted of islets were almost entirely CK19$^+$, with no insulin-positive cells nor insulin mRNA by qRTPCR (quantitative real-time PCR). These cells were expanded as monolayers, aggregated under serum-free conditions, and transplanted into normoglycaemic NOD (non-obese diabetic)/SCID (severe combined immunodeficiency) mice. Aggregation of purified duct with 0.1% cultured stromal cells induced insulin-positive cells (0.1% of CK19$^+$ cells), with a further increase to 1.1% 4 weeks after engraftment; insulin mRNA mirrored these changes. In these grafts, all insulin-positive cells were in duct-like structures. Some insulin-positive cells co-expressed duct markers (CK19 and CA19-9) and HSP27 (heat-shock protein 27), a marker of non-islet cells, suggesting the transition from duct. Thus purified primary duct cells from adult human pancreas can differentiate to insulin-producing cells when transplanted.

However, *in vitro* conditions may favour a plasticity of phenotype because of the lack of restraints normally present *in vivo*. One example of this would be the difference in the ability of acinar cells to transdifferentiate first to duct-like cells and then to islets, a process championed by Bouwens and co-workers [25–27]. Acinar-to-duct transdifferentiation with murine tissue has been shown *in vitro* using Cre-Lox lineage tracing [28,29], with Seino's group showing some insulin-positive cells [29]. Yet, using the same mice with the acinar-specific elastase 1 promoter in the Cre-LoxP system of lineage tracing, acinar cells were found not to contribute to *in vivo* new islet formation in adult mice or after pancreatic injury [4].

Our hypothesis of the mature duct epithelial cell as pancreatic progenitor

We have hypothesized that the sustained proliferation of the mature ductal epithelium (PDX-1 protein-negative) after partial pancreatectomy leads to an increased pool of less differentiated duct cells (PDX-1-positive, perhaps equivalent to a embryonic pancreatic epithelium) that can serve as pancreatic progenitor cells. This hypothesis is based mainly on our studies of 90% partial pancreatectomy in the young adult rat. Within 4 weeks of surgery, the 10% remnant pancreas regenerates to 27% of the pancreatic weight and 45% of the β-cell mass of sham-operated animals, essentially an 8-fold increase in β-cell mass, since there is a doubling of the β-cell mass in the sham animals over this time. This regeneration was accomplished by replication of the pre-existing cells and by formation of new lobes of pancreas that within 1–2 weeks were indistinguishable from the older ones [11]. These new lobes first appear approx. 60 h after surgery as discrete patches of proliferating ducts, which we termed foci of regeneration or focal areas. PDX-1 protein was transiently found in all duct cells following replication [30]. Immunostaining and RT (reverse transcription)–PCR data show the same cascade of transcription factors in these focal areas as in normal pancreas development as well as the loss of several mature phenotype duct markers [CAII (carbonic anhydrase II), mucin 5AC and others] in the proliferating cells. β-Cells in the islets in these developing foci expressed a number of duct genes that are not expressed in mature β-cells. So we hypothesized that differentiated (mature) pancreatic ductal cells can function as progenitors for new islets after birth, that with stimulus they replicate and regress to a less differentiated phenotype, and then can form new acini and new islets [6,30]. Rather than a transdifferentiation event, we think this is a normal regression to an earlier stage of a progenitor, a de-differentiation event.

Testing the hypothesis with duct-specific lineage tracing

Although much data support the concept of a ductal origin of new islets after birth, lineage-tracing experiments are considered the 'gold standard' of proof. Therefore, to test the hypothesis that duct cells are progenitors that can give rise to β-cells, we took a direct approach of genetically marking ductal cells. Genetically labelled tracing using the Cre-Lox system allows expansion of the reporter molecule without dilution [31]. In the offspring with the Cre transgene, a floxed stop codon before a reporter gene is excised in the cells that express the Cre, allowing the expression of the reporter gene in specific labelled cells and all their progeny. We generated transgenic mice in which the human

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CAII promoter drives expression of either Cre recombinase (CAII–Cre) or inducible Cre recombinase (CAII–CreER^{TM}). CAII is expressed throughout the ductal tree in the adult and has been used to distinguish pancreatic duct from the earlier embryonic tubular epithelium of pancreatic progenitors [32]. We characterized the developmental expression of CAII and found that only shortly before birth [E (embryonic day) 18.5] does it become expressed in ducts [33]. Using RT–PCR, CAII was shown to be present in pancreatic ducts, but not in FACS-sorted β-cells from adult MIP (mouse insulin I promoter)–GFP (green fluorescent protein) transgenic mice [34], even at 40 cycles of amplification [33].

As for any such model, we needed to validate the transgene expression [35]. After mating our CAII–Cre mice with a reporter mouse in which β-galactosidase is expressed after Cre excision, we found no β-galactosidase by immunostaining at E17.5, but found it expressed broadly throughout the ducts at birth; there were no β-galactosidase-positive insulin-positive β-cells, glucagon-positive α-cells or acini at birth. To exclude the possibility that the Cre was misexpressed in the β-cells we sorted using FACS GFP-positive β-cells from transgenic (CAII–Cre:MIP–GFP) mice. Even after 40 cycles of PCR amplification, no bands for CAII or Cre mRNA were seen from RNA obtained from these purified β-cells from 1-day-old, 2- and 4-week-old transgenic mice. Additionally, by immunostaining, the only Cre-positive cells were in the ducts and ganglia; no Cre protein expression was found in islets. Thus the transgene expression of Cre was restricted to the pancreatic ducts.

Two sets of lineage-tracing experiments were then carried out. In the first, to determine whether there had been either neogenesis or new lobe formation during normal postnatal development, constitutively expressing duct-specific CAII–Cre R26R transgenic mice were analysed for marked islets on the day of birth and at 4 weeks of age. Although at birth, few if any β-galactosidase-positive islet cells were seen, at 4 weeks, β-galactosidase expression was found in many ducts, patches of acinar cells and 35–40% of the islets. In some lobes, as many as 50% of the acinar cells were marked, with few, if any, in other lobes; this pattern is consistent with new lobe formation. These data indicate that the formation of both new islets and newly differentiated acini from CAII-expressing ductal progenitors occurs during the neonatal period.

The second set of experiments tested whether pancreatic ductal cells retain the ability to serve as pancreatic progenitors in adulthood. Here we used the inducible CAII–CreER^{TM} mice in a model of regeneration: the ductal-ligation model. This model has the advantage of having regeneration of both acini and islets localized distal to the ligation, with little to none in the non-ligated portion [36,37], allowing a comparison of labelling in regenerated and non-regenerated tissue within the same pancreas. To avoid marking the neonatal pancreatic expansion seen in the previous experiments, we administered tamoxifen for 3 weeks from 4 weeks of age onwards. Ductal ligations were performed at 8 weeks of age, and the animals were killed at 10 weeks of age. We found a substantial number of islets genetically marked distal to the ligation (the ligated portion). Thus these data also support the concept of mature (CAII-positive) ductal cells acting as pancreatic progenitors in adult mice in response to injury.

Summary

Although the concept of β-cell replacement as a therapy for diabetes seems straightforward, one major obstacle for this therapy has been the limited amount of islet tissue available for transplantation. Our genetically labelled tracing experiments provide strong evidence that pancreatic progenitors within the ductal structures contribute substantially after birth and after injury. This rigorous demonstration of pancreatic progenitor cells from the adult pancreas should direct focus to the development of new techniques to expand, both *in vivo* or *in vitro*, such cells and differentiate them to β-cells for human β-cell replacement therapy. Knowledge that mature pancreatic duct cells can serve as islet progenitors has enormous implications for designing strategies for generating new islets both *in vitro* and *in vivo*.

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