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Slow and steady is the key to β-cell replication

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- **Diabetes is a chronic disease of failed glucose homeostasis**
- **Structure and function of the adult pancreas**
- **Diabetes is an attractive target for cellular replacement therapy**
- ***In vitro* differentiation of β-cells from embryonic stem cells**
- **β-cell maintenance and expansion**
- **Strong evidence that new β-cells come from old β-cells**
- **All β-cells contribute equally to islet growth and maintenance**
- **Putative pancreatic stem cells**
- **Reported evidence for bone marrow stem cells giving rise to β-cells**
- **Reported evidence for spleen stem cells giving rise to β-cells**
- **Reported evidence for ductal stem cells giving rise to β-cells**
- **Reported evidence for acini stem cells giving rise to β-cells**
- **Reported evidence for islet and/or pancreatic stem cells giving rise to β-cells**
- **β-cell turnover**
- **β-cell mass is dynamic**
- **β-cell replication changes with age**
- **β-cell replication increases during pregnancy**
- **β-cell replication increases in cases of increased blood glucose and/or insulin resistance**
- **β-cell replication is regulated by cell cycle genes**
- **The elusive circulating β-cell growth factor**
- **Increasing β-cell replication *in vivo***
- ***In vitro* culture of β-cells**
- **Summary**

Abstract

The β-cells of the pancreas are responsible for insulin production and their destruction results in type I diabetes. β-cell maintenance, growth and regenerative repair is thought to occur predominately, if not exclusively, through the replication of existing β-cells, not *via* an adult stem cell. It was recently found that all β-cells contribute equally to islet growth and maintenance. The fact that all β-cells replicate homogeneously makes it possible to set up straightforward screens for factors that increase β-cell replication either *in vitro* or *in vivo*. It is possible that a circulating factor may be capable of increasing β-cell replication or that intrinsic cell cycle regulators may affect β-cell growth. An improved understanding of the *in vivo* maintenance and growth of β-cells will facilitate efforts to expand β-cells *in vitro* and may lead to new treatments for diabetes.

Keywords: β-cell replication • diabetes • pancreatic stem cells

Diabetes is a chronic disease of failed glucose homeostasis

Typically diagnosed during childhood, type I diabetes is caused by the autoimmune destruction of the insulin-producing β-cells of the pancreas. Patients have an inability to maintain glucose homeostasis; left untreated, diabetes results in abnormally high blood sugar levels, severe weight loss and death. First reported in 1922, insulin is a life-sustaining treatment for type I diabetics [1]. Even when managed properly with daily insulin injections, frequent monitoring of blood glucose levels and strict diet control, type I diabetes still leads to long-term complications including kidney disease, blindness and limb amputations. Approximately 0.2% of all children and adolescents in America have type I diabetes [2]; it has been estimated that nearly one million people are affected in the U.S. [2]. Conversely, 90% of diabetics have type II diabetes, a disease of peripheral resistance to insulin and insulin insufficiency [3], which is often accompanied by lack of insulin secretion, β-cell death and insufficient β-cell mass [4, 5]. The specific cause of type II diabetes is unknown, though it is typically diagnosed in adulthood and is associated with obesity.

Treatment of diabetes involves great cost: the estimated expenditure associated with treating the 20.8 million Americans affected by diabetes is 132 billion dollars per year [11, 12]. Worldwide, the prevalence of diabetes is increasing

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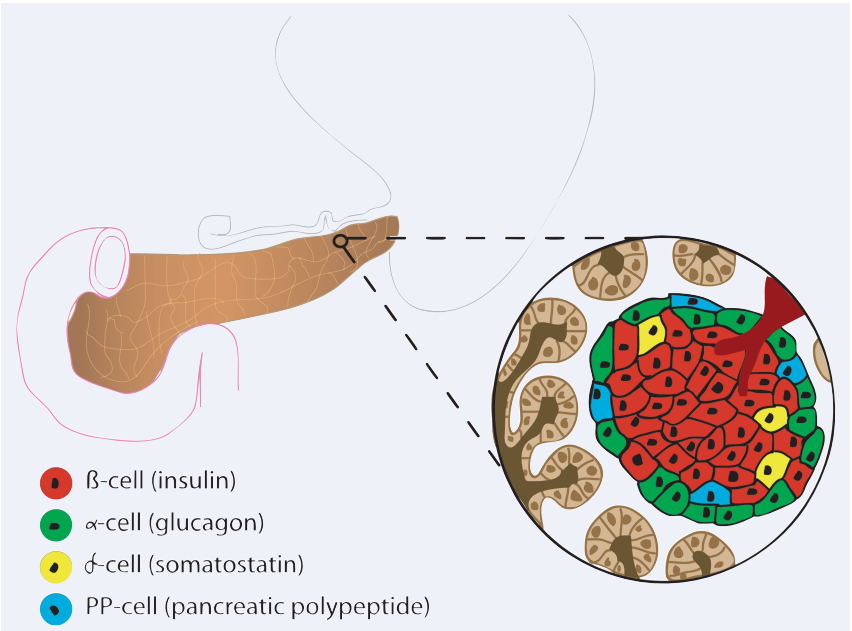

Fig. 1 Structure of the adult pancreas. Acini cells (light brown) surround duct cells (dark brown). Clusters of endocrine islets exist within the exocrine pancreas and contain four types of endocrine cells: β-cells (red), α-cells (green), δ-cells (yellow) and PP-cells (blue). Endocrine cells secrete hormones into blood vessels (dark red). In type I diabetes, β-cells are greatly reduced in number or completely absent.

and the total number of diabetics is projected to reach 366 million by 2030 [13].

In principle, a possible cure for type I diabetes might be an implantable device capable of performing the function of the destroyed β-cells: detecting blood glucose levels and releasing appropriate amounts of insulin into the blood stream in response. The development of a closed-loop mechanical device capable of both sensing glucose and secreting insulin has been difficult [14]. In lieu of such a mechanical cure, a biological cure must be twofold: preventing further immune attack and replacing the destroyed β-cells. Notably, given that both type I and type II diabetes are now understood to include reduced β-cell mass and function, β-cell replacement therapy may be a viable treatment option for both types of diabetes.

Structure and function of the adult pancreas

The adult pancreas functions as two independent organs: exocrine and endocrine pancreas. The exocrine pancreas comprises 95% of the total pancreatic mass. Embedded within the exocrine acinar cells are clusters of endocrine cells, called islets of Langerhans (Fig. 1).

The exocrine pancreas functions to produce secretory products critical to proper digestion: digestive enzymes, including trypsin, lipase, amylase and carboxypeptidase, and bicarbonate. The enzymes are synthesized and secreted from the exocrine acinar cells, and a network of pancreatic ducts drains these enzymes to the duodenum. Bicarbonate is secreted from the epithelial cells lining these pancreatic ducts. Acinar cells can be identified by digestive enzyme expression, while duct cells can be identified by their expression of cytokeratins and lectins such as those bound by *Dolichos Biflorus* Agglutinin.

Within an endocrine islet (each typically comprised of 100–1000 endocrine cells), approximately 60% (human) to 80% (mouse) of the cells are β-cells [15], which secrete insulin into the bloodstream. The remaining cell types within the islets are α-cells (secrete glucagon), δ-cells (secrete somatostatin) and PP-cells (secrete pancreatic polypeptide) (Fig. 1). Insulin functions to decrease blood glucose levels and its effects include increased glycogen synthesis in liver and muscle cells as well as increased fatty acid synthesis in fat cells. β-cells are also characterized by the expression of C-peptide, a byproduct of de novo insulin production, Glut2, a glucose transporter, and Pdx1, a transcription factor required for β-cell function. Conversely, glucagon functions to increase blood glucose levels and has complementary effects, including increased glycogenolysis and subsequent glucose release from liver and muscle cells as well as lipolysis and fatty acid release from fat cells. Somatostatin has a short half-life and its role is a predominantly paracrine effect to refine blood sugar regulation by the islet: when insulin levels are high and blood glucose low, somatostatin inhibits insulin release and, when insulin is low and blood glucose high, it inhibits the release of glucagon. Additionally, somatostatin acts in the gut to inhibit gastrointestinal hormone secretion, pepsin secretion, motility, and blood flow. The function of pancreatic polypeptide remains unclear. New evidence suggests that a fifth endocrine cell type may exist in the islet: the ghrelin-secreting cell [16, 17]. Ghrelin is thought to inhibit glucose-stimulated insulin secretion by β-cells [18, 19] and may prove to be an important regulator of metabolism and obesity.

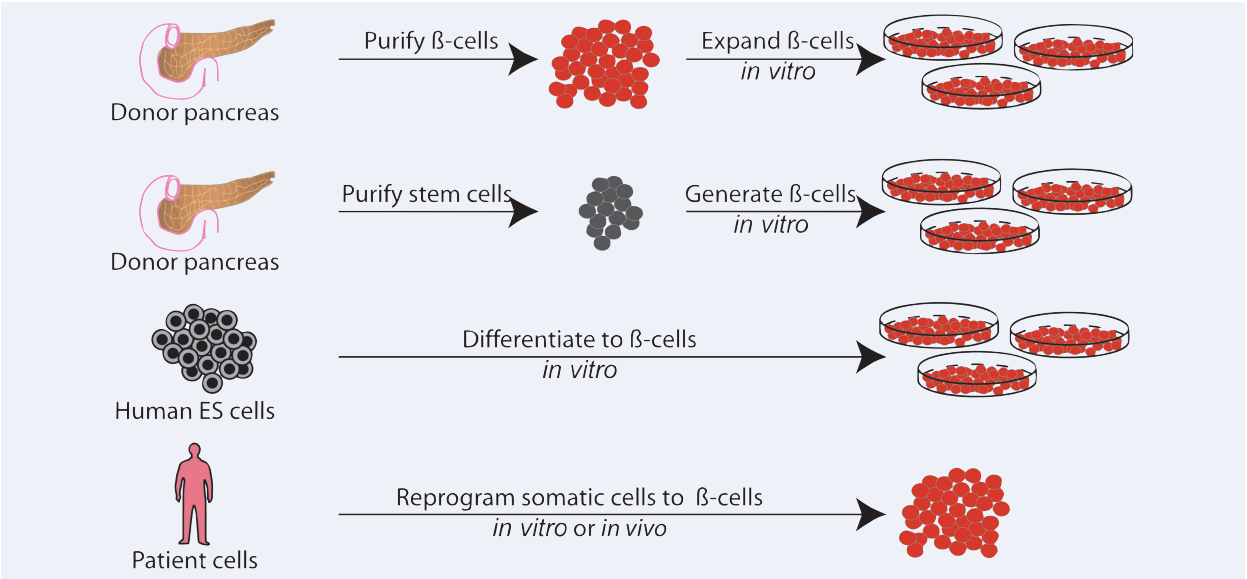

Fig. 2 Possible sources of β-cells for cell replacement therapy. First, more β-cells might be generated from existing β-cells through purification and *in vitro* expansion. Second, β-cells might be generated *via* a pancreatic stem cell that could be purified, expanded and differentiated *in vitro* to generate β-cells. Third, β-cells might be differentiated *in vitro* from embryonic stem cells. Fourth, β-cells might be directly reprogrammed from patient somatic cells using lentiviral expression of pancreatic β-cell transcription factors.

Diabetes is an attractive target for cellular replacement therapy

Diabetes, particularly type I diabetes, is an excellent candidate for cell replacement therapy because it is characterized by the destruction of a defined cell type, the insulin-secreting β-cell of the pancreas.

A clinical trial in 2000 demonstrated that grafting healthy human islets to diabetic patients and using a non-steroidal immunosuppression regime could result in insulin-independence [20]. The severe shortage of islets from cadaver [20] or live [21] donors has become an important limitation in the widespread clinical application of islet transplantation, as pancreatic tissue from two or more cadaver donors is typically required for therapy; 10,000 islet equivalents/kg in most recipients [20]. Islet grafts can survive and normalize glucose homeostasis for several years, though a more comprehensive follow-up study indicated that 76% of patients are once again insulin-dependent within 2 years of transplantation [22]. The majority of transplanted islets seemingly fail to engraft and become fully functional, though the cause of this failure remains unclear. Graft failure has been speculated to result from poor vascularization of the transplanted islets (perhaps resulting from non-optimal graft placement), persistent immune destruction of β-cells, toxicity of immunosuppressive drugs towards β-cells and/or insufficient transplanted islet mass [23].

Few diseases are caused by the loss of a single cell type, and fewer still have an existing transplantation protocol demonstrating any clinical success at curing the disease. It seems probable that with expanded access to pancreatic islets and/or β-cells, surgeons will find techniques to improve islet engraftment and function in diabetic patients. There are four obvious sources of β-cells for cellular replacement therapy (Fig. 2). First, β-cells might be differentiated *in vitro* from embryonic stem (ES) cells [24, 25]. Second, β-cells might be generated *via* a putative pancreatic stem cell following purification, expansion and differentiation *in vitro* to generate β-cells. Third, more β-cells might be generated from existing β-cells through purification and *in vitro* expansion. Fourth, β-cells might be directly reprogrammed from patient somatic cells, *via* viral expression of β-cell transcription factors [26]. Ultimately, cellular replacement therapy might prove a practical cure for type I diabetes, providing that a sufficient supply of islets or β-cells can be obtained.

In vitro differentiation of β-cells from embryonic stem cells

ES cells are capable of indefinite self-renewal and have the unique ability to form all cell types found in the adult. They may therefore provide a source of cells to replace those lost or absent in disease. ES cells hold the potential to be a limitless *in vitro* source of β-cells, though realizing this potential has proven more difficult than initially expected. Successful directed differentiation of ES cells to β-cells would have immediate clinical impact and has been the focus of intense research.

Though many have reported generating β-cells *in vitro* by selecting for the expression of β-cell genes [27, 28], or varying growth conditions [29–31], there are currently no reproducible means to

efficiently differentiate ES cells directly to insulin-positive cells without attempting stepwise recapitulation of embryonic development.

The most exciting early reports were a pair claiming to efficiently differentiate ES cells into insulin-producing cells that self-assemble to functional pancreatic islet-like structures [29, 30]. Though ES cell-derived insulin-positive cells underwent normal glucose-mediated insulin release, they contained 50 times less insulin per cell than normal islet cells [30]. Though these ES cell-derived 'β-cells' were insulin immunoreactive, no insulin1 mRNA or C-peptide was detected [32]. Furthermore, ES cells differentiated in the presence of fluoroscein isothiocyanate conjugated insulin showed substantial fluoroscein isothiocyanate fluorescence [32]. It is therefore important to keep in mind that ES-derived cells are capable of concentrating insulin present within the media and may falsely appear to be insulin-producing cells. Successful protocols will likely need to imitate the sequential events occurring *in vivo* during embryonic development and purify and enrich early precursors to the β-cell.

Now, three groups have independently reported directed differentiation of human ES cells into the pancreatic endocrine lineage by recapitulating embryonic development. ES cells were differentiated through a stepwise protocol, generating first definitive endoderm, then posterior foregut, pancreatic endoderm and finally hormone-expressing endocrine cells [24, 33, 34]. All groups generated Pdx1-expressing, insulin-secreting cells *via* a differentiation protocol designed to mimic embryonic development, though the three groups did not utilize the same growth factors. It is not clear whether the final differentiated cells are similar in all reports: both D'Amour *et al.* and Jiang *et al.* generated many double- or triple-hormone-positive cells (a characteristic never observed *in vivo*), and while Philips *et al.* generated fewer insulin-positive cells, like true β-cells, these cells were only insulin-positive and did not express additional endocrine hormones. All three groups used insulin secretion and C-peptide assays, which are good physiological tests for β-cell function. Additionally, D'Amour *et al.* and Jiang *et al.* used electron microscopy to show secretory granules present in the insulin-expressing cells and Phillips *et al.* demonstrated rescue of diabetic mice. More recently, the original group, with an optimized differentiation protocol, demonstrated that upon the transplantation of human ES-derived pancreatic endoderm into mice, glucose responsive endocrine cells, which properly express only one endocrine hormone, many β-cell transcription factors and the proinsulin processing enzymes, were generated [25]. These cells successfully maintained regulated serum levels of human insulin higher than those previously reported. Together, these papers represent a potentially important advance in the field of β-cell differentiation. Remaining challenges include making the differentiation process more efficient (producing a significant number of β-cells) and focusing on the physiological function of the end product.

β-cell maintenance and expansion

As recently as 20 years ago, the accepted dogma was that one was born with all the pancreatic β-cells one would ever have [35].

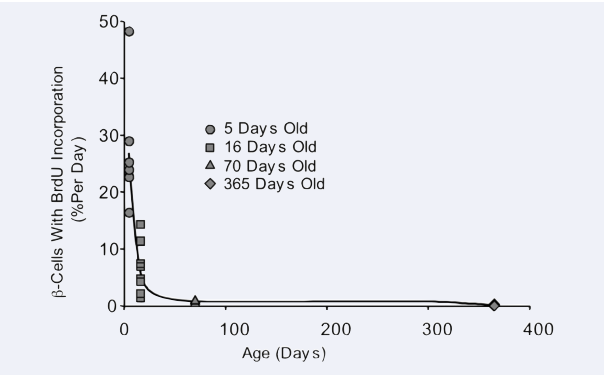

Fig. 3 β-cell replication rate declines as a function of age. BrdU incorporation studies clearly show that the rate of β-cell replication in the mouse declines with time. (From Teta *et al.*)

Now it is known that the homeostatic control of β-cell mass is based on the balance of cell proliferation and cell death. β-cell replication rates in rodents and human beings have been estimated *in vivo* by counting accumulated mitotic figures following colchicine treatment [36–38], 5-bromo-2′-deoxyuridine (BrdU) incorporation [39, 40], tritiated thymidine incorporation [41] or expression of the cell cycle marker Ki67 [42] (Fig. 3). β-cell replication is highest during late embryonic development and the neonatal period; it declines significantly throughout adulthood, leading many to propose than a stem cell pool maintains the adult β-cell population.

Strong evidence that new β-cells come from old β-cells

Lineage-tracing experiments demonstrate that pre-existing β-cells, rather than stem cells, are the major source of new β-cells in healthy and pancreatectomized mice [43]. In transgenic mice engineered such that the insulin promoter drives expression of tamoxifen-dependent Cre recombinase (RIP-CreER), a pulse of tamoxifen activates alkaline phosphatase expression specifically in β-cells. Cells generated after the pulse are only labelled if they are the progeny of existing (labelled) β-cells. Even after a chase period of up to a year in healthy and pancreatectomized mice, the proportion of labelled β-cells did not decrease, indicating that new β-cells arise from existing β-cells. Furthermore, following transient expression of diphtheria toxin in adult β-cells, the β-cell population recovers through the replication of surviving β-cells [44]. The evidence overwhelming suggests that under normal and injury conditions, most, if not all, new β-cells come from old β-cells (Fig. 4).

Furthermore, the forced cell cycle arrest of β-cells severely restricts postnatal β-cell mass [45–49], indicating that non–β-cells (such as putative adult stem cells) cannot maintain the β-cell population. Additionally, sequential thymidine analogue labelling demonstrated that β-cells are not maintained by specialized

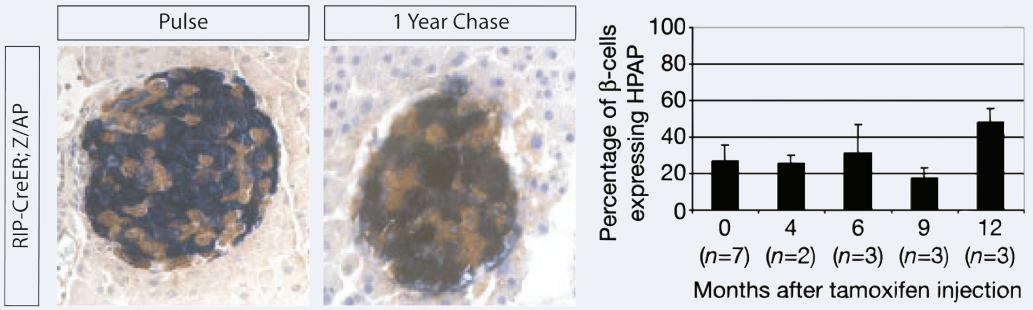

Fig. 4 New β-cells come from old β-cells. Genetic experiment that demonstrated β-cell replication is responsible for islet growth and maintenance. Expression of a Cre recombinase-estrogen receptor fusion protein (CreER) from the rat insulin promoter (RIP-CreER) permits permanent and heritable expression of alkaline phosphatase (AP) specifically in β-cells. Following a tamoxifen pulse to label β-cells, the same percentage of β-cells were labelled up to 1 year later. (From Dor *et al.*)

progenitors or stem cells during normal growth, neonatal growth or pregnancy [50]. Together, these results demonstrate that β-cell mass is predominately, if not exclusively, sustained through the replication of β-cells.

All β-cells contribute equally to islet growth and maintenance

Until recently, it remained unclear whether all β-cells contributed equally to growth and maintenance. Two possible models existed to explain the expansion of β-cells. The β-cell population could have been heterogeneous, comprised of both highly replicative cells and very slowly dividing, possibly postmitotic, cells. This would be consistent with the hypothesis that a subpopulation of insulin-expressing cells may maintain the entire pool, perhaps as unipotent adult stem cells [51] or by reversible dedifferentiation to a replicative state [52]. Alternately, the β-cell population could have been homogeneous, with all β-cells contributing equally to growth.

Through label retaining experiments, clonal analysis and sequential thymidine analogue labelling, it has been shown that the β-cell population in healthy adult mice is homogeneous, with all β-cells contributing equally to islet growth and maintenance [50, 53] (Fig. 5). Additionally, β-cells replicate homogenously during periods of increased replication such as pregnancy, neonatal growth and following pancreatectomy [50].

Putative pancreatic stem cells

Stem cells are defined by the ability to self-renew and differentiate into a variety of cell types. Some adult organs, including the intestine, skin blood, and parts of the brain, are maintained by stem cells [54–58]. In cases where the differentiated cells are postmitotic, such as erythrocytes and olfactory neurons, tissue turnover depends entirely on stem cell differentiation.

To explain the mechanism of β-cell maintenance and regenerative repair, it has been hypothesized that renewal occurs *via* adult stem cells residing in the bone marrow [59], spleen [60], pancreatic ducts [61, 62], acini [63] or islets [7–9, 64].

Most support for adult stem cells relies on indirect evidence such as transient Pdx1 or insulin expression outside the β-cell population. The most definitive and convincing experiment, a true lineage analysis demonstrating that cells that were genetically labelled with a heritable marker for either duct-, acini-, spleen- or bone marrow-specific identity can give rise to insulin$^+$ Pdx1$^+$ β-cells *in vivo* has not yet been performed.

Reported evidence for bone marrow stem cells giving rise to β-cells

There are numerous claims regarding the developmental plasticity of bone marrow cells to transdifferentiate to ectoderm, mesoderm and endoderm fates [65]. In this vein, it was reported that genetically marked bone marrow could form glucose-responsive, insulin-secreting cells *in vivo* [59]. Subsequently, many laboratories have been unable to reproduce these results and failed to find evidence that bone marrow cells give rise to β-cells [66–69]. It is now generally believed that bone marrow cells and/or haematopoietic stem cells are capable of giving rise to the cells of the blood lineage but not to other adult cell populations [70–72].

Bone marrow transplantation, however, has been associated with normalization of glycaemia and increased islet mass in diabetic mice [66, 67]. Diabetes is, to a great extent, an autoimmune

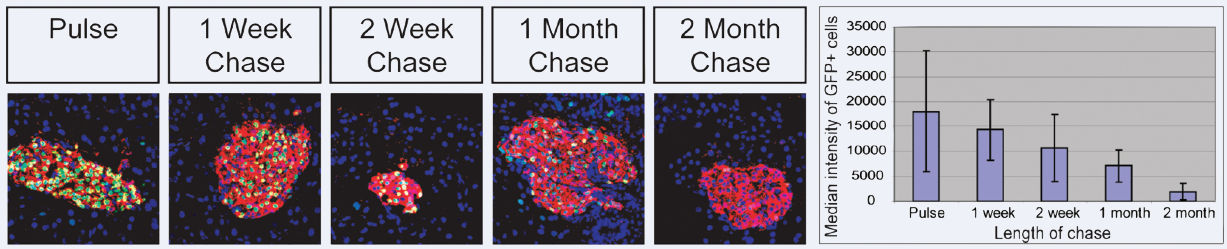

Fig. 5 All β-cells contribute equally to islet growth and maintenance. Genetic experiment that demonstrated homogeneous β-cell replication is responsible for islet growth and maintenance. Expression of a tetracycline transactivator permits inducible expression of histone2B-green fluorescent protein (H2BGFP) in β-cells. Following a pulse to label β-cells, all β-cells lost label equally during the chase period. (From Brennand *et al.*)

disease, and the homing of non-β-cell reactive haematopoietic cells to the islet may abrogate the original β-cell immune attack and thus permit β-cell recovery. Although unable to generate new β-cells, the apparent ability of transplanted bone marrow cells to initiate pancreatic recovery deserves continued investigation as a possible treatment for type I diabetes.

Reported evidence for spleen stem cells giving rise to β-cells

It was also claimed that spleen stem cells have the ability to give rise to new β-cells, based on the observation that infusions of splenocytes and complete Freund's adjuvant, when combined with temporary islet transplantation, reversed diabetes in new-onset diabetic non-obese diabetic (NOD) mice [60, 73]. Though it was suggested that splenocytes gave rise to new β-cells, it remained possible that the treatment regime facilitated an inhibition of autoreactive T cells and that the ultimate recovery was due to the replication of residual β-cells. More recently, three laboratories have independently found that following infusion of splenocytes and complete Freund's adjuvant, the recovered islets were entirely of host origin [74–76], indicating that recovery was due entirely to the expansion of residual β-cell mass in diabetic NOD mice. Notably, infusion with Freud's adjuvant alone was sufficient to dampen β-cell autoimmunity and permit recovery by the residual β-cells [75]. Therefore, reduction of the autoimmune β-cell destruction during early diabetes disease progression can result in recovery of β-cell function, an observation worthy of continued study.

Reported evidence for ductal stem cells giving rise to β-cells

There are reports that pancreatic ductal epithelial cells have the potential to dedifferentiate to a progenitor cell capable of producing new islets and acini; this is based largely on transient ductal Pdx1 expression and BrdU incorporation following pancreatectomy [61]. Following 90% pancreatectomy in the young adult rat, the pancreas recovers to 45% of the mass of the non-operated control (sham) within 4 weeks after surgery [37]. Immediately following surgery, a transient wave of DNA synthesis (as detected by BrdU incorporation) passes through the ducts as a threefold increase (to 15%) of $BrdU^+$ nuclei [77]. While sham animals have little to no BrdU incorporation and no ductal Pdx1 expression, 3 days following pancreatectomy, many duct cells express Pdx1 [77]. Furthermore, small clumps of branching structures project from the common pancreatic duct, which look as though they might be capable of differentiation into new pancreatic lobes of exocrine and endocrine cells.

Phenomena in two transgenic mouse lines are commonly cited as further evidence of ductal stem cells. In IFN-γ transgenic mice, Pdx1 can be detected in proliferating ducts, which appear to have new islets budding from them [78]. In Tgf-α overexpressing transgenic mice, Pdx1 expression is detected in the metaplastic ductal epithelium [79] and 6% of the epithelial cells in these metaplastic ducts express low levels of insulin [80].

In human beings and mice, islet-depleted pancreatic tissue, cultured under conditions believed to favour the expansion of ductal cells, forms three-dimensional cysts from which islet-like clusters budded [62, 81, 82]. Over weeks of culture in insulin-supplemented media, the insulin content and DNA content of the culture increased, implying that the predominantly ductal population may have expanded and given rise to β-cells [62, 81, 82]. Furthermore, these cells are capable of restoring normoglycaemia for up to 2 months when transplanted into diabetic mice [62, 82]. These studies, however, failed to definitively demonstrate that the ductal cell cultures were free of β-cell contamination. Additionally, with the observation that cultured cells can uptake insulin from media [32], one must be sceptical of claims of *in vitro* β-cell differentiation that are not supported by evidence that cells are also double positive for C-peptide and Pdx1.

Lineage tracing experiments, in which ductal cells are permanently and heritably labelled, have recently begun to investigate whether cells originating from the pancreatic ducts can contribute to the β-cell population. By using a duct-specific inducible Cre

recombinase (Carbonic Anhydrase II-CreER) to permanently and specifically label duct cells and their progeny, it has been shown that duct cells do not contribute to the adult β-cell population in healthy adult mice [83]. The authors suggested, however, that duct cells contribute to adult β-cell recovery following ductal ligation, though the percentage of labelled β-cells was not quantified, leaving some question as to the biological relevance of the observation. Moreover, there remains doubt concerning the fidelity of the transgenic CAII-CreER reporter used in these experiments to specifically label the duct cell population, as the report did not include images of pancreatic labelling immediately following tamoxifen administration.

Therefore, though numerous groups have brought forward evidence to suggest that ductal stem cells can differentiate to β-cells, to date, we feel that a convincing experiment proving the ability of duct cells to differentiate to β-cells is still missing.

Reported evidence for acini stem cells giving rise to β-cells

In addition to pancreatectomy, it has been reported that islet cell neogenesis is stimulated by partial duct ligation and regulated by growth factors such as gastrin and transforming growth factor (Tgf) [84]. Based on partial duct ligation experiments, an alternate model of pancreatic injury repair *via* transdifferentiation of acinar cells to islets has been suggested [85, 86].

Gastrin infusion following partial duct ligation results in a doubling of the β-cell mass as measured by morphometry [86]. This increase was not observed to be associated with increased β-cell proliferation, β-cell hypertrophy or reduced β-cell death, but instead with an increase in the percentage of single β-cells and small islet clusters. Colocalization of amylase and duct markers as well as amylase and insulin, was interpreted to suggest a direct transition from acinar to other pancreatic cell types [86, 87].

It has also been reported that cultured exocrine cells can give rise to rare insulin-secreting cells that are immunoreactive for C-peptide, Pdx1 and Glut2 [85, 88, 89], though it was not convincingly shown that the initial exocrine cell population was free of ductal or β-cell contamination. These cells are capable of restoring normoglycaemia when transplanted to diabetic mice, and hyperglycaemia recurs upon removal of the graft [85]. Two groups have independently completed cre-loxP lineage analysis of cultured acinar cells and found that cells expressing the predominantly exocrine markers amylase (Amylase-Cre) or elastase (Elastase-Cre) can give rise to insulin-positive cells [90, 91] or duct cells [91] *in vitro*. Conversely, it may simply be that a small percentage of β-cells were mislabelled by the duct reporter as these lineage studies failed to show that the ductal reporters were expressed exclusively in duct cells or that the putative β-cells generated by transdifferentiation coexpressed C-peptide and other β-cell markers. Further studies are required to convincingly demonstrate the ability of exocrine cells to transdifferentiate *in vitro.*

The ability of acinar cells to transdifferentiate *in vitro* does not necessarily denote that acinar cells can generate β-cells *in vivo*. Very recently, the first *in vivo* lineage tracing of acini cells failed to detect any contribution of acinar cells to the β-cell population, or, incidentally, the ductal epithelial population [92]. By using an acinar cell-specific inducible Cre recombinase (Elastase-CreER) to permanently and specifically label acinar cells and their progeny, it was shown that in healthy mice, as well as following pancreatectomy and ductal ligation, there is no detectable transdifferentiation of acini cells [92]. It is often difficult to reconcile *in vitro* and *in vivo* observations. In this case, the discrepancy likely reflects either the limitations of *in vitro* experiments or that exocrine cells have a more restricted plasticity *in vivo*. Regardless, preexisting acini cells have a substantial role in the maintenance and regeneration of new acinar cells but they do not appear to generate significant numbers of β-cells in mice *in vivo*.

Reported evidence for islet and/or pancreatic stem cells giving rise to β-cells

Several groups have identified rare pancreatic stem cells that can be grown clonally *in vitro* and induced to differentiate into many pancreatic cell fates, including β-cells [6–10]. These groups used different approaches, attempting to either prospectively identify pancreatic stem cells on the basis of their expression of known stem cell [6, 9] or embryonic pancreatic progenitor [10] genes or by screening for colony forming ability [7, 8]. It is not clear, however, whether the same subpopulation of cells is being isolated in each report, or what the physiological significance, if any, of these cells may be *in vivo*. The suggestion that insulin-negative cells can expand *in vitro* and give rise to insulin-positive cells is exciting, though at present, both the putative pancreatic stem cells, as well as the insulin-positive cells generated from them, require further characterization. Furthermore, it will be important to determine the identity and relationship to *in vivo* growth of these pancreatic colony-forming cells.

Nestin is a gene frequently reporter to be expressed in stem cells, and in islets, a distinct population of cells was reported that is nestin-positive but does not coexpress insulin, glucagon, somatostatin, PP, vascular, nerve or duct markers [9]. These nestin-positive cells can be purified and cultured *in vitro* for up to 8 months, grown clonally and can be induced to differentiate into cells expressing markers of pancreatic endocrine, exocrine and duct fate, as well as liver, fat and bone [6, 9]. Others reported that ES cells selected for their expression of nestin can differentiate *in vitro* into the four islet endocrine cell types [30]. In spite of this, there is not yet any evidence that nestin-positive cells can give rise to β-cells *in vivo*. In fact, two Cre/loxP lineage-tracing studies using Nestin-Cre drivers failed to find evidence that islet endocrine cells derive from nestin-expressing progenitors *in vivo*, either during embryogenesis or in adulthood [93, 94].

More recently, it was demonstrated that following pancreatic duct ligation, some pancreatic cells begin to express Neurogenin 3 (Ngn3), a transcription factor expressed in embryonic endocrine

progenitors [10]. These Ngn3$^+$ cells were termed facultative stem cells as they can be isolated from the injured mouse pancreas by flow cytometry and injected into explanted embryonic pancreas where they generate β-cells and other hormone-producing cells *in vitro*. Notably, the pancreatic cell type that initiates Ngn3 expression following ductal ligation has not been identified and the mechanism of facultative stem cell activation needs to be elucidated. It remains unclear what contribution, if any, these facultative stem cells have to normal pancreatic growth and repair, and whether these facultative stem cells persist in patients with diabetes.

By sorting cells based on cell surface marker expression, Suzuki *et al.* attempted to prospectively identify pancreatic stem cells. They purified a population of cells from whole adult pancreas that expresses the hepatocyte growth factor receptor c-Met but not haematopoietic or vascular markers [8]. These pancreatic cells formed clonal colonies at a frequency of ~0.03% *in vitro* and could differentiate into cells expressing markers consistent with pancreatic endocrine and exocrine fate, as well as liver, stomach and intestine. Similarly, another group reported that colony forming cells could be isolated from adult pancreatic islet or duct preparations at a frequency of ~0.02%, but did not characterize the cell surface marker or gene expression profiles of these colony forming cells. These single cell clones generated colonies of 2000–10,000 cells that expressed markers of neural, glial, pancreatic endocrine, exocrine and duct identities [7].

Notably, nearly all β-cells in healthy adult mice and human beings are generated through the replication of existing β-cells [42, 43, 50, 53]. The papers described in this section [6–10] describe rare cell populations and provide no evidence towards the *in vivo* role of these putative stem cell populations in β-cell growth and maintenance. The known rate of β-cell replication is sufficient to account for all new β-cells generated in neonatal and adult mice [42, 43, 50, 53]. To date, there is no evidence that in healthy adult mice or human beings, any stem cell contributes to the β-cell population.

β-cell turnover

The lifespan of an individual β-cell cannot be directly assayed. Instead, β-cell half-life is estimated based on the rate of cell proliferation and death within the population. The absolute value of both rates remains contentious as they vary greatly with age. The β-cell half-life has not been definitively quantified.

Based on β-cell mass measurements and an estimate of β-cell proliferation of 2% per day throughout adulthood, Finegood *et al.* calculated the β-cell lifespan to be 52 days [95]. This estimate is flawed in two respects: it is highly dependent on an accurate measurement of the β-cell replication rate and it assumes a constant rate of β-cell proliferation throughout adulthood. For example, if the replication rate approached a constant level of only 1% per day, the oldest β-cells would be 103 days old. Given that recent findings demonstrate that β-cell proliferation rates decline to less than 0.1% per day in 1-year-old mice [40], the fundamental assumption of a constant rate of β-cell replication throughout adulthood is erroneous. Likely, β-cells are much older than previously hypothesized.

The absolute death rate of β-cells is unknown. TUNEL analysis of wild-type β-cells consistently fails to identify apoptotic cells [40, 48, 96, 97]. It is unknown how, why or at what rate β-cells die *in vivo*. Very limited β-cell death, particularly in aged animals, is observed in healthy mice, indicating that β-cells may be very long lived. In adults, therefore, the slow rate of β-cell replication is perfectly countered by the extremely low rate of β-cell death. No adult stem cell is required to supplement β-cell replication in maintaining the β-cell population.

β-cell mass is dynamic

Although it is not generally maintained by stem cells, the β-cell mass is dynamic and can respond to environmental cues such insulin and glucose physiology [36, 98, 99]. β-cell replication increases during neonatal growth [95], pregnancy [100, 101], and in cases of insulin resistance [102]. β-cells are capable of replication and are therefore viable targets for *in vitro* or *in vivo* expansion, though the mechanism regulating β-cell expansion remains unclear.

At the moment, it remains unclear how many independent pathways are capable of affecting β-cell replication, and what extrinsic signals, if any, regulate these pathways. It is unknown whether the rate of β-cell replication changes in direct response to glucose and/or insulin levels, or whether a circulating factor affects β-cell growth. It is possible that many means of regulating β-cell growth exist and that changes in β-cell replication in response to age, pregnancy and insulin resistance occur by unrelated processes. A better understanding of the extracellular cues and the intracellular mechanisms responsible for altering rates of β-cell replication will facilitate efforts to expand β-cells *in vitro*.

β-cell replication changes with age

To compensate for increasing metabolic needs, β-cell number increases dramatically in the first year of life in rodents [43, 103]. The rate of β-cell replication changes significantly during the life of mice and rats: from ~20% per day in pups to ~10% per day at weaning, 2–5% per day in young adults and ~0.07% per day in aged 1-year-old mice [40, 50, 95]. The mechanism responsible for this decrease in β-cell replication with time is unknown. It is unclear whether this decrease in β-cell replication with age is an intrinsic property of β-cells or whether it occurs in response to decreasing levels of a circulating β-cell growth factor. The ability to restore neonatal β-cell growth rates to aged β-cells would be a substantial innovation.

β-cell replication increases during pregnancy

During pregnancy in mice, rats and human beings, rates of β-cell replication increase up to 1.5-fold [50, 100, 101, 104, 105]. Rates of replication increase during the second trimester and are

significantly decreased by parturition. Additionally, during pregnancy, β-cells show a twofold increase in glucose sensitivity and insulin secretion. The physiological cause of these changes remains poorly understood.

Several candidate factors, specifically hormones with lactogenic activity, might be involved in increasing β-cell replication during pregnancy and deserve further investigation. Pituitary prolactins and placental lactogens stimulate β-cell division and insulin secretion by islets isolated from mice, rats and human beings *in vitro* [106, 107]. Furthermore, transgenic mice with elevated levels of pituitary lactogens have 1.5-fold increased islet mass, whereas dwarf mice lacking pituitary lactogens have 10–40% decreased islet mass [105]. Finally, changes in islet cell proliferation and insulin secretion during pregnancy have been correlated to serum lactogen levels [104, 107]. It is therefore likely that circulating factors, specifically lactogens, affect β-cell mass during pregnancy.

Over the first 10 days postpartum, this increased β-cell mass reverses as a result of reduced β-cell proliferation and increased apoptosis [100, 108]. These changes are also correlated to lactation; lactating rats have lower blood glucose, lower circulating insulin levels and less β-cell mass than non-lactating rats [108, 109]. The cause of the rapid loss of β-cell mass following parturition remains unclear.

β-cell replication increases in cases of increased blood glucose and/or insulin resistance

β-cells can respond to environmental factors such as glucose and insulin [36, 98, 99], though it is often difficult to discern the individual effects of either glucose or insulin *in vivo* as the two cues generally occur in tandem. In rats, a 2- to 4-day infusion of glucose and/or insulin was claimed to result in a 50–75% increase in β-cell mass [36, 98] though in mice, a four-day glucose infusion was recently shown to induce a fivefold increase in β-cell replication but no significant increase in islet mass or number [99]. Glucose infusion increases β-cell replication in a dose-dependent manner, and though hyperglycaemia and hyperinsulinaemia occur immediately after infusion begins, β-cell replication increases only after a delay of several days, possibly resulting from an increased abundance and nuclear localization of a key cell cycle protein, cyclin D2, involved in β-cell replication [99].

It has previously been suggested that insulin or insulin growth factor (Igf) signalling may be responsible for regulating glucose-induced β-cell proliferation [110, 111]. Insulin and Igf signalling is mediated *via* two intracellular insulin receptor substrates (Irs1 and Irs2); null mutations in either Irs1 or Irs2 result in peripheral insulin resistance but only $Irs2^{-/-}$ mice are incapable enhancing β-cell replication to compensate for peripheral insulin resistance [112, 113]. Irs2 is believed to stimulate β-cell proliferation by activating phosphoinositide-3-kinase (PI3K), which activates the serine/threonine kinase Akt, which in turn inactivates glycogen synthase kinase 3 (Gsk3) and activates cell cycle inhibitors. Overexpression of Akt [114, 115] or inhibition of Gsk3 [116, 117] promotes β-cell replication and survival. Increased insulin signalling, either in tandem with or as a result of hyperglycaemia, can therefore directly affect genes known to be involved in β-cell replication.

In healthy human beings and rodents, timely insulin secretion generally prevents the occurrence of elevated glucose levels. In type II diabetes, however, peripheral insulin resistance leads to sustained hyperglycaemia, resulting in hyperinsulinaemia and islet hyperplasia [118]. It remains unclear whether the increase in β-cell mass that occurs in cases of insulin resistance is induced by hyperglycaemia, hyperinsulinaemia and/or a yet unidentified circulating factor [119].

Many mouse models have been developed with which to study the consequences of insulin resistance. Insulin receptor (IR)-null mice exhibit acute insulin resistance and die within 72 hours of birth [118]. Irs2-null mice are diabetic but incapable of β-cell proliferation in response to insulin resistance [112]. Irs1-null mice are insulin resistant but also severely growth retarded [120, 121]. It is doubly heterozygous $IR^{+/-}$ $Irs1^{+/-}$ mice that most closely model clinical cases of insulin resistance in human beings [102].

$IR^{+/-}$ $Irs1^{+/-}$ mice are highly insulin resistant and 40% develop diabetes between the ages of 4 and 6 months, despite massive hyperinsulinaemia (13-fold elevated plasma insulin levels) and pancreatic β-cell hyperplasia (2 to 30-fold increase in β-cell mass) [102]. Liver-specific insulin receptor knockout (LIRKO) mice exhibit dramatic insulin resistance, severe glucose intolerance, hyperinsulinaemia (fivefold elevated plasma insulin levels) and increased β-cell mass (sixfold) [122]. Finally, a β-cell specific knockout of the insulin receptor (βIRKO), results in insulin resistance but not increased β-cell replication, suggesting that β-cell IR-signalling is required for β-cell hyperplasia in response to insulin resistance [111].

Leptin receptor (Lepr)-null mice develop obesity due to overeating, decreased energy expenditure and excessive fat storage [123, 124]. The β-cell mass of $Lepr^{-/-}$ mice initially expands to compensate for increased peripheral insulin resistance, though β-cell mass ultimately decreases, resulting in severe hyperglycaemia [125]. There is also evidence from autopsies of human pancreas tissue to suggest a similar compensatory growth of β-cell mass in human cases of obesity [126].

β-cell replication is regulated by cell cycle genes

There is overwhelming evidence that the cyclin-dependent kinase4 (cdk4)-cyclinD complex is responsible for β-cell replication during neonatal and adult growth. Cdk4-cyclinD complexes phosphorylate proteins (including pRB, p107 and p130) responsible for cell cycle progression [127]. Cdk4-null mice are viable though they display reduced body and organ size; many tissues are unaffected, but these mice are diabetic due to proliferative defects in β-cells and infertile due to defects in spermatogenesis [128].

Cdk4 activity requires D-type cyclins (D1, D2, D3). D-type cyclin-deficient mice are fairly healthy, likely due to the structural similarity and redundant function of the D-type cyclins. Like

cdk4-null animals, cyclinD1$^{-/-}$ animals have somatic growth defects and cyclinD2$^{-/-}$ mice are infertile [129–131]. Cyclins D1 and D2, but not D3, are expressed in healthy β-cells [45]. CyclinD2 is required for the replication of endocrine cells but not for exocrine and duct cell replication: 14-day-old cyclinD2$^{-/-}$ mice have dramatically smaller islets and a fourfold reduction in β-cell mass [45]. Though adult cyclinD2$^{-/-}$ mice have glucose intolerance that progresses to diabetes by 12 months of age, cyclinD1$^{+/-}$cyclinD2$^{-/-}$ have greatly reduced β-cell mass after birth and severe diabetes by 3 months of age [46]. Therefore, cdk4 as well as cyclins D1 and D2 are essential for postnatal pancreatic growth.

Cdk inhibitors, such as p27Kip1, can also affect cell cycle progression in β-cells. Overexpression of p27 specifically in β-cells causes hyperglycaemia by four weeks of age and markedly reduced islet mass by 8 weeks of age, while deletion of the p27kip1 gene (Cdkn1b$^{-/-}$) ameliorates diabetes by increasing islet mass in both Irs2$^{-/-}$ and Lepr$^{-/-}$ mice [48]. Though no β-cell specific cell cycle gene has been identified, manipulation of the cell cycle within β-cells has the potential to restore islet mass and reverse the pathogenesis of diabetes.

An as yet unexplained observation is that embryonic and adult β-cell replication seem to be regulated independently. The forced cell cycle arrest of β-cells, either *via* the deletion of the cell cycle genes cyclinD2 [45], cdk4 [47], or the transcription factor FoxM1 [49], or *via* the overexpression of the cell cycle inhibitor p27^{Kip1} [48], severely restricts postnatal β-cell mass but not embryonic β-cell mass. Notably, Irs1 signalling seems to have a predominant role in embryonic, but not adult β-cell growth [112, 120, 121], whereas Irs2 signalling is essential for β-cell compensatory growth in adults, but not embryonic β-cell development [110, 112]. This distinct regulation of embryonic and postnatal β-cell replication leads one to speculate whether the signals responsible for regulating embryonic β-cell proliferation can be co-opted to increase adult β-cell growth.

β-cell mitogens stimulate progression through the G1 phase of the cell cycle, at least in part by stimulating activity of the cdk4-cyclinD complex. For example, prolactin and growth hormone are both known to stimulate STAT5 activation of cyclinD2 expression in mice [132, 133]. It remains to be seen whether the increased β-cell replication observed in pregnancy is also achieved through this mechanism, but evidence suggests that in pregnancy, prolactin not only increases STAT5 signalling, it also reduces the levels of the tumour suppressor gene menin, which is a known inhibitor of Cdk2 activity [134, 135]. Furthermore, evidence is now beginning to link increased cdk4-cyclinD activity to the increased β-cell replication observed in response to environmental cues such glucose infusion [99] and insulin resistance [48], though it remains unclear by what mechanism such external stimuli might affect cdk4-cyclinD activity.

The elusive circulating β-cell growth factor

One might hypothesize that a circulating factor, be it insulin, glucose or some as yet unidentified factor, must exist that is capable of increasing the rate of replication within the β-cell pool. The identification of such a factor, whether it be circulating or paracrine, would be a significant advance towards expanding β-cells for transplantation.

Several drugs developed to treat type II diabetes have been found to not only lower blood glucose levels, but also to increase β-cell replication. Glucagon-like peptide-1 (GLP-1) receptor agonists, notably Exendin-4, as well as glucose-dependent insulinotropic polypeptide (GIP) receptor agonists, not only reduce blood glucose but also increase β-cell replication [136]. A closer examination of the mechanism of action of these drugs may provide insights into the identity and/or nature of the elusive circulating β-cell growth factor.

Physiologically, both native GLP-1 and GIP are intestinal insulinotropic hormones, released to augment insulin secretion and reduce blood glucose. The ability of GLP-1R agonists to expand β-cell mass *via* stimulation of β-cell growth has been demonstrated in studies using β-cell primary cultures or cell lines, as well as in experiments using normal and diabetic rodents [137–139]. Though the mechanism by which GLP-1R agonists increase β-cell replication is still relatively unclear, it now seems that the effects are mediated through Pdx1, Irs2 and Akt [138, 140–142], while the mechanism of action of GIP receptor agonists is even less well understood but may occur *via* phosphatidylinositol 3-kinase (PI3K) [143]. Whether GLP-1 or GIP help to regulate β-cell replication in healthy mice remains a mystery, however, as the half life of these peptides in blood circulation is extremely short and estimated to be less than 90 seconds [144, 145] or 5 minutes [146, 147], respectively.

Circumstantial evidence now suggests that the increase in β-cell replication during pregnancy and insulin resistance is likely due to a circulating factor. Several hormones known to be upregulated during pregnancy, including prolactin and placental lactogen [104, 106, 107], have been associated with changes in β-cell proliferation *in vitro*. Additionally, the transplantation of wild-type islets into insulin-resistant mice leads to a marked increase in β-cell replication while transplantation of insulin-resistant islets into normal mice leads to reduced replication [119]. Parabiosis experiments, whereby the circulation systems of two animals are linked, are an excellent experimental model for the elucidation of the physiological conditions that result from circulating factors. Parabiosis of a pregnant and non-pregnant animal, for example, might allow clarification of whether a circulating factor is responsible for the increase in β-cell number during pregnancy, and/or the immediate decrease in β-cell mass thereafter, whereas parabiotic fusion between wild-type and insulin resistant animals could determine whether β-cell hyperplasia can be induced in wild-type animals. Furthermore, it would be interesting to screen for mutations that prevent β-cell hyperplasia in response to this putative circulating factor, which might help to elucidate the downstream signals responsible for increasing β-cell replication.

Clues towards identifying the genes downstream of this putative circulating factor may be found through the analysis of several forms of autosomal dominant, early onset, type II diabetes that exist in human beings. Maturity-Onset Diabetes of the Young (MODY) is

an inherited form of insulin resistance resulting from mutations in one of the following six genes: Pdx1, Hnf4α, Hnf1α, Hnf1β, NeuroD and glucokinase [148]. The transcription factor hepatocyte nuclear factor 4α (Hnf4α) regulates β-cell proliferation genes and Hnf4α-null mice fail to expand β-cells in response to physiological demands [149]. Furthermore, haploinsufficiency of Pdx1 limits β-cell hyperplasia in insulin resistant mice [96]. This suggests that at least two of the MODY genes, Hnf4α and Pdx1, may be downstream effectors of extrinsic signals working to increase β-cell replication.

Increasing β-cell replication *in vivo*

It has long been known that some type I diabetic patients have a small surviving population of β-cells [150]. It is now understood that in most diabetic patients, even those with long-standing type I diabetes, some β-cells persist and are continually destroyed [151]. This presents hope that should immunologists find the means to end the autoimmune attack of β-cells, type I diabetes may be reversed by replication of the surviving β-cells. Therefore, screening for factors that increase β-cell replication *in vivo* may have direct therapeutic benefits to diabetic patients.

β-cell replication can be induced under physiological conditions *in vivo.* These changes must be either extrinsic, occurring in response to signalling *via* a circulating or paracrine factor, or intrinsic, occurring in response to a cell autonomous effect. Therefore, screening of biological and/or chemical factors as well as gene misexpression studies should be pursued to elucidate methods to increase β-cell replication *in vivo*, which could then be applied towards β-cell expansion *in vitro*.

In vitro culture of β-cells

Attempts to expand β-cells *in vitro* have been difficult. Typically, purified islets divide slowly in culture, lose insulin expression and are gradually overtaken by insulin negative cells [152–155]. Recent data from three groups, however, suggested cultured human islets can dedifferentiate and expand, *via* an epithelial-mesenchymal transition, and then be directed to redifferentiate back toward a β-cell phenotype [9, 52, 156]. As cells expand, they express nestin and the mesenchymal marker vimentin, but not islet hormones [9, 156]. In all three studies, multiple passages were achieved and a variety of conditions were then used to force redifferentiation to β-cell fate. Notably, in all three studies, the insulin mRNA levels were only about 0.01% that of normal β-cells, suggesting that this redifferentiation is imperfect, at best.

A caveat with all of these studies is that the origin of the expanded cell is unclear. Even the purest islet preparations have many contaminating duct, acinar, stromal and endothelial cells, all of which proliferate actively. Consequently, this work was repeated in mice, where a clear lineage analysis of the expanded cell is possible. By heritably labelling β-cells with the Cre-lox system, Weinberg *et al.* followed the fate of β-cells cultured *in vitro* [157]. They confirmed that β-cells could undergo dedifferentiation to an insulin-, Pdx1- and glut2-negative state. However, dedifferentiated β-cells rarely proliferated and were eventually lost from the cultures, giving rise to cultures dominated by cells of a non-β-cell origin. It remains unclear what causes dedifferentiation of β-cells *in vitro*.

Lineage tracing of human β-cells, both *in vivo* and *in vitro*, remains an important challenge. A recent paper used dual lentiviral infection of adult human β-cells to label them with the Cre-lox system, and suggested that there may be substantial difference in the ability to culture human and mouse β-cells [158]. Though β-cells of both species cease expression of insulin within a few days of culture, this study suggests that dedifferentiated insulin-negative human, but not mouse, β-cells may be grown *in vitro* for up to fifteen passages [158]. This is an interesting finding that awaits validation by others and elucidation of the molecular mechanism. However, whether or not these dedifferentiated β-cells, have the ability to be easily differentiated back into functional glucose-sensing, insulin-releasing β-cells has not been demonstrated.

Likely, current β-cell culture conditions are not sufficient to support the maintenance of β-cell identity; therefore the majority of β-cells stop expressing insulin, Pdx1 and glut2. This loss of β-cell character may reflect the disruption of islet three-dimensional organization and/or a change in the cellular environment. Redifferentiation back to β-cell fate is a much more contentious claim and has not been convincingly demonstrated. Notably, the ability to directly reprogram exocrine pancreatic cells to functional β-cells *in vivo* [26], suggests that it may be possible to restore β-cell identity of these dedifferentiated cells by forcing the expression of certain key transcription factors.

Though a dynamic population capable of increased replication under specific physiological circumstances *in vivo*, there remains no robust method to expand β-cells *in vitro*. Given that β-cell maintenance *in vivo* occurs *via* the replication of differentiated β-cells, it is likely that critical factors responsible for maintaining β-cell identity and/or replication normally present in the adult pancreas are lacking in current *in vitro* culture systems. Screening of bioactive chemicals and co-culture substrates may elucidate conditions permissive for *in vitro* β-cell expansion. Perhaps co-culture of primary β-cells with exocrine or duct cell lines, and/or pancreatic mesenchyme, will facilitate maintenance of insulin expression *in vitro*. It is also possible that tight contacts between β-cells, such as those found in densely packed islets, are necessary to maintain β-cell identity and that dissociated islets will always cease insulin expression. Therefore, prior to initiating screens for chemical factors or cDNA transcripts that can significantly increase β-cell replication *in vitro*, one must first establish a culture system in which β-cells continue to express insulin.

To date, pancreatic stem cells have not been shown to contribute to normal pancreatic growth and maintenance. Instead, β-cell mass is predominately, if not exclusively, sustained through the replication of β-cells. Though the β-cell population undergoes increased replication in response to glucose, insulin resistance, pregnancy and modulation of cell cycle genes, adult β-cells generally replicate slowly and current efforts to expand β-cells *in vitro*

have met with limited success. Arguably the best hope for *in vitro* expansion of β-cells lies in obtaining a better understanding of the signals and factors responsible for β-cell expansion *in vivo*.

Summary

Caused by the autoimmune destruction of the insulin-producing β-cells, diabetes is an imminently curable disease. It has already been shown that islet transplantations can reverse insulin dependence, though cell replacement therapy to treat diabetes is currently limited by both the severe shortage of islets for transplantation and the poor survival and/or maintenance of islet cells following the existing transplantation protocol. The ability to differentiate mature β-cells from human ES cells and/or ability to expand adult β-cells from human pancreatic tissue might alleviate the shortage of islet cells, allowing surgeons to improve and optimize current islet transplant techniques. We hope that by better understanding the replication and differentiation of β-cells *in vivo*, it may one day become possible to generate vast numbers of β-cells *in vitro* with which to cure diabetes.

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