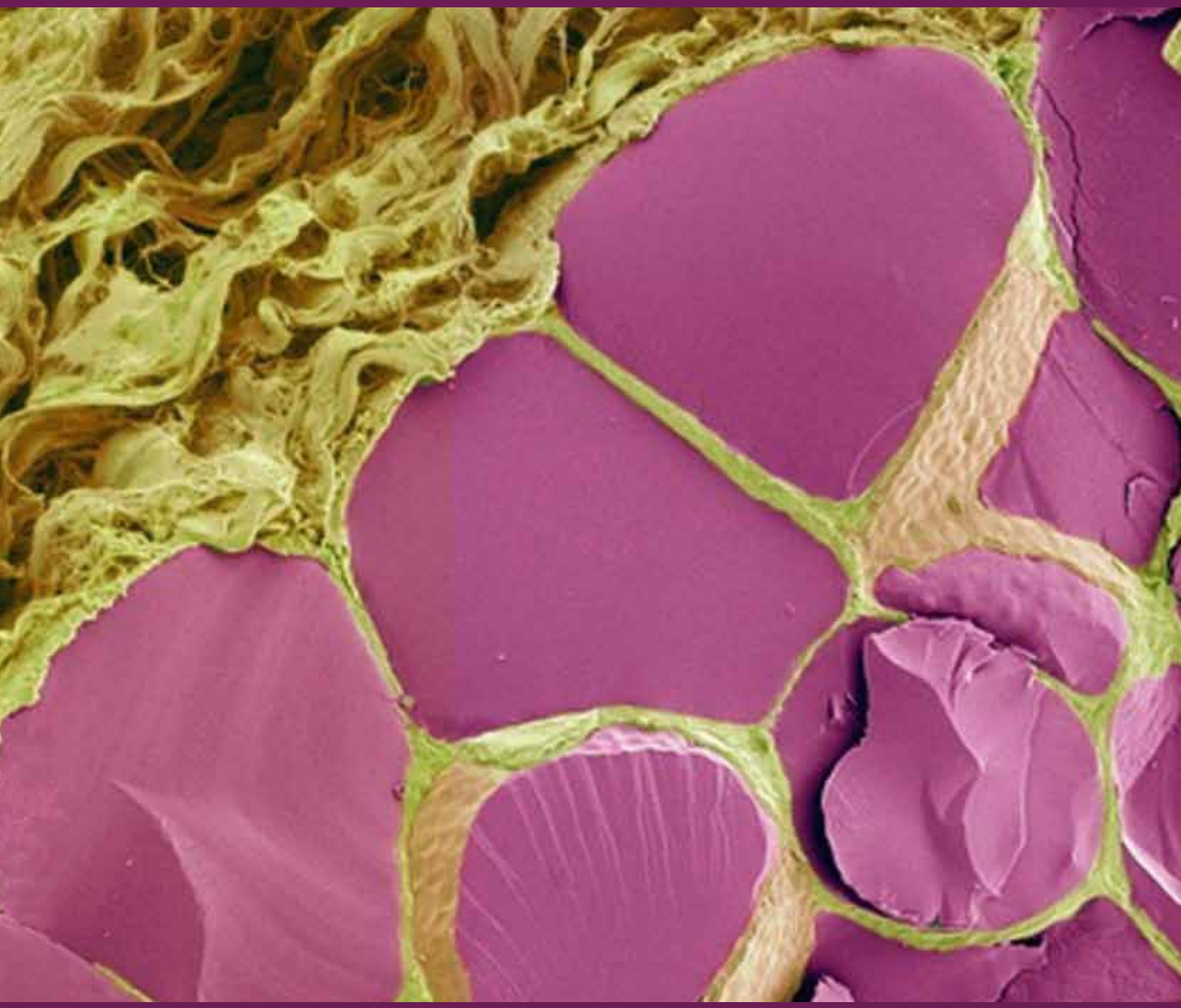
International Journal of Endocrinology

Islet Cell Biology, Regeneration, and Transplantation

Guest Editors: A. N. Balamurugan, Velayutham Kumaravel, Subbiah Pugazhenthi, and Bashoo Naziruddin



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Editorial Board

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Editorial

Islet Cell Biology, Regeneration, and Transplantation

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Research studies centered on the biology, regeneration, and transplantation of islets continue to shed significant understanding on the development of different forms of diabetes and provide further impetus for the quest to find a "cure." Diabetes is a manifestation of an inadequate mass of insulin-producing pancreatic beta-cells. While type 1 is characterized by complete loss of beta-cells due to autoimmune attack on them, type 2 is characterized by a relative deficiency of beta-cells due to a decreased compensation for insulin resistance [1, 2]. Restoring beta-cell mass and reversing diabetes can be accomplished by two approaches: either by endogenous regeneration of beta-cells or transplantation of beta-cells from exogenous sources; recent advancements in science and technology have facilitated progress in both. In the first approach, while efforts to expand mature beta-cells *in vitro* have been met with limited success, regeneration of beta-cells from embryonic and adult stem cells, or pancreatic progenitor cells, has shown promise [2]. Understanding the role of beta-cell-specific transcription factors in the transdifferentiation to beta-cell phenotype is critical to further progress. Pharmacological approaches, employing growth factors, hormones, and small molecules, have also been shown to boost beta-cell proliferation and function.

In the second approach, transplantation of isolated islets from cadaveric donor pancreas has proved to be an immediate and effective method for replacing depleted beta-cells in type 1 diabetic patients, allowing them to achieve independence from exogenous insulin administration [3, 4]. To preserve the transplanted beta-cell mass, however, islet transplant recipients require immunosuppression, which, under current regimens, are known to be beta-cell toxic. This limitation has ultimately led to poor long-term function of the transplanted islets and a disheartened medical community which is committed to providing a durable cure for patients.

In this special issue, substantial developments made in different research areas aimed at overcoming current limitations of islet regeneration and transplantation are presented. Of the numerous papers received from this open submission format, selected papers have been recommended for publication after peer reviews. This special edition presents a collection of exciting papers that describe strategies to improve availability of beta-cells and islets for transplantation, and also to improve their posttransplant survival.

It is clear that one of the major hurdles challenging further success in islet transplantation is the lack of suitable donor pancreases. This issue is compounded by poor long-term survival of allotransplanted islets. The review article by F.-C. Chou et al. summarizes many strategies developed to modulate immune response to transplanted islets. Gene therapy offers a powerful tool to engineer islet grafts to become resistant to apoptosis induced by inflammation and produce immunosuppressive molecules to attenuate T-cell response. In addition, the potential to develop patient-specific, autologous beta-cell replacement therapy by using iPSC-derived pancreatic beta-like cells is discussed. Key issues in this field which are presented in this paper include (i) duration and expression levels of targeted genes in islets, (ii) use of viral vectors for direct gene therapy that could lead to insertional mutagenesis and host immunogenicity,

and (iii) poor efficiency of differentiation of insulin-secreting cells from stem cells.

Other recent studies have shown that long-term function of allogeneic islet transplants could be improved by effective induction immunosuppression and control of inflammation [4]. Further improvement of long-term success will require control of autologous and allogeneic immune response against islet grafts. Induction of donor-specific tolerance is a "holy grail" pursued by transplant immunologists to improve survival of both solid organ and cell transplants. S. Bhatt et al. have presented a comprehensive review of the attempts to induce donor-specific tolerance. Since the current immunosuppressive regimen used in islet transplantation could be toxic to beta-cells, the future of islet transplantation is dependent on the development of tolerance-inducing therapies. A tolerizing regimen that selectively targets donor-reactive T cells while expanding populations of regulatory T cells will result in better outcomes. Further investigation into inherently tolerogenic cells such as hepatic stellate cells, sertoli cells, and mesenchymal stem cells will aid in the design of therapies.

Major causes of development of type 2 diabetes include excessive intake of food and lack of physical activity. Reduction in food intake which increases insulin sensitivity and improves glucose homeostasis is recommended to treat this metabolic disorder. The study by L. Belkacemi et al. investigated the effects of intermittent overnight fasting in streptozotocin-induced diabetic rats on glucose tolerance, plasma insulin, and homeostasis model assessment index. The study, which included an intermittent overnight fasting design (inspired by the daily fasting period during the Islamic Ramadan holiday), was recently reported to prevent the progressive deterioration of glucose tolerance otherwise taking place in sand rats exposed to a hypercaloric diet. The authors observed that the beta-cell mass, as well as individual beta-cell and islet area, was higher in intermittently fasting than in nonfasting STZ rats, and the percentage of apoptotic beta-cells was lower in the fasting STZ rats. Based on this study result, the authors proposed that intermittent fasting could represent a possible approach to prevent or minimize disturbances of glucose homeostasis in human subjects.

The paper by S.-T. Chen et al. investigated the complementary role of hyperglycemia and p27kip1 suppression on islet beta-cell regeneration in a syngeneic mouse model. Glucose has been postulated to regulate cyclin D2 in pancreatic islet beta-cells and play a dominant role in beta-cell compensation; however, it is not yet clear how glucose controls the cell cycle of islet beta-cells. It has been reported that the suppression of both cdk-inhibitors p27kip1 and p18INK4c, but not p27Kip1 alone, promotes endocrine tumor formation in rodents. In this study, they used shRNAs to silence p27Kip1 and used hyperglycemia as a complementary factor to examine the synergistic effect of glucose and p27Kip1 on the adaptation of adult mice islets. They transduced adult islets with lentivirus-carrying shRNA to silence 80% of p27Kip1 protein, and the resultant suppression of p27Kip1 expression lasted for over 96 hours after infection. The study results suggested that adult mouse islet beta-cells can replicate when the metabolic demands increase, and there is a synergistic effect of hyperglycemia and concurrent suppression of p27Kip1 on islet beta-cell replication.

Beta-cell mass is maintained at optimal levels in the body through a slow turn-over rate. In humans, it has been shown that beta-cell mass expands several folds from birth and through the first three years of childhood, but thereafter this initial period, beta-cell replication potential declines markedly until adulthood. A critical barrier to progress in the treatment of diabetes is the lack of small-molecule drugs to induce beta-cell regeneration. Small molecule-induced beta-cell proliferation in humans could be an important way to achieve this goal; such compounds could be used to restore beta-cell mass *in vivo* or alternatively provide methods for *ex vivo* expansion of beta-cell numbers before transplantation. In the review article by A. Amedeo Vetere and B. Bridget K. Wagner, the authors present an overview of the current trends involving small-molecule approaches to induce beta-cell regeneration. For further understanding about the physiological proliferative behavior of human beta-cells, we can start to identify the molecular switches that could be used to foster the proliferation of beta-cells in humans.

In another interesting investigation into beta-cell mass equilibrium, the review article by E. Tarabra et al. describes the cellular counter-forces of beta-cell proliferation, neogenesis, and hypertrophy to increase beta-cell mass, while apoptosis and atrophy (reduced cell size) decrease beta-cell mass. They proposed that postnatal beta-cell mass responds to changing metabolic demands, carried out by an interaction of beta-cell replication (proliferation and/or neogenesis) and apoptosis, and this process is regulated by different growth factors/nutrients. Specifically, this review elaborated on principal hormones and nutrients, as well as downstream signaling pathways regulating beta-cell mass in the adult. They also reviewed the role of miRNAs in beta-cell mass regulation. The most studied miRNA in this contest was miR-375 overexpression, which was reported to attenuate proliferation of beta-cells and glucose-induced insulin secretion. In ob/ob mice in which miR-375 was deleted, a marked decrease in beta-cell mass resulted in severe insulin-deficient diabetes not found in ob/ob miR-375+ mice. Therefore, it is becoming clear that miR-375 targets a suit of genes that negatively regulate cell growth and proliferation and that aberrant loss of this miRNA leads to dramatic reduction of beta-cell mass.

Interestingly, generation of patient-specific beta-cells could also provide for a revolutionary type of treatment for patients with diabetes. The review article by X. Wang et al. focused on the significant applications of patient-specific therapy which include the engineering of new beta-cells from a patient's own cells, and thus, the elimination of the life-long usage of immunosuppressants, bioincompatibility, and disease transmission inherent with donor cells. Of course, transcription factors for pancreatic stem cell development and differentiation of beta-cells play a critical role in this process for they are essential for tailoring the transplantable

beta-cells to function optimally. They concluded that the success of generating islet-like insulin-producing cell is largely achieved by building upon knowledge of the major steps in the differentiation of beta-cells during embryonic development of the pancreas. By applying multiple transcription factors, the available cells are coerced to differentiate into desired types in a unique delineation pathway, including across lineages, such as from fibroblasts into iPSs, or from one fully functional lineage to another, such as from fibroblasts into insulin-positive cells.

In conclusion, islet cell transplantation offers a potential cure for type 1 diabetes, but it is challenged by insufficient donor tissue and side effects of current immunosuppressive drugs [5, 6]. Therefore, alternative sources of insulin-producing cells and islet friendly immunosuppression are required to increase the efficiency and safety of this procedure. Beta-cells can be transdifferentiated from precursors or another heterologous (non-beta-cell) source to compensate for the reduced beta-cell function and insulin resistance experienced by diabetic patients. Fortunately, recent improvements in our understanding of islet cell biology and beta-cell regeneration have advanced the field of islet cell transplantation closer to our goal of finding a cure for diabetes [7].

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Review Article

Chemical Methods to Induce Beta-Cell Proliferation

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Pancreatic beta-cell regeneration, for example, by inducing proliferation, remains an important goal in developing effective treatments for diabetes. However, beta cells have mainly been considered quiescent. This "static" view has recently been challenged by observations of relevant physiological conditions in which metabolic stress is compensated by an increase in beta-cell mass. Understanding the molecular mechanisms underlining these process could open the possibility of developing novel small molecules to increase beta-cell mass. Several cellular cell-cycle and signaling proteins provide attractive targets for high throughput screening, and recent advances in cell culture have enabled phenotypic screening for small molecule-induced beta-cell proliferation. We present here an overview of the current trends involving small-molecule approaches to induce beta-cell regeneration by proliferation.

1. Introduction

Both type 1 (T1D) and 2 (T2D) diabetes involve loss of beta-cell mass and function. In T1D, autoimmune destruction of beta cells results in an absolute dependence on exogenous insulin, while the peripheral insulin resistance in T2D can lead to beta-cell decompensation and failure [1]. Overall beta-cell mass is regulated by various processes, including apoptosis, differentiation, neogenesis, and proliferation. Ways to increase beta-cell mass *in vivo* could provide new avenues for therapeutic development.

Beta-cell replacement is therapeutic for the treatment of T1D. The Edmonton protocol for islet transplantation demonstrated that patients could achieve insulin independence one year after the procedure. However, patients required islets from at least two donors, exceeding organ supply. In a follow-up study of 36 recipients of islet treated at nine transplantation centers [2] only one-third of patients were insulin-independent after two years. These results demonstrate that increasing beta-cell mass can result in insulin independence, but that we may need methods in addition to islet transplantation to achieve this goal.

It remains unclear whether beta-cell proliferation can be exploited to treat diabetes by inducing beta-cell regeneration. Beta-cell mass is maintained at optimal levels in the body through a slow turn-over rate. In humans, it has been shown that beta-cell mass expands several fold from birth and through the first three years of childhood, but that after this initial period, beta-cell replication potential declines markedly until adulthood [3].

Work in 2004 conclusively showed that new beta cells in the mouse arise from cell division of existing beta cells and not from a stem-cell population [4]. As mentioned, study of pancreatic samples from young human donors showed that replication is indeed responsible for beta-cell expansion, but only for a short period after birth [3]. However, an analysis of donors between 7 and 66 years old found beta cells positive for the proliferation marker Ki67 in every sample tested [5]. These observations support the hypothesis that beta cells have a physiological, albeit limited, capacity to proliferate.

A critical barrier to progress in the treatment of diabetes is the lack of small-molecule drugs to induce beta-cell regeneration. Small molecule-induced beta-cell proliferation in humans could be an important way to achieve this goal; such compounds could be used to restore beta-cell mass *in vivo*, or alternatively provide methods for *ex vivo* expansion of beta-cell numbers before transplantation. We present here

an overview of the current trends involving small-molecule approaches to induce beta-cell regeneration.

2. Physiological Mechanisms of Beta-Cell Proliferation

2.1. Glucose. Although beta-cell mass remains relatively constant throughout adulthood, there are a number of physiological stimuli that can promote or inhibit beta-cell proliferation; these naturally occurring conditions enable us to study them and identify novel targets for perturbation. First, glucose is a mitogen for beta-cell proliferation, with long- and short-term glucose infusion promoting beta-cell proliferation [6–8]. Human islets transplanted into nonobese diabetic mice also responded to glucose *in vivo* by proliferating [9]. Although the mechanism of glucose-induced proliferation has remained unclear, the role of, for example, the insulin receptor and insulin receptor substrate 2 have been shown to be important [10]. A recent study showed the importance of glycolytic flux on the stimulation of cell division [11]. After ablating the majority of beta cells in mice and inducing compensatory proliferation, the authors found that beta cells adjust their proliferation rate according to the rate of glycolysis. Glucokinase (GCK) phosphorylates glucose to glucose-6-phosphate in the first step of glycolysis. It serves as a glucose sensor and can regulate insulin secretion and beta-cell proliferation. Accordingly, mice deficient in GCK could not compensate for beta-cell ablation by proliferating, while GCK activator compounds increased this proliferation. Further evidence of GCK importance in beta-cell proliferation was provided by the identification of a rare variant (V91L) in the human glucokinase gene, in which the affinity of GCK for glucose was more than 8.5 times higher. These individuals have abnormally large islets, larger relative beta-cell area, and a high degree of proliferation, as determined by the number of Ki67-positive beta cells [12]. These studies point to GCK as an important target for small-molecule modulation.

2.2. Pregnancy. A well-studied phenomena associated with an increase in beta-cell mass is pregnancy. During pregnancy, the presence of the fetus creates an increased metabolic demand on the mother, resulting in increased glucose-stimulated insulin secretion, increased insulin synthesis, and a reversible increase in beta-cell replication [13, 14]. The peak of proliferation coincides with an increase in lactogen levels, such as prolactin, placental lactogen, and growth hormone [15]. Although early examination of cadaveric human islets showed an enlargement of islet size in pregnancy due to hyperplasia (from 72% beta cells per islet to 82% in pregnant women) [16], later studies showed that human islets respond to lactogens primarily by increasing insulin secretion [17] and that ductal neogenesis may in fact be occurring during pregnancy [18].

Despite the ambiguity of the adaptive response to pregnancy in humans, recent work has advanced our knowledge of this process in rodents. Work in 2007 showed that the increase in beta-cell proliferation in pregnant mice is mediated by the protein menin, encoded by the *Men1* gene [19]. Expression of menin in beta cells perfectly anticorrelates with the increase in BrdU incorporation in beta cells during pregnancy. Menin associates with the histone methyltransferase mixed-lineage leukemia (MLL), which in turn regulates expression of cell-cycle inhibitors p18 and p27. Overexpression of menin in islets causes a decompensation during pregnancy and loss of menin expression, and downstream consequences could be recapitulated in cell culture by the addition of prolactin. Consistent with these results, study of the transcription factor FoxM1 in islets found that pancreas-wide deletion of this gene resulted in loss of all proliferation during pregnancy and subsequent development of gestational diabetes [20]. These effects were accompanied by an increase in menin and p27 levels, suggesting an important role for FoxM1 and menin in the adaptation to pregnancy. Finally, another study of pregnancy in mice revealed that the expansion of beta-cell mass is at least partially regulated by serotonin signaling [21]. Tryptophan hydroxylase 1, the rate-limiting step in serotonin biosynthesis, is one of the most highly upregulated genes in pregnant islets, and the conclusion of the study was that serotonin acts as a paracrine signal to stimulate cell-cycle progression in beta cells. All these studies strongly suggest that researchers should pay particular attention to this physiological process when thinking about mechanisms to induce beta-cell regeneration.

2.3. Obesity. The loss of beta-cell mass has been reported in obesity and T2D [22, 23], although in some cases there is a compensatory increase in beta-cell mass [24]. Whether this adaptation is mediated by beta-cell proliferation is not well understood. Two lines of evidence point to regulation of proliferation in mouse obesity. First, the mouse model of hyperphagic obesity, A^y, was used to assess the levels of menin and downstream cell-cycle proteins. Relative to nonobese control littermates, A^y mice have much lower mRNA and protein levels of menin, p18, and p27 [19]. This loss of menin expression correlates with increased proliferation, though it was not shown explicitly. Second, an analysis of genes regulated in nondiabetic obese *ob/ob* mice revealed that cell-cycle genes were increased in expression. In particular, FoxM1 is an important regulator of beta-cell division and is increased in these obese mice [25]. Further, overexpression of *FoxM1* stimulated [^{3}H]-thymidine incorporation in whole mouse and human islets. These studies suggest that the response of beta cells to obesity may be quite similar to the response during pregnancy.

2.4. Aging. During aging, many tissues lose regenerative capacity, and the increase in expression of $p16^{INK4a}$ has been shown to be a marker of aging [26]. This phenomenon is also observed in islets [27, 28], and aged mouse islets express far more $p16^{INK4a}$ than exocrine tissue [29]. Mice with p16 deficiency had effects that depended on age: young mice were unaffected by the loss of the gene, while older mice lost any beta-cell proliferative capability. Similarly, $p27^{KIP1}$ protein accumulates in terminally differentiated beta cells during

embryogenesis [30], limiting their postnatal expansion. In humans, recent results reveal that these regulatory proteins are activated or inactivated according to the different beta-cell proliferative status. For example, p16 and p27 proteins were sporadically expressed in prenatal human beta cells, but were enriched in adult ones [31]. Thus, the effects of aging appear to be similar in rodent and human islets.

The mechanism of increased expression of cell-cycle inhibitors during aging is slowly becoming clearer. Aging in mice is associated with a decrease in expression of two repressors of the *Ink4a/Arf* locus. First, aging reduces expression of the polycomb group protein Bmi-1, resulting in increased recruitment of MLL and methylation of the locus, and increased p16 expression [32]. Accordingly, Bmi-knockout mice have increased p16 expression and decreased beta-cell mass. Second, aging decreases beta-cell expression of the histone methyltransferase enhancer of zeste homolog 2 (Ezh2). Conditional deletion of Ezh2 results in expression of p16 at a younger age and a decrease in beta-cell proliferation [33]. These studies show that the various adaptations of beta cells to the physiological stresses of hyperglycemia, obesity, pregnancy, and aging may all ultimately converge on regulation of the cell cycle in beta cells.

3. Cellular Targets for Proliferation

3.1. Cell-Cycle Proteins. From a molecular point of view, beta-cell proliferation is regulated by finely tuning protein kinases, kinase activators, or inhibitors [34–36]. Here, we will review relevant cellular proteins that could be targeted for high-throughput screening. In terms of positive regulation of beta-cell proliferation, a central role has been ascribed to cyclin D1 and D2 [37]. The role of cyclin D3 remains controversial in rodents [38], but its role seems to be more important in human pancreas [39]. Genetic manipulation through overexpression is effective in inducing beta-cell regeneration. One of the first pieces of evidence of induction in human beta cells came from overexpression of cdk-4 and cyclin D1 in islets [40]. Further, overexpression of the cyclin-dependent kinase cdk-6, either with or without cyclin D1, causes an increase in human beta-cell proliferation in both intact islets and dissociated islet cells [39, 41]. An important liability in targeting cell-cycle proteins chemically is their ubiquitous expression throughout the body. In fact, inhibition of cell-cycle kinases is an active area of research for cancer therapy [42]. It may be a daunting task to identify, for example, activators of key cell-cycle proteins that are specific only to beta cells. As discussed, many of the important physiological processes regulating beta-cell proliferation converge on the cell cycle, so this fundamental process may be too downstream for effective targeting by small molecules.

3.2. GSK-3β. Multiple lines of evidence indicate that the Wnt pathway is involved in beta-cell survival and proliferation. Activation of Wnt signaling in beta-cell lines or primary mouse islets has been shown to result in enhanced beta-cell proliferation [43–45], with upregulation of cell-cycle genes, including cyclins D1 and D2, and cdk-4 [45, 46]. Furthermore, increasing beta-catenin expression in islets causes an expansion of beta-cell mass *in vivo*, while upregulating axin expression, a negative regulator of Wnt signaling, blunts Wnt-stimulated gene expression and reduces beta-cell expansion [46].

In the absence of Wnt signaling, glycogen synthase kinase-3β (GSK-3β), adenomatous polyposis coli protein, axin, and beta-catenin form components of the "destruction complex," enabling GSK-3β to phosphorylate beta-catenin, targeting it for ubiquitination and degradation by the proteasome. GSK-3β inhibitors induce beta-cell proliferation; treatment of INS-1 cells with structurally unrelated GSK-3β inhibitors 1-azakenpaullone, CHIR99021, and 6-bromoindirubin-3'-oxime (BIO) increased proliferation rate in a dose-dependent manner, as measured by BrdU incorporation [47]. 1-Azakenpaullone and CHIR99021 were found to promote beta-cell replication in isolated rat islets. On the basis of these observations, novel derivatives of paullone were synthesized and assayed for their effect on beta-cell proliferation. Among those tested, 1-aza derivatives resulted in potent and selective GSK-3β inhibitors (Figure 1(a)). Within this series, 9-cyano-1-azapaullone (cazpaullone) showed the most efficacy in inducing beta-cell replication in both INS-1 cells and primary rat islets [48]. Work in human islets has revealed that GSK-3β inhibitors also promote proliferation, though the readout was total islet [^{3}H]-thymidine incorporation [49]. Rapamycin also reduced this proliferation, further pointing to activation of mTOR as a potential mechanism for inducing beta-cell proliferation. With pursuit of novel GSK-3β inhibitors, an active area of research [50], the question of the role of GSK-3β in beta cells will be answerable with ever more selective and potent compounds.

3.3. Glucokinase. As mentioned above, glucokinase (GCK) phosphorylates glucose to glucose-6-phosphate in the first step of glycolysis. It is the predominant hexose kinase in beta cells and plays an important role as a glucose sensor in beta cells. When fed a high-fat diet, wild-type mice showed marked beta-cell hyperplasia, while GCK heterozygous mice could not sufficiently increase beta-cell replication [55], leading to the conclusion that glucokinase plays a critical role in inducing beta-cell replication. To address this aspect, $GCK^{+/-}$ mice were fed with high-fat diet in the presence or absence of a glucokinase activator (GKA) for 20 weeks [56]. No significant differences in beta-cell proliferation were seen after chronic treatment, but after an acute 3-day treatment with GKA, beta-cell proliferation was markedly stimulated.

Potent proliferative effects of the glucokinase agonists GKA50 (Figure 1(b)) and LY2121260 were also observed in the INS-1 cell model [51]. Interestingly, GKA50, but not LY2121260, also prevented apoptosis in INS-1 cells under chronic high-glucose concentrations, probably by increasing the levels of glucokinase protein itself. Glucokinase agonists could play important role in promoting beta-cell proliferation and preventing apoptosis, and novel compounds are actively being sought for these purposes [57].

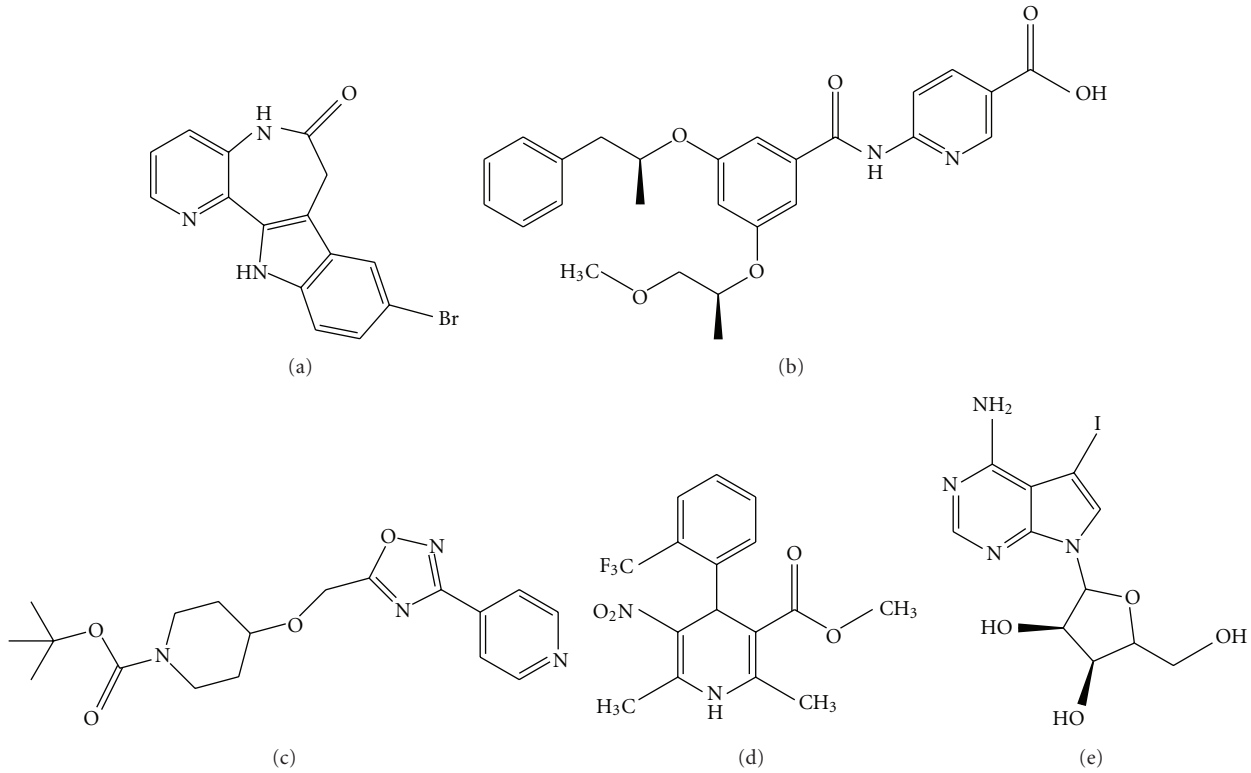

Figure 1: Chemical structures of small molecules reported to induce beta-cell proliferation. (a) 1-Azakenpaullone, a GSK-3β inhibitor [47]. (b) GKA50, a glucokinase activator [51]. (c) PSN632408, a GPR119 agonist [52]. (d) Bay K 8644, an L-type calcium channel activator [53]. (e) 5-Iodotubercidin, an adenosine kinase inhibitor [54].

3.4. GLP-1. Although much study has focused on glucagon-like peptide 1 (GLP-1) and its role in diabetes, important questions remain unanswered. For example, the ability of exendin-4 to induce beta-cell proliferation in rodents is well established [58–61], but whether this effect also occurs in humans has not yet been demonstrated [62]. A recent report showed an increase in the replication of human beta cells transplanted into mice treated with exendin-4, but only when the islets were from young donors [63]. Such observations raise questions about the differences, in terms of the molecular machinery and islet physiology, existing between murine models and humans. On the other hand, the fact that GLP-1 appears to activate Wnt in a TCF7L2-dependent manner [64] is encouraging for a translation to human biology, as single-nucleotide polymorphisms in TCF7L2 provide the strongest genetic associations with T2D [65]. It may be the case that such effects of GLP-1 activation are only observable when administered to a whole organism, and not in cell culture alone.

There is a great scientific and commercial interest in identifying GLP-1 mimetics and inhibitors of dipeptidyl peptidase 4, which degrades GLP-1 *in vivo* [66]. These compounds promote activation of the GLP-1 G protein-coupled receptor to stimulate insulin secretion and inhibit glucagon secretion, and also have the potential to increase beta-cell mass (see [67] for an extensive review). Despite some interesting successes, the development of small-molecule agonists of the GLP-1 receptor is still quite challenging and not yet fully developed.

3.5. GPR119. G protein-coupled receptor 119 (GPR119) is another attractive target for T2D therapy [68]. GPR119 was first identified as an orphan GPCR in various mammalian species [69]. This receptor is predominantly expressed in beta cells [70] and GLP-1-secreting intestinal L-cells [70]. The phospholipids lysophosphatidylcholine and oleoylethanolamide (OEA) are natural endogenous ligands for GPR119 [71, 72]; each of them increases intracellular cAMP levels and results in glucose-dependent insulin secretion. Gao et al. [52, 73] evaluated the effects of OEA and synthetic agonists (PSN632408 and AR231453) on murine beta-cell replication (Figure 1(c)) and found that all were able to increase the number of replicated beta cells compared to control animals. Similar results have been reported for other GPR119 agonists: AS1535907 [74] and APD597 [75]. These results provide interesting preliminary evidence that activating GPR119 may be a feasible strategy for treating T2D.

4. Chemical Screening and Beta-Cell Proliferation

In the past few years, attempts have been made to develop assays for high-throughput screening (HTS) to identify

small-molecule inducers of pancreatic beta-cell expansion. One of the first studies reported screening a heterocyclic library of 850,000 compounds for proliferation of the reversibly immortalized mouse cell line R7T1 [53]. This beta-cell line was immortalized using the SV40 T antigen under the control of the Tet-On system, such that the cells proliferate in presence of tetracycline but undergo growth arrest upon its withdrawal [76]. These cells express characteristic beta-cell markers (insulin 1, insulin 2, and Pdx1), secrete insulin, and restore euglycemia in streptozotocin-treated mice. Although not a true mimic of *in vivo* quiescent beta cells, this system can be considered a reasonable proxy to identify reentry into the cell cycle of growth-arrested cells as measured by intracellular ATP levels. To rule out false-positive tetracycline mimetics, the Tet-Off immortalized beta-cell line βTC-Tet, which proliferates only in the absence of Tet [76], was used as a counter screen.

Several structurally diverse, active compound classes were identified, including phorbol esters, dihydropyridines (DHP), and thiophene pyrimidines. In particular, the thiophene pyrimidines appeared to stimulate beta-cell proliferation by activating the Wnt signaling pathway. A piperazinyl derivative showed a dose-dependent induction of R7T1 proliferation with an IC_{50} of about 1.1 μM. The same compound was active in MIN6 and HIT-T15 beta-cell lines and in primary rat beta cells. Interestingly, this molecule turned out to be a potent GSK-3β inhibitor.

The dihydropyridine (DHP) class identified in this screen acts on L-type calcium channels. In beta cells, calcium channels play a key role in controlling glucose-stimulated insulin secretion and insulin production [77], and polymorphisms in some calcium channel-encoding genes are associated with both T1D and T2D [78–80]. However, it is also possible that these mutations lead to proliferative defects in beta cells. In fact, the $LTCC_{a1D}$ subunit knockout displayed a significant reduction of postnatal beta-cell proliferation [81], suggesting that calcium channel signaling is necessary for beta-cell replication. The most potent derivative identified (Bay K 8644; Figure 1(d)) resulted in a significant dose-dependent increase in proliferation of growth-arrested R7T1, HIT-T15 and MIN6 beta-cell lines, and primary rat beta cells. Data showing induction of human beta-cell proliferation, however, were less conclusive, suggesting that further study is required to identify inducers that are active in human islets.

Recently, we have developed a human islet cell-culture system [82] to screen for inducers of beta-cell proliferation directly in primary human cells. While very challenging, we have used this system to measure the percentage of $insulin^+/Ki67^+$ cells after compound treatment in 384-well plates. The major limitation is acquiring an adequate supply of human islets. A proof-of-concept screen using primary rat islets has also been reported [54]. Dissociated islets were seeded in 96-well plates and treated with a library of ~850 cell-permeable bioactive compounds. The primary screen measured the percentage of $PDX1^+/Ki67^+$ cells. Two adenosine kinase inhibitors: 5-iodotubercidin (5-IT; Figure 1(e)) and ABT-702 were screening hits, and both induced a two- to three-fold increase of the percentage of the $PDX1^+/Ki67^+$ cells compared to DMSO-treated controls. *In vivo* studies were carried out using ABT-702 due to its longer half-life; intraperitoneal injection of 21 mg/kg ABT-702 resulted in a two-fold increase in BrdU incorporation in $PDX1^+$ cells, but had no effect on either exocrine cells or hepatocytes. No data were presented in human islets, so it remains to be seen whether adenosine kinase plays an important role in human beta-cell proliferation.

Adenosine kinase is highly expressed in the liver and pancreas [83] and plays an important role in cellular metabolism [84]. To understand why adenosine kinase inhibitors induce beta-cell replication, islet cultures were treated with both 5-IT and various replication-pathway inhibitors. Only the p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580 increased 5-IT-dependent beta-cell replication, while rapamycin and the PI3K inhibitor wortmannin each suppressed beta-cell replication, suggesting 5-IT promotes beta-cell replication in an mTOR-dependent manner. The authors also observed an interesting link with glucose and GLP-1; the combination of 5-IT with high glucose, GLP-1, or exendin-4 induced the replication rate four- to five-fold. These results point to adenosine kinase as yet another possible target for beta-cell regeneration.

5. Future Directions: Cellular Targets for Screening

An impression that comes from the many studies performed on beta-cell proliferation is that there seem to be as many possible targets as there are studies. Because a potential goal in diabetes regenerative medicine is to induce proliferation of beta cells, it is important to find mechanisms that are very specific for beta cells. Otherwise, the risk for any compound that is identified is that it globally induces cell division, a potentially catastrophic consequence. In order to identify selective small molecules, it is likely that future directions should involve processes that are relatively specific to beta cells.

The adaptive response of beta cells to pregnancy provides several possibilities for cellular targets. First, serotonin is highly upregulated in beta cells during pregnancy [21]. Thus, activation of serotonin biosynthesis or signaling may be a novel and specific way to induce beta-cell proliferation. Increased knowledge of the mechanisms by which serotonin increases proliferation will help in this quest. Second, the protein menin is highly downregulated during pregnancy [19]. Menin is interesting because of its specificity towards beta cells, and its ability to act as a switch that can turn on or off beta-cell proliferation. Recently, these unique properties have been assessed in a hyperglycemic diabetic mouse model, where acute and temporally controlled deletion of *Men1* improves preexisting hyperglycemia in streptozotocin-treated mice, and reverses glucose intolerance in high-fat diet-fed mice by increasing proliferation of beta cells [85]. A very recent study reports screening for disruption of the menin-MLL complex [86], so it will be of great interest to determine whether these compounds also increase beta-cell proliferation.

The prospect of targeting menin and/or MLL highlights the likely important role epigenetics will play in

understanding beta-cell proliferation in the future. There is accumulating evidence that beta cells regulate their growth and fate by epigenetic mechanisms [32, 87]. Thus far, modulation of epigenetic status has not been fully exploited in beta-cell physiology and could represent an array of novel therapeutic targets. Indeed, some interesting examples of the potential of this approach are already available, particularly histone deacetylase inhibitors [88, 89]. A number of other cellular targets provide attractive models, including mTOR activation, as discussed above, and Pax4 activation [90].

Most of the studies in beta-cell biology, including the more recent high-throughput screening examples, rely heavily on murine animal and cell models. Although these studies provide the foundation for many advances in beta-cell research, unfortunately they often do not translate to similar results when applied to humans. The greatest challenge facing beta-cell regeneration approaches, particularly proliferation, is the requirement for treatments and mechanisms to work in both rodents and humans. As we understand more about the physiological proliferative behavior of human beta cells, we can start to identify the molecular switches that could be potentially used to foster the proliferation of beta cells in humans.

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Review Article

Genetically Engineered Islets and Alternative Sources of Insulin-Producing Cells for Treating Autoimmune Diabetes: Quo Vadis?

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Islet transplantation is a promising therapy for patients with type 1 diabetes that can provide moment-to-moment metabolic control of glucose and allow them to achieve insulin independence. However, two major problems need to be overcome: (1) detrimental immune responses, including inflammation induced by the islet isolation/transplantation procedure, recurrence autoimmunity, and allorejection, can cause graft loss and (2) inadequate numbers of organ donors. Several gene therapy approaches and pharmaceutical treatments have been demonstrated to prolong the survival of pancreatic islet grafts in animal models; however, the clinical applications need to be investigated further. In addition, for an alternative source of pancreatic β-cell replacement therapy, the *ex vivo* generation of insulin-secreting cells from diverse origins of stem/progenitor cells has become an attractive option in regenerative medicine. This paper focuses on the genetic manipulation of islets during transplantation therapy and summarizes current strategies to obtain functional insulin-secreting cells from stem/progenitor cells.

1. Introduction

Type 1 diabetes (T1D) is an autoimmune disease characterized by the progressive destruction of insulin-producing cells in the pancreatic islets by autoreactive T cells, which eventually leads to hyperglycemia. The disease accounts for about 10% of all cases of diabetes, occurs most commonly in people of European descent, and affects two million people in Europe and North America [1]. There is a marked geographic variation in the incidence, probably because the different populations vary in genetic susceptibility/resistance factors or in exposure to environmental triggers. For instance, a child in Finland (Northern Europe) is about 80 times more likely to acquire the disease than a child in China (Eastern Asia) [2]. The current global increase in incidence of 3% per year is well established [3, 4], and this rapid rise strongly suggests that environmental factors should be acting on susceptibility genes and contributing to the evolving epidemiology of T1D.

In patients with T1D, daily delivery of insulin by injection or a pump is crucial for metabolic control. However, this exogenous insulin delivery cannot achieve physiological control of blood glucose concentrations and also has the risk of causing hypoglycemic episodes. Moreover, a significant proportion of patients suffers chronic and degenerative complications, such as nephropathy, retinopathy, and vascular and heart disease [5, 6]. The appropriate treatment to achieve insulin independence for T1D is replacement of the β-cell mass, currently being accomplished through whole pancreas transplantation and islet transplantation. Transplantation of the whole pancreas is a standard treatment for diabetic patients, which can achieve insulin independence with a single donor [7]. Pancreatic islet transplantation is a safer and less invasive method than whole-organ transplant therapy, which causes thrombosis, pancreatitis, and peritonitis. However, the major drawback of islet transplantation compared with pancreas transplantation is the greater requirement for donors and the lower 5-year insulin independence rate [8].

Transplantation therapy should provide a better quality of life than current therapies and should help avoid complications. Unfortunately, immune-mediated destruction and inadequate numbers of donor organs for transplantation are the major obstacles to achieving insulin independence and long-term survival of grafts in this therapy. To circumvent those problems, genetically modifying islets to enhance their resistance to immune attack and explorations of alternative sources of insulin-secreting cells are being investigated intensively. In this paper, we will summarize the current knowledge regarding immunomodulatory therapy in islet transplantation and examine alternative sources of insulin-secreting cells for cell replacement therapy.

2. Mechanisms Involved in Islet Graft Rejection: Nonspecific Inflammation and the Contribution of T-Helper-Cell Subsets

The process of islet isolation also triggers a cascade of stressful events in the cells involving the induction of apoptosis or necrosis and production of proinflammatory molecules that negatively influence islet viability and function. Transplantation procedures such as collagenase-based islet isolation trigger proinflammatory cytokine and chemokine production by the islets. Proinflammatory cytokines such as interleukin- (IL-) 1β and tumor necrosis factor- (TNF-) α produced by islet-resident macrophages are toxic to islets and can induce the local production of reactive oxygen species (ROS) [9, 10]. By contrast, chemokine receptors such as chemokine (C-C motif) receptor (CCR)2, CCR5, and C-X-C chemokine receptor (CXCR)3, and their ligands are crucial to generate acute islet allograft rejection [11]. Taken together, the inflammatory cytokines, chemokines, and ROS contribute to the first line of attack to the islets, which can cause apoptosis and loss of function.

The second barrier against successful transplantation is recipient alloimmunity and autoimmunity. Previous reports have demonstrated that Th1 cells, type 1 cytotoxic $CD8^+$ T cells, and Th1-type cytokines such as interferon (IFN)-γ and IL-2 are commonly associated with graft rejection [12–15]. Th1 responses initiate allograft rejection by promoting cytotoxic T-cell activities and IFN-γ-mediated delayed-type hypersensitivity reactions, whereas Th2 responses cause allograft damage through the recruitment of eosinophils induced by IL-4 and IL-5. Moreover, cytokines produced by non-T-cell sources from the graft microenvironment, such as IL-7 produced by stromal cells and IL-15 produced by activated macrophages and endothelial cells, further support the idea of alloreactive T-cell proliferation [14, 16].

Recently, the role of subsets of Th cells in graft rejection has been reexamined after the identification and thorough characterization of Th17 cells. Several lines of evidence have demonstrated that Th17 cells have the capacity to cause rejection of cardiac allografts [17–19]. However, it is not clear whether Th1 and Th17 cells work synergistically or sequentially to cause graft rejection. In our laboratory, we have investigated the relative contribution of Th1 and Th17 cells in the autoimmune-mediated rejection of islets in a nonobese diabetic (NOD) mouse model. We have demonstrated that Th1 cells play a crucial role in the destruction of the islet graft, whereas Th17 cells constitute a much smaller population in the islet graft and might play only a minor destructive role in this model of autoimmune islet transplantation (Chou et al., manuscript in revision).

In summary, understanding the inflammatory factors that attack islets during the early phase and dissecting the role of the effector T-cell subsets in the rejection responses might contribute to developing target therapies to protect islets from inflammatory insults and to modulate T-cell responses.

3. Strategies to Protect Islets from Inflammatory Insults and T-Cell-Mediated Immunity

In autoimmune diabetes, pancreatic β cells suffer from inflammatory stress following T-cell-mediated destruction. Macrophages and/or dendritic cells in the islet microenvironment produce proinflammatory cytokines and free radicals, which induce β-cell damage. Activated T cells express death receptors and release cytotoxic molecules including granzyme B, perforin, or cytokines to further activate other immune cells and exacerbate β-cell death. Several preventive and therapeutic approaches have been demonstrated to protect β cells from immune attack, including the modulation of T-cell activity and inhibition of inflammatory responses in the islet microenvironment. To protect islets from immune attack, many gene targets that exhibit strong immunoregulatory effects and antiapoptotic effects have been introduced to the islets through different approaches: generation of transgenic mice using islet-specific promoters to carry genes of interest, delivery of genes into islets by viral vectors or transfection, and the administration of recombinant proteins and drugs.

4. Transgenic Overexpression of Regulatory Genes in Islets

Genetically manipulating islets by transgenic techniques was originally designed for study of the immunopathogenesis of autoimmune diabetes. This could help in dissecting the roles of different cytokines, death receptors, and major histocompatibility complex (MHC)/costimulation molecules in β-cell destruction (reviewed in [20]). Among the transgenic-mouse models that have been generated, some molecules display strong immunoregulatory functions and cytoprotective effects, which could be applied further in islet transplantation therapy (Table 1).

4.1. Cytokines and Cytokine Signaling. It is well established that proinflammatory cytokines and Th1-type cytokines are toxic to islets whereas IL-4 and transforming growth factor- (TGF-) β are postulated to be protective. Transgenic expression of IL-4 in β cells under the control of the insulin promoter in NOD mice suppresses insulitis and diabetes; however, islet expression of IL-4 is incapable of preventing islet rejection in diabetic recipients [21]. In other

Table 1: Transgenic overexpression of regulatory genes in the islets and their effects on islet transplantation.

Promoter	Gene of interest	Animal strain	Diabetic incidence	Effect on islets	Effects on islet transplantation	Reference
Human insulin	IL-4	NOD	Decreased	Protect islets from autoimmune destruction	No significant protective effect	[21]
Rat insulin	TGF-β	NOD	Decreased	Small clusters of micro-islet	N, and no protective effect when use pancreata in an allogeneic transplantation model	[22, 23]
Glucagon	TGF-β	NOD	Decreased	Morphologically normal, no other phenotypes mentioned	N	[24]
Rat insulin	TNF-α	NOD	Decreased	Massive insulitis	N	[25]
Human insulin	SOCS1	B6	B6 is not a diabetes-prone mouse strain	Not mentioned	Expression of SOCS-1 in islets delays allografts rejection (B6 to Balb/c) but cannot circumvent destruction of the islets by the recurrence of the tissue-specific autoimmune process of spontaneous diabetes (B6 to diabetic NOD)	[26]
Human insulin	PD-L1	NOD	Decreased	Protect from autoimmune destruction	No significant protective effect	[27]
Glial fibrillary acidic protein	PD-L1	NOD	Increased	Enhance the severity of insulitis	N	[28]
Rat insulin	PD-L1	B6	Induces T-cell-mediated spontaneous diabetes in B6 mouse	Induce insulitis	Accelerate allograft rejection	[29]
Human insulin	Single chain anti-CTLA-4 Fv	NOD	Decreased	Protect islets from autoimmune destruction	Prolong islet grafts survival in diabetic NOD mice	[30]
Rat insulin	CTLA-4-Ig	B6	B6 is not a diabetes-prone mouse strain	Morphologically normal	N, and transplantation of CTLA4-Ig transgenic pancreata combine with transient systemic CD4 T cell depletion in recipients enhance allograft acceptance	[31]
Human insulin	Thioredoxin	NOD	Decreased	Do not attenuate the development of insulitis	N	[32]
Human insulin	Heme oxygenase 1	NOD	Decreased	Protect islets from autoimmune destruction Resistant to inflammatory cytokine-induced apoptosis	Prolong islet grafts survival in diabetic NOD mice	[33]
Human insulin	DcR3	NOD	Decreased	Protect islets from autoimmune destruction	Increase the successful rate of implantation and prolong islet grafts survival in diabetic NOD mice	[34]
Human insulin	D6	NOD	Decreased	Protect islets from autoimmune destruction	N	[35]

NOD: Nonobese diabetic mouse, a spontaneous autoimmune diabetes mouse strain; SOCS-1: suppressor of cytokine signaling-1; PD-L1: programmed death 1 ligand 1; CTLA-4: cytotoxic T lymphocyte antigen 4; DcR3: decoy receptor 3; D6: an inflammatory CC chemokine decoy receptor; N: not tested.

studies, expression of TGF-β driven by an insulin promoter [22] or a glucagon promoter [24] protected islets from autoimmune destruction in NOD mice. However, the β-cell-specific expression of TGF-β changes the pancreatic architecture [22], and this TGF-β-expressing pancreatic tissue fails to inhibit allograft rejection [23]. In other aspects, the inhibition of toxic cytokine signaling in islets represents an attractive strategy in designing therapies to prevent islet destruction. Islets with transgenic expression of suppressor of cytokine signaling 1 (SOCS1) show delayed allograft rejection but cannot circumvent destruction of the islets by the autoimmune destruction [26].

4.2. Negative Costimulation Engagement. T-cell activation occurs through two important signals: one is the T-cell receptor recognizing a specific peptide MHC complex and the other is a costimulatory signal. Upon the T-cell activation, the expression of negative costimulatory molecules is induced. The programmed death (PD)-1 and cytotoxic T-lymphocyte antigen (CTLA)-4 are two important negative costimulatory molecules expressed on T cells, which control their effector functions, tolerance, and autoimmunity [36]. We have demonstrated that transgenic expression of PD-L1 (ligand of PD-1) [27] or a membrane-bound, agonistic single-chain anti-CTLA-4 Fv antibody (anti-CTLA-4 scFv) [30] on islets in NOD mice reduces the severity of insulitis and suppresses the development of diabetes. In an islet transplantation study, transgenic anti-CTLA-4 scFv prolonged islet graft survival and reduced the Th1 cell counts in islet grafts after transplantation into spontaneous diabetic NOD mice. However, the expression of PD-L1 on islets could not prolong graft survival [27]. The role of PD-L1 in the regulation of T-cell tolerance to islets needs to be further investigated because the transgenic expression of PD-L1 on islets in mice with a B6 background induced T-cell-mediated spontaneous diabetes, and the islets from transgenic mice displayed accelerated rejection in an allogeneic transplantation model [29].

4.3. Anti-Inflammatory, Antiapoptotic, and Antioxidative Molecules. Inflammatory cytokines such as IL-1β, TNF-α, and IFN-γ sensitize β cells to Fas-dependent and/or other death receptor-mediated apoptosis [37] and induce ROS formation in β cells. Because islets produce very low levels of antioxidative enzymes and are very sensitive to oxidative stress [38], the reduction of ROS levels in islets is crucial for maintaining the function and viability of islets. Others and we have demonstrated that β-cell-specific expression of the antiapoptotic and anti-inflammatory proteins, thioredoxin (TRX) [32] or heme oxygenase-1 (HO-1) [33], prevented autoimmune diabetes in NOD mice. Moreover, the islets from HO-1 transgenic mice survived longer in diabetic recipients, indicating that control of the initial inflammatory responses can promote graft survival. The roles of ROS scavengers in islet transplantation have also been investigated in transgenic mouse models. Thus, several β-cell-specific transgenic mice with different antioxidant enzymes have been generated (reviewed in [39]). In general, islets with transgenic antioxidative genes (e.g., catalase, glutathione peroxidase, metallothionein, copper/zinc superoxide dismutase, and manganese superoxide dismutase) are resistant to oxidative stress induced by chemicals [40–42] or hypoxia [43]; however, they are still sensitive to proinflammatory cytokines induced cytotoxicity [41]. Among these transgenic mice, islets from metallothionein transgenic mice showed prolonged survival of islet grafts in an allogeneic transplantation model [43].

In summary, most *ex vivo* studies have shown that overexpression of antioxidative genes in islets protects them from oxidative injury; however, the *in vivo* function and survival of these genetically modified islets in diabetic recipients have not produced overt success.

5. Genetically Engineering Islets by Transfection or Transduction

The direct delivery of protective and therapeutic genes to islet grafts can overcome many problems; for example, the therapeutic agents cannot be targeted locally and might have effects on other organs or tissues, causing unexpected side effects. By using gene therapy, islets can be manipulated by any vector system *ex vivo* without exposing the recipient to the vectors. Moreover, graft-specific gene therapy can provide prolonged, safe, and locally controlled gene expression. In this regard, *ex vivo* manipulation of islets by gene transfer systems becomes an attractive approach to protect grafts from immune attack. However, the gene delivery systems applied should be considered carefully. In general, nonimmunogenic vectors that cannot activate the host's immune response are used for long-term gene expression [44].

Many strategies have been proven to improve the function of islet grafts and protect grafts from immune attack (Table 2). These approaches include blockade of costimulation signals by CTLA-4-Ig [45, 46]; downregulation of Th1 responses by overexpression of galectin-9 (Chou et al., manuscript in revision); overexpression of antiapoptotic and antioxidative molecules such as B-cell lymphoma (Bcl)-2 [47], TRX [48], and superoxide dismutases (SODs) [49]; blockade of inflammatory cytokine signaling by overexpression of IL-1 receptor antagonist protein [50]; overexpression of anti-inflammatory cytokines such as TGF-β [51], IL-10 [52], and IL-4 [53].

In summary, these strategies significantly reduce apoptosis in islet grafts and prolong graft survival in diabetic recipients. However, the application of these protective genes to transplantation therapy has not been successful. In general, therapeutic targets that have paracrine actions would exert more marked biological effects than membrane-bound or intracellular molecules. Moreover, the efficiency of gene delivery to islets and the expression levels of target proteins in the microenvironment of grafts are closely linked to the protective effect in the grafts. Therefore, better results might have been obtained by using a "cocktail" therapy, for example, combining antiapoptotic and anti-inflammatory genes, which could display synergistic protective effects.

Table 2: Genetically engineered islets for transplantation therapy.

Vector type	Gene carried by vector	Effect on islet transplantation	Reference
Magnetic iron oxide nanoparticles	siRNA to caspase 3	Decrease cell apoptosis in recipients	[54]
Adenovirus	X-linked inhibitor of apoptosis protein (XIAP)	Increase successful rate of islet transplantation and reduce cell apoptosis in a syngeneic model	[55]
Gene gun transfection	CTLA-4-Ig	Prolong islet grafts' survival in an allogeneic model	[45]
Adenovirus/lentivirus	CTLA-4-Ig or TGF-β	Prolong islet grafts' survival in a xenogenetic model (rat to mouse)	[46]
Transfection by Lipofectin	Indoleamine 2, 3-dioxygenase (IDO)	Prolong islet grafts' survival in an allogeneic model	[56]
Adenovirus	TGF-β	Prolong islet grafts' survival in diabetic NOD mice	[51]
Adeno-associated virus	IL-10	Prolong islet grafts' survival in diabetic NOD mice	[52]
Adenovirus	IL-10	Combine with cyclosporin A, prolong islet grafts survival in an allogeneic model	[57]
Adenovirus	Bcl-2	Prolong islet grafts' survival and maintain functional islet mass in STZ-induced diabetic mice in a xenogenetic model (nonhuman primate to mouse)	[47]
Adenovirus	Manganese superoxide dismutase (MnSOD)	Prolong islet grafts' survival in STZ-induced diabetic NOD/SCID mice after challenge with diabetogenic splenocytes	[49]
Lentivirus	Thioredoxin	Prolong islet grafts' survival in diabetic NOD mice	[48]
Lentivirus	Galectin-9	Prolong islet grafts' survival in STZ-induced diabetic NOD/SCID mice after challenge with diabetogenic splenocytes	Chou et al., manuscript in preparation

6. Alternative Sources of Insulin-Producing Cells: Cell Replacement Therapy by Stem/Progenitor Cell-Derived Insulin-Producing Cells

Although islet transplantation is seen as a "cure" therapy for diabetes, this procedure is hampered by the limited number of donors for isolating islets. Many alternative approaches that can be applied to obtain insulin-secreting cells are being investigated intensively [58]. These include the following: (1) the production of surrogate cells by genetically modifying nonendocrine cells to secrete insulin in response to glucose challenge [59], (2) the transdifferentiation of nonendocrine stem/progenitor cells or mature cells to glucose-responsive adult tissues [60, 61], (3) the regulated differentiation of islet stem/progenitor cells to produce large numbers of mature, functional islets [62, 63], (4) the *in vitro* differentiation of stem cells to become insulin-secreting cells, and (5) the *in vitro* differentiation of induced pluripotent stem cells (iPSCs) derived from patients to form pancreatic β-like cells.

Stem cells can reproduce themselves (self-renew) and can differentiate into many cell types. These features make them an ideal focus for regenerative medicine. Besides, stem cells have strong immunosuppressive effects and can secrete many trophic factors that promote the regeneration of damaged tissues. Thus, stem cells have become an attractive alternative cell source to treat diabetes. There are many stem cell types available as a potential source for the generation of insulin-producing cells, including embryonic stem cells (ESCs), adult stem cells, and, most recently, the iPSCs.

Previous reports have demonstrated that ESCs can be induced to become insulin-secreting tissue with structures similar to pancreatic islets [64, 65], However, these cells are often unresponsive to glucose or produce lower levels of insulin compared with the endogenous β cells, which is insufficient to control normoglycemia in diabetic recipients in a mouse model [64]. ESCs have not yet been used therapeutically for treating diabetes mellitus in humans because the animal experiments have not progressed sufficiently to justify this approach; for example, the positive insulin staining of ESC-derived pancreatic-like tissue probably occurs by uptake of insulin from the culture medium [66], and the intermediate stages involved in the differentiation pathway are complicated and not fully understood [67]. In addition, the clinical applications of human ESCs are limited by ethical concerns, as well as the potential for teratoma formation.

In addition to the ESCs produced from embryos, mesenchymal stromal cells (MSCs) and iPSCs with fewer limitations or restrictions in ethical concerns in the research and clinical settings have become ideal cell types for regeneration therapies. Previous reports have demonstrated that MSCs can be differentiated into islet-like cell clusters, and these cell clusters can reverse the diabetic status after transplant into streptozotocin- (STZ-) induced diabetic recipients [68]. Moreover, MSCs themselves also have strong cytoprotective effects and immunosuppressive functions [69]. Several lines of evidence have demonstrated that cotransplantation of islets and MSCs produces superior outcomes to islet transplantation alone. In cotransplantation therapy, MSCs act as feeder cells that promote islet revascularization

[70] and improve graft function [71]. Besides, MSCs also express immunoregulatory molecules, which might help to reestablish peripheral tolerance in diabetic recipients and to prevent allorejection [72].

Recently, iPSCs generated from reprogrammed somatic cells have become an exciting alternative source of cells with pluripotent characteristics similar to ESCs [73]. This concept has been tested further using human skin fibroblasts [74] or cells from skin biopsies from patients with T1D [75] to generate iPSC-derived islet-like clusters. These cells can secrete C-peptide and respond to high concentrations of glucose, suggesting that functional β cells might eventually be generated from iPSCs. In further studies, transplantation of iPSC-derived pancreatic beta-like cells reversed hyperglycemia in diabetic mouse models [76]. These data provide an initial proof of principle for the potential clinical application of iPSCs. Besides, if iPSCs could be generated from patients with diabetes, they would have the same genotype as the recipient, avoiding the problem of immune rejection. Thus, iPSCs show promise for patient-specific regenerative therapy. Unexpectedly and surprisingly, autologous iPSCs reprogrammed from fetal fibroblasts by viral or nonviral genetic approaches elicit T-cell-dependent immune reactions in genetically identical mice, resulting in their rejection [77]. This is likely because of the abnormal expression of antigens in the iPSCs, leading to a breakdown of peripheral tolerance. Besides, there could be other unknown epigenetic differences between iPSCs and ESCs [78].

7. Conclusion

T1D is among the most amenable diseases for treatment with cell replacement therapy. Clinical trials of islet transplantation are showing remarkable success since the Edmonton protocol was developed [79], and this glucocorticoid-free immunosuppressive protocol was replicated successfully [80]. However, the long-term success of this procedure is limited by the effects of allograft rejection and recurrent autoimmunity. Moreover, the scarcity of organ donors also frustrates the treatment of T1D. To overcome these problems, researchers have developed many strategies to modulate the detrimental immune responses and have also explored the alternative sources of insulin-secreting cells. Gene therapy offers a powerful tool to engineer islet grafts to become resistant to inflammation-induced apoptosis, as well as modifying islets to produce immunosuppressive molecules to attenuate T-cell response. The use of stem cells in the generation of renewable and functional β cells is now a promising reality. Moreover, based on current knowledge about genomic cell reprogramming, it should be possible to develop patient-specific, autologous cell replacement therapy by using iPSC-derived pancreatic β-like cells.

Although these proofs of concepts for potential preclinical applications show a big breakthrough in this field, some issues need to be considered: (1) the duration and expression levels of targeted genes in islets, (2) the use of viral vectors for direct gene therapy raises the possibility of insertional mutagenesis (retroviruses and lentiviruses) and host immunogenicity (adenoviruses), and (3) the efficiency of differentiation of insulin-secreting cells from stem cells.

In conclusion, further investigations are required to develop the most potent graft-specific immunoregulatory therapies and to generate safe and stable sources of insulin-secreting cells for clinical islet transplantation or cell replacement treatments.

Abbreviations

T1D:	Type 1 diabetes
NOD mouse:	Nonobese diabetic mouse
PD-1:	Programmed death-1
CTLA-4:	Cytotoxic T-lymphocyte antigen-4
SOD:	Superoxide dismutase
STZ:	Streptozotocin
ESCs:	Embryonic stem cells
MSCs:	Mesenchymal stromal cells
iPSCs:	Induced pluripotent stem cells.

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Review Article

Tolerance-Inducing Strategies in Islet Transplantation

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Allogeneic islet transplantation is a promising approach for restoring normoglycemia in type 1 diabetic patients. Current use of immunosuppressive therapies for management of islet transplant recipients can be counterintuitive to islet function and can lead to complications in the long term. The induction of donor-specific tolerance eliminates the dependency on immunosuppression and allows recipients to retain responses to foreign antigens. The mechanisms by which tolerance is achieved involve the deletion of donor-reactive T cells, induction of T-cell anergy, immune deviation, and generation of regulatory T cells. This review will outline the various methods used for inducing donor-specific tolerance in islet transplantation and will highlight the previously unforeseen potential of tissue stromal cells in promoting islet engraftment.

1. Introduction

Diabetes mellitus is a disease characterized by metabolic abnormalities and the onset of hyperglycemia, which results from dysregulation of insulin. Insulin promotes the entry of glucose from the blood into the tissues. Diabetes affects approximately 25.8 million people of all ages, with the prevalence rising with age. Long-term complications of diabetes include nephropathy, retinopathy, neuropathy, and atherosclerosis [1]. The type II variant, or noninsulin-dependent form, arises from a resistance to insulin or inadequate production of insulin by the pancreatic β islets, eventually leading to islet dysfunction and desensitization to glucose. Type II diabetes accounts for 90–95% of all diagnosed cases of diabetes [1]. The current management for type II diabetes involves a change in lifestyle—weight loss and dietary modifications—and administration of glucose reducing agents. Type I diabetes, the more severe form of diabetes, affects 5% of the population [1] and results from autoimmune destruction of the pancreatic β islets. Since the islets fail to produce insulin altogether, the only treatment options for type I diabetes are exogenous delivery of insulin and pancreas or islet transplantation.

Despite improvements in the administration of insulin delivery and insulin supplying devices, maintenance of adequate and steady glucose levels with exogenous insulin therapy alone can be challenging and can cause episodes of hypoglycemia. Diabetic patients with suboptimal control of glycemia ultimately develop long-term complications. Currently the only real "cure" for type I diabetes is transplantation of the pancreas or isolated islets, which would result in insulin production closer to physiological conditions.

However, pancreas transplantation is generally considered only for severe, late-stage diabetics and is a significant surgical procedure requiring extensive immunosuppression [2–4]. Islet transplantation is, therefore, a more feasible alternative to pancreas transplantation. In comparison, islet transplantation has lower risks of morbidity and mortality and greater opportunities for *in vitro* manipulations of islets to optimize engraftment. The concept of transplanting pancreatic fragments to reverse diabetes was first proposed by the English surgeon Watson Williams in 1893 [5]. However, lack of knowledge about immune rejection and immunosuppression at the time prevented progress [2]. It was not until 1967 that Lacy and Ballinger demonstrated the first real advancement in islet transplantation. Using a rodent model in which recipients of islet allografts were induced to develop type I diabetes by administration of streptozotocin (STZ), mimicking autoimmune destruction of the pancreatic islets, Kemp et al. demonstrated that pancreatic islet cell

transplants could restore metabolic control and prevent long-term complications [6].

Translation from rodent to larger animal and human models had been hampered by difficulties in islet isolation from the pancreas, the lack of quality of isolated islets, and route of administration [7, 8]. The development of an automated method for human islet cell processing by Ricordi et al. in 1986 [9] and the discovery that islet clusters could be heterotopically implanted into the liver contributed to the first successful allogeneic islet transplantation in humans in 1989 by Lacy et al. [6, 10, 11]. Nevertheless, success rates remained low with only 10% of patients achieving insulin independence for greater than a year and demonstrating islet allograft rejection and recurrence of autoimmunity. Poor clinical outcomes were attributed to insufficient numbers of islets being transplanted and an ineffective immunosuppression regimen [12].

2. Immune Response to the Graft

The alloresponse is largely a T-cell-mediated response to the major histocompatibility complex molecules (MHCs) on the surface of donor tissues. Antigen presenting cells (APCs), such as dendritic cells (DCs), process and present donor peptides and molecules through MHCs to recipient T cells by the indirect pathway. Additionally, recipient T cells can recognize donor antigen directly on the surface of infiltrating donor-derived APCs through the direct pathway. T-cell receptors (TCR) on the surface of the recipient T cells recognize the peptide-MHC complex, initiating signaling cascades and activation of the T cells. In addition to this primary signal, additional interactions through costimulatory molecules on the T cells and APCs are required for full activation of the T cells. Upon activation, the T cells promote a series of proinflammatory events and initiate the activation of other cell types resulting in recruitment of leukocytes and humoral factors to the graft. The effector response includes the production of the cytokines IFN-γ and IL-2 by type 1 helper (Th1) $CD4^+$ T cells, the cytotoxic factors granzyme and perforin by $CD8^+$ T cells, and alloantibodies (Abs) by B cells [13].

3. Pitfalls of Immunosuppression

Current immunosuppressive therapies target T cells since they are the main culprits in rejection. Previously, the standard protocol for immunosuppression for islet transplantation consisted of a combination of calcineurin inhibitors (tacrolimus and cyclosporine), purine analogs (mycophenolate mofetil), and corticosteroids. Many of these agents proved to be diabetogenic, impairing insulin secretion, and lethal to the islets [14]. The advent of the Edmonton protocol in 1999 was a significant step in the field of islet transplantation.

The Edmonton protocol utilized a steroid-free therapy based on low-dose sirolimus, tacrolimus, and daclizumab (a humanized anti-IL-2 receptor α mAb). Furthermore, the protocol administered two infusions of islets from different donors to increase chances of engraftment. All 7 patients involved in the trial demonstrated insulin independence beyond 1 year [15, 16]. Despite the success of rapamycin-based therapies, they have their own shortcomings including increased risk of hyperlipidemia, hypertension, and pneumonia [15]. Work by Monti et al. reported that patients conditioned under the Edmonton protocol receiving infusion of cadaveric islets developed lymphopenia and displayed elevated serum levels of homeostatic cytokines, leading to the expansion of autoreactive cells [17].

While the results of multiple center clinical trials demonstrated that the protocol can provide short-term insulin independence and reduce incidences of acute rejection, the patients regressed back to insulin dependency, and graft function was lost in several patients within 5 years [18]. Chronic administration of immunosuppression of any kind will eventually lead to risk of infection, malignancies, and drug toxicity [19]. Maintenance immunosuppression can, therefore, be detrimental rather than beneficial to transplant recipients. Side effects of immunosuppression often outweigh the benefits of islet transplantation.

The risks associated with immunosuppressive agents prompted investigation into tolerance inducing therapies, with the goal being to achieve indefinite graft survival without dependency on long-term immunosuppression while preserving host immunity to other alloantigens. Tolerance induction has been challenging in both large animal and human models due to the complex nature of the alloresponse. Tolerance at both the central and peripheral stages involves clonal deletion of alloreactive T-cells, T cell anergy, immune deviation, and induction of regulatory T cells.

4. Central Tolerance

Central tolerance refers to lack of responsiveness to self through deletion of self-reactive T cells in the thymus—the site of T-cell maturation and selection [20]. Donor-specific tolerance can be achieved using strategies similar to those used for preventing autoimmunity. Intrathymic tolerance (IT) can be induced by intrathymic inoculations of recipient APCs pulsed with allopeptides. Alloantigens, when presented in the context of self-APCs result in donor-specific unresponsiveness and promote tolerance. However, the clinical applications of the IT model face many challenges as it is an invasive technique and may have limited potential in adults, since the thymus involutes with age, compromising the tolerance process [21, 22].

An alternative and perhaps more effective method for achieving central tolerance involves the generation of hematopoietic chimerism, which is developed through bone marrow (BM) transplantation. Prospective transplant recipients are conditioned with total body irradiation (TBI) or partial myeloablation prior to BM transplantation in order to make room for the transplanted bone marrow. BM cell transplantation enables the reconstitution of the recipient hematopoietic compartment with donor hematopoietic stem cells. Donor-reactive T cells are then deleted through central

regulation and maintained by peripheral mechanisms. The use of TBI for prolonging islet allograft survival was originally proposed by Britt et al. [23] and later shown by other groups to prevent the onset of diabetes in nonobese diabetic (NOD) mice [24]. Though combined islet and hematopoietic cell transplant seems promising, the inherent risks associated with the process have limited its applications. TBI or myeloablation prior to receipt of the BM increases the risk of infection and malignancies. Additionally, BM transplantation is met with the risk of graft versus host disease (GVHD) [25, 26]. Mixed allogeneic chimerism is emerging as a safer method to fully allogeneic chimerism. Mixed chimerism can be achieved in allogeneic BM recipients conditioned with sublethal total body irradiation with the aid of costimulatory blockade or anti-CD4 and anti-CD8 monoclonal (m)Abs [27–29]. NOD mice receiving nonmyeloablative conditioning accepted allogeneic islets and displayed a reversal of autoimmunity, suggesting successful establishment of mixed chimerism [30]. Similarly, NOD mice that received low-dose irradiation, cyclophosphamide (CY), and two intravenous infusions of BM from WT mice showed high levels of donor-cell chimerism and effectively eliminated host donor-reactive lymphocytes after transfer of splenocytes from overtly diabetic NOD mice [31, 32]. BM infusion at the time of islet transplantation may, therefore, be used to induce donor-specific tolerance to islet allografts.

5. Tolerance through T-Cell Depletion Strategies

Another strategy in promoting tolerance involves the depletion of alloreactive $CD4^+$ and $CD8^+$ T cells prior to transplantation. Depletion of alloreactive T cells would promote a hyporesponsive environment and peripheral mechanisms of anergy, thus driving the shift towards tolerance [33, 34]. Depletion can be achieved through TBI, lymphocyte depleting Abs, and pharmaceutical agents. Anti-CD3 mAb has been used for development of mixed chimerism in murine NOD models with few side effects, achieving the same results as myeloablation while bypassing the risk of GVHD [35, 36]. Anti-CD3 mAb is proposed to downmodulate the TCR complex, induce apoptosis of alloreactive T cells, increase production of the immunoregulatory cytokine TGF-β, and promote the development of regulatory T cells [37, 38]. Anti-CD3 is effective in inducing tolerance in models of both syngeneic and allogeneic islet transplantation, enabling long-term engraftment [34, 39]. Antithymocyte globulin (ATG) is also a potent inducer of T-cell depletion. Its mechanisms of action are poorly understood, though. Administration of ATG alone or in combination with other agents prolonged allograft survival in various models [40–43]. Islet allograft survival was significantly improved in a nonhuman primate model with administration of ATG and the B-cell-depleting CD20 mAb, rituximab [44]. Taken together these findings suggest that lymphoid-depleting agents may not be effective as a monotherapy but may be useful in combination with other tolerance-inducing therapies.

6. Tolerance Induction through Costimulatory Blockade

Tolerance can also be achieved by interfering with costimulatory interactions to inhibit the secondary signal required for full T-cell activation. Suboptimal signaling renders the cells anergic. The B7-CD28 pathway is a key pathway in T-cell activation, survival, and function. Blockade of the B7 (CD80, CD86) receptor on APCs with CD28 on T cells modulates the immune response. In rodent models, B7-CD28 blockade through administration of inhibitory CTLA4-Ig led to prolonged allograft survival and tolerance [45–48]. In nonhuman primates, however, CTLA-Ig treatment alone led to moderate improvements in allograft survival but failed to induce tolerance [47–49].

Similar findings have been reported with CD40-CD154 blockade. Ligation of the CD40 receptor on APCs with CD40 ligand (CD154) on T cells enhances TCR signaling and effector responses [50, 51]. CD154-deficient mice displayed impaired antigen-specific T-cell responses and priming. Coadministration of anti-CD154 mAb and inactivated donor lymphocytes resulted in long-term survival of islet allografts in an STZ-induced rodent model and nonhuman primate models with pancreatectomy [7, 52, 53]. Anti-CD154 mAb downregulated CD28 on donor lymphocytes, thereby inhibiting CD28-CD80 interactions between donor APCs and recipient T cells [54]. Anti-CD154 mAb treatment has been shown to result in "indefinite" survival in islet, BM, and cardiac allograft models [53, 55–57].

Whether true tolerance can be achieved with anti-CD154 mAb alone or in combination with BM transplantation, donor-specific transfusion (DST), or conventional immunosuppression is debatable. Anti-CD154 mAb therapy is met with mixed results. Kenyon et al. reported that rhesus monkeys treated with the humanized anti-CD154 mAb (hu5c8) prior to transplantation with periodic maintenance therapy achieved long-term survival and improved function of intrahepatic islet allografts with little to no side effects [7]. In contrast, Kirk et al., using a similar regimen, found that while hu5c8 therapy prevented acute renal allograft rejection in rhesus monkeys, recipients developed donor-specific Abs and showed signs of chronic allograft nephropathy [58]. A similar result has been reported by Azimzadeh et al. using a primate cardiac allograft model [59]. Although anti-CD154 mAb therapy may allow for the manipulation of donor-specific responses and assist in the induction of tolerance, the consequences of administering anti-CD154 mAb need to be evaluated further. There are strong indications that anti-CD154 mAb therapy results in thromboembolic complications in nonhuman primate models [60]. Discrepancies in outcomes may be due to timing of administration, dosage, duration, and dependent on the animal model.

7. DCs in Tolerance

DCs play a critical role in provoking the immune response by mediating the uptake, processing, and presentation of antigen from the microenvironment to naive T cells in

the secondary lymphoid tissues. However, DCs also act as regulators in the thymus and periphery by eliminating self-reactive T cells and preventing autoimmunity [21]. Therefore, DCs may be key to achieving central and peripheral tolerance by helping shape the immune response. The immunogenic versus tolerogenic nature of DCs is dependent on the maturation of the DCs and the subset. Manipulation of DCs may, therefore, serve as a therapeutic tool in the design of tolerogenic regimens [61, 62]. Murine DC subsets are characterized and categorized based on their surface markers and origin, such as myeloid DCs ($CD11c^+CD8\ \alpha^+$, $CD11c^+CD8\ \alpha^-$), plasmacytoid (p)DCs ($CD11c^-B220^+Gr\text{-}1^+$), and Langerhans cell-derived DCs from the epidermis ($CD11c^-$). Human DCs fall primarily into two categories: monocytes and pDCs ($CD4^+CD8^-CD11c^-$). Differences in murine and human DC surface markers make comparisons difficult [63].

Whereas mature myeloid DCs upregulate MHC class II and the costimulatory molecules CD40, CD80, and CD86, immature DCs downregulate these markers and are potent inhibitors of allospecific T-cell responses [64]. The lack of stimulatory molecules allows immature DCs to induce antigen specific hyporesponsiveness in T cells [65]. Immature DCs also vary in their stimulatory activity. $CD11b^+CD8\ \alpha^-$ DCs induce a Th2 phenotype (IL-4, IL-5, IL-10, and IL-13), while $CD11b^-CD8\ \alpha^+$ DCs induce a Th1 phenotype (IL-2 and IFN-γ) through secretion of IL-12 [63, 66]. In addition to skewing the Th1/Th2 profile, tolerogenic DCs have been shown to promote allograft acceptance by deletion and anergy of alloreactive T cells and induction of donor-specific regulatory T (Treg) cells [67–69].

The ability of tolerogenic DCs to mediate anergy and proliferative arrest of alloreactive T cells has been demonstrated by Munn et al. pDCs produce indoleamine 2,3-dioxygenase (IDO), which catabolizes the essential amino acid L-tryptophan, and triggers the integrated stress pathway within antigen specific T cells and suppresses their proliferation and function [70]. The exact mechanism by which these tolerogenic DCs inhibit alloreactive T cells is not well understood but is thought to involve the activation of the general control nondepressible-2 (GCN2) kinase pathway. The GCN2 pathway is activated in response to an accumulation of uncharged tRNAs that results from amino acid deprivation [71]. Transfer of tolerogenic IDO producing immature DCs from primary tolerant recipients into murine cardiac allograft recipients enhanced allogeneic T cell apoptosis and Treg-cell development resulting in prolonged graft survival [67]. Ochando et al. showed that treatment with DST and anti-CD154 mAb prompted pDCs to migrate to the allograft and subsequently induced Treg development in the lymph nodes. Furthermore, pDCs isolated from tolerogenic mice promoted $CD4^+CD25^-Foxp3^-$ T cells to convert into $CD4^+CD25^+Foxp3^+$ Treg cells *in vitro* [72].

DC maturation can be limited through addition of cytokines (IL-10 and TGF-β) and costimulatory blockade [73, 74]. In mice, BM cells cultured in the presence of granulocyte macrophage colony-stimulating factor (GM-CSF) alone tended to acquire an immature phenotype—expressing low levels of MHC class II and costimulatory molecules. In contrast, addition of IL-4 to the GM-CSF culture led to DC maturation and high levels of MHC class II, CD40, CD80, and CD86 expression [75–77]. DCs can be restricted to an immature state through pharmacological interventions as well. *In vitro*, rapamycin conditioned BM-derived DCs suppressed the development of alloreactive $CD4^+$ T cells but allowed for the proliferation and functioning of Tregs [78]. Lutz et al. examined the tolerogenic potential of immature myeloid DCs and found that the transfer of these immature myeloid DCs induced a state of T-cell unresponsiveness and resulted in a significant improvement in cardiac allograft survival [75].

In addition to extrinsic influences, DC maturation is also governed by NF-κb signaling. NF-κb regulates expression of MHCs and costimulatory molecules. Lu et al. inhibited NF-κb activity within DCs by constructing a decoy double-stranded oligodeoxynucleotide (ODN) that selectively inhibited expression of costimulatory molecules while permitting the expression of MHCs, thus preventing DC maturation. Genetic engineering of DCs allows for expression of molecules that play a role in the inhibition of T-cell responses. Administration of DCs deficient in NF-κb activity prevented the onset of diabetes in NOD mice [79]. Recipients of virally transduced DCs expressing CTLA4-Ig, IL-10, or FasL displayed improved pancreatic islet and cardiac allograft survival [80–83]. The therapeutic potential of these engineered DCs is limited by the fact that the cells may become activated or mature *in vivo* through exposure to the virus and proinflammatory stimuli [80].

8. Myeloid-Derived Suppressor Cells (MDSCs) in Tolerance

MDSCs are emerging as important regulators of tolerance. Originally identified for their role as suppressors in cancer [84, 85], MDSCs are comprised of heterogeneous myeloid cell populations: subsets of DCs, macrophages, and granulocytes. MDSCs in mice are characterized based on expression of the markers CD11b, Gr-1, Ly6C, and Ly6G. MDSCs can be divided even further by their nuclear morphology as mononuclear, monocytic MDSCs ($CD11b^+Gr\text{-}1^{high}Ly6G^-Ly6C^{high}$) and polymorphonuclear, granulocytic MDSCs ($CD11b^+Gr\text{-}1^{int}Ly6G^+Ly6C^{low}$) [86]. Their variability can even be extended to their function and production of immunosuppressive components arginase-1 (Arg-1), inducible nitric oxide synthase (iNOS), and reactive oxygen species (ROS), with suppressive function depending on the environment and pathological condition [87–89]. MDSCs make up a relatively small proportion in healthy mice but expand and accumulate in the lymphoid tissue of tumor bearing mice, remaining in an immature state and inhibiting antitumor responses [90, 91]. The generation and expansion of MDSCs is dependent on inflammatory cues [92, 93]. The inflammatory molecules vascular endothelial growth factor (VEGF) and GM-CSF have been linked to the accumulation of MDSCs [94, 95]. Additional proinflammatory cytokines IL-1β and IL-6 have been shown to contribute to the development of MDSCs. IL-1β secreting tumors had greater

accumulation of MDSCs with improved lifespan resulting in aggressive tumor growth [96–98].

The ability of MDSCs to suppress T-cell responses and expand Treg cells has been demonstrated in various models of autoimmunity, infection, and cancer [99–101]. The mechanisms by which MDSCs impart their suppressive effect include production of iNOS and arginase-1. Arginase-1 depletes L-arginine resulting in downregulation of the TCRζ chain and inhibition in production of cell cycle regulator proteins [102, 103]. iNOS promotes phosphorylation of the Janus kinase 3 and STAT5 pathway and inhibits MHC class II expression and T-cell proliferation [104–106]. MDSCs also produce ROS and peroxynitrates, which inhibit protein tyrosine phosphorylation through nitration of tyrosine residues [88, 107]. Increased production of these factors was observed in tumor models and related to T-cell unresponsiveness [108].

As suggested in tumor models, MDSCs may be useful in transplant settings by suppressing alloreactive T-cell responses and prolonging graft survival. In a murine kidney allograft model, Dugast et al. demonstrated that recipients with long-term surviving grafts exhibited an accumulation of $CD3^-$MHC $II^-CD11b^+CD80/86^+$ cells in the blood and graft. Isolation and characterization of the cells revealed myeloid-like morphology. These cells showed strong suppressive activity against activated T cells, with suppressive activity related to the increased number of cells and dependent on production of iNOS. The phenotypic and functional analysis of these cells fits the criteria for MDSCs. Inhibition of the MDSC suppressor molecule iNOS by aminoguanidine resulted in rejection of kidney allografts [109]. MDSCs also have implications in skin transplant models. Heme oxygenase-1 (HO-1) secreting MDSCs facilitated tolerance in recipients of skin allografts through T-cell suppression and IL-10 production [110]. Our group has recently shown that cotransplantation of MDSCs with islet allografts reduces $CD8^+$ effector T-cell responses and results in the expansion of antigen specific Tregs in the draining lymph nodes (dLNs), spleen, and peripheral blood, translating to markedly improved islet allograft survival. MDSC-mediated suppressor functions were dependent on the inhibitory B7-H1 (PD-L1)-PD1 pathway. The protective effect imparted by Tregs was negated in recipients receiving B7-$H1^{-/-}$ MDSCs. Frequencies of $CD4^+Foxp3^+$ cells were markedly reduced in all compartments in B7-$H1^{-/-}$ MDSC recipients. Direct evidence for the role of B7-H1 in MDSC-induced Treg development was demonstrated through *in vitro* coculture of donor BALB/C T cells with DCs or MDSCs from WT or B7-$H1^{-/-}$ MDSCs. WT MDSCs expanded $Foxp3^+$ cells, whereas MDSCs deficient in B7-H1 exhibited reduced capacity for Treg induction. Further evidence was shown in B6 mice intravenously injected with OVA-specific $CD4^+$ T cells with subsequent footpad injection of OVA-pulsed WT or B7-$H1^{-/-}$ MDSCs. Examination of the popliteal LNs showed an increase in the frequency of $CD4^+Foxp3^+$ cells in recipients of OVA-pulsed WT MDSCs compared to recipients of OVA-pulsed B7-$H1^{-/-}$ MDSCs. Therefore, it appears that MDSCs require B7-H1 to exert their immunoregulatory activity and to induce Tregs [111, 112].

9. Tregs in Tolerance

The presence of $CD4^+CD25^+Foxp3^+$ regulatory T cells is correlated with improved graft outcomes and tolerance [113–116]. Tregs act as surveillance for the immune system, and depletion of Tregs results in lymphoproliferation and autoimmune syndrome [117–119]. Biopsies taken from grafts of tolerant recipients had greater infiltration of Tregs compared to those from rejecting grafts [120]. While Treg cells only make up 5–10% of the mature T-cell population, they are potent in numbers [121]. Lymphodepletional therapies and costimulatory blockades alone can do little in terms of promoting tolerance; therefore, strategies that promote Treg cells and their functions will improve chances of engraftment. Unfortunately, many immunosuppressive agents, especially those targeting the IL-2 receptor and IL-2, inhibit Treg development and function since IL-2 signaling is critical to T cell survival and proliferation [122]. The ideal scenario would minimize immunosuppressive therapy and focus on expanding the endogenous Treg population or generation of antigen-specific Tregs, thereby inducing tolerance without the need for immunosuppression.

There is evidence that apoptotic cells have the ability to influence Treg development by way of DCs [123]. Apoptotic cells suppress inflammatory responses by emitting inhibitory signals to DCs and other phagocytes. Adopt transfer of donor apoptotic cells imparted an immature phenotype on DCs, which in turn inhibited $CD4^+$ T cell activation and IL-2 and IFN-γ production. Combination with CD40-CD154 blockade led to prolonged cardiac allograft survival through induction of Tregs [124].

It is speculated that Tregs impart their suppressive function through direct interactions with cells (engagement with CTLA4-Ig), production of soluble factors and inhibitory cytokines TGF-β and IL-10, and cytokine deprivation [125, 126]. Zhang et al. [127] provided mechanistic insight into how Tregs exert their suppressive effect using an islet allograft model. Tregs in the blood migrated to the allograft through the guidance of the chemokines CCR2, CCR4, and CCR5. Upon activation, they traveled to the dLNs where they inhibited DC migration and antigen-specific T-cell migration and response in the dLNs and islet allografts [127]. Similar observations were made by Golshayan et al. Alloantigen specific Tregs were expanded *in vitro* and maintained their suppressive function *in vivo*. When transferred into recipients of skin allografts, they trafficked to the secondary lymphoid organs and accumulated in the graft dLNs and within the allograft itself. The donor-specific Tregs delayed graft rejection in the absence of immunosuppression. Tregs infiltrated skin allografts early on in the immune response and suppressed rejection by inhibiting alloreactive T-cell responses. In the presence of Tregs, $CD4^+$ T cells produced far less IFN-γ and did not accumulate in the secondary lymphoid tissues [113]. The suppressive features of Tregs make them candidates for therapeutic use in islet transplantation.

The differential effects of immunosuppressive therapies on Treg development make it difficult to determine the optimal combination of agents that promote Treg activity, while inhibiting Teff functions. Transient depletion of dividing T

cells with anti-CD25 mAb altered the homeostatic balance and created space for *de novo* expansion of Tregs. Anti-CD25 mAb recipients displayed tolerance to islet allografts, unlike control-treated recipients [128]. These data suggest that not all T-cell depleting therapies may have an effect on Tregs or that Tregs may adapt by downregulating CD25. In contrast, Li et al. showed that anti-CD25 mAb treatment prevented tolerance of liver allografts by reducing the ratio of $CD4^+CD25^+$ Tregs to $CD3^+$ T cells [129]. The Teff/Treg ratio determines the outcome of the graft; therefore, expansion of Treg populations and deletion of effector T-cell populations are crucial to tolerance induction. Zheng et al. have shown that combined treatment of rapamycin and agonistic IL-2/Fc and antagonistic mutant IL-15/Fc selectively targeted alloreactive T cells while preserving Tregs [130]. When administered with other calcineurin inhibitors, beneficial effects were lost [122, 131, 132]. Thus, not all agents within a family necessarily exert the same effect.

One of the current limitations of Treg therapy is the inability to generate sufficient numbers of antigen-specific Tregs for therapeutic outcomes. The absence of reliable markers for human Tregs makes isolation and purification difficult. Isolated T cells would have to be expanded *in vitro*, but expansion may not be restricted to Treg populations specifically. Additionally, Tregs demonstrate a great degree of plasticity and have the potential of converting to an effector phenotype *in vivo* [133]. The risks associated with Treg therapy warrant further investigation and require technical advancement before application in humans.

10. Organ Stromal Cells in Tolerance

The contribution of organ stromal cells in the regulation of the immune response is understudied. Our group has extensively investigated the influence of these populations in the liver on islet transplantation. The liver is unique in that it is one of few organs with inherent tolerogenic properties [134–136]. A number of factors have been attributed to the tolerogenic state of liver allografts including increased B-cell infiltration, production of soluble MHC class I antigens, involvement from stromal cells, and presence of Tregs [112, 137–139]. The importance of liver stromal cells is highlighted by the fact that while liver transplantation in mice results in indefinite acceptance, transplantation of hepatocytes is rejected [140, 141].

We have focused on a population of stromal cells in the liver called the hepatic stellate cells (HSCs), which have been known to have a primary role in liver fibrosis and repair following hepatic injury. Additional features of these cells are their participation in the storage of vitamin A (retinoid) droplets, and their ability to produce TGF-β in response to inflammation [142–144]. However, little is known about the involvement of HSCs in immune regulation. HSCs, from both mice and humans, have been shown to act as nonprofessional APCs and upregulate MHCs, CD40 and CD80, and inhibitory PD-L1, in response to proinflammatory cytokines [137, 139]. Jiang et al. found that HSCs can also expand $CD4^+CD25^+Foxp3^+$ cells in an IL-2-dependent manner [145]. Therefore, it is conceivable that these cells possess tolerogenic qualities. Addition of HSCs into an MLR culture at an HSC to T ratio of 1 : 20 resulted in 80–90% inhibition of T-cell response [137]. *In vivo* inhibition was demonstrated in mice by cotransplantation of HSCs with islet allografts. A prolongation in survival in >60% of islet allografts was observed without immunosuppression. This was associated with elimination of antigen-specific T cells and enhanced MDSC and Treg development [112, 146, 147]. It appears that HSCs exert their effects primarily by inducing MDSCs. The ability of HSCs to promote MDSCs was verified *in vivo* by analyses of mononucleocytes infiltrating the islet grafts. Cotransplantation with HSCs led to an accumulation of MDSCs, instead of DCs, as seen in islet alone grafts. *In vitro* evidence confirmed that addition of HSCs to BM-derived DC culture promoted the development of $CD11b^+CD11^-c$ cells displaying suppressive functions. MDSC induction was abolished in IFN-$\gamma^{-/-}$ recipients, demonstrating the additional dependency of an inflammatory environment for MDSC development [112, 146]. MDSCs have been shown to induce Treg development as well [148]. Increased Treg levels were observed with islet/HSC transplantation. We, therefore, suspect that HSCs recruit MDSCs to the islet allografts and promote engraftment upon inflammation. Benten et al. have shown the tolerogenic role of HSCs in promoting hepatic engraftment in a rat hepatic allograft model. Hepatic transplantation led to the activation of HSCs and a series of genetic and phenotypic changes within the HSCs. Prior depletion of HSCs impaired hepatocyte acceptance [149]. HSCs demonstrate potent immunomodulatory properties and can influence the development of suppressor cells. With further study, HSCs may be implemented into tolerance inducing therapies.

Analogous to HSCs, Sertoli cells (SCs) within the seminiferous tubules of the testis also exhibit suppressive features. The immunoprivileged SCs have been exploited for protection of various transplanted tissues [150–154]. The immunoprotective capabilities of SCs can be extended across allogeneic and xenogeneic barriers [155, 156]. Transplantation of SC xenografts alone into NOD mice altered the cytokine milieu in the pancreas and induced a regulatory environment, inhibiting IL-6, IL-10, and IFN-γ production while promoting TGF-β and the regulatory enzyme indoleamine. TGF-β produced by the SCs was responsible for the generation of autoantigen-specific regulatory T cells. Recipients displayed reversion of diabetes with the SC xenografts [157]. It has also been shown that SCs have the ability to influence and inhibit T-cell responses and complement activation, although the mechanisms are not well defined [158–160]. Selawry et al. were the first group to demonstrate the application of the immunomodulatory SCs for inducing tolerance in islet transplantation [158, 161, 162]. Cotransplantation of SCs and islet allografts induced long-term graft survival, with recipients remaining normoglycemic for at least 60 days after transplantation without systemic immunosuppression [150, 157]. The transfer of xenogenic neonatal porcine SCs (NPSCs) at the time of islet transplantation was shown to prolong islet allograft survival in nonimmunosuppressed rats in a dose-dependent manner

(MST = 16.33 ± 1.53 days versus islets alone group, 5.67 ± 0.94 days). Examination of grafts showed reduced lymphocyte infiltration and increased expression of Bcl-2 compared to recipients receiving islets alone, suggesting that NPSCs may also be regulating the expression of immunoprotective genes [163]. There is also evidence that NPSCs can suppress the upregulation of CD40 expression on DCs in response to LPS stimulation, thereby preventing full activation of the DCs and inducing the development of tolerogenic DCs [160]. The efficacy of NPSCs has even been demonstrated in human models of xenotransplantation. Transplantation of porcine islets and NPSCs into type 1 diabetic patients led to a reduction in insulin dependence and maintenance of metabolic control for up to 4 years without immunosuppression in half of the 12 patients involved in the study [164]. Cotransfer of SCs at the time of transplantation may, therefore, provide protection for alloislet transplants and improve chances of engraftment.

The BM also contains a rich source of immunomodulatory stromal cells referred to as mesenchymal stem cells (MSCs). MSCs have the capacity to develop into various types of mesodermal tissues and exhibit properties of self-renewal [165]. MSCs alter the cytokine profile of DCs, naive and effector T cells, and NK cells in response to IFN-γ, downregulating the production of IFN-γ and TNF-α in the microenvironment and inducing a more tolerogenic phenotype. IFN-γ-induced expression of the immunosuppressive factors TGF-β, hepatic growth factor (HGF), IL-10, prostaglandin E2, matrix metalloproteinases (MMPs), and IDO account for the inhibition of alloresponses [166–168]. Furthermore, MSCs were found to promote the expansion of Tregs [169–171]. Characterization of human MSCs has been a challenge, and the results are somewhat conflicting [165, 172]. Fiorina et al. found that in their NOD model, murine bone-marrow-derived MSCs were positive for the stem cell markers CD29, CD44, CD73, CD105, and CD166 and negative for hematopoietic lineage markers CD45 and CD90.2 and costimulatory molecules CD40, CD80, and CD86. Interestingly, the MSCs expressed high levels of PD-L1, and it was speculated that the level of PD-L1 expression enabled the immunosuppressive functions of MSCs [169].

The ability of MSCs to modulate T-cell responses and influence tissue rejection suggests a therapeutic role for inducing tolerance in islet transplantation. The negligible expression of MHC II and absence of costimulatory molecules on the cell surface allow MSCs to escape immune recognition. However, the immunomodulatory effects of MSCs observed *in vitro* have been difficult to replicate *in vivo*, as evidenced in baboon skin allograft and rat cardiac allograft models [173, 174]. Allogeneic MSC infusion has been effective in preventing the onset of diabetes in prediabetic mice and reversing hyperglycemia in diabetic mice [169, 175]. In islet transplantation models, MSCs demonstrate the ability to migrate to the pancreatic islets and exert an immunosuppressive effect in the graft microenvironment [176, 177]. MSCs have also been shown to influence pancreatic vascularization and remodeling following transplantation [178]. Although the mechanisms by which MSCs exert their immunosuppressive effects remain elusive, Ding et al. suggest that one possible strategy involves the production of MMP-2 and MMP-9. In culture, MSCs mediated T-cell proliferative arrest and hyporesponsiveness by downregulating CD25 on the surface of T cells. Downregulation of CD25 was dependent on the production of MMP-2 and MMP-9 by MSCs, since inhibition of MMP via the thiirane gelatinase inhibitor SB-3CT restored surface expression of CD25 and T-cell responses. Administration of syngeneic MSCs at the time of syngeneic islet transplantation prevented rejection in murine STZ-induced diabetic Rag-2/γ(c)KO recipients reconstituted with $CD4^+CD25^-$ T cells and resulted in a rapid return to normoglycemia. In contrast, recipients under the same conditions treated with the MMP-2 and MMP-9 inhibitor SB-3CT became diabetic within 30 days (MST = 30 days) [167]. In theory, MSC therapies sound promising in the induction of tolerance but require further evaluation before clinical application.

11. Concluding Remarks

The future of islet transplantation depends on the development of tolerance inducing therapies. While temporary immunosuppression can be advantageous, the long-term risks outweigh the benefit. Tolerance suggests freedom from insulin dependency and an improvement in the patient's overall quality of life. A tolerizing regimen that utilizes techniques that selectively target donor-reactive T cells while expanding populations of regulatory T cells will result in better outcomes. Further investigation into inherently tolerogenic cells in the body such as HSCs, SCs, and MSCs will aid in the design of therapies. Though many challenges still remain, the progress made in the animal models of tolerance holds great promise for humans.

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Review Article

Generation of Transplantable Beta Cells for Patient-Specific Cell Therapy

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Islet cell transplantation offers a potential cure for type 1 diabetes, but it is challenged by insufficient donor tissue and side effects of current immunosuppressive drugs. Therefore, alternative sources of insulin-producing cells and isletfriendly immunosuppression are required to increase the efficiency and safety of this procedure. Beta cells can be transdifferentiated from precursors or another heterologous (non-beta-cell) source. Recent advances in beta cell regeneration from somatic cells such as fibroblasts could circumvent the usage of immunosuppressive drugs. Therefore, generation of patient-specific beta cells provides the potential of an evolutionary treatment for patients with diabetes.

1. Introduction

Type 1 diabetes is one of the most common chronic diseases in children and adolescents caused by autoimmune destruction of insulin-producing beta cells of islets of Langerhans. Thus patients depend on insulin injection all their life. The majority of young patients depend on life-long treatment with insulin injections to control hyperglycemia. However, an exogenous supply of insulin often leads to severe hypoglycemia-related complications. Hence, insulin therapy saves life but is not a cure. On the other hand, beta-cell replacement therapy by transplantation may offer a cure because transplantation of functional beta cells can reestablish glucose-responsive insulin secretion and provide optimal control to prevent hypoglycemia when insulin is secreted [1–9]. Whole-pancreas transplantation can restore endogenous insulin production, but it has rarely been carried out in children with diabetes due to the risk of perioperative morbidity related to the damage by digestive enzymes from the exocrine pancreas during the surgical procedure. In contrast, islet-cell transplantation provides insulin-producing beta cells in a relatively noninvasive manner. It becomes a more feasible option for young recipients. In fact, much progress has been made in islet-cell transplantation following the success of the Edmonton protocol that emphasizes both a sufficient amount of donor islets and steroid-free immunosuppressive regimens [3, 4, 8, 9]. However, the requirement of 2 to 4 donors to reverse diabetes results in a considerable lack of transplantable islets. The destruction of transplanted islets by the cytotoxicity of immunosuppressive drugs further worsens this shortage [7]. In this regard, use of an alternative source of beta cells is a key to bridge the gap between cell supply and demand. Therefore, a major goal of diabetes therapy is to promote the formation of new beta cells. In consideration of elimination of immunosuppressants, autologous cells may offer a safer alternative. Ideally, a patient-specific approach can enhance the success and safety of islet transplantation.

The pancreas is fundamental to the regulation of nutritional homeostasis. The pancreas is composed of exocrine and endocrine compartments. The former consists of acinar and ductal cells that produce and transport digestive enzymes into the duodenum, and the latter of the islets of Langerhans that make hormones for adaptive glucose metabolism. Each islet can secret five hormones (glucagon, insulin, somatostatin, ghrelin, and pancreatic polypeptide),

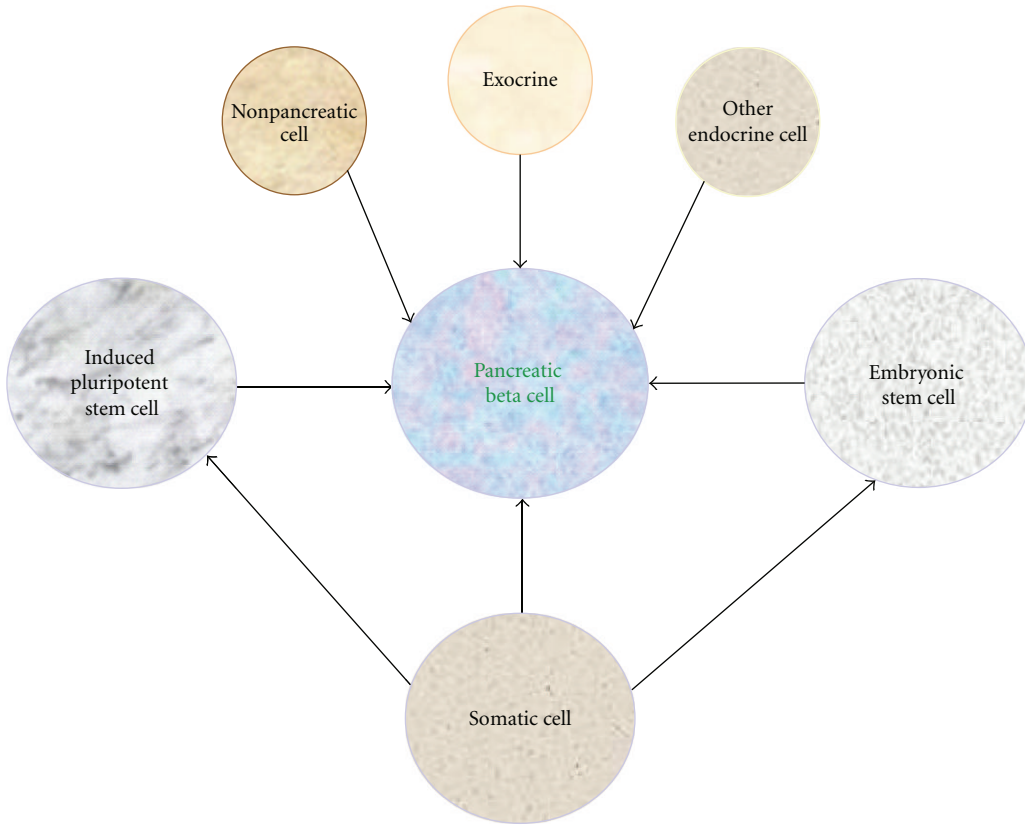

Figure 1: Generation of pancreatic beta cells. New beta cells can be generated by manipulation of different cell sources, such as from other endocrine, exocrine, and nonpancreatic cells, induced pluripotent stem cells, embryonic stem cells, and somatic cells.

which are produced by alpha, beta, delta, epsilon, and PP cells, respectively [10].

There is a great interest in developing novel sources of transplantable beta cells for replacement therapy. Adult beta cells possess a limited capacity to replicate under normal physiologic conditions. However, beta-cell mass expands during times of metabolic changes such as during pregnancy and obesity [11, 12]. Beta cells can also be regenerated after the destruction of existing beta cells, such as by chemical treatment with streptozotocin or the partial removal of pancreas by a surgical procedure [13, 14]. In theory, new beta cells could arise through differentiation of progenitors or other nonbeta cells (Figure 1). Embryonic stem cells have the ability to differentiate into any cell type. For this reason, they are considered as an ideal starting material [15–18]. Some nonpancreatic cells, including hepatic cells, can also differentiate into insulin-positive cells [19, 20]. Non-endocrine pancreatic cells, such as ductal and acinar cells, may retain a degree of plasticity to differentiate into other cell types, including beta cells [21–24]. Beta cells can also be transdifferentiated from other endocrine cells, such as alpha cells [25–27].

Recent advances in stem cell biology have established the feasibility of converting one cell type into another [28–31]. This breakthrough directs autologous cell therapy that drives the transdifferentiation of readily available cells, such as fibroblasts, into therapeutically desirable cells, such as blood, neuron, cardiomyocyte, and islet-like cells. Significant applications of such patient-specific therapy include the engineering of new beta cells from patients' own cells, and the elimination of the life-long usage of immunosuppressants, bioincompatibility, and disease transmission coupled with donor cells. Transcription factors for pancreatic stem cell development and the differentiation of beta cell play a critical role in this process.

2. Transcription Factors Determine the Development of Beta Cells

Transcription factors have been recognized as the key mediators of cellular identity. Cell-specific gene expression is controlled at the transcriptional level and in large part by the interface among multiple transcription factors interacting with the promoter and/or enhancer regions of target genes. Many attempts have been made towards generating functional beta cells. The importance of transcription factors in pancreatic development has been well characterized in knockout and transgenic mice using the reverse genetic approach of loss- and gain-of-function of the target genes (Figure 2, [32–35]).

The pancreas develops from dorsal and ventral budding of the foregut boundary beginning around at embryonic day 9 (E9) in mice and at around 3 weeks postfertilization in

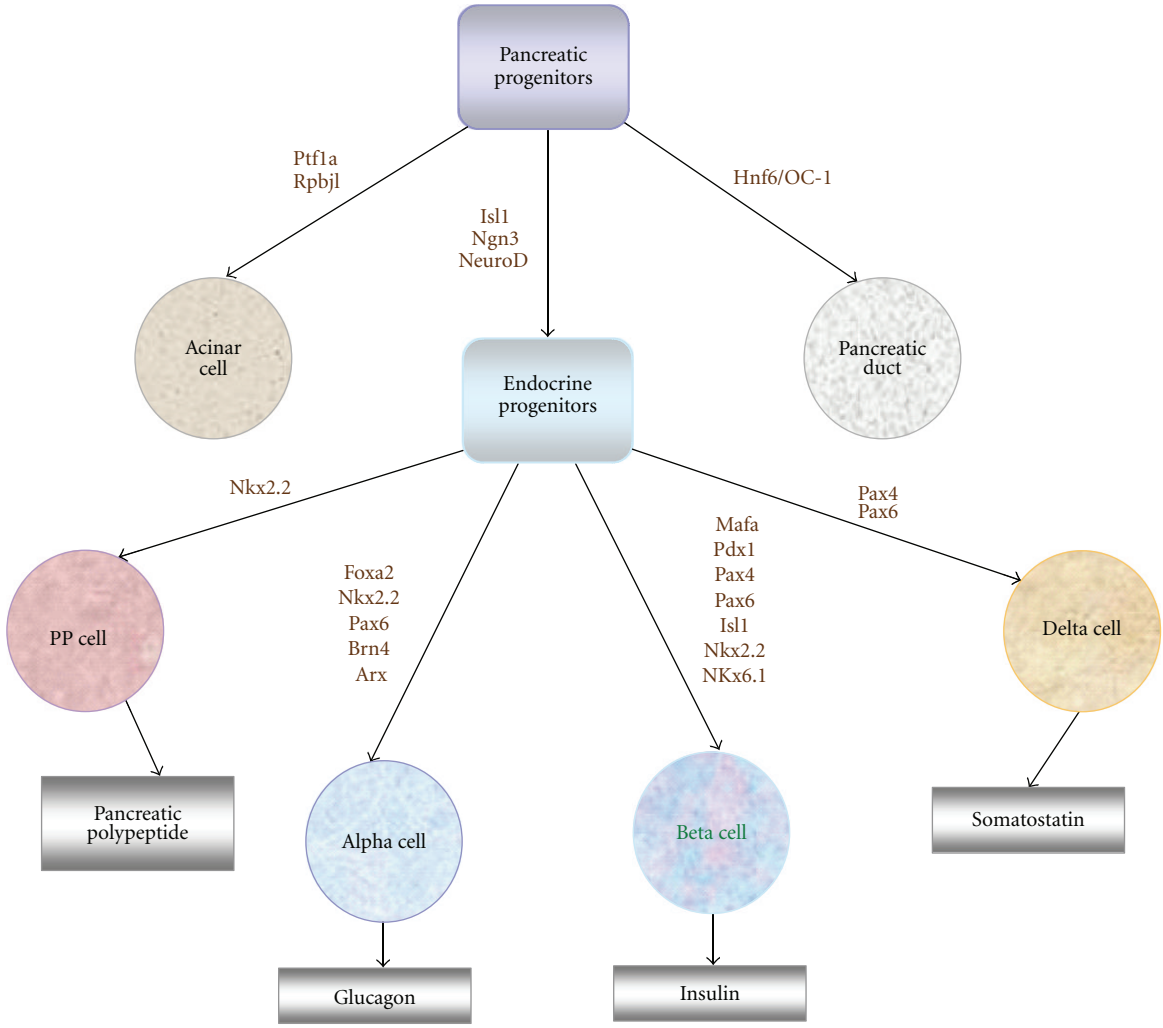

Figure 2: Development of pancreatic beta cells and the associated transcription factors that required for lineage specificity in various steps. Alpha-, beta-, delta-, and pp-cells secrete glucagon, insulin, somatostatin, and pancreatic polypeptide, respectively.

humans [36]. Both loss- and gain-of-function approaches have confirmed the key role of pancreatic and duodenal homeobox 1 (Pdx1), also known as insulin promoter factor 1, in pancreas development. Genetics lineage tracing has determined the multipotent ability of Pdx1-positive cells, which give rise to all pancreatic cell types [18, 19]. The expression of Pdx1 in abundantly restricted in beta cells in adulthood, although it exhibits a broad and dynamic expression pattern during the developmental stage. The absolute requirement of Pdx1 in normal pancreatic development was well established in Pdx1-deficient mice, which completely lack a pancreas [32, 33]. Furthermore, this the phenotype of pancreatic aplasia was also observed in either heterozygous or homozygous Pdx1 mutations [34]. In humans, mutations in one allele of the Pdx1 gene are associated with adult-onset diabetes (a form of monogenic diabetes), while homozygous mutations in Pdx1 result in pancreatic aplasia [34]. The function of Pdx1 is also demonstrated in studies of gain-of-function. Several studies have shown that nonbeta cells could be transdifferentiated into functional beta cells by overexpression of Pdx1 [25]. Ectopic overexpression of Pdx1 activates pancreatic genes in nonpancreatic cells, and these Pdx1-expressing liver cells can ameliorate streptozotocin-induced diabetes [20]. Therefore, this gene plays an important role in the initial stages of pancreatic development, and it has a great potential as a manipulative target for generating insulin-producing beta cells.

Endocrine versus nonendocrine cell fates appear to be governed by the differential synthesis of the basic helix-loop-helix (bHLH) transcription factors, Ngn3 (neurogenin 3), Hes1 (hairy and enhancer of split 1), and NeuroD1 (neurogenic differentiation 1). Ngn3 is characterized as an endocrine cell-specifying factor, the expression of which is essential for all endocrine-cell development [37, 38]. Lineage analysis of Ngn3-derived cells reveals that all endocrine cell types transiently express Ngn3 during a narrow window in the developing mouse embryo. Ngn3 is detectable at E13.5, peaks at E15.5, and disappears entirely thereafter. No Ngn3-positive cells are found at birth or in adult pancreas, suggesting a role in a particular stage of the pancreas

development. In fact, Ngn3-deficient mice proceed along nonendocrine cell fates, confirming its central role as an endocrine progenitor [37, 38]. One key target gene of Ngn3 is NeuroD1, which appears to lie immediately downstream of Ngn3, based on the fact that no NeuroD1 can be found in Ngn3-deficient mice and that expression of Pdx1 is unaffected in NeuroD1 knockout mice. The expression of NeuroD1 can be found in pancreatic endocrine cells and other nonpancreatic tissues, such as the intestine and the brain. The function of NeuroD1 is to activate the insulin gene and drive further differentiation into functional islet cells. In fact, the development of islet cells is arrested at a premature stage in NeuroD1-deficient mice, demonstrating a critical role in early islet formation [39].

The transition from endocrine to individual cell types occurs in two distinct steps. The primary transition features clusters of first-wave glucagon-positive and insulin-negative cells at E9.5. Insulin-positive cells appear during the secondary transition period around E13.5 to E15.5. These cells proliferate and form islet cells soon after birth. The transition from the first to the second stage is not random, but rather each one of the specific endocrine lineages is directed by additional transcription factors. The paired homeobox transcription factors 4 and 6 (Pax4 and Pax6) provide an additional level of specificity for pancreas. Mice deficient for Pax4 display absence of beta and delta cells, suggesting its role in directing the fate of those cells [40]. Pax6 knockout mice fail to develop glucagon-secreting cells, signifying its role in the commitment towards alpha-cell lineage [41].

Arx expression is restricted to islet at mature stage although it is expressed in the pancreas during development. Arx appears to be downstream of Ngn3 based on the fact that it is not expressed in Ngn3-deficient pancreata [42]. Arx-deficient mice die prematurely due to severe hypoglycemia, indicating a possible role in alpha cell development. In fact, a complete loss of alpha cells in the pancreas from Arx knockout mice was observed, confirming that Arx is necessary for alpha cell development [42]. Overexpression of Arx in either Pdx1-positive progenitor cells or insulin-producing cells leads to an increase or conversion in alpha cells, suggesting that Arx is sufficient to turn beta cells towards alpha cell identity [43].

Brain4 (Brn4), a POU domain transcription factor, is only expressed in glucagon positive cells of the adult rat pancreas, suggesting a potential role in alpha cell lineage [44, 45]. Ectopic expression of Brn4 in beta cells leads to the conversion of glucagon-positive cells in both the mantle and the centre of the islet, which led to coexpress insulin and glucagon, suggesting that Brn4 might play a role in early development of glucagon-expressing cells [44]. However, Brn4 is not essential for alpha fate as Brn4-null mutant mice show no significant impact on glucagon or insulin gene expression, biosynthesis, or secretion [45].

The insulin promoter region contains binding sites for both the NK homeobox factors Nkx2.2 and Nkx6.1, indicating that these factors may direct differentiation of insulin-producing beta cells. Nkx6.1 exhibits distinct expression patterns during early and late embryonic stages. It is expressed exclusively in the beta cells by the end of gestation, although it can be stained broadly in the pancreas at E10.5. Mice deficient in Nkx6.1 have no insulin-positive beta cells but are normal for other islet cell types, demonstrating its central role in final differentiation of beta cells [46]. Unlike Nkx6.1, Nkx2.2 can be detected in alpha and PP cells in addition to beta cells. Mice deficient for Nkx2.2 display a complete beta-cell loss and reduced numbers of alpha and PP cells, suggesting its preferential roles in these three cell lineages during pancreatic development [47].

Although certain transcription factors hold specificity, other transcription factors may be required in multiple stages of beta-cell development, and several transcription factors interact with each other to maintain normal pancreatic function. Leucine zipper transcription factors Mafa and Mafb are preferentially expressed within the beta and alpha cells of the adult pancreas, respectively [48, 49]. The transcription of insulin and glucagon is regulated by Mafa and Mafb, respectively. Mice deficient in Mafb exhibit reduced numbers of both insulin- and glucagon-positive cells during pancreatic development, suggesting its essential role in both alpha- and beta-cell differentiation [49]. Mice deficient in Mafa display normal beta-cell development early on, but they develop beta-cell dysfunction and diabetes as adults [48]. This normal phenotype of early beta-cell development in Mafa knockout animals is thought to be due to the compensation from Mafb. Mafb is also found to be expressed in nearly 90% of insulin-producing cells in addition to alpha cells, although this expression ability of Mafb-insulin double positive cells relies on Nkx6.1 expression, in large part due to the lack of insulin-positive cells when Nkx6.1 is knocked down [46, 49]. Forced expression of multiple transcription factors can induce the capacity of differentiation from cell type to another. Experimental data show that combination of the three transcription factors Ngn3, Pdx1, and Mafa directs the reprogramming of differentiated pancreatic exocrine cells from adult mice into islet-like cells [22].

The knowledge on transcription factors is believed to be essential for tailoring the transplantable beta cells to function optimally. First, multiple transcription factors instead of one direct specific cell lineages so that combinatorial interactions obviously are essential for the final pattern of certain cell types. Second, although transcription factors direct the formation of new beta cells, other factors, such as growth factors and transcriptional coactivators are, also needed.

3. Endocrine Progenitor Cells

Although the turnover of adult human beta cells is rare, reprogramming may be easier if the starting cell type shares a common developmental history with the desired cell type. The transcription factor Ngn3 directs the formation of the endocrine alpha and beta cells [37, 38]. The development of alpha cells is earlier than that of beta cells. After the endocrine cell types start developing, the glucagons-positive alpha cells become the first to be detectable as early as E9.5. This is followed by the existence of insulin-producing cells, which

coexpress glucagon. The fully differentiated beta and alpha cells can be found by E14 [37, 38].

Once fully functional endocrine cells are developed, cell-cell contacts between alpha and beta cells are the greatest among others [25, 26, 37]. For example, the contact rate of alpha and beta cells is much higher than that of alpha-alpha or beta-beta cell contact. This enormous contact between beta and alpha cells suggests a close relationship between these two cell types. In fact, experimental data reveal that alpha cells can serve as a novel source of beta-cell progenitors. Alpha-to-beta cell conversion has been demonstrated independently using different damage models [26, 27]. Detailed studies are needed to clarify the factors mediating the conversion process and the possibility of application to humans.

4. Nonendocrine Progenitor Cells

During development, endocrine cells, such as beta cells, and exocrine cells, such as ductal and acinar cells, derive from the same progenitor cells. Adult ductal epithelium has been considered as the most likely source of beta-cell progenitors. This consideration is based on the fact that embryonic insulin-producing cells arise from pancreatic ductal epithelial progenitor cells using morphological staining of beta cells, which are frequently existing in the duct or nearby and also in lineage-tracing studies [24, 50]. It has been found that significant beta-cell neogenesis results from ductal ligation, but this could not be confirmed in other studies [24, 50]. Therefore, the plasticity of ductal cells as progenitors to differentiate into beta cells is still under debate.

Similarly, mouse pancreatic acinar cells can be converted into beta cells using three transcription factors [22]. The newly generating insulin-positive cells were detected by irreversible genetic lineage-tracing techniques and are confirmed to derive from acinar cells [22, 23]. Therefore, both ductal and acinar cells in the exocrine compartment have the potential to be an alternative source for differentiation into beta cells.

5. Nonpancreatic Cells

The liver and the pancreas share a common progenitor cell during embryonic development [19, 20]. Therefore, the liver has been examined as an alternative source for generating beta cells [19, 20]. In fact, insulin-positive cells have been produced either in a non-lineage-restricted manner in the presence of high glucose and nicotinamide, or by tissue-specific expression of Pdx1 by adenoviral transfection and with addition of special soluble factors for beta-cell growth [19, 20].

6. Inducible Pluripotent Stem Cells (iPSs)

Stem cells possess great plasticity to differentiate into any cell type. However, adult multipotent stem cells are extremely rare and difficult to isolate. Induced pluripotent stem cells (iPSs) are similar to embryonic stem cells and are inducible *ex vivo*. iPSs can be reprogrammed by defined factors from somatic cells [51–54]. Under special conditions, such as the addition of growth factors and multiple transcription factors, iPSs can differentiate into some desired cell types. The significance of this patient-specific conversion would be the avoidance of the risk of immunological rejection.

The desired cell types can be reprogrammed from abundant somatic cells by two major pathways, either directly or indirectly. In the former, beta cells are derived directly from somatic cells, such as fibroblasts [28–31]. They can also be generated through an iPS stage or as the so-called indirect pathway. Novel findings show that islet-like insulin-producing cells can be converted from iPSs, demonstrating the possibility of using patient-specific iPSs as a source of transplantable beta cells for cell-replacement therapies in diabetes. The fact that iPSs can be generated by defined factors using somatic cells makes this strategy possible. Directed reprogramming refers to as direct conversion of beta cells from somatic cells without a multipotent or pluripotent intermediate, mostly directed by a combination of the addition of special growth factors and overexpression of key transcription factors. Fibroblasts can be reprogrammed directly into insulin-producing cells, neurons, cardiomyocytes, and blood-cell progenitors, demonstrating the possibility of converting from one defined cell type to another. Both direct and indirect pathways for regenerating new beta cells have a great impact on patient-specific therapy in the future [28–31].

7. Conclusion

The success of generating islet-like insulin-producing cell is largely achieved by using our knowledge of the major steps in the differentiation of beta cells during embryonic development of the pancreas. The multiple transcription factors have been applied to coerce available cells to differentiate into desired types in a unique delineation pathway, including across lineages, such as from fibroblasts into iPSs, or from one fully functional lineage to another, such as from fibroblasts into insulin-positive cells. However, major safety issues are still a concern. For example, lentiviruses that are used in the expression of defined factors are unlikely to gain acceptance for transplantation into human patients due to their ability to integrate into the host genome. One of the goals of clinical trials is to balance both safety and efficiency. In terms of safety, some alternate, safer methods, such as the use of nonintegrable viruses, transferring plasmids, soluble proteins of reprogramming mediators, and repeated transfection of mRNA encoding the defined factors, are being tested as an alternative substitute for lentiviruses [52–54]. Although the experiments have been carried out as proof of principle, low efficiency is still an issue for large-scale production in therapeutic applications. Despite the promising progress in the search for alternative sources of insulin-producing beta cells, functionality of new generating cells that replace the same type *in vivo* should be measured considerably. These new transplantable beta cells must mimic extremely closely the function of the normal beta cells

if long-term normoglycemia is hoped to be achieved. This is particularly challenging to the production of true insulin-producing cells, due to the enormous complexity of the beta cell. Therefore, it is of great importance to understand in detail the mechanisms that control the origin and fate of beta cells if we hope to reach the ultimate goal of finding a cure for diabetes.

Abbreviations

Pax:	The paired homeobox transcription factors
Pdx1:	Pancreatic and duodenal homeobox 1
bHLH:	Basic helix-loop-helix
Ngn3:	Neurogenin 3
Hes1:	Hairy and enhancer of split 1
NeuroD1:	Neurogenic differentiation 1
Nkx:	The NK homeobox factors.

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Review Article

A Simple Matter of Life and Death—The Trials of Postnatal Beta-Cell Mass Regulation

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Pancreatic beta-cells, which secrete the hormone insulin, are the key arbiters of glucose homeostasis. Defective beta-cell numbers and/or function underlie essentially all major forms of diabetes and must be restored if diabetes is to be cured. Thus, the identification of the molecular regulators of beta-cell mass and a better understanding of the processes of beta-cell differentiation and proliferation may provide further insight for the development of new therapeutic targets for diabetes. This review will focus on the principal hormones and nutrients, as well as downstream signalling pathways regulating beta-cell mass in the adult. Furthermore, we will also address more recently appreciated regulators of beta-cell mass, such as microRNAs.

1. Introduction

The endocrine cells of the pancreas, located in the islets of Langerhans, are responsible for blood glucose homeostasis, secreting hormones with differing and even opposing effects on blood glucose disposal. Beta-cells, the most numerous islet cells, secrete the hormone insulin which reduces blood glucose levels by increasing peripheral uptake of glucose and by suppressing release of glucose from the liver. Conversely, islet alpha-cells secrete the hormone glucagon which can increase blood glucose levels. Glucagon mainly acts on the liver where it promotes glycogenolysis, releasing glucose from breakdown of glycogen stores and gluconeogenesis. Optimal control of blood glucose levels depends on delicate changes in insulin production and secretion by the pancreatic beta-cells and on their capacity for large increases in secretion after meals, requiring large stores of insulin. It is of critical importance that islets maintain an adequate beta-cell mass in response to various changes.

Recent evidence has revealed that beta-cell replication plays a central role in maintaining adult beta-cell mass [1]. In addition, rates of beta-cell proliferation change dynamically according to metabolic demand throughout life [2]. However, replication of pre-existing beta-cells is not the only available mechanism for generating new beta-cells. In fact, a reasonable body of evidence supports the existence of four other potentially important contributors to adult beta-cell mass regulation: (i) differentiation from stem cells precursors, (ii) transdifferentiation from a non-beta-cell differentiated precursor, (iii) whole islet neogenesis on the plus side and apoptosis on the negative, and (iv) changes in beta-cell size [3, 4]. However, the relative contribution of these processes in maintaining and expanding beta-cell mass is at present not well defined and varies between species [5–7]. During adult life, the beta-cell mass may have to adapt in the face of increased demands due to increases in body mass, pregnancy, or even loss of insulin sensitivity of peripheral tissues. If such compensatory adaptation is inadequate, then glucose homeostasis will be compromised and result in chronically elevated blood glucose, or diabetes [8, 9].

It is well known that beta-cells proliferate extensively during late embryonic development, but the rate of replication slows during postnatal life. During adult life, beta-cell proliferation is detected between 0.5% and 2% [10] gradually declining with age [11]. Remarkably, this low rate of baseline proliferation can be increased significantly in response to pregnancy or obesity and is regarded as an adaptive mechanism in response to increasing systemic insulin demand.

Although important roles of insulin [12] and glucose [13] in beta-cell compensation have been suggested, the mechanism underlying this process is not well understood.

In recent years, various groups have identified microRNAs (miRNAs) small molecules of noncoding RNA that are able to regulate protein expression that contribute to beta-cell dysfunction and diabetes onset [14–18]. However, the role of these miRNAs is not yet fully understood.

Type 2 diabetes (T2D) is characterized by hyperglycaemia resulting from impaired insulin secretion and/or impaired insulin action in peripheral tissues [19]. T2D constitutes one of the greatest pandemics of our time, with 220 million people currently diagnosed [20], and 439 million people expected to be affected by 2030 [21]. Importantly, there is substantial evidence that beta-cell dysfunction plays a major role in the pathology of T2D. For this reason, great efforts are being made in order to develop new therapeutic strategies, such as beta-cell replacement or regenerative medicine.

However, despite progress, most diabetic patients will still die prematurely as a direct result of their disease, its complications, or sometimes even its treatments. In fact, although one may hope that GLP-1 analogues and improved lifestyle may eventually translate into a slowing of T2D progress, clinical trials data have been generally disappointing and confirm that the disease continues to progress [22–25]. To date, no effective treatments for T1D based on slowing or reversing the natural history of the disease exist. Thus we must rely on treatments that can maintain or restore blood glucose levels, and evidences that disease progression can be significantly arrested are scanty. Thus, all T1D and over time most T2D patients will require exogenous insulin (or in rare cases an islet transplant). It is not all negative, and short-term benefits (usually progression from prediabetes to overt diabetes) have been reported in small studies for metformin and acarbose with the common factor likely to be weight loss. The similar success of supervised weight loss programs supports this view. Trials with the PPARg ligand rosiglitazone have also suggested that the progression of T2D may be retarded, but as the drug has been withdrawn, due to an increased risk of cardiovascular disease, this remains interesting rather than clinically useful. In the largest study of T2D to date (UKPDS-UK Prospective Diabetes Study (UKPDS) Group), treatments did not slow progression of patients to additional drugs in order to maintain blood glucose levels at target levels [26].

Furthermore, beta-cells from donor pancreases are in such short supply that transplants can be provided only to a limited number of patients. Ultimately, to cure diabetes, missing beta-cells that must be replaced, and in practical terms this would need to be done from within or a limitless source of beta-like cells must be developed for transplants. One way forward is to "create" beta-cells from alternative cell sources (neogenesis, transdifferentiation, stem cells, etc.). Such an approach requires further knowledge of the mechanisms that regulate pancreatic beta-cell mass.

This review briefly outlines current knowledge of significant factors/nutrients regulating beta-cells mass, and their signal transduction pathways, with greater focus on postnatal regulation and the role of a new class of beta-cell mass regulators: the microRNAs.

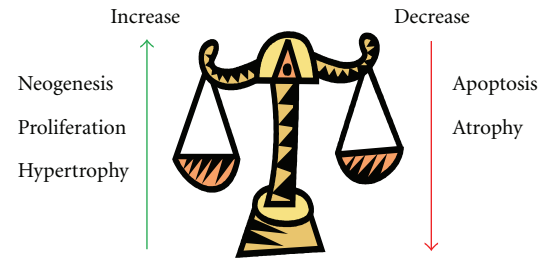

Figure 1: Beta-cell mass equilibrium. Beta-cell proliferation, neogenesis, and hypertrophy (enlarged cell size) increase beta-cell mass, while apoptosis and atrophy (reduced cell size) decrease beta-cell mass.

2. Postnatal Beta-Cell Regulation

How Do Beta-Cells Maintain Their Normal Mass? Beta-cell mass is increased by neogenesis (differentiation from beta-cell precursor cells), proliferation, and cell hypertrophy (increased cell size) and is decreased by beta-cell death, primarily through apoptosis, and beta-cell atrophy (decreased cell size, Figure 1). Several studies have revealed that the primary mechanism by which new beta-cells arise during adulthood is through proliferation of existing beta-cells rather than neogenesis [7, 27]. Thus, organisms born with reduced beta-cell mass have fewer beta-cells available to enter the cell cycle later in life. Under normal circumstances during adulthood, beta-cells are a slowly renewing population, with steady low levels of proliferation and apoptosis. However, beta-cell mass continuously expands over the lifespan of an organism [28], likely due to age-related increases in body weight and insulin resistance. A large number of hormones and nutrients have been shown to affect beta-cell mass, as extensively reviewed by Nielsen et al. [29]. As a result, the number of molecular pathways that have been implicated in beta mass regulation is also large. Many studies have employed animal models, and it is important to note that significant differences between species have been observed [30].

2.1. Regulation of Beta-Cell Mass by Hormones, Growth Factors, and other Factors. Beta-cell mass regulation is modulated by various environmental factors and nutrients including glucose, insulin, amino acids, fatty acids, and various other growth factors/hormones, such as IGF-I, IGF-II, glucagon-like peptide-1 (GLP-1) 1, glucagon, gastroinhibitory peptide (GIP), somatostatin (SST), HGF and betacellulin, gastrin, cholecystokinin (CCK), growth hormone (GH), prolactin (PRL), placental lactogen (PL), and leptin, amongst others (Table 1). These growth factors and nutrients can affect a variety of beta-cell functions—suppress/stimulate beta-cell replication, survival, mass expansion, and differentiation through different intracellular pathways discussed later.

Glucose. Glucose is thought to be the most important determinant of beta-cell mass equilibrium [31–36]. Glucose is not only the pathological hallmark of diabetes, but it is also a potential contributor to further decline in beta-cell mass

Table 1: Hormones, growth factors, and other factors regulating beta-cell mass.

	Inhibitors	Stimulators
Metabolites	Glucose, FFA	Glucose, FFA, amino acids
Cytokines	IL-1, IFN-g, TNF-a, leptin	GH, PRL, PL
Growth factor family	HGF	IGF-I, IGF-II, insulin, TGF-a, betacellulin, HB-EGF, aFGF, VEGF, PDGF, HGF
Placental hormones		placental lactogen, prolactin
Glucagon family		GLP-1, GIP, glucagon
Somatostatin family	Somatostatin	
CGRP family	IAPP/amylin	
Gastrin family		Gastrin, CCK
TGF-b family	TGF-b, follistatin	Activin A
Neurotrophins		NGF, NT-3
Neurotransmitters	(Nor)epinephrine	Acetylcholine
Lectins		Reg/INGAP/PSP/PTP
Adhesion molecules		Integrin a6b1, Cx43, Cx36
Drugs	Diazoxide	Nicotinamide, SU
Toxins	Streptozotocin, alloxan	

Modified from Nielsen et al. [29]. aFGF, acidic fibroblast growth factor; CCK, cholecystokinin; Cx. Connexin; HB-EGF, heparin-binding EGF-like protein; HGF, hepatocyte growth factor/scatter factor; IAPP, islet amyloid polypeptide; IFN, interferon; IL, interleukin; INGAP, islet neogenesis- associated peptide; NGF, nerve growth factor; NT-3, neurotrophin-3; PDGF, platelet-derived growth factor; PSP, pancreatic stone protein; PTP, pancreatic thread protein; SU, sulfonylurea; TGF, transforming growth factor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

through what has been termed glucotoxicity. Thus, in the face of evolving demand for insulin, if adaptation (functional or numeric) fails, then blood glucose levels rise, sequentially resulting in IGT and then T2D. Once glucose levels exceed the safe threshold, then further beta-cell death will likely arise [37].

It has been reported that hyperglycaemia for a short time in rats induces beta-cells to enter the cell cycle accompanied by ~50% increase in beta-cell number by neogenesis [33, 38] and suppression of beta-cell apoptosis [33, 36]. In another study using prolonged periods of glucose infusion, an increase of beta-cell mass was also reported, although the origin of these cells has been disputed. While some authors describe proliferation of new beta-cells formed by neogenesis of precursor cells [36], other groups report an increase in beta-cell replication and hypertrophy as well as neogenesis, leading to sustained effects on beta-cell mass even after glucose infusion is stopped [35, 39].

To support the mitogen effect of glucose on beta-cells, Porat and colleagues demonstrated in an animal model an increased replication rate of beta-cells correlated with an increased blood glucose level. Moreover, this effect was mediated by a specific enzyme involved in glucose metabolism (glycolysis) in beta-cells, glucokinase (GCK). In fact, it was demonstrated that GCK has a key role in beta-cell proliferation through membrane depolarization [40].

In addition, glucose has been shown to promote beta-cell survival by suppressing a constitutive apoptotic program *in vitro* [41]. Moreover, it was reported that mild hyperglycaemia can affect beta-cell phenotype [37] with a progressive loss of beta-cell differentiation markers (Pdx-1, BETA2/NeuroD, NKX6.1, and HNF-1β) and genes involved in glucose sensing (GLUT-2 and glucokinase) [37, 42]. On the other hand, some genes that are normally suppressed in beta-cells (e.g., c-Myc and its gene target, lactate dehydrogenase-A) are increased. This proapoptotic effect of glucose may also involve altered calcium homeostasis [43], activation of caspases by cytokines, such as IL-1β which is secreted by human islets in the presence of high glucose and leads to beta-cell apoptosis [44] or Fas-Fas-Ligand interactions [44], reactive oxygen species [45], and c-Myc [46, 47]. Intriguingly, c-Myc has been shown to trigger apoptosis through all of these pathways in other systems, including in particular the activation of some caspases (caspase 8 and 9), Fas-Fas-Ligand signalling, and reactive oxygen species (reviewed in [48]).

Therefore, paradoxically, it appears that glucose may have both an inhibitory and stimulating effect on beta-cell apoptosis. However, it appears that both the level and duration of hyperglycaemia are crucial in determining the fate of the beta-cell. For example, prolonged hyperglycaemia appears to have a proapoptotic effect, a process referred to as glucose toxicity. Chronic hyperglycemia may thus be injurious to beta-cells and contribute to the development of T2D [49]. Increased beta-cell apoptosis and reduced beta-cell mass have been observed in humans with T2D also, in keeping with the preclinical data in [6, 50–53]. This difference in beta-cell mass between T2D patients and matched controls is particularly evident when those are matched for obesity (obese patients without T2D have increased beta-cell mass compared to lean controls).

Insulin. The effect of *insulin* on beta-cell mass has not been completely clarified. Various authors have shown in various *in vivo* studies that insulin alone can stimulate beta-cell mass, whereas other authors have found that it promotes growth only in the presence of hyperglycaemia [34, 36, 54–56].

In a physiological condition, it seems reasonable that during a period of increased insulin demand, insulin itself could stimulate beta-cell growth, creating a positive feedback loop. Further studies have suggested a positive role of insulin in cell regeneration [57, 58].

Genetically altered mice in which the insulin receptor was knocked out exhibited a decreased beta-cell mass in adults and diabetes onset [59]. Insulin resistance, leading to hyperinsulinemia, stimulates an increase in beta-cell mass.

In contrast, as previously described [60, 61], insulin can also impose negative effect on beta-cell mass and/or proliferation. For example, rats with insulinomas resulting in hyperinsulinemia and hypoglycaemia were found to have smaller beta-cells, as well as reduced beta-cell numbers (in the islets without tumour), suggesting that a "sensor" mechanisms exist that can co-ordinate beta-cell mass across islets and possibly even distal grafts [62]. Increased rates of beta-cell apoptosis were evident [60, 61], but interestingly, beta-cell mass regenerated within days of tumour excision [63]. This demonstrates that excess insulin with a negative feedback is able to inhibit beta-cell growth.

These observations indicate that insulin can control beta-cell population dynamics, although the mechanism is still unclear.

Free Fatty Acids. Another proposed stimulus for beta-cell mass regulation has been free fatty acids (FFAs). It is well documented that chronic high levels of FFAs, which are often accompanied by obesity, contributes to the pathophysiology of insulin resistance and T2D [64, 65].

It is reported that acute FFA exposure stimulates insulin secretion, while prolonged FFA exposure decreases glucose-stimulated insulin secretion [66, 67] and induces insulin resistance and beta-cell dysfunction in both animal models [68] and humans [69–72].

Animal studies have been conflicting with some arguing that FFAs have a positive trophic effect on beta-cells, whilst others suggest the contrary. However, precise molecular mechanisms linking FFA to beta-cell dysfunction have yet to be fully elucidated.

It was reported that a prolonged *in vitro* exposure of isolated islets or insulin-secreting cell lines to elevated levels of fatty acids is associated with inhibition of glucose-induced insulin secretion [67, 73, 74], impairment of insulin gene expression [75–78], and induction of cell death by apoptosis both *in vitro* [79–88] and *in vivo* studies [80].

Importantly, *in vitro* [76, 88, 89] and *in vivo* [90, 91] studies have provided evidence that lipotoxicity only occurs in the presence of concomitantly elevated glucose levels. A number of studies have shown that fatty acids can induce beta-cell death by apoptosis in the presence of high glucose [88]. *In vitro*, saturated fatty acids induce beta-cell apoptosis [82, 86, 88], whereas unsaturated fatty acids are usually protective [81, 82, 88].

Recently, it was observed by various authors that oxidative stress is implicated in the pathogenesis of beta-cell dysfunction induced by FFAs. It has been suggested that increased reactive oxygen species (ROS) levels are the important trigger for beta-cell dysfunction. Under diabetic conditions, ROS is increased in many tissues and organs and cause various forms of tissue damage in patients with diabetes. It is considered that enhanced ROS generation may act as a link between FFA and beta-cell dysfunction [92].

Glucagon-Like Peptide-1. Glucagon-like peptide-1 (GLP-1) is produced and secreted in response to fat and glucose from intestinal L-cells, located in the distal ileum and colon.

Preclinical studies reveal potential benefits of GLP-1 receptor agonist treatment in individuals with T2D, which include the promotion of beta-cell proliferation [93] and reduced beta-cell apoptosis [94]. These preclinical results indicate that GLP-1 could be beneficial in treating patients with T2D.

However, the role of endogenous GLP-1 in stimulating beta-cell proliferation is less clear. Data regarding the regenerative role of GLP-1 and related agonists are controversial.

However, there is strong evidence to suggest that GLP-1 receptor activation can protect beta-cells from apoptosis [94–97] and equally convincing evidence of a beta-cell mitogenic effect of GLP-1 [93, 94, 98, 99].

It was reported that exogenous GLP-1 treatment enhances beta-cell replication in normoglycaemic rats [100] and mice [101–105].

However, while there are reports of GLP-1-mediated islet neogenesis [98, 106–109], recent statements by researchers examining the *in vivo* effects of GLP-1 call into question a neogenic response [110–112].

GLP-1 treatment stimulates beta-cell replication in multiple rodent models of obesity-induced diabetes, including both intact [113, 114] and defective leptin signalling [94, 115–117] models. In addition, some authors have demonstrated that exogenous GLP-1 treatment promotes beta-cell replication in models of beta-cell regeneration and obesity-induced diabetes [118, 119].

Moreover, GLP-1 prevents beta-cell death in mouse models of beta-cell loss, in particular, in both unstressed mice [102] and in streptozotocin-induced beta-cell toxicity [95, 120]. In summary, exogenous GLP-1 treatment stimulates beta-cell proliferation in multiple rodent species and models of beta-cell mitogenesis. Reports of human beta-cell proliferation are, however, sparse. Data to support a role for endogenous GLP-1 signalling in beta-cell proliferation are less clear.

Glucose-Dependent Insulinotropic Polypeptide. Glucose-dependent insulinotropic polypeptide (GIP) is a gut hormone synthesized and secreted from intestinal K-cells, which reside in the duodenum and jejunum.

It was previously reported that if GIP promotes beta-cell proliferation during obesity, then GIP inhibition should reduce beta-cell mass [121].

However, it is unclear whether the primary effect of GIP antagonism is on the adipocyte or the beta-cell. Few studies implicate GIP in *in vivo* beta-cell proliferation [119, 122]. However, one report demonstrated that GIP treatment increases islet size and number in the Leptin ob/ob mouse

[123]. The latter study did not discriminate between beta-cell proliferation, apoptosis, or islet neogenesis. In a human patient, elevated fasting plasma GIP correlated with islet cell hyperplasia [124]. Despite the limited *in vivo* evidence, *in vitro* experiments demonstrate that GIP enhances beta-cell mitogenesis [125–128].

Furthermore, the role of GIP in the reduction of beta-cell apoptosis *in vivo* is more clearly defined than its role in beta-cell proliferation.

Various studies have also demonstrated that exogenous GIP treatment prevents beta-cell apoptosis during severe obesity [123, 129] and streptozotocin-induced diabetes [120, 129].

Insulin-Like Growth Factor I and II. The role of *insulin-like growth factor I (IGF-I) and insulin-like growth factor II (IGF-II)* in beta-cell mass regulation has long been accepted [130–132].

IGF-I has been shown to increase the number of replicating beta-cells in rodent islets by up to 6% of the islet cell population [133–135] and to induce differentiated pancreatic beta-cell growth [133]. However, IGF-I appears only to be effective at inducing beta-cell proliferation under physiologically relevant glucose concentration range: 6 mM to 18 mM [136]. Thus, IGF-I potentiates the mitogenic effect of glucose on beta-cell proliferation.

Interestingly, overexpression of IGF-I in beta-cells is associated with increased beta-cell proliferation, but not mass [137]. In contrast, transgenic mice overexpressing IGF-II exhibit an increase in beta-cell mass due to augmented proliferation [138]. Taken together, there is clear evidence that the IGF molecules are important factors in beta-cell proliferation and mass.

Seemingly paradoxically, beta-cell-specific IGF-I knockout mice showed a delayed onset of T2D induced by a high-fat diet, accompanied by enlarged pancreatic islets, increased insulin mRNA levels, and preserved sensitivity to insulin [139]. This is likely due to secondary increases in insulin and others. Moreover, given the dominant role of circulating IGFs on tissue mass homeostasis in other systems, the significance of this is uncertain. Concerning IGF-II, it has been reported that mice overexpressing this hormone display an increase in beta-cell mass, hyperinsulinemia, and increased glucose-stimulated insulin secretion [140]. These results suggest that IGF-II may have some potential as an islet growth factor.

Cholecystokinin. Cholecystokinin (CCK) is able to stimulate beta-cell proliferation *in vivo*, as reported by different authors. In fact, various studies have demonstrated that the administration of CCK-8 after streptozotocin treatment in rats expands beta-cell mass via proliferation, in association with fasting plasma insulin increasing, and fasting plasma glucose reduction [141].

At the same time, it was reported that CCK enhances rat beta-cell proliferation *ex vivo*. In fact, neonatal rat beta-cells proliferate in culture after CCK-8 treatment [142]. Similarly, in a rat model, loss of CCK resulted in decreased islet size and reduced beta-cell mass through increased beta-cell death [143]. However, overexpression of *Cck* in mouse and human islets has no effect on replication [143].

Parathyroid Hormone-Related Protein. A recent study has demonstrated that acute and systemic administration of the first 36 amino acids of *parathyroid hormone-related protein* (PTHrP (1–36)) in rodents can stimulate beta-cell proliferation and enhance beta-cell mass *in vivo*, without negatively affecting beta-cell function or survival [144]. Previous studies have demonstrated that RIP-PTHrP transgenic mice producing the full-length PTHrP (1–139) in beta-cells show an increase in beta-cell mass, proliferation and islet number, and improvement of glucose homeostasis [145–147]. In addition, *in vitro* studies (in rodent insulinoma cell lines and primary human beta-cells) have shown that PTHrP (1–36) is sufficient to enhance not only proliferation, but also function, increasing insulin mRNA and protein, and augmenting glucose-stimulated insulin secretion [148–150].

However, it is notable that *PTHrP* knockout mice have normal beta-cell mass as compared to controls, suggesting that PTHrP may not be crucial for normal beta-cell mass *in vivo.* However, the explanation of this result should be found on the possibility that the PTHrP was absent in islets but it could be produced in other islet cell types, resulting in a paracrine compensatory mechanism [151].

These findings suggest that PTHrP may provide a possible target for gene therapeutic strategies designed to increase beta-cell mass and function.

Ghrelin. Ghrelin is a stomach-derived hormone involved in glucose metabolism and has been shown to regulate cellular differentiation and proliferation in various systems. In beta-cells, ghrelin treatment has been shown to increase expression of insulin and PDX1, as well as beta-cell replication in a diabetes model [152]. This suggests a potential to increase beta-cell mass and function.

Moreover, it has been recently reported that both acylated- and unacylated-ghrelin can on the one hand promote cell proliferation while on the other inhibit apoptosis of beta-cells, including human pancreatic islets induced by serum starvation and/or cytokine's stimulation [153, 154].

Menin. Menin is a tumour suppressor encoded by the *Men1* gene. Mice with heterozygous deletion for *Men1* develop pancreatic hyperplasia [155] that coincides with hyperinsulinemia and hypoglycaemia [156], with an increased islet cell growth through a progressive reduction in expression of some cell growth suppressors, such as p18 and p27 [157]. It was reported that the regulation of p18 and p27 by menin is through histone methylation [158, 159]. Moreover, mutation of *Men1* can induce acceleration in S-phase entry and consequently an enhancement of cell proliferation in pancreatic islets [160]. Recently, it has been reported that menin is also able to increase caspase-8 expression [161] and that caspase-8 is critical for the maintenance of beta-cell mass under physiological conditions [162].

More recently, menin has been demonstrated to play an important role during pregnancy, but this will be discussed below.

Leptin. *Leptin* is one of the most important cytokines produced by the *ob* gene and secreted by adipose tissue, and it plays an important role in controlling food intake/satiety and body energy balance (51) through the leptin-receptor (Ob-R).

Ob-R is also present in pancreatic tissue, and it has been proposed that leptin may increase beta-cell proliferation of insulin-secreting cell line [163].

Leptin has also been reported to prevent pancreatic beta-cells from inducible apoptosis, and this may partially account for islet hypertrophy in obese rodents and patients. Leptin may exert its antiapoptotic effects on pancreatic beta-cells. This was associated with a reduction of triglyceride accumulation, an inhibition of nitric oxide production, and a restoration of Bcl-2 expression in the face of fatty acid insult. This may be a possible mechanism for its antiapoptotic effect [164–166].

In the past, it was reported that leptin acted via the JAK/STAT and MAPK pathways and has been shown to increase beta-cell proliferation in cell line studies and foetal pancreatic tissue [167, 168].

However, recent findings indicate that leptin mainly acts through the PI3K-AKT signalling pathway in beta-cell proliferation [169–171]. All these results suggest that leptin has a protective role on beta-cell function. However, the effect of leptin on apoptosis has been conflicting. *In vitro*, prolonged exposure to leptin has been associated with impaired beta-cell function, and increased rates of apoptosis [172].

Furthermore, in contrast to the observations in rodent animal, leptin is likely to have a deleterious effect on human islet function [173]. For instance, it was reported that in isolated islets from mice, leptin was able to increase cell number through the suppression of apoptosis [164] meanwhile in human islets *in vitro* leptin impairs insulin secretion and induces apoptosis of beta-cells in the presence of 20 mM glucose via activation of c-JNK [174].

Overall, leptin is likely to exert diverse effects in the regulation of pancreatic beta-cell function and proliferation. Therefore, further research is still required to clarify its distinct role in various conditions.

Visfatin. *Visfatin* is an adipokine secreted by adipose tissue [175], elevated in T2D [176]. This novel compound can mimic the effects of insulin, by binding insulin-receptor, and exerts protective effects on pancreatic beta-cells acting via the PI3K/AKT and MAPK pathways [175]. Through these pathways, visfatin would appear to have the potential to regulate plasma glucose levels, as well as beneficial effects on beta-cell mass.

Overall, visfatin may have a protective effect on pancreatic beta-cell mass, but further research is necessary to clarify its distinct roles.

Placental Hormones. These hormones (especially *placental lactogen* and *prolactin*) are principally responsible for the changes in beta-cell mass during pregnancy (reviewed in [177, 178]). These hormones stimulate beta-cell proliferation in isolated islets, and beta-cell mass is reduced 26–42% in receptor deficient mice [179] as described in a later.

Connexin. Beta-cell-cell interactions are mediated by *connexins (Cxs)*. These proteins form the gap junction domains of cell membranes. Cxs have been shown to affect cell proliferation and survival in different cell types [180–182]. In particular, it was reported that the Cx36 isoform is the only one expressed in rodent beta-cells [183]. A recent study performed by Klee et al. [184], using Connexins-inducible/knockout transgenic mouse models, reported a key role in beta-cell mass regulation by demonstrating that the native Cx36 does not alter islet size or insulin content, whereas the Cx43 isoform increases both parameters, and Cx32 has a similar effect only when combined with the GH.

Bone Morphogenetic Proteins. As recently reported, there are some new mediators that are responsible of proliferation/apoptosis of beta-cells. Bone morphogenetic proteins (BMPs) are growth factors involved in the pancreas mass homeostasis both during embryonic development and adult life [185, 186]. The binding of these molecules, in particular BMP1 and BMP4, to their specific receptors induce, the phosphorylation and nuclear translocation of SMADs. In addition, there were identified different target genes able to regulate different transcription factors (ID1, ID2, ID3, ID4) and proteins involved both in the proliferation and apoptosis processes (including GK, IRS, E2F, SMAD7/9, HES-1). This is the reason why BMPs have a double effect, both proliferative and proapoptotic.

2.2. Signalling Pathways in Beta-Cell Mass Homeostasis. An ever expanding literature suggests that the above-described hormonal and nonhormonal factors are able to regulate beta-cell mass by cell cycle regulatory proteins and a range of key signalling pathways. Of particular interest in the light of recent data are mitogenic and apoptotic signalling pathways regulated by the transcription factor c-Myc [46], downstream targets involved in G_1/S transition [187–195], and various relevant pathways, including PI3K-AKT and MAPK.

In particular, the PI3K-AKT pathway is an important mediator of beta-cell mass increase [5, 136, 196]. In fact, PI3K-AKT is known to play a major role in preventing c-Myc apoptosis in many cell types [197] and to promote hypertrophy, hyperplasia, and neogenesis [5].

Phosphatidylinositol 3-Kinase/AKT Pathway. There are many signal transduction pathways involved in the regulation of beta-cell mass, with phosphatidylinositol 3-kinase (PI3K) and AKT signalling being one of the best defined [198]. This pathway is activated by factors, such as glucose, insulin, IGFs, and GLP-1 [136, 199], and is able to regulate beta-cell size, proliferation, and apoptosis. Following ligand-receptor binding, insulin receptor substrate (IRS) is phosphorylated and is able to interact with various downstream targets. The most important target of IRS is PI3K, which in turn can activate PDK1 (via a secondary messenger) [200], resulting in the activation of AKT [200, 201]. AKT plays diverse roles in the beta-cell, including activation of glycogen synthase kinase-3 (GSK3, described later), and phosphorylation and nuclear exclusion of Forkhead transcription factor O1

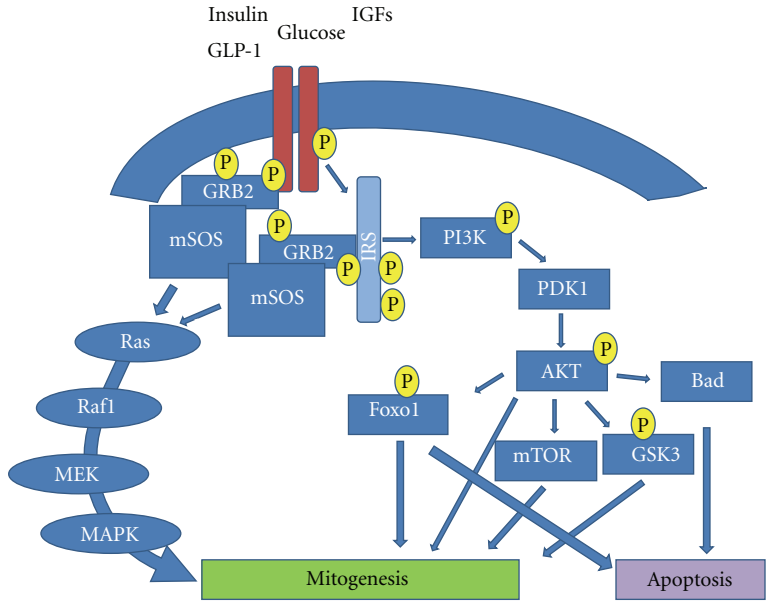

Figure 2: Schematic representation of PI3K/Akt and MAPK signalling. Mechanisms of some growth factors modulate beta-cell replication through proliferation or apoptosis. These factors on one hand stimulate PI3K, AKT, MAPK, and increase beta-cell proliferation. On the other hand, stimulate AKT, FOXO1, and other proapoptotic molecules resulting in induction of apoptosis.

(FOXO1) [202], ultimately leading to cell growth/replication and suppression of apoptosis [191] (described in Figure 2).

This is further evidenced in *IRS2* $^{-/-}$ mice, whereby beta-cell proliferation was achieved by ablation of one allele of *FOXO1* [203].

It was reported that PDX1 transcription is regulated positively by forkhead transcription factor A2 (FOXA2) but negatively by FOXO1 [204, 205]. In fact, these 2 forkhead transcription factors share common DNA-binding sites in the *Pdx1* promoter, FOXO1 competes with FOXA2 for the same binding site resulting in the inhibition of *Pdx1* transcription [204]. Moreover, nuclear translocation of FOXO1 excludes PDX1 from the nucleus, or vice versa [204, 206]. In addition, constitutively active nuclear FOXO1 has been shown to inhibit beta-cell proliferation in the face of insulin resistance [207]. This serves as a second mechanism by which FOXO1 inhibits beta-cell proliferation.

Recently, studies of beta-cells exposed to low nutrition revealed an additional beneficial role of FOXO1 activation—beta-cell proliferation without any changes in apoptosis compared to the control group [208]. Moreover, this study revealed that PI3K and MAPK signalling pathways are dampened and that the induction of CyclinD1 expression by activated FOXO1 in low nutrition is responsible for the improved proliferation of beta-cells [208].

Various studies using animal models to evaluate the role of different molecules on the PI3K/AKT pathway and beta-cell mass regulation have been performed.

Both IRS1 and 2 are critical for beta-cell growth and survival (previously mentioned), and they occupy a key position between the insulin receptor and downstream signaling elements. *IRS1* $^{-/-}$ knockout mice develop severe insulin resistance [209, 210] and compensate for insulin resistance by increasing beta-cell mass [211]. Although *IRS2* $^{-/-}$ mice are born with only a slightly reduced beta-cell mass, they later develop diabetes due to a marked increase in spontaneous apoptosis and reduced survival of beta-cells [209]. It, therefore, appears that IRS2 plays an important role in maintaining beta-cell homeostasis.

Furthermore, the role of AKT has been studied in beta-cells using genetically altered mice in which AKT is knocked out or overexpressed. AKT knockout mice displayed an increase of blood glycaemia and insulin resistance, although there have been conflicting reports on the effect on compensatory beta-cell growth [212]. Overexpressing AKT in transgenic mice led to a significant increase in beta-cell mass [5, 213]. In addition, AKT has been shown to promote beta-cell survival *in vivo* [214], suggesting an important role for AKT in beta-cell mass regulation.

The role of FoxO1 on signal transduction was further evidenced in different mouse models. It was demonstrated that constitutively active form of FoxO1 protects β cells from oxidative-stress-induced damage [197], and using another mouse model, it was demonstrated that deletion of one allele of FoxO1 resulted in a marked increase in the number, but not the size [209].

The role of PDX1 in beta-cell biology has been predominately examined in knockout studies. However, inactivating mutations in one *Pdx-1* allele have yielded conflicting results, although a "critical" level of PDX-1 is thought to be required in order to maintain beta-cell mass that [215, 216].

GSK3. Glycogen synthase kinase-3 (GSK3), a constitutively active serine/threonine kinase, was the first substrate shown to be phosphorylated by AKT (8).

Mice overexpressing a constitutively active form of GSK3 in beta-cells display reduced levels of PDX-1 protein [217]. *In vitro* studies confirmed the role of GSK3 in regulating PDX-1 protein stability, for example, in MIN6 cells, GSK3 has been shown to phosphorylate PDX-1 leading to its proteasomal degradation. These results highlighted a new mechanism, whereby signalling through the PI3K/AKT/GSK3 pathway regulates beta-cell growth [217]. In addition to the modulation of PDX-1 stability, GSK3 can also regulate beta cell proliferation through phosphorylation and degradation of cell cycle proteins, such as cyclin D1 and/or p27 [218, 219]. Other targets of GSK3 include numerous transcription factors involved in the cell cycle including c-Myc and c-Jun [220]. Therefore, in response to different growth factors, AKT prevents GSK3 phosphorylation of cyclin D, thereby promoting cell cycle progression and subsequent mitogenesis (Figure 2).

Mitogen-Activated Protein-Kinase (MAPK) Pathway. IRS can activate the RAF-MEK-ERK pathway (aka the mitogen-activated protein-kinase (MAPK) pathway, Figure 2) which plays a crucial role in development, cellular differentiation, and mitogenesis [221]. Firstly, IRS engages the adaptor molecule growth factor receptor-bound protein-2 (Grb2) via its SH2 domain [222, 223]. In turn, Grb2 binds to the murine Son-Of-Sevenless-1 protein (mSOS), which is able to activate RAS protein [222, 223]. It then, associates with RAF serine kinase [224], which subsequently phosphorylates mitogen activated protein kinase-kinase (MEK) resulting in the activation of ERK/MAPK [223]. Activated ERK/MAPK can then activate other protein kinases or migrate to the nucleus to activate transcription factors, such as c-JUN, c-MYC and c-FOS, important in the cell cycle [225].

However, the RAS/MAPK pathway can also be activated independently of IRS via direct binding of Grb2 to the receptor's SH2 domain [222, 223].

Janus Kinase/Signal Transducers and Activators of Transcription Pathway. Another important pathway in beta-cell biology is the JAK/STAT pathway. Both growth hormone (GH) and prolactin have been shown to mediate their effects via this pathway [226, 227]. Once activated, Janus kinases (JAKs) are able to phosphorylate signal transducers and activators of transcriptions (STATs) which enter the nucleus to regulate transcription of target genes. In particular, JAK2 and STAT-5 have been shown to regulate beta-cell growth and survival [226–228], as well as to reduce apoptosis [229]. More recently, it was recognized that the JAK/STAT pathway is regulated by Suppressors of cytokine signaling (SOCS). These suppressor proteins inhibit STAT phosphorylation by binding to phosphorylated JAKs, or compete with STATs for phosphotyrosine binding sites on cytokine receptors. Furthermore, SOCs facilitate the degradation of JAKs [230] (Figure 3). Its effects have resulted in reduced beta-cell proliferation in a sex-dependent manner [231].

c-Myc. The cellular proto-oncogene, c-*Myc*, encodes the protein c-Myc whose key biological function is to promote

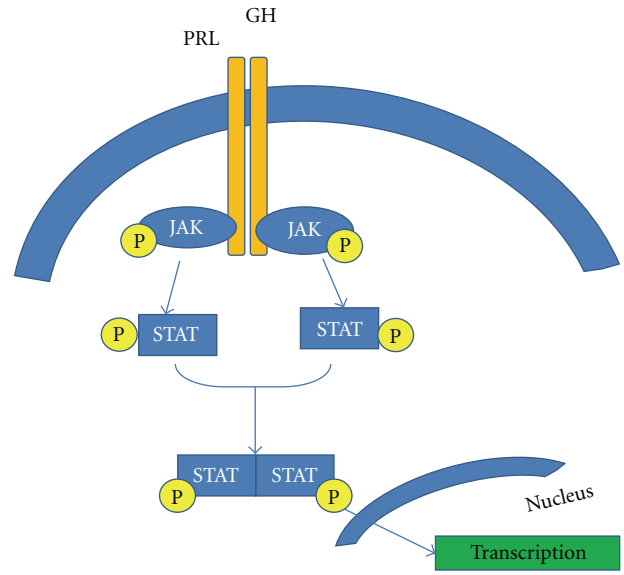

Figure 3: Schematic representation of JAK/STAT signalling. Mechanisms through prolactin and growth hormone modulate beta-cell proliferation. These hormones via prolactin receptor activate JAK, which is able to phosphorylate STAT. After phosphorylation, two molecules of STAT bind together and translocate into the nuclei where elicit their transcriptional function.

cell cycle progression. However, this enigmatic protein appears to be a key player in various other biological processes, such as differentiation, cell death, and angiogenesis.

Recent studies by several laboratories, including our own, support the notion that c-Myc may have a central role in the regulation of beta-cell mass required for replication (during the G_1/S transition), cell growth, and apoptosis [227].

It is likely that blood glucose levels influence the regulation and expression of c-Myc in beta-cells. In fact, it was observed that expression of c-Myc was stimulated by high glucose in a Ca^{2+}-dependent manner and also by cAMP [232]. High glucose also increases c-Myc mRNA levels in rat islets *in vitro* [233]. Therefore, taken together, these studies support the opinion that high blood glucose levels may activate beta-cell replication through c-Myc expression.

Besides, the actual mechanism by which hyperglycaemia activates c-Myc is fully clear. It is known that cell signalling of c-Myc pass through cyclin E-CDK2 activity early in the G_1 phase of the cell cycle, an essential event in c-Myc-induced G_1-S progression [234, 235].

In particular, it is well known that CCND2 (which encodes cyclin D2) and CDK4 are direct target genes of c-MYC [236, 237]. Expression of CCND2 and CDK4 leads to sequestration of p27, the CDK inhibitor, in cyclin D2–CDK4 complexes [236]. The subsequent degradation of p27 has been shown to involve two other c-MYC target genes, CUL-1 and CKS [237]. By preventing the binding of p27 to cyclin E-CDK2 complexes, c-MYC allows inhibitor-free cyclin E-CDK2 complexes to become accessible to phosphorylation by cyclin activating kinase (CAK). Increased CDK2 and

CDK4 activities would result in Rb hyperphosphorylation and subsequent release of E2F from Rb.

However, the final result of c-Myc activation on beta-cell is peculiar and differs in various tissues. For example, in contrast of skin, the predominant effect of activating c-MYC in pancreatic beta-cells of adult transgenic mice ($cMYCER^{TAM}$) is apoptosis and not proliferation, resulting in development of diabetes [46, 238].

However, concerning cell signalling mechanism involved in cMYC-induced apoptosis, D cyclins do not appear to be required for c-MYC-induced apoptosis *in vitro* [239], indicating that these two major functions of c-MYC (proliferation and apoptosis) may involve, at least in part, distinct sets of downstream mediators.

Calcineurin/Nuclear Factor of Activated T-Cells. More recently a new important pathway able to regulate beta-cell mass and function has been identified. The key components of this pathway are Ca^{2+}/calmodulin-dependent protein phosphatase 2-b (or calcineurin-b) and nuclear factor of activated T cells (NFAT).

In beta-cells calcineurin is activated by an increase of cytosolic Ca^{2+}, once activated, it can dephosphorylate NFAT transcription factors, which translocate into the nucleus where bind to consensus NFAT binding elements on specific gene promoters resulting in the activation of gene transcription [240]. On the contrary, some specific NFAT kinase can phosphorylate NFAT proteins and then inactivate and exporting them from nucleus.

In particular, it was reported in transgenic NFAT activated mice that NFAT in beta-cells can induce proliferation activating transcription of genes coding for cyclin D1, cyclin D2, and CDK4 [159].

More recently, another possible way to regulate the beta-cell mass has been reported. In fact, it was observed that calcineurin/NFAT pathway is implicated in the glucose regulation of IRS-2 gene expression. In particular, NFAT activated can bind to IRS-2 promoter enhancing its transcription and it was prevented by specific inhibition of NFAT [241]. This positive effect on beta-cell survival observed was also previously described [242]. Unfortunately, until now, the mechanisms involved in the regulation of beta-cell cycle by calcineurin are partially understood.

The important role of calcineurin/NFAT on beta-cell proliferation was highlighted using different transgenic models. Using mice with beta-cell-specific deletion of the Calcineurin phosphatase regulatory subunit, Calcineurin b1 (Cnb1), has shown a reduction of beta-cell function, proliferation and mass, and it coincided with reduced expression of normal regulators of beta-cell proliferation. Similarly, long-term activation of calcineurin induces impaired glucose tolerance by alterations in beta-cell mass, and the activation of calcineurin signalling negatively affects proliferation and survival of beta-cells [243].

On the contrary, in normal adult beta-cells, conditional NFAT activation has shown to promote expression of cell-cycle regulators resulting in an increase of beta-cell proliferation and mass, resulting in hyperinsulinaemia [159].

2.3. Cell Cycle/Molecular Pathways of Beta-Cell Proliferation. The critical point in the beta-cell cycle seems to be the G_1S "checkpoint." At this point, the cell is committed to DNA replication, and it is regulated by the retinoblastoma protein (Rb). Rb is critical because it has the ability to stall the cell cycle. Rb itself is inactivated by a group of serine threo nine protein kinases, the cyclin-dependant kinases (CDKs) including CDK4/6 and CDK2, which in turn are negatively regulated by cyclin-dependent kinase inhibitors (CDKIs).

(i) *Rb* is a member of a family of pocket proteins which is able to block G_1S transition by binding to E2F transcriptional activator proteins. When liberated from Rb, E2F transcription factor elicits its activity in controlling the transition from G_1 to S phase.

Mice deleted for *E2F-1* display defective pancreatic growth and islet dysfunction [187]. In *E2F-1* and *E2F-2* double knockout mice, both exocrine and endocrine glands had atrophied by 3 months of age, a feature not seen in single knockouts at the same time point. Diabetes subsequently followed, along with an increased rate of apoptosis [182].

(ii) The *cyclin-dependent kinase* CDK4 and 6 are activated by Cyclin D (in particular, D1 and D2) whereas CDK2 is activated by Cyclin E. Both Cyclins D1 and D2 are essential for normal postnatal islet growth. In adult islets, global deletion of cyclin D2 fails to stimulate adequate compensatory upregulation of cyclin D1 within islets and drastically damages postnatal beta-cell proliferation, islet mass, and decreases glucose tolerance [7, 189]. The importance of cyclin D1 has been highlighted using transgenic mice that overexpress cyclin D1. In this animal model, an increase in both beta-cell mass and proliferation was shown [190].

CDK4 has also been shown to be a key regulator of beta-cell cycle [244]. Knockout studies have shown a very specific phenotype, with beta-cell hypoplasia and severe diabetes [191, 194, 245], whilst studies using an activating mutation in CDK4, rendering it resistant to p16, have resulted in pancreatic hyperplasia, which demonstrated normal physiology [191, 192]. Other studies have shown up to a three-fold increase in beta-cell proliferation with CDK4 overexpression [191, 193].

(iii) The CDKs are inhibited by two groups of CDK inhibitors (CDKIs) which are also expressed in islets, *INK4 proteins* (p16, p15, p18, p19 inhibit CDK 4 and CDK6), and *CIP/KIP proteins* (p21, p27 and p57). Whilst INK4 family proteins promote cell cycle arrest, p21 and p27 are integrated into the cyclin D/Cdk4 complex that is able to arrest cell cycle progression through dephosphorylation of Rb protein [246], on the other hand, they are able to inhibit cyclin E/A CDK2 complex resulting in a block of phosphorylation of Rb protein [247].

Various studies have looked at the effects of CDKIs on islets. In $p21^{-/-}$ mice, islets were phenotypically

and metabolically comparable to their wildtype counterparts, possibly due to upregulation of p57, whereas loss of function of p57 has been associated with hyperinsulinaemia at infancy [248]. Actively inhibiting the effect of p18, using knockout mice resulted in beta-cell hyperplasia of up to 40% that appears to be CDK4 dependent [245].

In contrast, p27 has been shown to partially reverse the islet phenotype independent of CDK4. Overexpression of p27 in beta-cells of transgenic mice impairs beta-cell proliferation, resulting in decreased beta-cell mass and diabetes [195]. Furthermore, $p27^{-/-}$ mice increases beta-cell proliferation doubling their beta-cell mass at birth, and this expansion was accompanied by increased insulin secretion [249].

3. Role of MicroRNAs on the Beta-Cell Mass Regulation

MicroRNAs (miRNAs) are small 19–23 nucleotide noncoding RNA molecules that act as posttranscriptional regulators of different genes involved in various cellular processes, such as apoptosis, differentiation, and proliferation. This regulation pass through Generally, miRNAs can regulate protein synthesis either by repressing translation and/or by degradation through deadenylation of mRNA targets [250, 251].

The role of miRNAs in beta-cell mass regulation is not yet fully understood. The most studied miRNA in this contest is miR-375. It was reported that its overexpression attenuates proliferation of beta-cells and glucose-induced insulin secretion [16–18]. Indeed, ectopic expression of miR-375 in diabetic pancreatic beta-cells results in increased susceptibility to fatty acid induced apoptosis [16]. Moreover, it was reported that in ob/ob mice in which miR-375 was deleted, they develop a marked decrease in beta-cell mass, which results in a severe insulin-deficient diabetes not found in ob/ob mice [15]. Therefore, it is becoming clear that miR-375 targets a suit of genes that negatively regulate cell growth and proliferation and that aberrant loss of this miRNA leads to dramatic reduction of beta-cell mass [15].

Furthermore, it was reported that overexpressions of both miR-34a and miR-146a are involved in programmed cell death, in particular, in apoptotic processes. In contrast, reducing miR-34a or miR-146a levels did not affect cell survival.

In fact, prolonged beta-cell exposure to palmitate (FFA) and proinflammatory cytokines changes/increases the expression of miR-34a and miR-146a, and treatment with anti-miR34a or anti-miR148 diminished the number of death cells in the presence of apoptotic stimulus.

The effect on the regulation of apoptosis may be due to the capacity of miR-34 to control the expression of the anti-apoptotic protein Bcl2 [252], meanwhile miR-146a may control the apoptotic process through the regulation of the NF-kB pathway [253].

4. Beta-Cell Mass Plasticity

In addition to maintaining beta-cell mass under normal circumstances, as just discussed, an organism must also be able to alter its beta-cell mass in accordance with insulin's requirements. In particular conditions, such as pregnancy and obesity, beta-cell mass enlargement is observed [2]. However, when compensatory beta-cell mass expansion is inadequate, gestational diabetes in the case of pregnancy and T2D in the case of obesity are the results.

Although the majority of humans do not become diabetic in these circumstances, a significant part of the population is predisposed to beta-cell failure, for currently unknown reasons. The precise mechanism of beta-cell mass maintenance and expansion, that is, proliferation, neogenesis, or increase in size, has been elucidated only in part [178, 254, 255].

4.1. Reversible Beta-Cell Mass Expansion during Pregnancy. Different studies in rodents found a two- to five-fold increase in beta-cell mass during gestation [255, 256] and demonstrated an involvement of both beta-cell hypertrophy [178, 256, 257] and proliferation [256, 257]. Van Assche et al. [254] have reported that in humans during pregnancy the beta-cell mass increased 2.4-fold compared with nonpregnant women. Unfortunately, human data are limited to few studies using a small number of subjects. Butler et al. [258] have reported an approximately 1.4-fold increase in beta-cell mass using 18 women. In contrast to rodents, beta-cell mass expansion in humans was achieved by the formation of new islets or islet neogenesis [258].

The main stimuli for beta-cell proliferation during pregnancy are placental lactogen (PL), prolactin (PRL), growth hormone (GH) [178, 259], and serotonin [260]. Postpartum, in rodents, beta-cell mass returns to normal levels within 10 days through increased beta-cell apoptosis, decreased beta-cell proliferation, and reduced beta-cell size [255].

Placental Lactogen. Placental lactogen (PL) hormone has been implicated as the primary factor responsible for the enhanced islet mass and function that occur during pregnancy [178, 259]. PL interacts with receptors in the PRL/GH receptor family, stimulating the JAK-2/STAT-5 intracellular signalling pathway [261, 262].

Prior studies performed *in vitro* and over the short term suggested that PL is a more powerful islet mitogen than GH or PRL [178, 263]. These data were confirmed in *in vivo* studies using transgenic mice expressing mouse PL-1 under the control of the rat insulin promoter (RIP-mPL1) [259, 264]. The expansion of beta-cell mass in RIP-mPL1 was attributed principally to a two-fold increase in beta-cell proliferation and a 20% increase in beta-cell size (hypertrophy). Interestingly, islet number was not significantly increased when compared to wild-type mice [259]. These findings were supported by a PL receptor knockout study, which showed a significant reduction in beta-cell mass [265]. Therefore, PL could offer a novel therapeutic target to treat diabetes.

Prolactin. Prolactin (PRL), a hormone, acts through its specific receptor, prolactin-receptor (PRLR) to induce beta-cell proliferation *in vitro* [263]. Targeted deletion of the PRLR reduces beta-cell mass and mildly impairs insulin secretion [265]. The pivotal role of the PRLR for beta-cell adaptation during pregnancy was demonstrated using pregnant mice heterozygous for the PRLR null mutant. These mice exhibited reduced beta-cell proliferation, and impaired glucose tolerance [266]. It has been extensively demonstrated in different *in vitro* studies that PRL binding to PRLR is able to activate different signaling pathways such as STAT5, MAPK, and IRS/PI3K resulting in enhancing beta-cell mass during pregnancy [267–269]. It is currently unclear whether PRL and/or PL stimulate these pathways *in vivo*.

Menin. More recently, menin (a tumour suppressor encoded by the *Men1* gene) has been linked to the regulation of beta-cell proliferation during pregnancy [157]. Expression of menin, p18, and p27 are reduced during pregnancy in maternal islets, thus leading to islet mass expansion to meet the increased metabolic demand. After birth, menin returns to normal. Interestingly, menin is regulated by prolactin [157], supporting menin as an important mediator of beta-cell proliferation during pregnancy. It has also been proposed that, during pregnancy, activation of JAK2 and AKT, in response to prolactin, leads to increased p21 expression, whereas menin and p18 expression are suppressed. It is well known that AKT and p21 induce beta-cell proliferation, meanwhile the downregulation of menin and p18 allows for enhanced beta-cell proliferation [270].

Serotonin. A recent study by Kim et al. [260] shows that beta-cells, like serotoninergic neurons, are able to synthesize, store, and secrete serotonin (5-HT) as well as express the specific serotonin receptors (5-HTR2B and 5-HTR1D). These authors reported that, in pregnant mice, 5-HTR2Bexpression increased significantly during midgestation (day 6 to 16) and normalized at the end of gestation, whereas 5-HTR1D expression increased at the end of gestation (day 17) and postpartum. Notably, increased 5-HTR2Bexpression closely correlated with the period of increased beta-cell proliferation, and increased 5-HTR1Dexpression correlated with the cessation of beta-cell proliferation and regression of beta-cell mass. Moreover, it was reported that in pregnant mice stimulation with high concentrations of prolactin induces a strong increase in the serotonin expression compared with nonpregnant control mice.

These results suggest that during pregnancy lactogenic signalling induces serotonin expression and synthesis in islets able to stimulate beta-cell proliferation through the 5-HTR2B pathway. Shortly before parturition, expression of 5-HTR2B decreases and 5-HTR1D expression increases, resulting in the reduction of beta-cell proliferation and beta-cell mass. In this way, beta-cell mass returns rapidly to prepregnancy levels.

4.2. Compensation for Body Mass. Excessive increase in body weight leads to obesity. Obesity is linked/correlated with insulin resistance and associated with compensatory physiological response at the level of beta-cell mass increase. As previously described, when compensatory beta-cell mass expansion is inadequate, T2D in the case of obesity is consequential.

Diet-induced obesity results in insulin resistance and beta-cell mass expansion in humans and mice. In nondiabetic animal models of obesity, for example, the C57Bl/6 mouse strain which is notoriously susceptible to these effects, there is a 2.2-fold increase in beta-cell mass and proliferation after 4 months of a high-fat diet versus a control diet [271]. Meanwhile, in the Zucker diabetic fatty (ZDF) *fa/fa* rat, beta-cell mass increased four folds compared with lean controls [79]. There is also evidence for increased beta-cell mass in nondiabetic obese humans [51].

However, these mice eventually become diabetic and lose their beta-cell mass due to increased beta-cell apoptosis and reduced beta-cell proliferation.

In genetic models of obesity and insulin resistance, there is a compensatory expansion of beta-cell mass. For example, in mice lacking a functional leptin receptor (db/db mice), beta-cell mass exhibits two-fold beta-cell mass increase [115]. The Zucker rat (*fa/fa*) also has a homozygous mutation in the gene encoding the leptin receptor. ZDF rats exhibit increased beta-cell mass and increased beta-cell proliferation prior to the onset of diabetes, but increased beta-cell apoptosis prevents them from adequately expanding their beta-cell mass after the onset of diabetes, despite continued high rates of beta-cell proliferation [79]. This phenotype contrasts with that observed in nondiabetic Zucker fatty (ZF) rats, which possess the same mutation as ZDF rats and also become obese and insulin resistant but do not develop diabetes due to sufficient beta-cell mass expansion through increased beta-cell proliferation, neogenesis, and hypertrophy [79].

5. Conclusions

On the basis of available research findings that we have discussed above, we can propose that (i) postnatal beta-cell mass sizes in response to changing metabolic demands, (ii) is carried out by an interaction of beta-cell replication (proliferation and/or neogenesis) and apoptosis, and (iii) this process is regulated by different growth factors/nutrients that interact between them.

Actually, our present knowledge to the understanding of proliferation, neogenesis, and apoptosis is still incomplete.

The balance between apoptosis and replication seems to be pivotal in a right beta-cell mass maintenance. Failure in this balance results in beta-cell dysfunction and consequently diabetes onset. Because of the worldwide diffusion of this pathology, new therapies able to restore the original beta-cell mass and its functionality are under continuous development. Islet transplantation is already under clinical investigation but for the foreseeable future it will be very restricted in application due to the limited supplies of human cadaveric islets for transplantation. As a result, there is intense interest in developing other sources of new beta-cells and identification of new molecules able to restore beta-cell mass in diabetics.

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Research Article

Synergistic Effect of Hyperglycemia and p27^{kip1} Suppression on Adult Mouse Islet Beta Cell Replication

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The complementary role of hyperglycemia and p27^{kip1} suppression on islet beta cell regeneration was investigated in a syngeneic mouse model. p27^{kip1} gene silencing was performed by infecting islets of C57BL/6 with shRNA lentiviral particles. At 54 hours after viral infection, p27^{kip1} protein content in cultured targeting islets was 22% of that in freshly isolated islets. Six days after transplantation to diabetic mice, targeting islet graft had considerably more cells with Ki67-staining nuclei than nontargeting islets. The mice in the targeting-islet group had a significantly shorter duration of temporary hyperglycaemia than mice in the non-targeting-islet group. The long-term *ex vivo* beneficial effect of p27^{kip1} silencing on graft function was also indicated by the significantly higher cumulative cure rate for diabetes in mice receiving 200 targeting islets than that in mice receiving 200 non-targeting islets. Our data suggest that hyperglycemia and persistent p27^{kip1} suppression have a synergistic effect on islet beta cell replication in adult mice.

1. Introduction

Blood glucose homeostasis can be disturbed by many clinical conditions such as pregnancy and obesity and cause β-cell mass expansion to meet the increased demand of insulin [1, 2]. However, the mechanism of action underlying the expansion of β-cells is currently unclear [3–6]. Although glucose has been postulated to regulate cyclin D2 in pancreatic islet beta cells and play a dominant role in beta cell compensation, it is not yet clear how glucose controls cell cycle of islet beta cells [7–10]. A previous study showed that the suppression of both cdk-inhibitors p27^{kip1} and p18^{INK4c}, but not p27^{Kip1} alone, promotes endocrine tumour formation in rodents [11, 12]. Similarly, although cyclin D is important for cell proliferation, its overexpression does not trigger beta cell replication [13]. It suggests that adult islets are under multi-points control to regulate cell cycles for beta cell replication.

Our previous study revealed that primary islets of adult mice respond to chemical-mechanical digestion and purification procedures by markedly increasing cyclin B1 but reducing p27^{Kip1} protein level [14]. During the following 7 days of cultivation, maintenance of high level of cyclin B1 but rapid restoration of p27^{Kip1} of islets cultured in medium containing high glucose was noted [14]. The rapid restoration of p27^{kip1} level of cultured islets may explain the reason why cell number did not increase for fetal rat islet beta cells that are cultured in a medium containing 200 mg/dL glucose for 7 days [15]. Since the p27^{Kip1} is an important G1/G0 checkpoint for the progression of cell cycle, it is interesting to study whether persistent suppression of p27^{kip1} can enhance islet beta cell proliferation in a hyperglycaemic milieu. Since data obtained from experiments using cell lines, fetal islets or islets of knock-out mice to elucidate the role of p27^{kip1} and glucose on beta cell replication may not be suitable for applying to adult islets, in this study, we used primary islets of adult mice to investigate the complementary role of hyperglycemia and persistent p27^{kip1} suppression on beta cell regeneration.

2. Materials and Methods

Chemicals including Tris(hydroxyl-methyl)-aminomethane (Tris), histopaque-1077, type XI collagenase, and antibody

against β-actin (AC-15) were obtained from the Sigma Chemical Company (St. Louis, Mo, USA). RPMI-1640 medium was purchased from GIBCO Invitrogen, Life Technologies, Inc. (Grand Island, NY, USA). Antibodies against cyclin B1 (GNS1), cyclin D1 (DCS-6), and $p27^{kip1}$ (DCS-72.F6) were obtained from Thermo Fisher Scientific (Fremont, CA, USA), and antibody against FoxM1 (4G3) was purchased from Millipore Corporation (Bilerica, Mass, USA). Rabbit against mouse Ki67 polyclonal antiserum (ab15580) was purchased from Abcam Inc. (Cambridge, MA, USA).

2.1. Animal Care and Induction of Diabetes. Male C57BL/6 mice (8–12 weeks old) were obtained from a local breeder and were administered a single intraperitoneal injection of streptozotocin 200 mg/kg body weight to induce diabetes. Mice with whole blood glucose levels remaining at >360 mg/dL for more than 2 weeks were used as diabetic recipients. Three to 5 mice were housed in each cage and fed standard pelleted food and tap water ad libitum. The animal room had an automatic lighting cycle with 12 h of light and 12 h of darkness. The animals were treated humanely in accordance with the laboratory animal guidelines of Chang-Gung Memorial Hospital.

2.2. Islet Isolation. Pancreatic islets were isolated from C57BL/6 mice by collagenase digestion, enriched on a histopaque density gradient, and finally hand picked. Briefly, after administering sodium amobarbital to the mice to induce anaesthesia, we distended the pancreas of each nonfasted healthy mouse with 2.5 mL RPMI-1640 medium containing 1.5 mg/mL collagenase and then excised and incubated these in a 37°C water bath. The islets were purified using a density gradient and were hand-picked under a dissecting microscope. Isolated islets with diameters of 150 μm were collected and separated into groups of 50 islets per group. To minimize the influence of batch-to-batch variation in islet function on the experimental observations, each batch of islets isolated from 8–10 mice in a single day were separated into equal groups and transplanted into an equal number of mice in both the control and experimental groups on the same day.

2.3. Renal Subcapsular Transplantation. The islets were centrifuged (500 g for 90 seconds) in a PE-50 tube connected to a 200 μL pipette tip. With the recipient mouse under amobarbital anaesthesia, the left kidney was exposed through a lumbar incision. A capsulotomy was performed in the lower pole of the kidney, and the tip of the PE-50 tube (Clay Adams, Parsippany, NJ, USA) was advanced beneath the capsule toward the upper pole, where the islet graft was implanted using a Hamilton syringe. The capsule was left unsutured. The retroperitoneal cavity was closed using a 2-layered suture.

2.4. Targeting and Nontargeting Lentiviral Transduction to Silence $p27^{kip1}$ in Islets. We transduced islets with small hairpin RNAs (shRNAs) to silence $p27^{kip1}$ and used nontarget shRNAs as controls and then examined the effect of $p27^{kip1}$ silencing on the adaptation of adult mice islets. Briefly, freshly isolated islets, at a concentration of 50 islets per well, were plated in a 96-well tissue culture plate with 150 μL of RPMI-1640 medium containing 8 μg/mL of polybrene. Lentivirus was then incubated with islets at 20 multiplicity of infection (MOI) for 24 h at 37°C. The next day, the islets were transferred to new plates, washed twice, and further cultured at 37°C, with the medium being changed on a daily basis. At 72 h following isolation, the islets were collected for transplantation. $p27^{kip1}$ gene silencing was performed by infecting the islets with 4 clones of MISSION shRNA Lentiviral Particles (Sigma, Saint Louis, MO, USA) that are designed to target cyclin-dependent kinase inhibitor 1B ($p27^{kip1}$). These 4 clones were TRCN0000071063, (CCGGCGCAAGTGGAATTTCGACTTTCTCGAGAAAGTCGAAATTCCACTTGCGTTTTTG); TRCN0000071064, (CCGGCCGGCATTTGGTGGACCAAATCTCGAGATTTGGTCCACCAAATGCCGGTTTTTG); TRCN0000071066, (CCGGCCTTTAATTGGGTCTCAGGCACTCGAGTGCCTGAGACCCAATTAAAGGTTTTTG); TRCN0000071067, CCGGCCCGGTCAATCATGAAGAACTCTCGAGAGTTCTTCATGATTGACCGGGTTTTTG.

2.5. The Effect of $p27^{kip1}$ Silencing on Islet Cell Cycle Protein In Vitro. At 24 hours of transduction, equal batches of targeting and non-targeting islets (about 300 islets per batch) were washed 3 times with ice-cold phosphate-buffered saline and lysed with a buffer containing 150 mmol/L NaCl, 0.2 mmol/L ethylenediamine-tetraacetic acid 20 mmol/L Tris-HCl (pH 7.4), and 0.5% sodium dodecyl sulfate supplemented with complete protease inhibitor cocktail (Roche Diagnostic Deutschland GmbH, Mannheim, Germany). After 20-minute incubation at 4°C, the lysate was centrifuged at 13,000 ×g for 15 minutes at 4°C, and the supernatant was collected as the total islet protein content. Total islet proteins separated on a 12% sodium dodecyl sulfate polyacrylamide gel were electrophoretically transferred onto nitrocellulose membranes. After Ponceau S staining to check transfer efficiency, the membrane was blocked for 2 hours at room temperature with 10% nonfat dry milk (NFDM) in Tris-buffered saline (pH 7.4) containing 0.1% Tween-20 (TBST). It was then incubated overnight at 4°C in NFDM-TBST containing 2 μg/mL each of antibodies against β-actin (housekeeping), $p27^{kip1}$ (G1/G0 checkpoint), cyclin D1 (G1/S), cyclin B1 (G2/M), and FoxM1, washed 3 times with TBST, and incubated for 1.5 h at room temperature with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G antibody (0.1 μg/mL NFDM-TBST). After 3 washes with TBST, the bound antibodies were detected by using the VisGlow chemiluminescent kit obtained from Visual Protein Biotechnology (Taipei, Taiwan) and Kodak BioMax MS films. The cell cycle proteins were expressed as the ratio of band density of each protein over that of β-actin in the same batch of total islet proteins.

2.6. The Effect of $p27^{kip1}$ Silencing on Islet Glucose-Stimulation Insulin Secretion In Vitro. Function of islets transduced with shRNAs to silence $p27^{kip1}$ or the non-target shRNA control were evaluated by glucose-stimulation insulin secretion.

Table 1: Effect of $p27^{Kip1}$ silencing on the expression of cell cycle proteins in adult mouse islets. Cellular lysates of freshly isolated mouse islets and cultured islets at 54 and 96 h after infection with lentivirus to silence $p27^{Kip1}$ were separated on 12% sodium dodecyl sulfate-polyacrylamide gel and electrophoretically transferred onto a nitrocellulose membrane, which was then incubated with antibodies against β-actin, $p27^{Kip1}$, cyclin B1, cyclin D1, and FoxM1. Cellular lysates of control islets were collected at 24, 54, and 96 h after infection with virus carrying non-target shRNAs for the determination of $p27^{Kip1}$ protein level. Bound antibodies were detected by using a chemiluminescence kit. The levels of cell cycle proteins were expressed as the ratio of the band density of each protein over that of β-actin of the same batch of islet proteins. The protein content of freshly isolated islets was used as the standard for comparison to express the changes in the levels of cell cycle proteins after virus infection. Data were expressed as mean ± standard error. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.005$ when the indicated mean was analyzed using the single t-test.

Hours after viral infection	$p27^{Kip1}$/actin non-targeting control	$p27^{Kip1}$/actin targeting	B1/actin targeting	D1/actin targeting	FoxM1/actin targeting
0	1	1	1	1	1
24	0.56 ± 0.11^{a}, $n = 6$				
54	0.80 ± 0.18, $n = 4$	0.22 ± 0.06^{b}, $n = 4$	9.40 ± 2.42^{a}, $n = 4$	0.97 ± 0.11^{a}, $n = 4$	1.31 ± 0.10^{b}, $n = 4$
96	0.95 ± 0.24, $n = 4$	0.19 ± 0.03^{c}, $n = 4$	3.26 ± 0.85^{a}, $n = 4$	0.93 ± 0.12^{b}, $n = 4$	1.88 ± 0.46^{a}, $n = 4$

After 24 h of transduction and at 3 and 7 days of culturing, batches of 30 islets per well were sequentially exposed to different concentrations of glucose. Islets were incubated in 100 mg/dL glucose for 60 min. After the medium was collected, the same batch of islets was then stimulated with 360 mg/dL of glucose for 60 min. The net insulin secretion from the glucose stimulation test (GSIS) was calculated as the difference of the insulin content between the medium containing 360 mg/dL and medium containing 100 mg/dL glucose. The stimulation index (SI) was considered as the ratio of insulin content of the medium containing 360 mg/dL of glucose over that of the medium containing 100 mg/dL of glucose for the same batch of islets.

2.7. The Short-Term Ex Vivo Effects of $p27^{kip1}$ Silencing on Islets Cell Replication and Function. To study the short-term effect of $p27^{kip1}$ silencing on islet cell function and replication, mouse islets transduced with shRNAs to $p27^{kip1}$ or the non-target shRNA control were transplanted to both normoglycemic healthy mice and mice with streptozotocin-induced diabetes. Whole blood glucose and body weight were measured every other day. Six days after transplantation, the islet grafts were removed for immunohistochemical examinations. The relative number of cells in the islet graft undergoing replication was estimated by the relative numbers of cells with nuclei staining positive for Ki67 for an average of 250 cells per graft ($n = 4$ per group).

2.8. The Long-Term Ex Vivo Effects of $p27^{kip1}$ Silencing on Islets Cell Function. To study the long-term effects of p27 silencing on the function of islet grafts, mouse islets transduced with shRNAs to $p27^{kip1}$ or the non-target shRNA control were transplanted to mice with streptozotocin-induced diabetes 24 hours after transduction and 48 hours after incubation in the culture medium. We transplanted 200 islets per recipient to study the effect of p27 silencing on islet function *in vivo*. After transplantation, the body weight and whole blood glucose level in blood samples drawn from a tail vein were measured 2 to 3 times a week for 12 weeks, and the duration of temporary hyperglycaemia was considered as the period of time between transplantation and 2 consecutive measurements of whole blood glucose level below 200 mg/dL. At the end of the 12-week observation period, left nephrectomy was performed on all mice to remove the islet graft. All nephrectomized mice were kept alive for 2 weeks to confirm graft function, and then, the pancreas was removed for measuring the insulin content.

2.9. Measurement of Insulin Content. Insulin content in the pancreas remnant and the islet graft was determined by using the acid-ethanol method. Briefly, the pancreas or graft-bearing kidney from nonfasted mice was removed randomly between 8 and 10 AM on the indicated date, homogenized in an acid-ethanol solution, and stored overnight at 4°C. After centrifugation at 2400 rpm for 30 min, the supernatant was collected and stored at −20°C. The pancreas remnant and islet grafts were then homogenized again in a fresh aliquot of acid-ethanol solution and the insulin was re-extracted overnight. After centrifugation, the supernatant was collected and pooled with the first extracted sample. Finally, the insulin concentration was measured using radioimmunoassay.

2.10. Statistical Analysis. Data are expressed as means ± standard error. Statistical differences between means were analyzed using a paired or unpaired Student's t-test, as appropriate. The cumulative cure rate in groups of the long-term effect study was assessed using the Kaplan-Meier method. The log rank test was used to analyze differences in the cure rates between groups of mice. A value of $P < 0.05$ was considered significant.

3. Results

Table 1 reveals that the lentivirus carrying $p27^{Kip1}$-targeting shRNA effectively decreased the expression of $p27^{Kip1}$ protein in adult mouse islets at 54 and 96 h after infection and a representative western blot was shown in Figure 1. The $p27^{Kip1}$ protein content in cultured islets at 54 hours after viral infection was only 22% that in freshly isolated islets, and the suppression of $p27^{Kip1}$ protein was maintained even after 96 hours of infection. Table 1 shows that the suppression

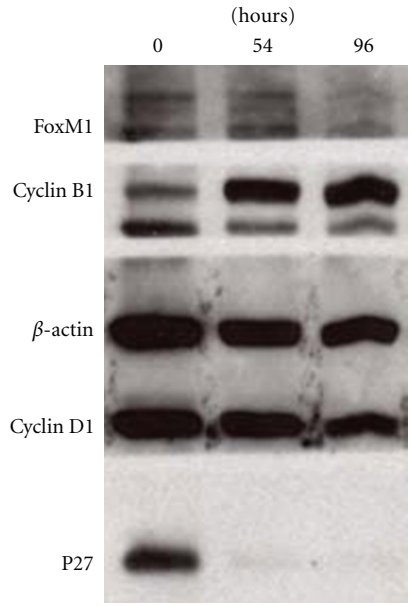
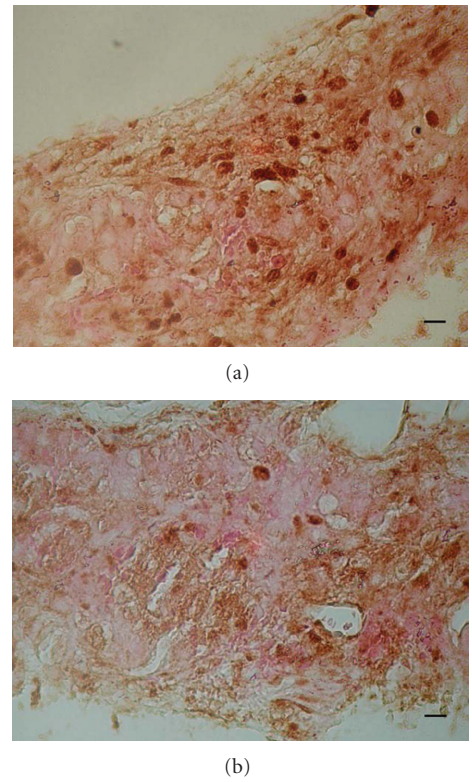


Figure 1: Effect of $p27^{kip1}$ silencing on the expression of islet cell cycle proteins. Total islet cell protein contents of targeting islets were prepared right after isolation (0 hr) and at 54 and 96 hours (hrs) after viral infection; separated on a 12% SDS gel and electrophoretically transferred onto nitrocellulose membranes. After checking transfer efficiency, the membrane was blocked and incubated overnight in buffer containing 2 μg/mL each of rabbit antibodies against mouse $p27^{kip1}$, cyclin D1, β-actin, cyclin B1, and FoxM1; incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G antibody. The bound antibodies were detected by using chemiluminescent kit and film development. The molecular weight is 27 kD for p27, 36 kD for cyclin D1, 43 kD for β-actin, 62 kD for cyclin B1, and 84 kD for FoxM1.



(a)

(b)

Figure 2: Short-term *ex vivo* effect of $p27^{Kip1}$ silencing on islet cell proliferation in diabetic syngeneic recipients. Batches of 50 of targeting (a) or non-targeting (b) transduced islets were implanted in the subcapsular space of the left kidney of B6 mice with streptozotocin-induced diabetes, and grafts were removed 6 days later for immunohistochemistry by using the anti-Ki67 antibody. The deep-colored oval structures represent positively stained nuclei. The black scale bar indicates a length of 10 micrometers.

of $p27^{Kip1}$ expression was accompanied by the increment in the levels of B1 and FoxM1 proteins, which suggest that the reduced $p27^{Kip1}$ expression allows islets cells to enter the cell cycle. The control islets infected with the non-targeting lentivirus had a $p27^{Kip1}$/actin ratio of 0.56 ± 0.11 ($n = 6$), 0.80 ± 0.18 ($n = 4$), and 0.95 ± 0.24 ($n = 4$) at 24, 54, and 96 hours after infection, respectively, in comparison with the ratios obtained for the freshly isolated islets.

To study the effect of lentivirus transfection on islets function, the glucose-stimulated insulin secretion (GSIS) and stimulation index (SI) of islets were measured at 3 and 7 days after targeting and non-targeting viral infection. Table 2 shows that the ratio and net difference of insulin secretion between 360 mg/dL and 100 mg/dL glucose of cultured islets with targeting did not differ from that of islets with non-targeting viral infection.

To investigate the short-term effect of $p27^{Kip1}$ silencing on islet cell proliferation, batches of 50 each of targeting and non-targeting transduced islets were implanted in the subcapsular space of the left kidney of normal healthy mice and B6 mice with streptozotocin-induced diabetes; 6 days later, the grafts were removed and immunohistochemistry was performed. When the mice with streptozotocin-induced diabetes were used as recipients, the number of nuclei staining positive for Ki67 was 0.35 ± 0.12 and 0.12 ± 0.07 ($n = 4$, $P < 0.05$) for grafts of targeting and non-targeting islets, respectively. Figure 2 shows the typical results of immunohistochemistry of islets grafts of the targeting group (Figure 2(a)) and the non-targeting group (Figure 2(b)). In normal healthy mice, anti-Ki67 antibody staining was hardly detectable in the nuclei of islet graft cells from both the targeting and non-targeting groups (data not shown). For targeting and non-targeting mice in the diabetic recipient groups, the whole blood glucose level was 373 ± 16 mg/dL and 360 ± 6 mg/dL ($n = 4$, $P > 0.05$) at day 0 and 412 ± 20 mg/dL and 429 ± 46 mg/dL ($n = 4$, $P > 0.05$) at day 6 after implantation, respectively. The body weight for targeting and non-targeting mice in the diabetic recipient groups was 23.0 ± 0.8 g and 23.3 ± 1.3 g ($n = 4$, $P > 0.05$) at day 0 and 24.6 ± 0.3 g and 24.2 ± 1.0 g ($n = 4$, $P > 0.05$) at day 6 after transplantation respectively.

To study the long-term effect of $p27^{Kip1}$ silencing on islet graft function, batches of 200 isogeneic islets with targeting or non-targeting transduction were implanted in the subcapsular space of the left kidney of B6 mice with streptozotocin-induced diabetes, and the whole blood glucose and body

Table 2: The effect of $p27^{kip1}$ silencing on islet glucose-stimulation insulin secretion *in vitro*. After 24 h of transduction and at 3 and 7 days of culturing, batches of 30 islets per well were sequentially exposed to different concentrations of glucose. Islets were incubated in 100 mg/dL glucose for 60 min. After the medium was collected, the same batch of islets was washed and then stimulated with 360 mg/dL of glucose for 60 min. The net difference of insulin secretion from the glucose stimulation test (GSIS; ng/islet × 60 min) was calculated as the difference of the insulin content between the medium containing 360 mg/dL and medium containing 100 mg/dL glucose. The stimulation index (SI) was considered as the ratio of insulin content of the medium containing 360 mg/dL of glucose over that of the medium containing 100 mg/dL of glucose for the same batch of islets.

	Time	Targeting islet	Non-targeting islet control	*P* value
GSIS (ng/islet × 60 min)	Day 3	0.17 ± 0.02 ($n = 6$)	0.20 ± 0.02 ($n = 10$)	>0.05
	Day 7	0.24 ± 0.09 ($n = 6$)	0.17 ± 0.02 ($n = 12$)	>0.05
SI	Day 3	2.6 ± 0.3 ($n = 6$)	2.8 ± 0.4 ($n = 10$)	>0.05
	Day 7	2.4 ± 0.3 ($n = 6$)	2.6 ± 0.3 ($n = 12$)	>0.05

Table 3: Effect of $p27^{Kip1}$ silencing on the period of temporary hyperglycaemia and insulin contents of islet graft and pancreatic remnant. Batches of 200 isogeneic islets with targeting and non-targeting (control) transduction were implanted in the subcapsular space of the left kidney of B6 mice with streptozotocin-induced diabetes. After transplantation, blood glucose was measured, and the duration of temporary hyperglycemia was the period between transplantation and the stable restoration of normoglycemia (<200 mg/dL). The graft-bearing kidneys were removed 12 weeks after transplantation for the measurement of insulin contents. The pancreatic remnants were removed 2 weeks after nephrectomy for measuring the insulin content. [a]$P < 0.05$ when the indicated means were analyzed using unpaired Student's *t*-test.

No. of Islets	Lentivirus	*N*	Period of temporary hyperglycaemia (days)	GIC (μg)	PIC (μg)
200	Targeting	13	16.5 ± 2.9[a]	1.75 ± 0.18	0.22 ± 0.07
200	Control	14	25.9 ± 3.5[a]	1.54 ± 0.17	0.22 ± 0.05

weight were measured. As shown in Figures 3(a) and 4, all treated diabetic mice that received 200 islets converted to normoglycemia, and the mice in the targeting group had significantly shorter temporary hyperglycaemia period than mice in the non-targeting group (16.5 ± 2.9, $n = 13$ versus 25.9 ± 3.5, $n = 14$, $P < 0.05$ in Table 3). The long-term *ex vivo* beneficial effect of $p27^{Kip1}$ silencing on graft function was also indicated by the significantly higher cumulative cure rate for diabetes in mice receiving 200 targeting islets than that in mice receiving 200 non-targeting islets ($P < 0.05$) (Figure 4). At the end of the 12-week observation period, the grafts were removed to confirm the hypoglycaemic effect. Three days after the removal of the graft-bearing kidney, the mice showed rapid elevation of whole blood glucose level and a significant decrease in body weight (Figures 3(a) and 3(b)). There was no difference between the targeting and non-targeting groups in terms of insulin contents of the graft and pancreatic remnant at 12 weeks after transplantation (Table 3).

4. Discussion

In this study, we transduced adult islets with lentivirus-carrying shRNA to silence 80% of $p27^{Kip1}$ protein, and the resultant suppression of $p27^{Kip1}$ expression lasted for over 96 hours after infection. The transduction and the subsequent $p27^{Kip1}$ suppression did not influence islet functions in terms of glucose-stimulated insulin secretion. It has been demonstrated that suboptimal number of islet tissue was insufficient to achieve normoglycemia in diabetic recipient; graft beta cell replication was increased initially but not by 18 days and after despite persistent hyperglycemia, and beta cell mass fell progressively [16]. Therefore, in this study, 50 islet tissues per recipient are used for short-term transplantation experiment to render islet beta cell a higher replication rate in diabetic recipient; 200 islet tissue is used for long-term experiment in order to shorten the temporary hyperglycemia days and avoid falling beta cell mass by persistent hyperglycemia. In the short-term *ex vivo* study, when normoglycemic healthy mice were used as recipients, neither control islets nor islets with $p27^{Kip1}$ silencing had detectable cells with nuclei staining positive for Ki67, at 6 days after transplantation. On the contrary, adult islets with $p27^{Kip1}$ silencing expressed more nuclei and higher density of positive Ki67 staining than the control islets, at 6 days after the islets were transplanted to mice with streptozotocin-induced diabetes. Our data suggest that $p27^{Kip1}$ suppression alone does not enhance islet cell proliferation in normoglycemic mice but hyperglycemia and persistent $p27^{Kip1}$ suppression have a synergistic effect on islet cell replication in terms of nuclei staining positive for Ki67. To further confirm the complementary effect of hyperglycemia and $p27^{Kip1}$ silencing on beta cell replication, we transplanted adult mouse islets with or without $p27^{Kip1}$ silencing to diabetic mice and followed up all treated mice for 3 months. The beneficial effect of $p27^{Kip1}$ silencing on graft replication was indicated by the significantly shorter temporary hyperglycemic period and the significantly higher cumulative cure rate for diabetes in mice receiving targeting islets than that of mice receiving the same number of non-targeting islets.

During the early G1 phase of the cell cycle, cyclin D-cyclin dependent kinase 4/6 catalyzes phosphorylation of retinoblastoma protein (pRB) and the phosphorylated pRB

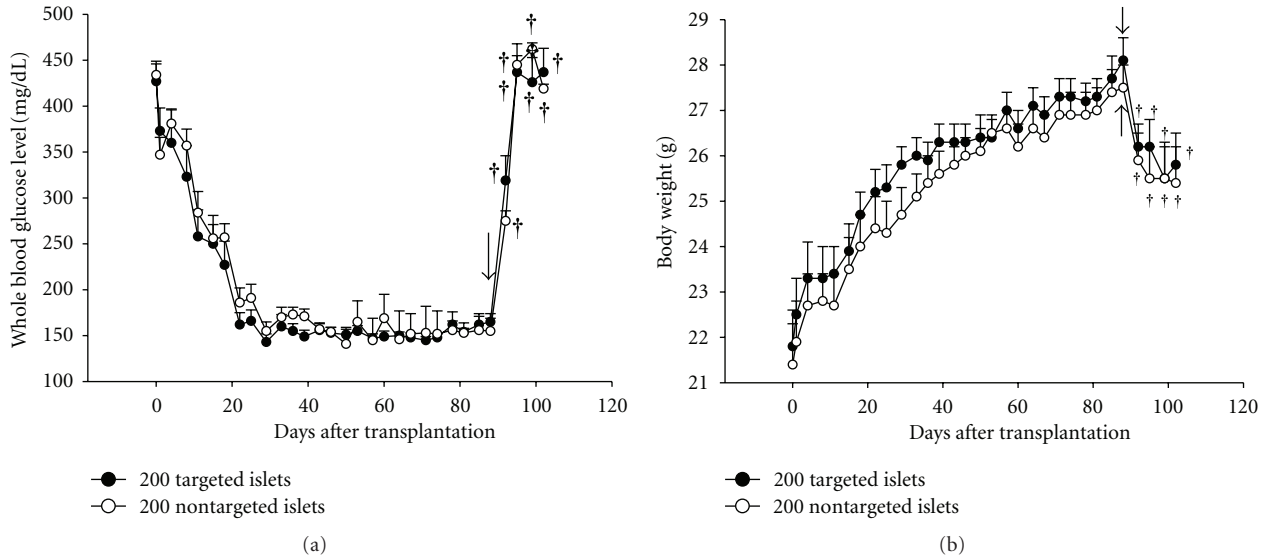


Figure 3: Long-term *ex vivo* effect of $p27^{Kip1}$ silencing on the blood glucose level (a) and body weight (b) in a syngeneic mouse transplantation model. Each mouse with streptozotocin-induced diabetes was transplanted with 200 mouse islets transduced with either shRNAs to $p27^{kip1}$ or the non-target shRNA control under the left renal capsule at day 0. After transplantation, blood glucose level and body weight were measured. At 84 days following transplantation, the graft-bearing kidneys of all mice were removed by nephrectomy (solid arrow), and all mice were kept alive for 2 weeks before being sacrificed. In Figures 3(a) and 2(b), $^{*}P < 0.05$, $^{\dagger}P < 0.01$ indicates the marked mean compared to that on day 84 in the same group using paired Student's *t*-test.

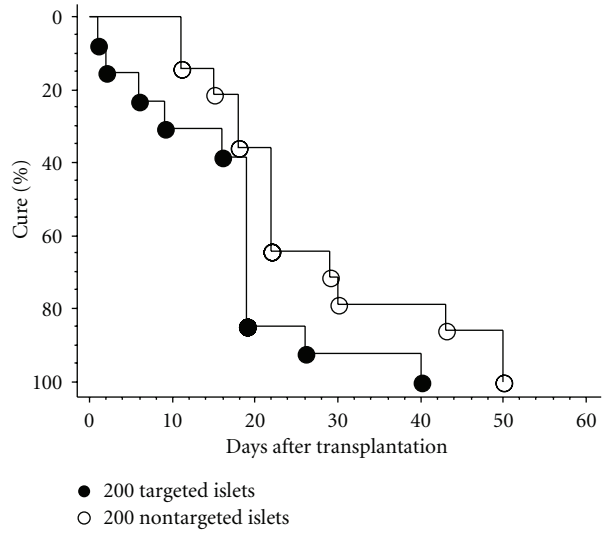


Figure 4: Curves of cumulative diabetes mellitus cure rate for syngeneic islet transplantation. Each streptozotocin-induced diabetic mouse was transplanted with 200 mouse islets transduced with either shRNAs to $p27^{kip1}$ or the non-target shRNA control implanted underneath the left renal capsule at day 0. Cure of diabetes was defined as the stable restoration of normoglycemia (<200 mg/dL glucose). Percent cure 50 (%) stands for half number of the transplanted diabetic mice becoming normoglycemia. The beneficial effect of $p27^{kip1}$ silencing on the function of islet grafts was reflected by the high cumulative cure rate of diabetes. The difference between two groups was analyzed with the log rank test. The value of the comparison for targeting versus non-targeting was $P < 0.05$.

inhibits anaphase-promoting complex- (APC/C-) mediated polyubiquitination and subsequent proteolysis of skp2, which results in increase of skp2 and decrease of $p27^{Kip1}$ protein [17]. The degradation of $p27^{Kip1}$ is essential for cells to enter G1/S phases for replication and the resultant adaptive expansion of pancreatic beta cells [11, 12]. During S-G2-M phases of each beta cell division, glucose downregulates cyclin D2 expression that will reduce phosphorylated pRB and cause decrease of skp2 and increase of $p27^{Kip1}$ protein [10, 17]. The increment of $p27^{Kip1}$ protein during S-G2-M phases of each beta cell division prevents cells from immaturely entering the next cycle [17]. In late G2 phase, the amount of B1 protein in Xenopus oocytes is 20- to 30-fold higher than in G1 and a threshold level of cyclin B1

protein must be reached before mitosis can proceed [18]. Once initiated, progression through mitosis is dependent on the degradation of several cell cycle regulatory proteins by APC/C, including cyclin B1 and skp2, which again prevents cells inappropriately entering other phases during mitosis [19, 20]. Our previous study revealed that isolated islets of adult mice persistently express high level of cyclin B1 especially when the culture medium contains high glucose [14]. In this study, we used shRNAs to silence $p27^{Kip1}$ and used hyperglycemia as a complementary factor to examine the synergistic effect of glucose and $p27^{Kip1}$ on the adaptation of adult mice islets. Although the mechanism of action of the synergistic effect of hyperglycemia and $p27^{Kip1}$ silencing on adult islet beta cell replication is not yet clear, we hypothesize that the persistent suppression of $p27^{Kip1}$ by lentivirus-carrying shRNA on adult islet cells will drive more cells into the G1/S phase and the high glucose-mediated persistent elevation of cyclin B1 will prepare more cells to be ready to enter mitosis and increase beta cell replication.

In conclusion, our data suggested that adult mouse islet beta cells can replicate when the metabolic demands increase and there is a synergistic effect of hyperglycemia and concurrent suppression of $p27^{Kip1}$ on islet beta cell replication.

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Research Article

Intermittent Fasting Modulation of the Diabetic Syndrome in Streptozotocin-Injected Rats

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This study investigates the effects of intermittent overnight fasting in streptozotocin-induced diabetic rats (STZ rats). Over 30 days, groups of 5-6 control or STZ rats were allowed free food access, starved overnight, or exposed to a restricted food supply comparable to that ingested by the intermittently fasting animals. Intermittent fasting improved glucose tolerance, increased plasma insulin, and lowered Homeostatis Model Assessment index. Caloric restriction failed to cause such beneficial effects. The β-cell mass, as well as individual β-cell and islet area, was higher in intermittently fasting than in nonfasting STZ rats, whilst the percentage of apoptotic β-cells appeared lower in the former than latter STZ rats. In the calorie-restricted STZ rats, comparable findings were restricted to individual islet area and percentage of apoptotic cells. Hence, it is proposed that intermittent fasting could represent a possible approach to prevent or minimize disturbances of glucose homeostasis in human subjects.

1. Introduction

Overabundant food intake with chronic positive energy balance leads to obesity and type 2 diabetes, whilst reduction in food intake, by increasing insulin sensitivity and improving glucose homeostasis, is currently recommended in the treatment of these metabolic disorders [1–4]. Such a caloric restriction may include a relative decrease of food intake [5–7] or otherwise either a total short [8, 9] or prolonged [10] fasting.

Intermittent overnight fasting, inspired by the daily fasting period during the Ramadan, was recently reported to prevent the progressive deterioration of glucose tolerance otherwise taking place in sand rats exposed to a hypercaloric diet [11–13]. The major aim of the present study was to investigate whether a comparable benefit of intermittent fasting may prevail in streptozotocin-induced diabetic rats.

2. Materials and Methods

2.1. Streptozotocin-Induced Diabetes. Eight to 10 weeks after birth, female Wistar rats (Charles River, Wilmington, MA, USA) were injected intraperitoneally, after overnight starvation, with streptozotocin (STZ, 65 mg/kg body wt.) freshly dissolved in a citrate buffer (50 mM, pH 4.5). These rats were given access during the night after the injection of streptozotocin to a solution of saccharose (10 g/100 mL) to prevent possible hypoglycemia. Control rats were injected with the citrate buffer. Five days after the injection of streptozotocin, the glycemia was measured with the help of glucometer (Lifescan Benelux, Beerse, Belgium) in blood obtained from caudal vein. Only those rats displaying a glycemia in excess of 16.7 mM were kept for further investigations.

2.2. Starvation and Restricted Food Supply. In order to compare the effects of an intermittent fasting, mimicking

the Ramadan fasting, to that of a caloric restriction, the experimentation in both control and STZ rats was conducted over two successive periods. Twenty days after the injection of either streptozotocin (STZ rats) or the citrate buffer vehicle (control rats), the rats were either given free access to food throughout the experimental period (NF: nonfasting rats), deprived of food and water from 5 p.m. to 8 a.m. (IF: intermittently fasting rats) or given access from 5 p.m. onwards to an amount of food comparable to that ingested by the IF rats (CR: calorie-restricted rats). Relative to the food intake in NF rats, such a caloric restriction represented a 20% decrease in food intake in the control animals and a 40% decrease of food intake in the STZ rats.

2.3. Body Weight and Food Intake. The initial body weight was measured before the injection of streptozotocin or its citrate buffer vehicle, 7 days thereafter, 20 days thereafter, on day 4, 7, 11, 14, 18, 21, and 27 of the final 30 days experimental period and at sacrifice, after overnight starvation. Likewise, food intake was measured 15 to 20 days (6 measurements) after injection of STZ or its vehicle in 6 control rats and 3 groups of 5-6 STZ rats, and daily (26 measurements) during the last 30 days experimental period.

2.4. Intraperitoneal Glucose Tolerance Test (IPGTT). An IPGTT [14, 15] was conducted in all rats on day 10, 20, and 29 of the final 30 days experimental period, after overnight starvation. A solution of D-glucose (20%, w/v) in distilled H_2O was intraperitoneally injected in conscious rats in order to deliver 2 g D-glucose per kg body weight. The glycemia was measured by a glucometer before and 30, 60, and 120 min after the administration of D-glucose in blood samples obtained from a caudal vein. The total and incremental areas under the glycemic curve (AUC) were computed in each individual experiment.

2.5. Sacrifice. At the end of the experimental period, the rats were sacrificed after overnight starvation and under anesthesia provoked by the intraperitoneal injection of a solution containing ketamine and xylocaine. Blood samples were obtained from the heart and placed in heparinized tubes, the plasma being then separated by centrifugation and stored at −80°C. The plasma D-glucose [16] and insulin [17] concentrations were measured by methods described in the cited references. These measurements were used to calculate the insulinogenic index (i.e., the ratio between the plasma insulin concentration, expressed as mU/L, and the difference between the plasma D-glucose concentration, expressed as mM, and 4.0 mM, considered as the threshold value for stimulation of insulin secretion by the hexose) and the HOMA index (i.e., the product of the plasma insulin concentration, expressed as μU/mL, times the plasma D-glucose concentration, expressed as mM). The pancreas were either used for the isolation of islets or fixed for immunohistochemical examination.

2.6. Insulin Secretion In Vitro. Groups of 4 islets each, obtained by the collagenase procedure [18], were incubated at 37°C for 90 min in 0.5 mL of a salt-balanced medium [19] containing bovine serum albumin (5 mg/mL) and equilibrated against a mixture of O_2/CO_2 (95/5, v/v). The insulin released by the islets during incubation and their final insulin content were measured by radioimmunoassay [17].

2.7. Immunohistochemical Study. For immunodetection of insulin, pancreatic rehydrated paraffin sections were blocked 1 h at room temperature with 1 : 20 normal goat serum (Vector Laboratories, Burlingame, CA, USA) in PBS for nonspecific reactions. The slides were incubated with primary anti-insulin (12018, Sigma-Aldrich, St Louis, MO, USA) mouse monoclonal antibody overnight at 4°C at a concentration of 1/3000 in normal goat serum (1/20 in PBS). The secondary antibody, Rhodamine Red X-conjugated goat anti-mouse IgG (H + L) (115-295-146, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was applied at a dilution of 1/200 in PBS/normal goat serum for 30 min at room temperature. The slides were mounted, and DNA was counterstained with DAPI (In Vitrogen, Merelbeke, Belgium). The staining patterns were observed with an Axioplan and recorded with an Axiocam (Carl Zeiss, Oberkochen, Germany).

2.8. β-Cell Mass Assessment. Pancreatic sections were stained for insulin using standard ABC-DAB technique [20]. The slides were incubated overnight at 4°C with the first antibody: anti-insulin (Santa Cruz Biotechnology, Inc., CA, USA) at dilution 1 : 500 in PBS with appropriate blocking serum at a dilution of 1/20. Purified immunoglobulins (IgG) (Sigma-Aldrich, St Louis, MO, USA) from nonimmunized rabbit were used as negative controls. The slides were further incubated with the secondary biotinylated antibody: goat anti-rabbit IgG (H+L) (BA-1000, Vector Laboratories) at a dilution of 1/300 in PBS for 30 min, at room temperature. β-cell mass was measured by point-counting morphometry on these immunoperoxidase-stained sections [21]. The measurement was performed on live using Leica Microsystems microscope (Heerbrugg, Switzerland). A grill of 110 points was used to assess insulin positive stained islet on each field.

2.9. Individual β-Cell Area. Individual β-cell area was determined by using image J logician on immunofluorescence stained sections of pancreas used for β-cell apoptosis assessment. The β-cell area was calculated from the ratio between individual area and β-cell nuclei number within the area taken in consideration.

2.10. Glucagon Immunodetection. For glucagon immunodetection, the same procedure as that described for insulin immunodetection by the ABC-DAB technique [20] was used. The sole difference consisted in the first antibody, that is, antiglucagon (A0565, Dako, Carpinteria, CA, USA) used at dilution 1 : 400.

2.11. Apoptosis Detection. The quantification of β-cell apoptosis by the TUNEL method was performed using the *in situ Cell Death Detection kit*, POD (Roche Diagnostics,

Vilvorde, Belgium). At the end of this procedure, the pancreatic sections were rinsed with PBS and eventually exposed overnight at 4°C to primary anti-insulin antibody (see above) followed by exposition for 30 min at 20°C to the Rhodamine Red X-conjugated secondary antibody (1/200 dilution). The apoptotic index represents the ratio between positive and total nuclei of insulin-producing cells in each islet.

2.12. Presentation of Results. All results are presented as mean values (±SEM), together with the number of separate determinations *(n)*. The statistical significance of differences between mean values was assessed by use of Student's *t*-test.

3. Results

3.1. Body Weight. At day zero of the last 30 days experimental period, the mean body weights of the control and STZ rats did not differ significantly ($P > 0.52$) from one another, with an overall mean value of 223 ± 4 g ($n = 33$). Over the 2 weeks following the injection of either streptozotocin or the citrate buffer vehicle, the changes in body weight averaged +17.6 ± 4.0 g ($n = 16$) in control rats, as distinct ($P < 0.005$) from −2.0 ± 5.0 g ($n = 17$) in STZ rats. Over the last 30 days of the experiments, the changes in body weight failed to differ significantly, whether in the control or STZ rats, when comparing IF animals to NF animals, with overall mean values of +33.8 ± 2.7 g ($n = 10$) in the control rats and −15.9 ± 4.4 g ($n = 11$) in the STZ rats. Expressed as a daily change in body weight, these two mean values were not significantly different ($P > 0.4$ or more) from those recorded in the same type of rats (control or STZ) over the 2-week period following the injection of streptozotocin or the citrate buffer vehicle. Over the last 30 days of the experiments, the gain in body weight was much lower ($P < 0.04$ or less) in the CR animals than in the IF animals, with mean values of +6.0 ± 3.1 g ($n = 6$) in control rats and −47.5 ± 8.7 g ($n = 6$) in STZ rats (Figure 1). A comparable situation ($P < 0.07$ or less) prevailed when considering the changes in body weight over the entire experimental period.

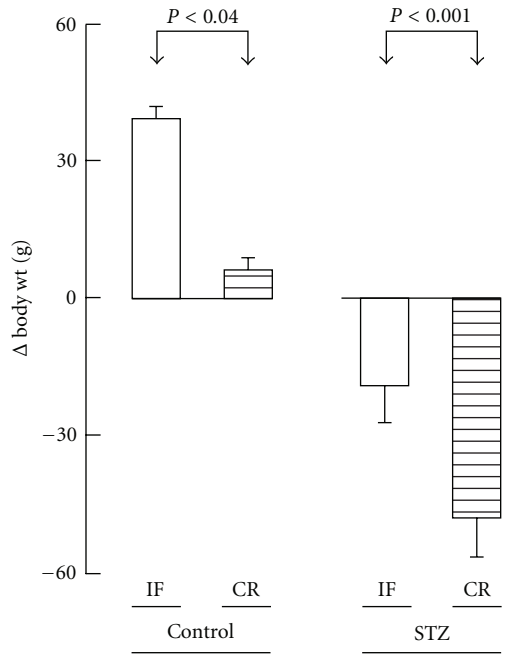

Figure 1: Comparison between the changes in body weight over the last 30 days experimental period in IF and CR control or streptozotocin rats. Mean values (±SEM) refer to 5-6 individual measurements.

Table 1: Food intake (g/day per rat).

Rats		Control period (last 6 days)[a]	Experimental period (30 days)[b]
Control	NF		20.1 ± 0.3 (5)
	IF		15.3 ± 0.5 (5)
	CR	18.7 ± 0.1 (6)	15.0 ± 0.1 (6)
STZ	NF	44.5 ± 1.8 (5)	46.0 ± 0.7 (5)
	IF	42.9 ± 0.9 (6)	25.8 ± 0.6 (6)
	CR	44.2 ± 1.6 (6)	26.5 ± 1.0 (6)

[a]Each individual value represents the mean of 6 successive determinations.
[b]Each individual value represents the mean of 26 determinations.

3.2. Food Intake. As indicated in Table 1, the food intake over the last 6 days of the control period (day 15 to day 20 after the injection of STZ or its solvent), was more than twice higher ($P < 0.001$) in the STZ rats than in the control animals. Such a difference persisted when comparing fed control and STZ rats over the 30 days experimental period. In the IF and CR rats examined during the last 30 days experimental period, the food intake was again almost twice higher ($P < 0.001$) in STZ rats than in control animals.

The individual values considered in Table 1 represented the mean of 6–26 measurements in each rat. During the last 6 days of the control period, the variation coefficient (SD/mean) for the 6 successive measurements made in each rat amounted to 7.3 ± 0.6% ($n = 23$) in control and STZ rats. Likewise, over the last 30 days of the present experiments, the variation coefficient for the 26 measurements made during this period averaged 9.9 ± 0.8% ($n = 5$) and 9.1 ± 1.1% ($n = 5$) in NF control and STZ rats, respectively, as compared ($P < 0.001$) to 15.5 ± 0.3% ($n = 5$) and 16.5 ± 0.3% ($n = 6$) in IF control and STZ rats.

3.3. IPGTT. The paired difference between the glycemia at min 30 and min zero of the IPGTT was comparable ($P > 0.77$) in control rats (6.31 ± 0.62 mM; $n = 48$) and STZ rats (6.68 ± 1.15 mM; $n = 52$).

The profile of glycemia during the IPGTT conducted in control rats is illustrated in Figure 2. The total AUC averaged in the IF and CR control rats, respectively, 94.0 ± 3.9% ($n = 16$) and 96.1 ± 2.8% ($n = 17$) of the mean corresponding values recorded on the same day in the fed control rats (100.0 ± 5.0%; $n = 15$). None of these mean values differed significantly from one another ($P > 0.35$ or more). However, as documented by the data listed in Table 2, the incremental AUC tended to be lower in IF and CR control rats than in fed control rats. Thus, the values recorded in IF and CR

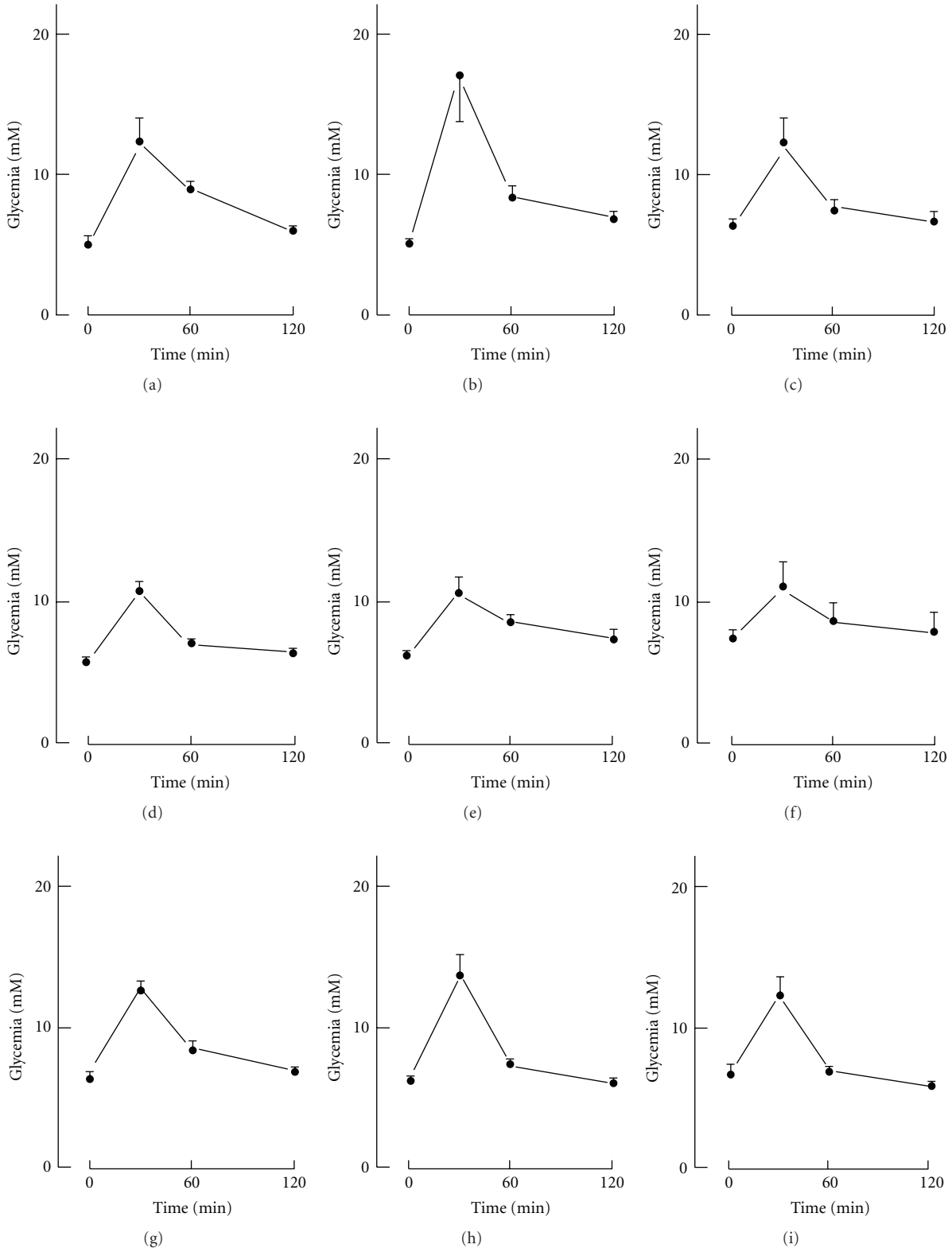

Figure 2: Glycemic profile during IPGTT conducted on day 10 (left), 20 (middle), and 29 (right) of the final experimental period in NF (upper panels), IF (middle panels), and CR (lower panels) control rats. Mean values (±SEM) refer to 5-6 individual experiments.

Table 2: IPGTT glycemic data in control rats.

Rats		Day	Time zero (mM)	Total AUC (mM·min)	Incremental AUC (mM·min)	(n)
Control	NF	10	5.12 ± 0.59	1,027 ± 73	412 ± 120	(5)
		20	5.21 ± 0.27	1,177 ± 121	552 ± 104	(5)
		29	6.44 ± 0.38	998 ± 104	225 ± 77	(5)
Control	IF	10	5.79 ± 0.36	927 ± 28	233 ± 49	(6)
		20	6.16 ± 0.42	1,014 ± 49	275 ± 28	(5)
		29	7.50 ± 0.49	1,061 ± 97	161 ± 113	(5)
Control	CR	10	6.54 ± 0.32	1,081 ± 34	295 ± 52	(5)
		20	6.21 ± 0.30	1,044 ± 30	299 ± 42	(6)
		29	6.64 ± 0.74	959 ± 56	160 ± 75	(6)

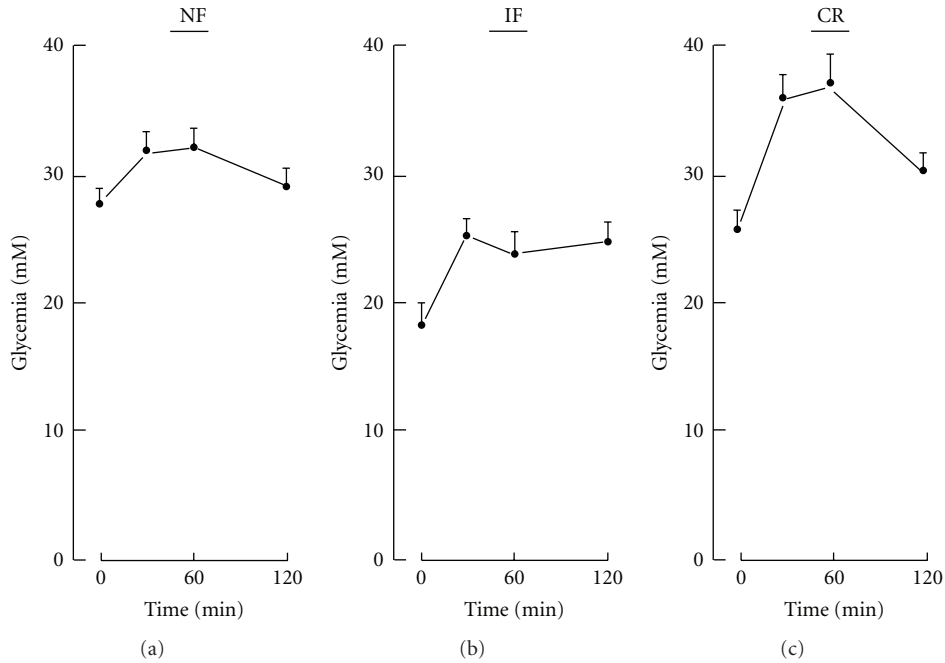

Figure 3: Glycemic profile during IPGTT conducted in NF (a), IF (b), and CR (c) STZ rats. Mean values (±SEM) refer to 15–20 individual experiments.

control rats, respectively, averaged 59.1 ± 15.3% ($n = 16$) and 65.3 ± 9.5% ($n = 17$) of the mean corresponding values recorded on the same day in the fed control rats (100.0 ± 15.1%; $n = 15$). Such a difference only achieved statistical significance ($P < 0.04$) when comparing the overall mean value recorded in both IF and CR rats (62.3 ± 9.5%; $n = 33$) to that found in the fed control rats.

In the STZ rats, the results of the IPGTT were closely comparable in 4 groups of NF animals examined 20 days after the injection of streptozotocin or on day 10, 20, and 29 of the final experimental period. Hence, these results were pooled together. Likewise, the results of the IPGTT conducted on day 10, 20, and 29 of the final experimental period were pooled together in either the IF or CR STZ rats (Figure 3). The time zero glycemia was lower ($P < 0.007$ or less) in IF rats than in either NF or CR rats, no significant difference ($P > 0.24$) being observed between the latter two groups of STZ rats (Table 3). Likewise, the total AUC was lower ($P < 0.001$) in IF rats than in either NF or CR rats, which failed to differ significantly ($P > 0.21$) from one another. The incremental area, however, was not significantly different ($P > 0.08$ or more) in IF rats, as compared to either NF or CR rats, being only significantly higher ($P < 0.02$) in the CR rats than in the NF diabetic rats.

Table 3: IPGTT glycemic data in STZ rats.

Rats	Time zero (mM)	Total AUC (mM·min)	Incremental AUC (mM·min)	(n)
NF	27.84 ± 1.17	3,681 ± 155	279 ± 153	(20)
IF	17.97 ± 1.85	2,825 ± 174	668 ± 149	(15)
CR	25.38 ± 1.70	3,988 ± 194	943 ± 205	(17)

3.4. Plasma D-Glucose, Insulin Concentrations, and Insulinogenic and HOMA Indices at Sacrifice. The plasma D-glucose

Table 4: Plasma D-glucose and insulin concentrations at sacrifice.

Rats		Plasma D-glucose (mM)	Plasma insulin (μU/mL)	Insulinogenic index (mU/mmol)	HOMA (mM·μu/mL)
Control	NF	8.61 ± 0.88 (5)	38 ± 6 (5)	8.63 ± 2.08 (5)	336 ± 73 (5)
	IF	8.12 ± 1.08 (5)	32 ± 2 (5)	9.44 ± 3.42 (5)	264 ± 45 (5)
	CR	7.80 ± 0.72 (6)	45 ± 2 (5)	14.61 ± 4.51 (5)	336 ± 68 (5)
STZ	NF	35.80 ± 2.47 (5)	16 ± 2 (5)	0.48 ± 0.08 (5)	548 ± 57 (5)
	IF	22.91 ± 4.30 (6)	22 ± 5 (6)	1.17 ± 0.51 (6)	419 ± 45 (6)
	CR	26.35 ± 4.20 (6)	25 ± 5 (6)	1.16 ± 0.52 (6)	592 ± 129 (6)

concentration was about 3-4 times higher in STZ rats than in control rats (Table 4). In the control rats, it was comparable ($P > 0.49$ or more) in NF, IF, and CR animals (Table 4). In the STZ rats, however, the overall mean value found in the IF and CR animals (24.63 ± 2.91; $n = 12$) was significantly lower ($P < 0.04$) than that recorded in the NF STZ rats.

The plasma insulin concentration was about twice lower in STZ rats than in control animals. In the latter animals, the mean values recorded in either the IF or CR animals did not differ significantly ($P > 0.26$ or more) from that found in the NF animals. Such was also the case ($P > 0.15$ or more) in the STZ rats.

As illustrated in Figure 4, no significant correlation was observed between plasma insulin and D-glucose concentration in the 15 control animals ($r = +0.0416$; $P > 0.1$), whilst a highly significant negative correlation between these two variables prevailed in the 17 STZ rats ($r = -0.6892$; $P < 0.004$). Covariance analysis, however, indicated that the two regression lines failed to differ significantly from one another in either their slope ($F = 0.567$; $f = 1, 28$; $P > 0.25$) or elevation ($F = 2.156$; $f = 1, 28$; $P > 0.1$).

The insulinogenic index was much higher ($P < 0.001$) in control rats (10.60 ± 1.84 mU/mmol; $n = 15$) than in STZ animals (0.90 ± 0.21 mU/mmol; $n = 17$). In each of these two sets of rats, no significant difference was found between NF, IF or CR animals. At the most, there was a trend ($P < 0.09$) towards a higher value for the insulinogenic index in IF and CR diabetic rats (1.17 ± 0.34 mU/mmol; $n = 12$) than in the NF STZ rats (0.48 ± 0.08 mU/mmol; $n = 5$).

The HOMA index for insulin resistance did not differ significantly in the 3 groups of control rats, with an overall mean value of 312 ± 35 mM·μu/mL ($n = 15$). Such was also the case in the STZ rats, with an overall mean value of 518 ± 51 mM·μu/mL ($n = 17$) significantly higher ($P < 0.004$) than that recorded in the control animals.

3.5. Pancreatic Islet Data. The release of insulin by islets prepared from control rats and incubated for 90 min at 8.3 mM D-glucose averaged 87.3 ± 12.7 μU/islet ($n = 40$). As illustrated in Figure 5, the concentration-response relationship for insulin output at increasing concentration of the hexose was comparable in NF, IF, and CR control rats. At the most, there was a trend towards higher mean values in islets prepared from IF and CR control rats as distinct from NF control rats and incubated at 2.8 mM and 8.3 mM D-glucose. However, a significant difference between the mean values recorded at each D-glucose concentration in each type of control rats (NF, IF, and CR) was only observed once ($P < 0.02$) among nine comparisons.

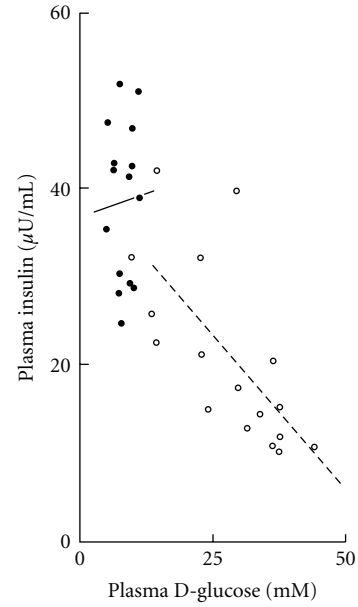

Figure 4: Comparison between plasma insulin and D-glucose concentrations found at sacrifice in control (closed circles and solid line) and STZ (open circles and dashed line) rats. The two oblique lines correspond to the regression lines.

The final insulin content of the islets prepared from control rats failed to differ significantly after incubation at 2.8, 8.3, or 16.7 mM D-glucose. Pooling all available data, it averaged, relative to the overall mean value recorded in each experiment after incubation at the three hexose concentrations, 96.0 ± 5.5% ($n = 39$), 98.8 ± 3.8% ($n = 40$), and 105.0 ± 3.3% ($n = 40$) in islets first exposed to 2.8, 8.3, and 16.7 mM, respectively. None of these mean values differed significantly from one another. Likewise, no significant difference was observed between the mean values for the insulin content of the islets prepared from NF control rats (347.2 ± 18.0 μU/islet; $n = 30$) and either IF control rats (303.0 ± 14.0 μU/islet; $n = 30$) or CR control rats (385.1 ± 16.4 μU/islet; $n = 29$). In a further experiment conducted in IF control rats, the insulin content again failed to differ significantly from that recorded in the fed control rats after incubation at either 2.8 or 16.7 mM D-glucose. Only the mean value found in this further experiment in the islets from IF control rats after incubation at 8.3 mM happened to

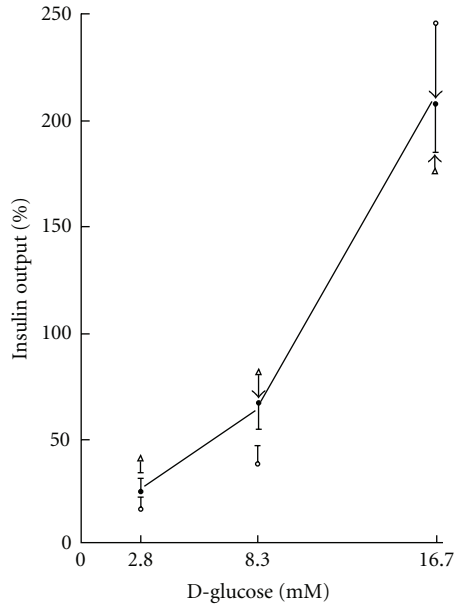

Figure 5: Insulin output by islets from NF control rats (open circles), IF control rats (closed circles), and CR control rats (open triangles) incubated at increasing concentrations of D-glucose. All results are expressed relative to the overall mean values recorded at the three concentrations of the hexose in each type of rats. Mean values (±SEM) refer to 10 (NF and CR rats) or 20 (IF rats) separate measurements, the SEM bar ending by an arrow whenever it exceeded the space to the next mean value. The solid line refers to the overall mean value recorded in the three groups of rats at each hexose concentration.

be lower ($P < 0.01$) than that otherwise recorded under the same experimental condition in the fed control rats.

The release of insulin (μU/islet per 90 min) by islets prepared from STZ rats averaged, at 2.8, 8.3, and 16.7 mM D-glucose, respectively, 2.08 ± 0.91 ($n = 28$), 4.01 ± 1.01 ($n = 36$), and 7.18 ± 1.70 ($n = 36$). It was thus significantly higher ($P < 0.02$) at 16.7 mM D-glucose than at 2.8 mM D-glucose. As judged from the mean values for insulin output and content measured in each experiment, the release of insulin represented $32.7 \pm 11.7\%$ ($n = 4$), $43.0 \pm 19.4\%$ ($n = 5$), and $62.6 \pm 32.1\%$ ($n = 5$) of the final insulin content of the islets after incubation at 2.8, 8.3, and 16.7 mM D-glucose, respectively. The insulin content of the islets prepared from STZ rats, expressed relative to the mean value found in each experiment in islets first incubated at 8.3 and 16.7 mM D-glucose ($9.7 \pm 1.6\,\mu$U/islet; $n = 70$), averaged $165.5 \pm 27.8\%$ ($n = 28$) after exposure to 2.8 mM D-glucose, $105.1 \pm 20.1\%$ ($n = 35$) after exposure to 8.3 mM D-glucose, and $94.8 \pm 17.4\%$ ($n = 35$) after exposure to 16.7 mM D-glucose. Such a progressive decrease in insulin content as a function of the concentration of the hexose during incubation was validated by the significant difference ($P < 0.03$) found between the highest and lowest of these three percentages. In these respects, no significant difference was observed between NF, IF and CR diabetic animals.

Both the insulin output and islet insulin content were dramatically lower in STZ rats than in control animals. For instance, over 90 min incubation at 8.3 mM D-glucose, the mean insulin output by islets from STZ rats did not exceed $4.01 \pm 1.01\,\mu$U/islet ($n = 36$), as distinct from a mean value of $87.3 \pm 12.7\,\mu$U/islet ($n = 40$) in control animals. Likewise, after 90 min incubation at 2.8 mM D-glucose, the insulin content of the islets did not exceed $5.9 \pm 1.3\,\mu$U/islet ($n = 28$) in STZ rats, as distinct ($P < 0.001$) from $331.5 \pm 20.8\,\mu$U/islet ($n = 29$) in control animals.

3.6. Islet Immunochemistry. The detection of insulin-producing cells by the ABC-DAB technique yielded comparable images in NF, IF and CR control rats (Figures 6(a), 6(b), and 6(c)). In the STZ rats, however, the same technique revealed a severe decrease in insulin staining, such a decrease being apparently most pronounced in the NF animals (Figures 6(d), 6(e), and 6(f)). The immunodetection of glucagon-producing cells, by a comparable ABC-DAB technique is illustrated in Figure 7. In the NF, IF, and CR control rats, the glucagon-producing cells were typically located at the periphery of the islets. In the STZ rats, however, an apparently increased number of glucagon-producing cells seemed to invade the center of the islets.

The relative value occupied by β-cells in serial sections of the whole pancreas did not exceed $0.20 \pm 0.05\%$ ($n = 9$) in STZ rats, as compared ($P < 0.001$) to $1.06 \pm 0.07\%$ ($n = 9$) in control animals. The values recorded in the IF and CR control rats did not differ significantly ($P > 0.23$) from those found in the NF control rats (Table 5). In the STZ rats, however, both the relative and absolute values for β-cell mass were higher ($P < 0.07$ or less) in IF than NF animals, such not being the case when comparing CR and NF STZ rats. When multiplied by the weight of the pancreas measured after 5 min evaporation of formol, the total β-cell mass appeared somewhat lower in IF control rats (5.7 ± 1.3 mg; $n = 3$) than in either NF control rats (11.1 ± 1.5 mg; $n = 3$; $P < 0.05$) or CR control rats (9.6 ± 1.0 mg; $n = 3$; $P < 0.08$), with an overall mean value (8.8 ± 1.0 mg; $n = 9$) one order of magnitude higher ($P < 0.001$) than that recorded in the STZ rats (1.5 ± 0.4 mg; $n = 9$). In these experiments, the mean pancreatic weight failed to differ significantly ($P > 0.66$) in control animals (0.84 ± 0.09 g; $n = 9$) and STZ rats (0.79 ± 0.04 g; $n = 9$). In both cases, however, and relative to the mean values found in NF animals ($100.0 \pm 2.8\%$; $n = 6$), those recorded in the IF rats ($68.9 \pm 7.5\%$; $n = 6$) were significantly lower ($P < 0.02$) than those measured in the CR rats ($99.9 \pm 8.0\%$; $n = 6$).

The latter data were in fair agreement with the direct measurement of pancreas wet weight at sacrifice with a paired ratio between the values obtained by direct measurement at sacrifice and those reached after fixation averaging $97.7 \pm 4.2\%$ ($n = 17$). Even when expressed relative to body weight, the pancreatic wet weight at sacrifice represented in the IF rats no more than $80.8 \pm 5.5\%$ ($n = 6$; $P < 0.06$) of that recorded in the CR rats ($100.0 \pm 6.6\%$; $n = 7$) of the same group (control or STZ rats).

As indicated in Table 6, the individual β-cell area averaged in the STZ rats $135.8 \pm 5.2\%$ ($n = 60$; $P < 0.001$) of the mean corresponding value found in control animals

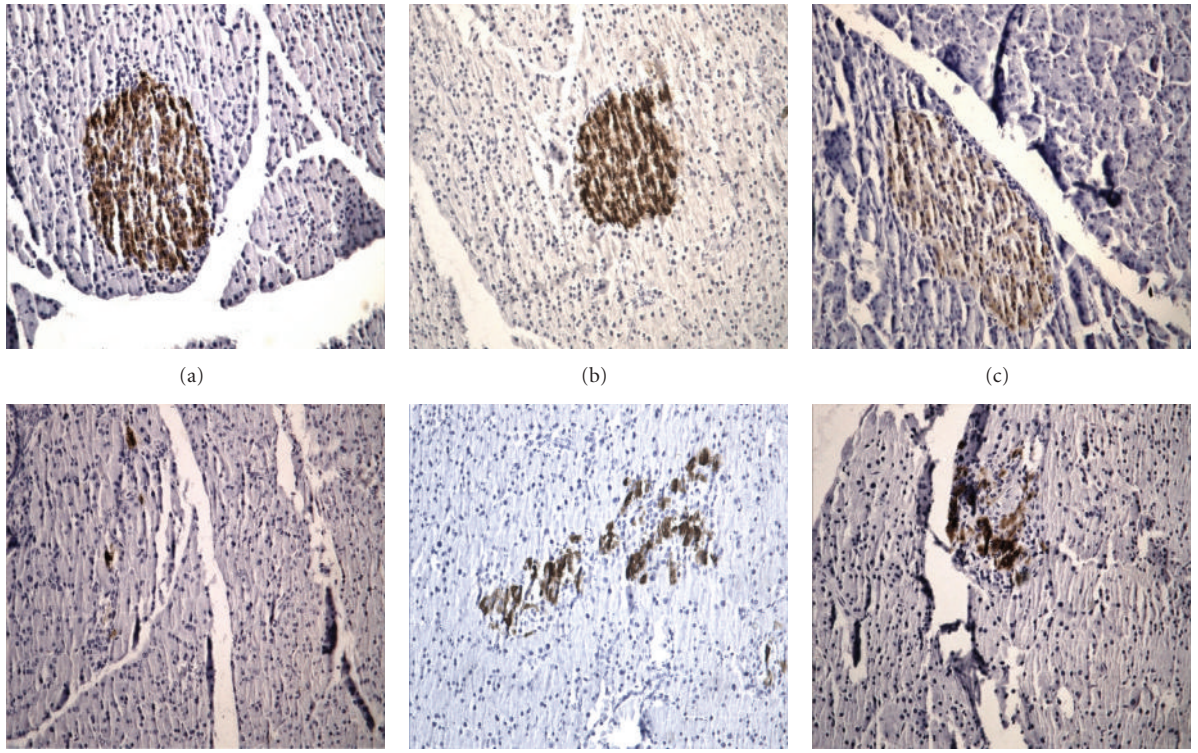

Figure 6: Immunodetection of insulin by the ABC-DAB technique in NF (a, d), IF (b, e), and CR (c, f) control (a, b, c) and STZ (d, e, f) rats.

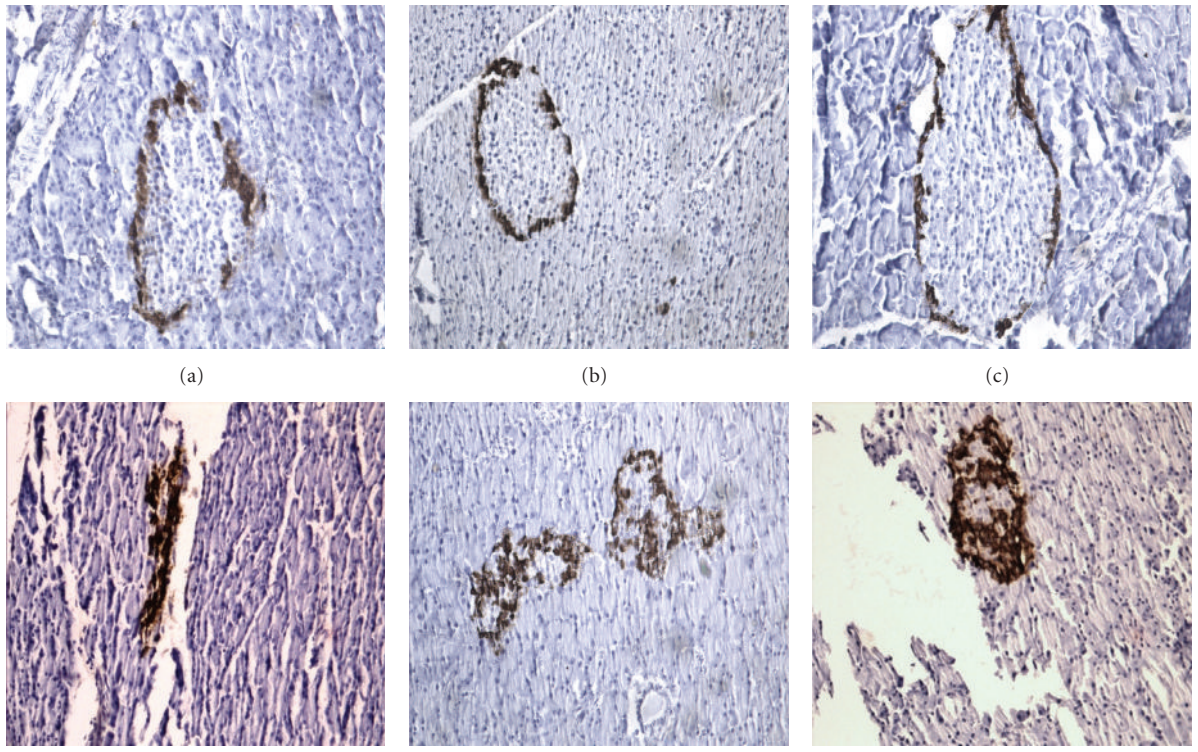

Figure 7: Immunodetection of glucagon by the ABC-DAB technique in NF (a, d), IF (b, e), and CR (c, f) control (a, b, c) and STZ (d, e, f) rats.

Table 5: Relative and absolute values for total β-cell mass.

Rats		Relative volume (‰)	Pancreas weight (g)	β-cell mass (mg)
Control	NF	10.76 ± 0.73 (3)	1.03 ± 0.06 (3)	11.12 ± 1.46 (3)
Control	IF	10.39 ± 2.04 (3)	0.55 ± 0.05 (3)	5.67 ± 1.28 (3)
Control	CR	10.56 ± 0.70 (3)	0.92 ± 0.13 (3)	9.58 ± 0.95 (3)
STZ	NF	1.11 ± 0.08 (3)	0.81 ± 0.01 (3)	0.90 ± 0.06 (3)
STZ	IF	3.71 ± 0.88 (3)	0.68 ± 0.03 (3)	2.55 ± 0.64 (3)
STZ	CR	1.31 ± 0.55 (3)	0.88 ± 0.06 (3)	1.20 ± 0.57 (3)

Table 6: Individual β-cell and islet area.

Rats		Sample	Individual β-cell area (μm^2)	Individual islet areas ($mm^2 \times 10^3$)
Control	NF	1	116.2 ± 3.0 (10)	23.72 ± 2.67 (10)
		2	102.4 ± 4.5 (10)	21.49 ± 1.85 (10)
Control	IF	1	123.6 ± 8.5 (10)	24.15 ± 3.13 (10)
		2	130.9 ± 8.7 (10)	14.50 ± 1.50 (10)
Control	CR	1	119.5 ± 8.3 (10)	23.30 ± 2.69 (10)
		2	117.3 ± 6.7 (10)	24.12 ± 1.61 (10)
STZ	NF	1	157.1 ± 25.2 (10)	1.11 ± 0.23 (10)
		2	131.1 ± 11.9 (10)	1.57 ± 0.30 (10)
STZ	IF	1	192.5 ± 14.0 (10)	3.34 ± 0.55 (10)
		2	186.4 ± 12.1 (10)	5.63 ± 1.18 (10)
STZ	CR	1	145.4 ± 8.5 (10)	5.94 ± 1.13 (10)
		2	156.8 ± 8.4 (10)	4.20 ± 1.18 (10)

(100.0 ± 2.3%; $n = 60$) exposed to the same feeding schedule (NF, IF, or CR). Whether in control rats or STZ rats, the mean β-cell area was significantly higher ($P < 0.04$ or less) in IF animals than in NF and/or CR animals. Thus, relative to the corresponding mean value found in the NF animals of the same group (control or STZ), that is, 100.0 ± 5.0% ($n = 40$), the values recorded in CR and IF animals averaged, respectively, 107.0±3.1% ($n = 40$; $P > 0.24$) and 123.9±4.3% ($n = 40$; $P < 0.001$), the latter mean value being also significantly higher ($P < 0.003$) than the former one.

As also documented in Table 6, the individual islet area represented in STZ rats 14.8 ± 1.9% ($n = 60$; $P < 0.001$) of that found in the control animals (100.0 ± 4.5%; $n = 60$) exposed to the same feeding schedule (NF, IF or CR). In the control animals, such a mean islet area (expressed as $mm^2 \times 10^3$) was not significantly different in NF rats (22.6 ± 1.6; $n = 20$), IF rats (19.3 ± 1.9; $n = 20$), and CR rats (23.7 ± 1.5; $n = 20$). In the STZ animals, however, it increased from 1.34 ± 0.19 ($n = 20$) in the NF rats to 4.50 ± 0.69 ($n = 20$; $P < 0.001$) in the IF rats and to 5.07 ± 0.82 ($n = 20$; $P < 0.001$) in the CR rats, the latter two mean values failing to differ significantly ($P > 0.59$) from one another.

Figure 8 illustrates the immunodetection of β-cells using rhodamine-labelled secondary antibody, and Figure 9 the immunodetection of apoptotic β-cells by the TUNEL procedure. In the control animals, the percentage of apoptotic islet β-cells was comparable in NF, IF, and CR rats, with an overall mean value not exceeding 4.31 ± 0.10% ($n = 15$), as distinct ($P < 0.001$) from 14.31 ± 1.49% ($n = 15$) in STZ rats (Table 7). In the latter rats, the values recorded in IF and CR animals (11.45 ± 0.76%; $n = 10$) appeared lower ($P < 0.004$) than that found in the NF animals (20.03 ± 2.90%; $n = 5$), but the total number of β-cells examined in the NF STZ rats (101 ± 1 cells; $n = 5$) was much lower ($P < 0.001$) than that examined in the IF and CR rats (663 ± 62 cells; $n = 10$). Incidentally, even the latter value remained much lower ($P < 0.001$) than the total number of β-cells examined in the control animals (2,236 ± 161 cells; $n = 15$).

4. Discussion

In the light of a prior study conducted in sand arts [11–13], the major aim of the present experiments was to investigate the potential benefit of intermittent fasting in STZ-induced diabetic rats. Parallel experiments were here conducted in control animals.

As expected, the glycemia (or plasma D-glucose concentration) and the total AUC during an IPGTT were much higher in STZ rats than in control animals. Even the incremental AUC during the IPGTT was higher ($P < 0.006$) in STZ rats (608 ± 105 mM·min; $n = 52$) than in control animals (287 ± 29 mM·min; $n = 48$) despite a comparable initial increment in glycemia 30 min after the injection of D-glucose. Also, as expected, the plasma insulin concentration, the insulinogenic index, the secretion of insulin by isolated islets, their insulin content, the relative volume occupied by the β-cells in serial sections of the whole pancreas and the individual islet area were much lower in STZ rats than in control animals. Incidentally, a positive secretory response to D-glucose was still observed in isolated pancreatic islets from STZ rats, this coinciding with a progressive decrease in their final insulin content after incubation at increasing concentrations of D-glucose. Such a decrease was not observed, however, in pancreatic islets from control animals. Last, the HOMA for insulin resistance and percentage of apoptotic β-cells were also significantly higher in STZ rats than in control animals.

In terms of morphological findings, there was, as a rule, little to distinguish between NF, IF, and CR control animals. At the most, there was a trend ($P < 0.02$) towards a higher individual β-cell area in IF than in NF control rats. Moreover, the pancreatic wet weight and, hence, total β-cell mass appeared lower ($P < 0.04$ or less) in IF control rats than in NF control rats. A comparable situation ($P < 0.02$) prevailed when comparing the pancreatic wet weight in IF STZ rats and NF STZ rats.

In the STZ rats, the major other changes attributable to differences in feeding schedule concerned, in terms of morphological findings, the relative and absolute values for β-cell mass, the individual β-cell and islet area and the percentage of apoptotic β-cells. In the IF STZ rats, the relative and absolute values for β-cell mass, as well as the individual β-cell area and islet area, were all higher than in NF STZ rats, whilst the percentage of apoptotic cells appeared lower in IF than NF STZ rats. In the CR STZ rats, comparable findings

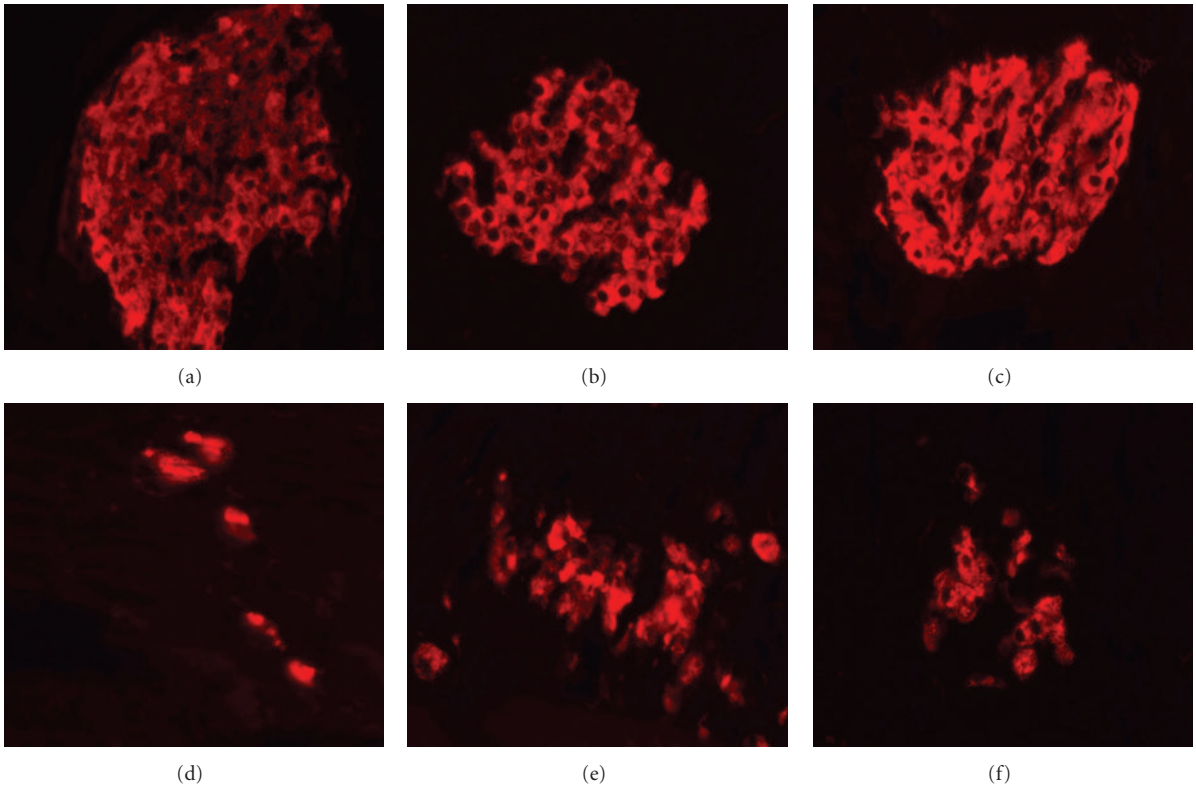

Figure 8: Immunodetection of insulin using rhodamine-conjugated secondary antibody in NF (a, d), IF (b, e), and CR (c, f) control (a, b, c) and STZ (d, e, f) rats.

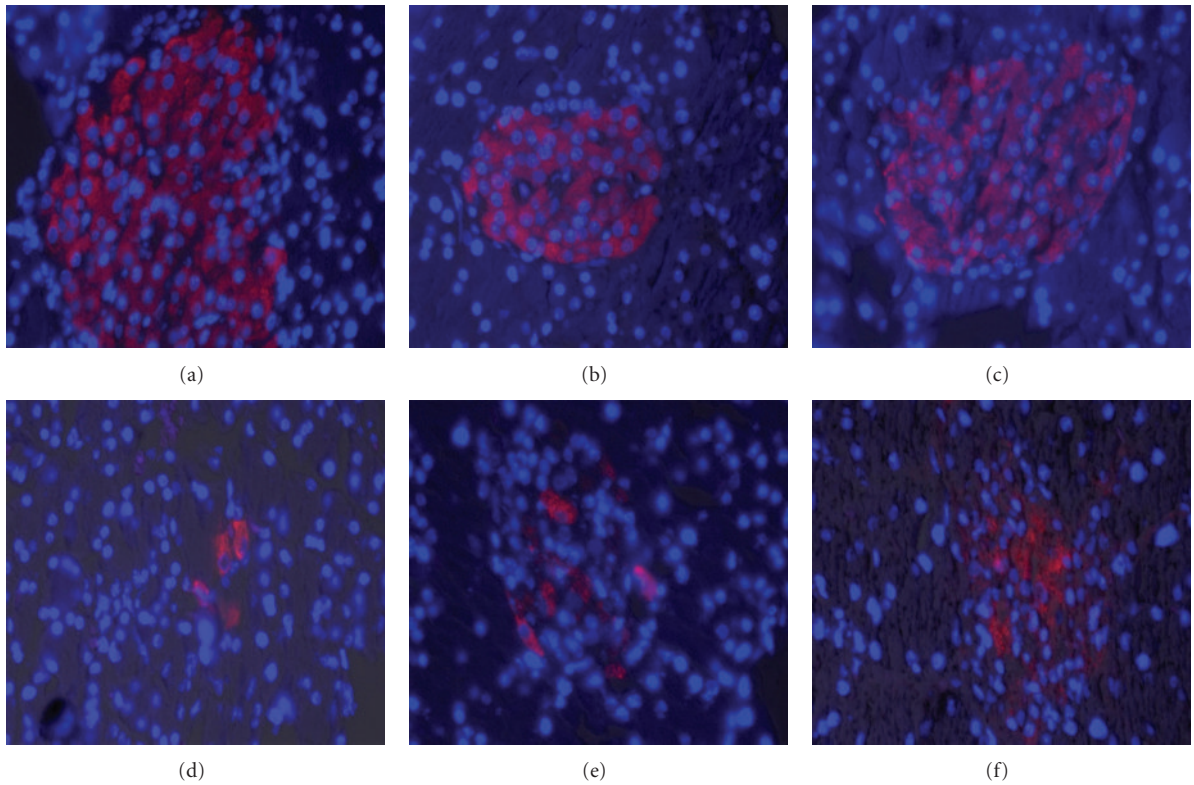

Figure 9: Detection of apoptotic β-cells by the TUNEL technique in insulin-stained pancreatic islet cells from NF (a, d), IF (b, e), and CR (c, f) control (a, b, c) and STZ (d, e, f) rats.

Table 7: Number and percentage of apoptotic β-cells.

Rats		TUNEL plus cell number	Total cell number	Apoptotic β-cells (%)
Control	NF	113 ± 14 (5)	2,452 ± 325 (5)	4.65 ± 0.21 (5)
Control	IF	70 ± 7 (5)	1,732 ± 152 (5)	4.02 ± 0.10 (5)
Control	CR	107 ± 12 (5)	2,525 ± 219 (5)	4.26 ± 0.07 (5)
STZ	NF	19 ± 2 (5)	101 ± 17 (5)	20.03 ± 2.90 (5)
STZ	IF	68 ± 5 (5)	643 ± 66 (5)	10.68 ± 0.38 (5)
STZ	CR	90 ± 23 (5)	683 ± 113 (5)	12.22 ± 1.46 (5)

were restricted to the individual islet area and percentage of apoptotic cells.

The much higher percentage of apoptotic β-cells in STZ rats, as compared to control animals, is likely attributable to two major factors. First, the β-cell cytotoxic effect of STZ should not be ignored. According to Morimoto et al. [22], apoptosis of β-cells is already detected 6 hours after the injection of STZ, even before the onset of hyperglycemia. In this respect, it should be kept in mind that 3 to 14 days after STZ administration, infiltration of the islets by mononuclear cells takes place, eventually resulting in the removal of apoptotic cells by unspecific macrophages [23, 24]. Hence, the present data may well underestimate the apoptosis index otherwise prevailing during the first days after STZ administration. A second factor consists of the hyperglycemia resulting from STZ administration, since *in vitro* exposure of pancreatic islets to high concentrations of glucose also induces β-cell apoptosis [24].

The latter process may account, in part at least, for the apparent differences between IF and CR STZ rats, in terms of both relative β-cell mass and individual β-cell area. Thus, according to the data listed in Tables 3 and 4, the glycemia (or plasma D-glucose concentration) after overnight starvation, expressed relative to the mean corresponding values found in NF STZ rats averaged in the IF STZ rats 64.9 ± 5.7% ($n = 21$), as distinct ($P < 0.01$) from 86.6 ± 5.6% ($n = 23$) in the CR STZ rats. The mean values for both the total and incremental AUC during the IPGTT were also higher in CR STZ rats than in IF STZ rats (Table 3). Such differences in glucose homeostasis coincided with higher mean values for both the relative volume of β-cells and their individual area in IF STZ rats as distinct from CR STZ rats (Tables 5 and 6). Thus, the mean relative value of β-cells was almost twice higher in IF than CR STZ rats, whilst the mean individual β-cell area represented in the IF STZ rats 125.4 ± 6.0% ($n = 20$; $P < 0.002$) of that found in CR STZ rats (100.0 ± 4.0%; $n = 20$). Despite the vastly different magnitude of the IF/CR ratio for these two variables, the difference between CR and IF STZ rats remained highly significant ($P < 0.005$) when pooling together the results recorded for each of these variables.

The individual β-cell area was also always significantly higher in STZ rats than in control animals exposed to the same dietary schedule (Table 6). These converging findings concerning differences in individual β-cell area as a function of glucose tolerance are reminiscent of the hypertrophy of β-cells found either *in vitro* after exposure to a high concentration of D-glucose [25] or *in vivo* in rats which became hyperglycemic after partial pancreatectomy [26] and currently ascribed to a compensatory mechanism in residual β-cells no more susceptible to undergo mitosis [27].

The latter consideration is not meant to deny that in addition to β-cell hypertrophy, an increase in β-cell number, possibly attributable to transdifferentiation of glucagon-producing to insulin-producing cells [28, 29], may participate in the difference in relative or total β-cell mass between NF and IF STZ rats, as also suggested by the total cell numbers listed in Table 7.

A beneficial effect of intermittent fasting from 5 p.m. to 8 a.m. in STZ rats was documented by a decrease in glycemia at time zero of the IPGTT, a decrease in the total glycemic AUC during the IPGTT, a lower plasma D-glucose concentration at sacrifice after overnight starvation, and a trend towards a higher plasma insulin concentration and insulinogenic index and a lower HOMA index at sacrifice. For the latter three variables, the geometric means of the relevant variable (plasma insulin concentration, insulinogenic index, and inverse of HOMA index) yielded a significant difference ($P < 0.025$) between NF (100.0 ± 6.9%; $n = 15$) and IF (164.4 ± 29.5%; $n = 18$) STZ rats. Furthermore, no significant adverse effect of intermittent fasting ($P > 0.49$) was observed in terms of the changes in body weight of the STZ rats over the 30 days final experimental period, when comparing NF animals (−12.4 ± 2.8 g; $n = 5$) to IF rats (−18.8 ± 7.9 g; $n = 6$).

A different situation prevailed in the calorie-restricted STZ rats. No statistically significant beneficial effects of caloric restriction in the STZ rats was observed when comparing NF to CR diabetic animals. Moreover, the decrease in body weight observed in the STZ rats during the final 30 days experimental period was 2.5 to 3.8 times higher ($P < 0.001$) in CR rats than in IF and NF animals, respectively.

Even in control rats, the gain in body weight was much lower in CR animals than in IF ones (Figure 1). This coincided with lower mean values for the plasma insulin concentration, insulinogenic index, and HOMA index in IF control rats than in CR control rats examined at sacrifice after overnight starvation (Table 4). Thus, for these three variables, the values recorded in IF control rats averaged 71.6 ± 8.6% ($n = 15$; $P < 0.06$) of the mean corresponding values found in CR control rats (100.0 ± 11.5%; $n = 15$). Since such distinctions between IF and CR control rats could not be ascribed to any difference in either food intake or the responsiveness to D-glucose of isolated pancreatic islets incubated *in vitro*, they suggest a more stressful situation in CR control rats than in IF control animals. To a large extent, a comparable situation may prevail in CR as distinct from IF diabetic animals.

In conclusion, therefore, the present study allows to extend to streptozotocin-induced diabetic rats, the proposal that intermittent fasting exerts a beneficial effect on glucose tolerance [11–13]. In our opinion, such a dietary approach merits to be also considered as a possible approach to prevent or minimize, if not correct, disturbances of glucose homeostasis in human subjects.

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