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Article

Control of Pancreatic β Cell Regeneration by Glucose Metabolism

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SUMMARY

Recent studies revealed a surprising regenerative capacity of insulin-producing β cells in mice, suggesting that regenerative therapy for human diabetes could in principle be achieved. Physiologic β cell regeneration under stressed conditions relies on accelerated proliferation of surviving β cells, but the factors that trigger and control this response remain unclear. Using islet transplantation experiments, we show that β cell mass is controlled systemically rather than by local factors such as tissue damage. Chronic changes in β cell glucose metabolism, rather than blood glucose levels per se, are the main positive regulator of basal and compensatory β cell proliferation in vivo. Intracellularly, genetic and pharmacologic manipulations reveal that glucose induces β cell replication via metabolism by glucokinase, the first step of glycolysis, followed by closure of K_{ATP} channels and membrane depolarization. Our data provide a molecular mechanism for homeostatic control of β cell mass by metabolic demand.

INTRODUCTION

The fundamental problem of organ size control can be divided into (1) what are the cellular origins of a given organ (i.e., stem cells versus differentiated cells that retain a replicative potential), and (2) what are the signals that determine organ size homeostasis? A classic distinction in the latter topic is between organs that are controlled by systemic factors, and organs that are controlled by local signals (Conlon and Raff, 1999).

Insulin-producing β cells of the endocrine pancreas operate to maintain blood glucose levels within a narrow range by secreting insulin in response to glucose. Insufficient functional β cell mass is the underlying cause of type 1 and a major contributor to type 2 diabetes, underscoring the importance of understanding β cell dynamics (Butler et al., 2003; Muoio and Newgard, 2008). In healthy adult mice, as well as in mice recovering from a diabetogenic injury, new β cells are derived by replication of pre-existing β cells (Brennand et al., 2007; Dor et al., 2004; Georgia and Bhushan, 2004; Meier et al., 2008; Nir et al., 2007; Teta et al., 2007). This situation implies that signals controlling β cell number must act by modulating the survival and/or proliferation of differentiated β cells.

Although it has been known for decades that food intake or glucose infusion increases β cell replication in mice (Alonso et al., 2007; Bonner-Weir et al., 1989; Chick, 1973; Chick and Like, 1971) and that insulin resistance states result in a compensatory increase in β cell mass (Kulkarni et al., 2004), the precise mechanisms regulating these processes are still controversial (Butler et al., 2007; Heit et al., 2006), as emphasized by recent reviews (Halban et al., 2010; Martens and Pipeleers, 2009; Sachdeva and Stoffers, 2009). Specifically, controversy persists as to the relative importance of local versus systemic signals, the latter of which can be neural or circulating (Imai et al., 2008). There is evidence that circulating factors control β cell proliferation, including the observation of β cell hyperplasia in mice lacking insulin receptors in the liver (Imai et al., 2008; Kulkarni et al., 2004; Michael et al., 2000; Okada et al., 2007), and the demonstration that a circulating factor in insulin resistant animals induces β cell proliferation in islet grafts (Flier et al., 2001).

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The identity of blood-borne β cell mitogens remains unknown. Glucose is a particularly attractive candidate since it can increase the rate of β cell proliferation in vitro (Kwon et al., 2004) and during short periods of glucose infusion in rodents (Alonso et al., 2007; Bonner-Weir et al., 1989; Paris et al., 2003). However, insulin, fatty acids, and incretin hormones have also been proposed, particularly for situations of insulin resistance where circulating glucose levels are not measurably elevated. Furthermore, in situations of β cell destruction, such as seen in type 1 diabetes, local inflammatory responses could in theory be negative or positive regulators of regeneration (Duffield et al., 2005; Ehses et al., 2007; Tessem et al., 2008).

Here we examine the mechanisms regulating β cell proliferation in vivo, using a combination of surgical, pharmacologic, and genetic approaches. We find that basal as well as compensatory β cell proliferation rates are controlled to a large extent systemically, and that β cell glucose metabolism is a key positive regulator of the process. We then demonstrate that the mitogenic effect of glycolysis is transmitted by modulation of ATP-sensitive potassium channels. These findings may have important therapeutic implications in both type 1 and type 2 diabetes.

RESULTS

Functional β Cell Mass Controls β Cell Proliferation

Islet Transplantation Reduces Basal and Compensatory Proliferation of Endogenous β Cells

We have previously developed a transgenic mouse system for conditional ablation of β cells, based on doxycycline-induced diphtheria toxin expression in Insulin-rtTA;TET-DTA (βDTA) mice (Nir et al., 2007). Using this system we showed that ablation of ~80% of β cells in adult mice, resulting in diabetes, is followed by a slow (~6 weeks) return to normoglycemia and regeneration of β cell mass, due to increased proliferation of surviving β cells (Nir et al., 2007). To determine if this compensatory proliferation persists in the absence of hyperglycemia, we used islet transplantation to normalize blood glucose in diabetic βDTA mice. We transplanted 500 wild-type islets under the kidney capsule of transgenic mice and 3 weeks later added doxycycline to the drinking water for 1 week to kill endogenous β cells. In the presence of wild-type islet grafts, mice showed normal glucose tolerance and normal serum insulin levels in the fasting state and following glucose challenge (see Figure S1 available online). As expected, apoptotic β cells were found in endogenous islets at a frequency similar to transgenic mice bearing no grafts, and the architecture of islets was disrupted as previously described (Figure S1). Thus, islet transplantation uncouples transgene-mediated pancreatic β cell ablation from the physiological outcome of hyperglycemia.

Hyperglycemic βDTA transgenic mice showed an approximately 6-fold increase in endogenous β cell proliferation rate 7 days after the addition of doxycycline (Figure 1A). Strikingly, the rate of β cell proliferation in transgenic mice grafted with normal islets was 50% lower than that in transgenic, hyperglycemic βDTA mice bearing no grafts. Interestingly, we observed that wild-type mice transplanted with wild-type islets had a similar decrease in endogenous β cell proliferation, to about 50% of their basal rate of proliferation (Figure 1A), without any change in blood glucose level (Figure S1). These results provide clear evidence for systemic control of both basal and compensatory β cell proliferation. Furthermore, while blood glucose is a key driver of β cell proliferation (see below), the results suggest that adaptive proliferation of β cells does not require overt hyper- or hypoglycemia.

β Cell Proliferation in Transplanted Islets Correlates with Functional β Cell Mass

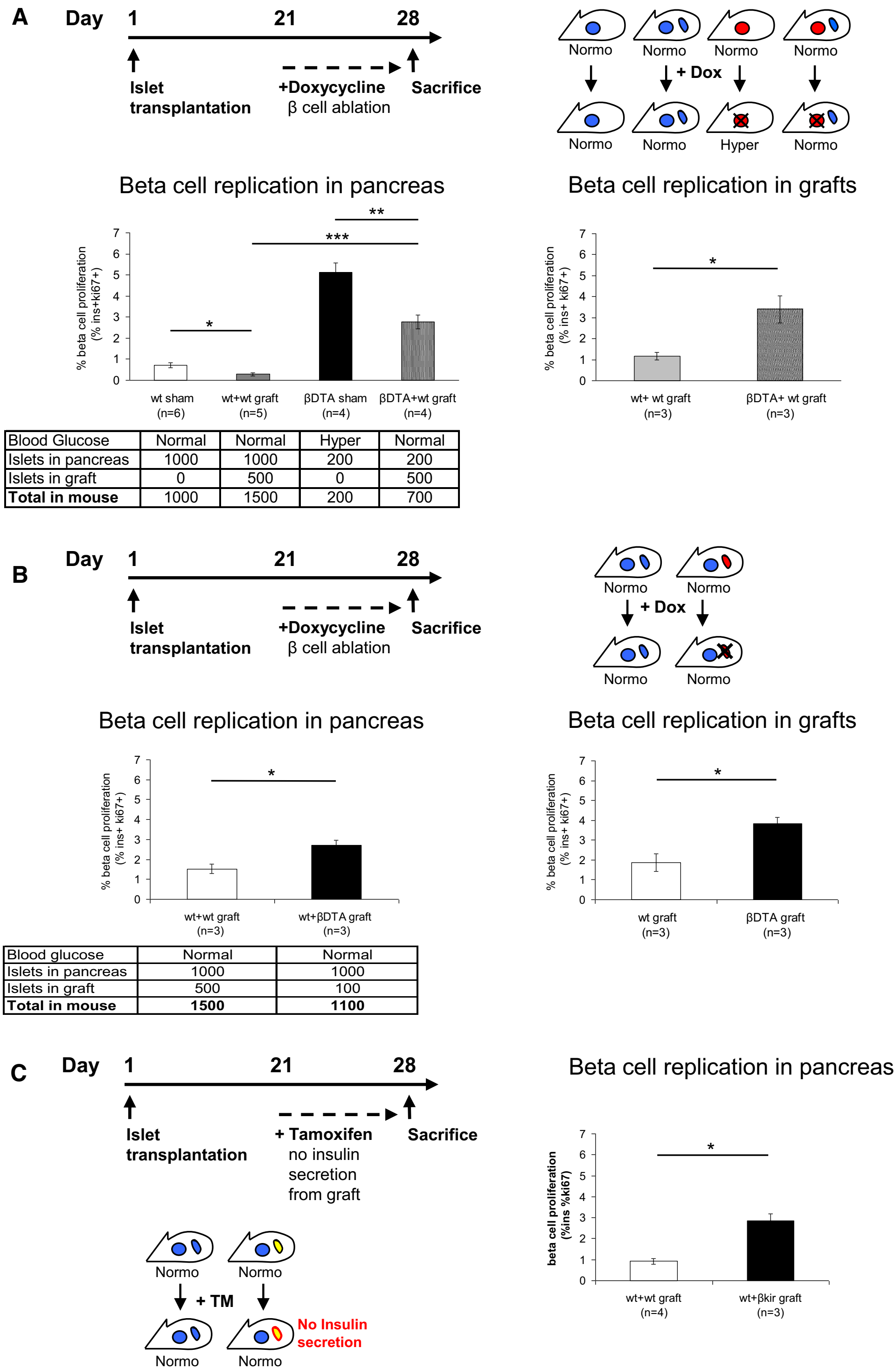
Despite the glucose-normalizing effect of islet grafts, endogenous β cells in βDTA mice bearing islet grafts had a replication rate almost 3-fold higher than normal (Figure 1A). This could reflect local cues for replication (e.g., destruction of islet architecture) or suboptimal β cell mass in these mice. To distinguish between these possibilities, we examined β cell replication in islet transplants. Grafted wild-type β cells had a higher replication rate in βDTA hosts compared with wild-type hosts (Figure 1A). This supports the concept that systemic factors, rather than local tissue damage, are responsible for compensatory β cell replication in βDTA mice.

To gain more insight into the control of compensatory β cell proliferation, and to exclude the influence of local, pancreatic factors, we performed reciprocal experiments where transgenic βDTA islets were transplanted under the kidney capsule of wild-type mice. As expected, the administration of doxycycline caused extensive destruction of grafted βDTA islets, but no hyperglycemia was observed due to the presence of a normal complement of endogenous β cells (Figure S2). As shown in Figure 1B, the rate of β cell proliferation in grafted βDTA islets was higher than in grafted wild-type islets. To determine if this reflects a systemic or a local trigger, we examined β cell proliferation in the pancreas of recipients (wild-type in both cases). Strikingly, endogenous β cell replication rate was higher in recipients of βDTA islets than in recipients of wild-type islets. These results further support the idea that systemic factors control β cell proliferation. Since grafted islets are not innervated, systemic regulation is likely blood-borne.

These experiments suggest, but do not prove, that grafted islets reduce β cell proliferation in the pancreas indirectly, by secreting insulin which maintains net peripheral glucose uptake, while reducing the workload on host β cells without a measurable perturbation of circulating glucose levels. To examine this notion, we used islets from insulin-CreER; Rosa26-loxP-stop-loxP-Kir6.2-V59M mice (βKir). Upon tamoxifen injection, βKir β cells express a mutant Kir6.2 subunit of the K_{ATP} channel, which prevents glucose-induced membrane depolarization and insulin secretion (Girard et al., 2009). If grafted islets reduce endogenous β cell proliferation by the (indirect) action of secreted insulin, then dysfunctional βKir islets should fail to impact replication. Tamoxifen injection of NOD/SCID mice transplanted with 500–700 βKir islets caused transient hyperglycemia, likely because a considerable portion of islet mass in these mice suddenly became dysfunctional (Figure S2). Figure 1C shows that endogenous β cell replication in hosts of βKir islets was higher than in hosts of wild-type islets. This result strongly supports the idea that it is the secretion of insulin from grafted islets that reduces β cell replication in the pancreas by decreasing the workload on endogenous β cells (see below).

Relationship of Blood Glucose to β Cell Proliferation

To obtain independent evidence regarding the impact of glucose metabolism on β cell regeneration, we plotted β cell proliferation



Blood Glucose	Normal	Normal	Hyper	Normal
Islets in pancreas	1000	1000	200	200
Islets in graft	0	500	0	500
Total in mouse	1000	1500	200	700

Blood glucose	Normal	Normal
Islets in pancreas	1000	1000
Islets in graft	500	100
Total in mouse	**1500**	**1100**

Figure 1. Islet Transplantation Shows Systemic Regulation of Compensatory and Basal β Cell Replication and a Positive Effect of Glucose

(A) βDTA mice grafted with wild-type islets. Top, schematic of experiment (left) and expected blood glucose levels after the addition of doxycycline and β cell ablation (right). Circles, native pancreas; ovals, transplanted islets; blue, wild-type; red, βDTA. Bottom, β cell replication in the pancreas (left) and in grafts (right). Table under graph provides estimated numbers of islets per mouse. Error bars represent standard error.

(B) Wild-type mice engrafted with wild-type and βDTA islets. Top, schematic of experiment. Bottom, β cell replication in the pancreas (left) and in grafts (right). Table under graph provides estimated numbers of islets per mouse. Error bars represent standard error.

(C) Wild-type mice engrafted with βKir islets (yellow before tamoxifen-induced expression, red after tamoxifen). Left, schematic of experiment. Right, β cell replication in the pancreas. Error bars represent standard error.

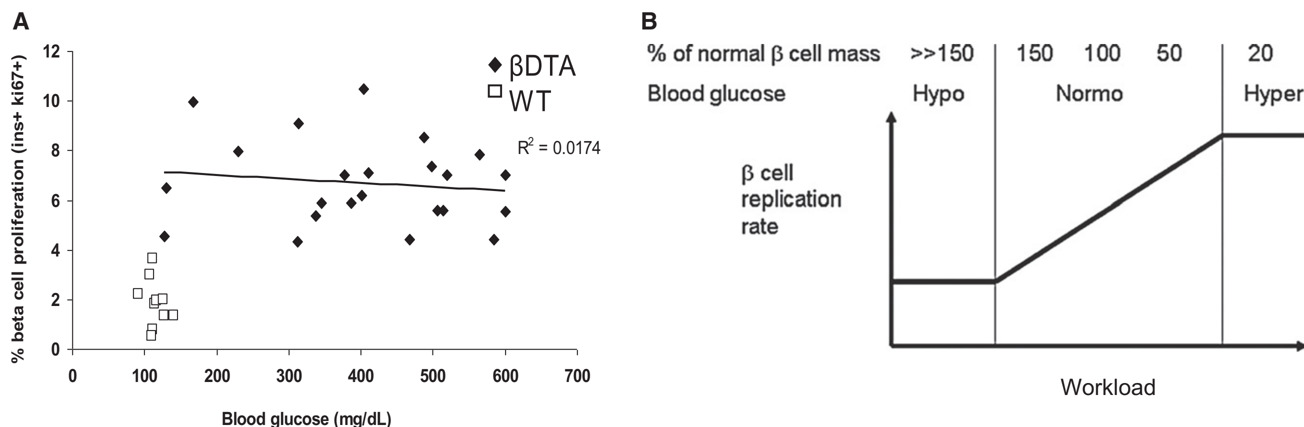


Figure 2. Relationship between Blood Glucose and β Cell Replication Rate

(A) β cell replication rate as a function of blood glucose levels at sacrifice. Open symbols, wild-type mice; closed symbols, βDTA mice. Note a positive effect of glucose on replication rate, but no relationship between glucose levels and β cell replication rate within the transgenic group.

(B) Proposed model for the effect of glucose, via workload of β cells, on β cell replication rate. The model explains how different normoglycemic conditions may cause different β cell replication rates.

as a function of blood glucose measured at sacrifice in wild-type and βDTA mice. As previously reported, β cell replication was higher in βDTA mice than in littermate controls (Nir et al., 2007) (Figure 2A). Surprisingly, within the βDTA group there was no correlation between the level of blood glucose and the rate of proliferation. Rather, βDTA mice had an ~3-fold increase in β cell replication rate regardless of glucose levels, and even when circulating glucose levels were within the normal range, suggesting that even when β cell destruction is too mild to cause overt hyperglycemia, maximal compensatory β cell proliferation is triggered.

Working Hypothesis: Proliferation Is Regulated by β Cell Glycolytic Flux

To explain the results presented above, we hypothesized that β cell replication is controlled by the workload imposed on an individual β cell in order to maintain euglycemia, as predicted by control theory in a feedback-regulated system (Astrom and Murray, 2009). We further hypothesized that this workload (i.e., insulin secretory demand) is sensed by the β cell as the rate of glycolysis (Figure 2B). In such a system, glucose oscillations entrain insulin secretion oscillations, which in turn regulate peripheral glucose utilization, and thus circulating glucose levels (Palumbo and De Gaetano, 2010). The net insulin secretion per β cell, as regulated by glucose metabolism, is the β cell "workload." According to this model, when the total body insulin requirement remains constant, any reduction of β cell mass will increase the workload on each remaining β cell and as a result will trigger a compensatory proliferation. If β cell loss is small, enhanced insulin secretion will prevent a measurable increase in blood glucose level, but proliferation will nevertheless be stimulated. When β cell loss reaches the point of causing hyperglycemia, β cell replication rate is presumably stimulated maximally, hence a further rise in glucose level does not increase replication. In the other direction, an excess of functional β cells (as in wild-type mice bearing islet grafts) would lead to a reduced glycolytic flux per β cell, and thereby a reduced replication rate.

This model explains how a homeostatic response to β cell deficiency or excess can be mounted before detectable hyperglycemia or hypoglycemia develops.

Glucokinase Controls β Cell Function, Proliferation, and Survival

Glucose Metabolism Is Required for Stimulation of β Cell Proliferation

To directly examine the hypothesis that glucose triggers β cell proliferation via glycolysis, and not, for example, via protein glycosylation, we deleted glucokinase (GCK) in β cells of adult mice. GCK catalyzes the rate-limiting step of glucose metabolism in β cells, and is a central regulator of glucose-stimulated insulin secretion. Its absence is expected to reduce glucose flux in β cells and as a consequence reduce insulin secretion and increase blood glucose levels (Magnuson et al., 2003). Thus, GCK deficiency in β cells uncouples extracellular glucose levels from intracellular metabolic flux. Previous genetic studies of GCK used either heterozygous mice in which one copy of the gene remains intact and deficiency is not β cell specific, or a β cell-specific deletion which led to early postnatal lethality, precluding detailed analysis (Postic et al., 1999; Terauchi et al., 2007). To overcome these limitations, we employed a tamoxifen-inducible deletion of GCK specifically in β cells of adult mice. Tamoxifen injection of adult insulin-CreER;GCK$^{loxP/loxP}$ mice (βGCK) led to a 3-fold decrease in islet GCK mRNA levels, indicating efficient deletion of the gene (data not shown). Tamoxifen-treated βGCK mice developed severe hyperglycemia and hypoinsulinemia (Figure 3A), consistent with the inability of mutant β cells to sense glucose and secrete insulin. Staining for phospho-AMPK, a sensitive marker of cellular energy stress (high AMP/ATP ratio) (Hardie et al., 2006) showed a striking increase in the intensity of p-AMPK in βGCK islets (Figure 3B). These data are consistent with energy stress and reduced glucose flux in mutant β cells. We conclude that while βGCK mice exhibit circulating hyperglycemia, their β cells behave as if exposed to hypoglycemia, reflecting the blunted glycolytic flux.

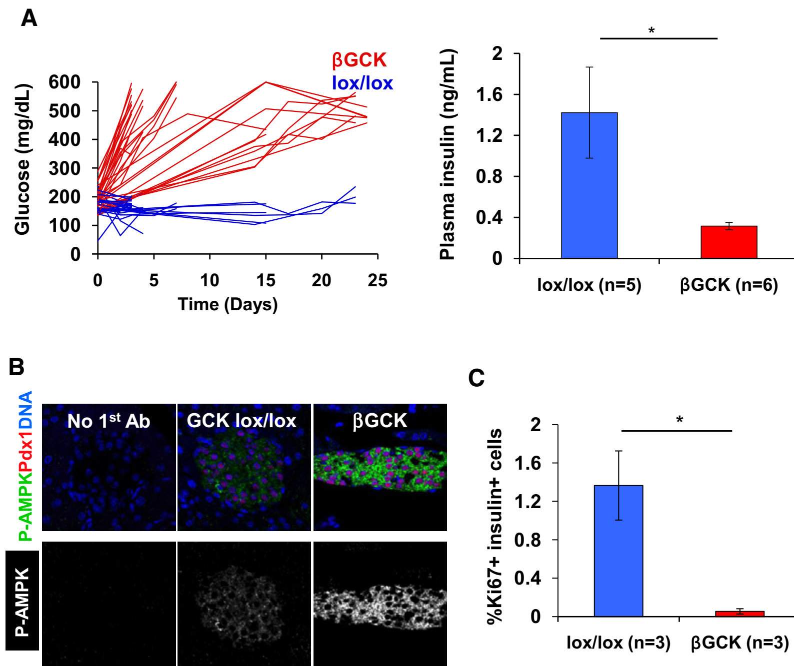


Figure 3. Deletion of Glucokinase in Adult β Cells Reduces β Cell Replication Rate

(A) Blood glucose (left) and serum insulin levels (right) 9 days after tamoxifen injection of insulin-CreER; $GCK^{lox/lox}$ mice (βGCK, red) or $GCK^{lox/lox}$ controls (blue). Measurements were taken in the fed state. Error bars represent standard error.

(B) Increased phosphorylation of AMPK in βGCK islets, 9 days after tamoxifen injection, providing evidence for low intracellular energy charge despite high blood glucose levels. Original magnification, 800×.

(C) Reduced β cell replication rate in βGCK islets, 9 days after tamoxifen injection. Error bars represent standard error.

Staining for Ki67 revealed a dramatic drop in β cell proliferation rate in βGCK islets (Figure 3C). This indicates that the mitogenic effect of glucose is mediated by glucose metabolism, and that glucose flux is a key regulator of basal β cell proliferation. We also observed increased β cell apoptosis in βGCK mice (Figure S3), and reduced β cell size (data not shown). As a result of these changes, total β cell mass was reduced 2-fold 2 months after GCK deletion (Figure S3). Since the dependence of β cell proliferation on GCK could simply reflect the minimum cellular energy requirements for replication, we performed additional experiments (see below), which suggest that this is not the case and support the hypothesis that GCK, via its control of glycolysis in β cells, is a major determinant of β cell proliferation.

Glucokinase Activation Boosts β Cell Proliferation

If suppressed β cell proliferation after GCK ablation were simply the result of cellular starvation, enhancing the activity of GCK above normal in wild-type animals would not be expected to affect the β cell proliferation rate. If, however, GCK controls proliferation by regulating the rate of glycolysis, similar to its role in insulin secretion, increasing the activity of GCK would be predicted to boost β cell proliferation. Pharmacologic activators of GCK are a novel class of drugs being developed for the treatment of type 2 diabetes which act by increasing the glucose affinity and maximum velocity of GCK and hence improve insulin secretion (Matschinsky et al., 2006). As previously reported (Grimsby et al., 2003), administration of a specific GCK activator (GKA) to wild-type mice led to hypoglycemia, that persisted for more than 24 hr (Figure 4A). Despite circulating hypoglycemia, GKA is expected to increase the rate of glycolysis in β cells. To test this prediction, we measured glucose oxidation in isolated islets incubated in the presence of GKA in various concentrations of glucose. As shown in Figure 4B, GKA significantly increased glucose oxidation in all glucose concentrations tested. Moreover, glucose oxidation in GKA-treated islets at 3 mM glucose was higher than oxidation in control islets at 5 mM, showing that GKA uncouples glycolysis from extracellular glucose. Consistent with the in vitro glucose oxidation data, mice treated with GKA showed reduced staining for p-AMPK in islets, suggesting that GKA increased energy charge in β cells in vivo (Figure S4).

Seventeen hours after administration of GKA, the fraction of replicating β cells had doubled as reflected in the number of Ki67+ β cells (Figure 4C), the number of β cells that incorporated the thymidine analog BrdU (Figure 4D, Figure S5), and staining for the mitotic marker phophorylated histone H3 (Figure S5). When GKA was given to βGCK mutant mice, blood glucose levels still decreased, likely reflecting the activation of GCK in the liver or residual islet GCK (data not shown); however, β cell proliferation remained low (Figure 4E), indicating a cell-autonomous role of GCK in β cell proliferation. These results show that the rate of β cell proliferation in vivo is controlled by GCK and can be both decreased and increased in response to altered GCK activity. Finally, the islet transplantation experiments described above suggested that in βDTA mice undergoing β cell regeneration, enhanced glycolysis is responsible for increased replication. Our model predicts that the addition of GKA, which artificially increases Vmax of GCK, will further increase the rate of β cell replication in hyperglycemic βDTA mice. As shown in Figure 4F, this is indeed the case: GKA led to a moderate yet significant increase in the rate of β cell replication in diabetic βDTA mice.

Glucokinase-Regulated β Cell Proliferation Is Mediated by Plasma Membrane Depolarization

We next investigated how mitogenic signals are transmitted by GCK. Glucose flux could, in principle, act directly on signaling molecules such as AMPK. Alternatively, it could act via the glucose sensing/insulin secretion pathway, where increased ATP/ADP ratio closes K_{ATP} channels and causes membrane depolarization. To examine if K_{ATP} channels are necessary for the mitogenic signal of GCK, we treated mice simultaneously with GKA and diazoxide, a K_{ATP} channel opener. As expected, diazoxide led to a transient hyperglycemia (due to the prevention of insulin secretion), which was unaffected by GKA (Figure 5A). Importantly, diazoxide neutralized the mitogenic effect of GKA (Figure 5A), suggesting that closure of K_{ATP} channels and depolarization were necessary for GCK-regulated replication.

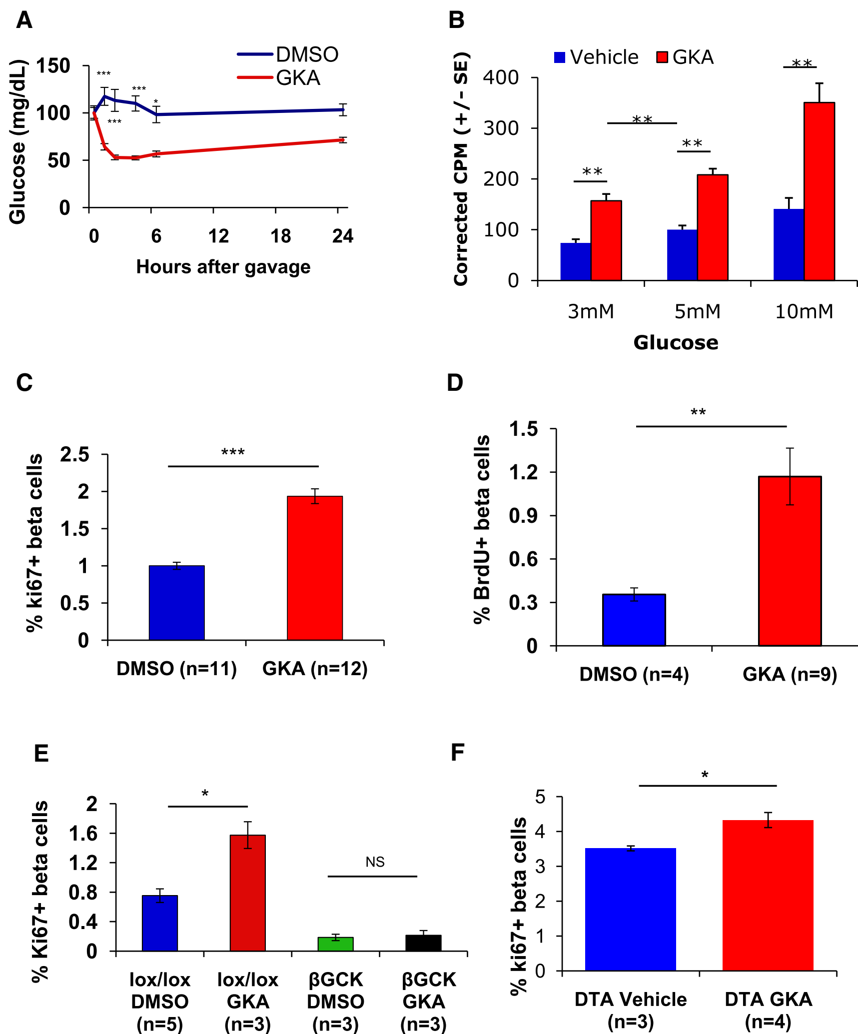


Figure 4. Effects of Glucokinase Activator on β Cells In Vivo

(A) Reduced blood glucose levels following a single oral administration of GKA (50 mg/kg) or vehicle (DMSO) to wild-type mice. n > 10 mice in each group. Error bars represent standard error.

(B) Increased glucose oxidation in wild-type islets exposed to GKA at different glucose levels. Error bars represent standard error.

(C) Increased β cell replication, measured as fraction of Ki67+ β cells, 17 hr after administration of GKA or vehicle (DMSO) to 6-week-old wild-type mice. n refers to number of mice analyzed; for each mouse, >2000 β cells were counted. Error bars represent standard error.

(D) Increased incorporation of BrdU in β cells of mice treated with a single dose of GKA. Error bars represent standard error.

(E) GKA-induced β cell replication is abolished in mice deficient for GCK in β cells (green and black bars). NS, not significant. n = number of mice analyzed. Error bars represent standard error.

(F) GKA moderately increases the fraction of replicating β cells in hyperglycemic βDTA mice. Error bars represent standard error.

To substantiate this conclusion, we took advantage of transgenic mice expressing a Cre-activated mutant of the K_{ATP} channel Kir6.2 (βKir) described above. Acute activation of the mutant channel in β cells caused hyperglycemia, and led to a decrease in β cell proliferation rate. Furthermore, administration of GKA failed to induce proliferation in βKir mice (Figure 5B). This supports the view that β cell replication depends on a signaling pathway involving glucokinase and membrane depolarization. We then performed a reciprocal experiment, where βGCK mice were injected with glyburide, a K_{ATP} channel blocker that induces membrane depolarization. As expected, acute administration of glyburide reduced blood glucose in βGCK mice, albeit not to normal (Figure 5C). Strikingly, glyburide acutely rescued β cell proliferation in βGCK mice (Figure 5C). This finding strongly suggests that reduced β cell proliferation in βGCK mutants is not simply a result of cellular energy deficit but rather the result of reduced membrane depolarization. However, it also demonstrates that glucose flux per se is needed to achieve optimal proliferation, since glyburide stimulation resulted in only partial recovery of replication, increasing it to the same level found in glyburide-treated wild-type mice (Figure 5C).

In both of these models, membrane depolarization occurred in the face of decreased glycolytic flux, due to GCK deficiency in the former and ambient hypoglycemia in the latter. We further tested if glyburide can increase β cell replication in the face of increased glucose flux using the βDTA mouse model. Acute administration of glyburide decreased glucose levels of βDTA mice somewhat, demonstrating that the drug could further stimulate insulin secretion (Figure 5D). Importantly, the frequency of β cell replication in βDTA mice almost doubled upon injection of glyburide, indicating that forced membrane depolarization, in the presence of increased glycolytic flux, can further stimulate proliferation.

All these studies were performed on 4- to 6-week-old mice, an age that corresponds to young adulthood in man. To determine whether GCK-induced β cell replication is age dependent, we administered GKA to 6-month-old mice. We observed the expected age-dependent decrease in basal proliferation, but interestingly, GKA administration resulted in a 2- to 3-fold increase in proliferation in all age groups (data not shown).

DISCUSSION

Our experiments show conclusively that the basal proliferation rate of adult β cells in vivo and their regeneration following injury are controlled by systemic factors. Local factors, such as the presence of dead cells or disrupted islet architecture, appear to play only a minor role, if any. Thus, the control of β cell number is analogous to that found for other systemically controlled tissues such as blood (e.g., erythrocytes) (Stanger, 2008), liver

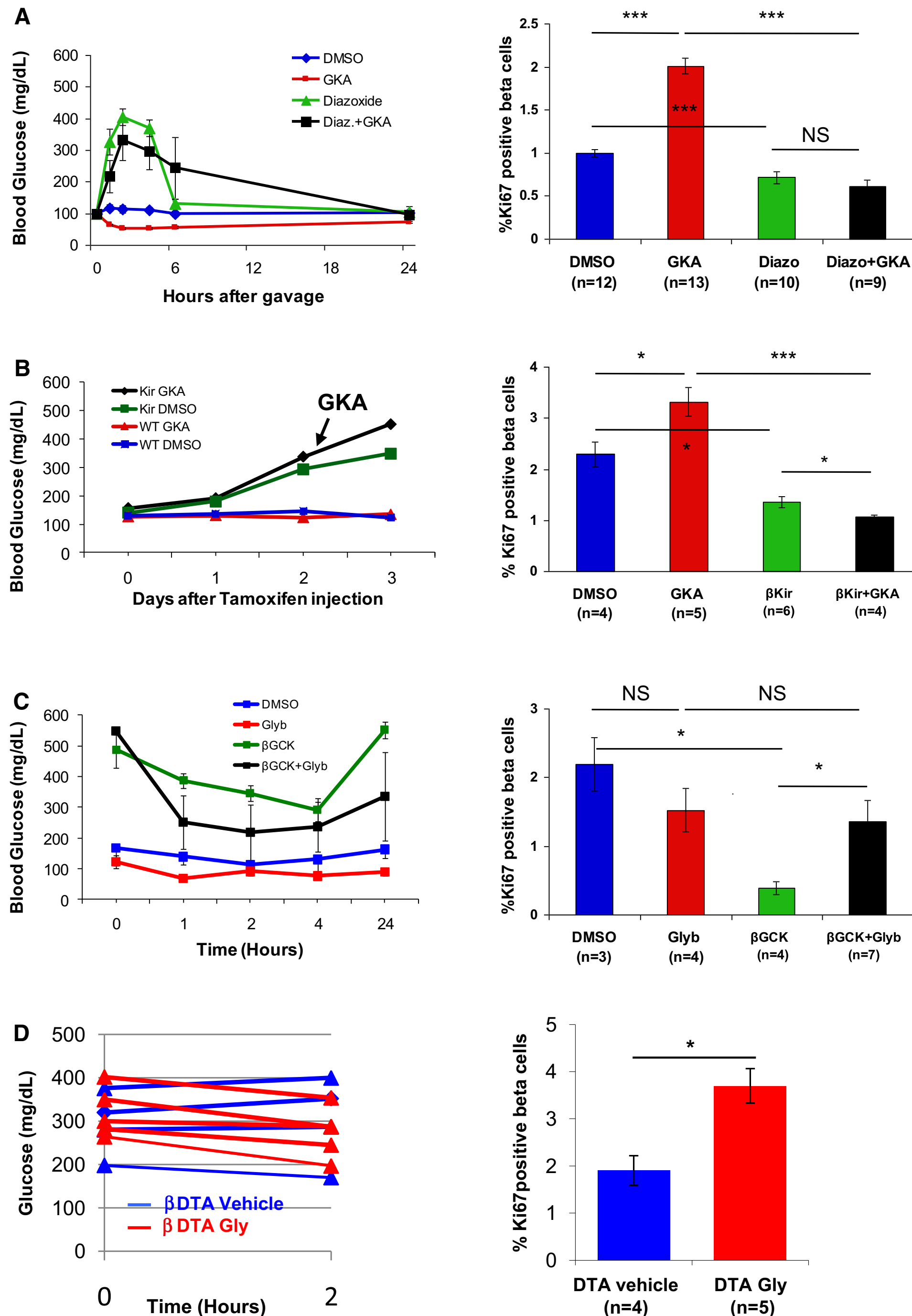


Figure 5. Dependence of β Cell Replication Downstream of Glucokinase on Membrane Depolarization

(A) Left, effect of diazoxide (40 mg/kg) on blood glucose levels from injection to sacrifice 24 hr later. Right, diazoxide abolishes GKA-induced β cell replication in wild-type mice. n = number of mice analyzed. Error bars represent standard error.

(B) Left, effect of transgenic expression of Kir6.2 V59M in adult β cells (βKir) on blood glucose levels. Right, the Kir6.2 mutation reduces basal β cell replication and abolishes GKA-induced β cell replication. Tamoxifen was injected on day 0 to activate the mutant gene. GKA and vehicle were administered on day 2. Error bars represent standard error.

(C) Left, blood glucose levels in βGCK mutants and $GCK^{lox/lox}$ littermate controls in response to glyburide. Right, acute rescue of β cell replication in βGCK mutants by glyburide (Glyb). Glyburide was given by oral gavage at 20 mg/kg. Error bars represent standard error.

(D) Left, effect of glyburide on blood glucose in diabetic βDTA mice. Right, glyburide increases the fraction of Ki67+ β cells in diabetic βDTA mice. Error bars represent standard error.

(Conboy et al., 2005; Sakai, 1970), thymus (Conlon and Raff, 1999), and muscle (Conboy et al., 2005).

Our data strongly suggest that the key systemic factor controlling β cell replication is glucose. They further demonstrate that the control of β cell number relies on determination of functional β cell mass, capable of insulin secretion. Thus, the individual β cell senses the organism's insulin needs according to the workload placed on it. Grafted islets therefore reduce β cell replication in the host pancreas indirectly, by releasing insulin, lowering the workload on endogenous and transplanted β cells. Supporting this view, grafted, nonfunctioning βKir islets failed to reduce host β cell replication. Our conclusion is consistent with that of a recent study that examined β cell replication in the setting of autoimmunity (Pechhold et al., 2009).

This observation adds β cells to the short list of mammalian tissues whose size is known to be controlled by a feedback loop directly related to their function. This paradigm is classically illustrated by erythrocytes, whose total number is controlled by erythropoietin, a hormone that is produced when erythrocytes fail to deliver sufficient oxygen to tissues. In the context of endocrine organs, thyroid homeostasis provides another classic example of feedback control of organ size: thyrocyte replication depends on TSH concentration, which in turn is negatively regulated by thyroid hormone (serving as a sensitive indicator of thyroid function). Finally, recent studies showed that the flux of bile acids through the liver, serving as a signal for workload, controls hepatocyte proliferation and liver mass homeostasis (Huang et al., 2006).

We further show that β cell replication is controlled by the rate of intracellular glucose metabolism, which we refer to as the workload. Physiologically, this model explains how β cell number can be finely adjusted according to the organism's needs, without deviating from the normoglycemic range.

In terms of molecular signaling, we find that glucose metabolism controls β cell replication via K_{ATP} channels and membrane depolarization. Our experiments indicate that both glycolysis and membrane depolarization are necessary for the mitogenic effect of glucose metabolism. This is consistent with previous reports on glibenclamide-induced β cell proliferation (Guiot et al., 1994).

What determines if enhanced glycolysis causes insulin secretion alone, or both secretion and replication? We speculate that the decision is temporally controlled, such that a short pulse of glucose metabolism, as would happen after a meal, will trigger secretion but not replication, while more persistent activation of the pathway (indicating an organismal need for more β cells), will trigger replication. Experiments examining this hypothesis are ongoing.

Our results are consistent with a recent study of mice globally heterozygous for glucokinase, which found that two copies of GCK were necessary to achieve β cell hyperplasia in response to high-fat diet, and identified IRS2 as a critical component in the GCK-dependent mitogenic response to a high-fat diet (Terauchi et al., 2007). However, this paper concluded that normal levels of glucose oxidation are not necessary for compensatory β cell replication, which is not supported by our finding that the rate of glycolysis in β cells, even when uncoupled from blood glucose levels, is the critical driver of basal and compensatory β cell replication (via its effect on membrane depolarization).

While we have shown here that glucose is the key driver of β cell proliferation, toxic effects of glucose on β cells are well recognized and are thought to be important in the pathogenesis of diabetes. What is the relationship between glucose-induced replication and glucotoxicity? We have recently reported on enhanced replication as well as enhanced apoptosis in β cells of a human patient bearing an activating mutation in glucokinase (Kassem et al., 2010). This suggests that toxic effects of glucose are also mediated through glycolysis, like the mitogenic effects, and shows that glucose can trigger simultaneously replication and apoptosis. While in this clinical case, as well as in βDTA mice, the net effect of glucose metabolism was expansion of β cell mass, it is possible that under other settings the net effect of glucose is different. It will be important to identify the molecular pathways leading from glycolysis to β cell replication and apoptosis and their divergence points.

Our study addresses the mechanisms that control the replication of differentiated β cells. While there is strong evidence that replication is the major determinant of β cell mass in mice and men, under certain circumstances β cells could be generated from other cells (neogenesis), including differentiation of duct cells (Inada et al., 2008; Xu et al., 2008) or reprogramming of alpha cells (Thorel et al., 2010). It will be interesting to determine if glucose metabolism has a role in β cell neogenesis or whether entirely different pathways control this process, as occurs in embryonic development.

Our data have several clinical implications. First, if glucose-driven glycolysis is the key mitogenic trigger for β cells, reducing circulating glucose levels to normal or subnormal levels by the administration of exogenous insulin may reduce β cell proliferation rate due to reduced workload. While the normalization of blood glucose in diabetic patients is clearly beneficial for the patients and for β cell survival and function, the antimitogenic effects of this correction may have a long-term impact on β cell mass. Second, glucokinase activators, upcoming drugs aimed at improving the control of blood glucose in type 2 diabetes, could have beneficial effects on β cell number. We predict that non-tissue-specific GKAs will be mitogenic to β cells, while liver-specific GKAs will decrease β cell replication if they effectively achieve euglycemia while decreasing demand for insulin. Whether GKAs increase β cell proliferation in humans remains to be proven, but this idea is strongly supported by the observation that patients bearing an activating mutation in the glucokinase gene have hyperplastic islets (Cuesta-Munoz et al., 2004; Kassem et al., 2010).

In conclusion, we identify a simple mechanism for homeostasis of β cell proliferation and mass. β cells adjust their proliferation rate according to the rate of glycolysis; this provides a system for sensitive measurement of organismal demand for β cells, while normoglycemia is maintained. The same homeostatic mechanism appears to be responsible for the control of β cell number during healthy adult life as well as during regeneration following injury. Our findings following genetic and pharmacologic manipulation of β cell K_{ATP} channel activity suggest that the downstream mechanism by which glucose metabolism triggers proliferation is similar to the mechanism regulating insulin secretion. Further research is required to fully characterize this pathway and to identify points at which novel therapeutic interventions can be developed, aimed at boosting β cell mass for the cure of diabetes.

EXPERIMENTAL PROCEDURES

Transgenic Mice

Transgenic/knockout mouse strains used in this study included insulin-rtTA, TET-DTA (βDTA) (Nir et al., 2007), insulin-CreER (Dor et al., 2004), Pdx1-CreER (Gu et al., 2002), GCK loxP/loxP (βGCK) (Postic et al., 1999), and Rosa26-LSL-Kir6.2 V59M (βKir) (Girard et al., 2009). The joint ethics committee (IACUC) of the Hebrew University and Hadassah Medical Center approved the study protocol for animal welfare. The Hebrew University is an AAALAC International-accredited institute.

Drugs

Mice were administered with the following drugs: glucokinase activator Ro28-1675 (Grimsby et al., 2003), 50 mg/kg; glyburide (Sigma), 20 mg/kg; diazoxide (Sigma), 40 mg/kg. All these drugs were dissolved in DMSO and given by oral gavage at 10 μl/gr body weight. Doxycycline was given in the drinking water at 400 μg/ml for 7 days. Tamoxifen was dissolved in corn oil and administered subcutaneously via a single injection of 8 mg/mouse.

Assays

Islet transplantation was performed as described before (Molano et al., 2003). Graft recipients were C57/Bl6 males (wild-type or βDTA, Figure 1A) aged 6–7 weeks. Recipients of βDTA or βKir islets were NOD/SCID males aged 7–8 or 8–10 weeks, respectively. Comparisons of β cell replication rate were performed between control and experimental mice of the same strain, sex, and age. Islet donors were males aged 9–12 weeks. Donors of βDTA or βKir islets were of the same strain, sex, and age as donors of wild-type islets in the same experiments. Grafts were composed of 500–700 hand-picked islets. Glucose and insulin levels were measured in the fasted state, unless stated otherwise. The rate of glucose oxidation was determined by measuring the formation of $^{14}CO_2$ from D-[U^{14}C] glucose as previously described (Malaisse et al., 1974). Briefly, isolated islets from C57/B6 mice were incubated overnight in standard culture medium. The next day, islets were divided into batches of 40 islets and incubated in 100 μl KRBB solution containing either 3, 5, or 10 mM glucose, 0.5% w/v BSA and 0.5, 1, or 2 μCi of D-[U^{14}C] glucose (PerkinElmer), respectively. Incubation was performed in 0.5 ml tubes placed inside 20 ml glass scintillation vials fitted with airtight rubber seals (Altech). An eppendorf tube placed inside the scintillation vials contained 200 μl of hyamine (PerkinElmer) to absorb CO_2 produced. Islets were incubated for 60 min at 37°C with continuous shaking, with either GKA or vehicle. Metabolism was stopped by injecting 50 μl of 3M perchloric acid into the incubation tube through the rubber seal. After another 1 hr incubation at room temperature, the amount of radioactive CO_2 in the hyamine tube was determined by liquid scintillation counter.

To determine β cell replication rate, at least 2000 insulin-positive cells were counted per mouse and scored for the percentage of Ki67-positive cells. Glucose tolerance tests, insulin measurements, immunostaining, calculation of β cell mass, and TUNEL assays (Roche) were as described before (Nir et al., 2007; Weinberg et al., 2007). In all statistical analyses, *$p < 0.05$; **$p < 0.01$; ***$p < 0.005$, NS, $p > 0.05$.

Antibodies

Primary antibodies used in this study included rabbit anti-Ki67 (NeoMarkers, 1:200), guinea pig anti-insulin (Dako, 1:500), mouse anti-glucagon (BCBC, 1:800), rabbit anti-p-AMPK (Cell Signaling, 1:100, requires amplification with TSA kit, NEN), and goat anti-pdx1 (a gift from Chris Wright, 1:2500). Secondary antibodies were from Jackson Immunoresearch.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and can be found at doi:10.1016/j.cmet.2011.02.012.

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REFERENCES

Alonso, L.C., Yokoe, T., Zhang, P., Scott, D.K., Kim, S.K., O'Donnell, C.P., and Garcia-Ocana, A. (2007). Glucose infusion in mice: a new model to induce beta-cell replication. Diabetes *56*, 1792–1801.

Astrom, K.J., and Murray, R.M. (2009). Feedback Systems: An Introduction for Scientists and Engineers (Princeton, NJ: Princeton University Press).

Bonner-Weir, S., Deery, D., Leahy, J.L., and Weir, G.C. (1989). Compensatory growth of pancreatic beta-cells in adult rats after short-term glucose infusion. Diabetes *38*, 49–53.

Brennand, K., Huangfu, D., and Melton, D. (2007). All beta cells contribute equally to islet growth and maintenance. PLoS Biol. *5*, e163. 10.1371/journal.pbio.0050163.

Butler, A.E., Janson, J., Bonner-Weir, S., Ritzel, R., Rizza, R.A., and Butler, P.C. (2003). Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. Diabetes *52*, 102–110.

Butler, P.C., Meier, J.J., Butler, A.E., and Bhushan, A. (2007). The replication of beta cells in normal physiology, in disease and for therapy. Nat. Clin. Pract. Endocrinol. Metab. *3*, 758–768.

Chick, W.L. (1973). Beta cell replication in rat pancreatic monolayer cultures. Effects of glucose, tolbutamide, glucocorticoid, growth hormone and glucagon. Diabetes *22*, 687–693.

Chick, W.L., and Like, A.A. (1971). Effects of diet on pancreatic beta cell replication in mice with hereditary diabetes. Am. J. Physiol. *221*, 202–208.

Conboy, I.M., Conboy, M.J., Wagers, A.J., Girma, E.R., Weissman, I.L., and Rando, T.A. (2005). Rejuvenation of aged progenitor cells by exposure to a young systemic environment. Nature *433*, 760–764.

Conlon, I., and Raff, M. (1999). Size control in animal development. Cell *96*, 235–244.

Cuesta-Munoz, A.L., Huopio, H., Otonkoski, T., Gomez-Zumaquero, J.M., Nanto-Salonen, K., Rahier, J., Lopez-Enriquez, S., Garcia-Gimeno, M.A., Sanz, P., Soriguer, F.C., et al. (2004). Severe persistent hyperinsulinemic hypoglycemia due to a de novo glucokinase mutation. Diabetes *53*, 2164–2168.

Dor, Y., Brown, J., Martinez, O.I., and Melton, D.A. (2004). Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. Nature *429*, 41–46.

Duffield, J.S., Forbes, S.J., Constandinou, C.M., Clay, S., Partolina, M., Vuthoori, S., Wu, S., Lang, R., and Iredale, J.P. (2005). Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair. J. Clin. Invest. *115*, 56–65.

Ehses, J.A., Perren, A., Eppler, E., Ribaux, P., Pospisilik, J.A., Maor-Cahn, R., Gueripel, X., Ellingsgaard, H., Schneider, M.K., Biollaz, G., et al. (2007). Increased number of islet-associated macrophages in type 2 diabetes. Diabetes *56*, 2356–2370.

Flier, S.N., Kulkarni, R.N., and Kahn, C.R. (2001). Evidence for a circulating islet cell growth factor in insulin-resistant states. Proc. Natl. Acad. Sci. USA *98*, 7475–7480.

Georgia, S., and Bhushan, A. (2004). Beta cell replication is the primary mechanism for maintaining postnatal beta cell mass. J. Clin. Invest. *114*, 963–968.

Girard, C.A., Wunderlich, F.T., Shimomura, K., Collins, S., Kaizik, S., Proks, P., Abdulkader, F., Clark, A., Ball, V., Zubcevic, L., et al. (2009). Expression of an activating mutation in the gene encoding the KATP channel subunit Kir6.2 in mouse pancreatic beta cells recapitulates neonatal diabetes. J. Clin. Invest. *119*, 80–90.

Grimsby, J., Sarabu, R., Corbett, W.L., Haynes, N.E., Bizzarro, F.T., Coffey, J.W., Guertin, K.R., Hilliard, D.W., Kester, R.F., Mahaney, P.E., et al. (2003). Allosteric activators of glucokinase: potential role in diabetes therapy. Science *301*, 370–373.

Gu, G., Dubauskaite, J., and Melton, D.A. (2002). Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors. Development *129*, 2447–2457.

Guiot, Y., Henquin, J.C., and Rahier, J. (1994). Effects of glibenclamide on pancreatic beta-cell proliferation *in vivo*. Eur. J. Pharmacol. *261*, 157–161.

Halban, P.A., German, M.S., Kahn, S.E., and Weir, G.C. (2010). Current status of islet cell replacement and regeneration therapy. J. Clin. Endocrinol. Metab. *95*, 1034–1043.

Hardie, D.G., Hawley, S.A., and Scott, J.W. (2006). AMP-activated protein kinase–development of the energy sensor concept. J. Physiol. *574*, 7–15.

Heit, J.J., Karnik, S.K., and Kim, S.K. (2006). Intrinsic regulators of pancreatic beta-cell proliferation. Annu. Rev. Cell Dev. Biol. *22*, 311–338.

Huang, W., Ma, K., Zhang, J., Qatanani, M., Cuvillier, J., Liu, J., Dong, B., Huang, X., and Moore, D.D. (2006). Nuclear receptor-dependent bile acid signaling is required for normal liver regeneration. Science *312*, 233–236.

Imai, J., Katagiri, H., Yamada, T., Ishigaki, Y., Suzuki, T., Kudo, H., Uno, K., Hasegawa, Y., Gao, J., Kaneko, K., et al. (2008). Regulation of pancreatic beta cell mass by neuronal signals from the liver. Science *322*, 1250–1254.

Inada, A., Nienaber, C., Katsuta, H., Fujitani, Y., Levine, J., Morita, R., Sharma, A., and Bonner-Weir, S. (2008). Carbonic anhydrase II-positive pancreatic cells are progenitors for both endocrine and exocrine pancreas after birth. Proc. Natl. Acad. Sci. USA *105*, 19915–19919.

Kassem, S., Heyman, M., Glaser, B., Bhandari, S., Motaghedi, R., Maclaren, N.K., Garcia-Gimeno, M.A., Sanz, P., Rahier, J., Rodriguez-Bada, P., et al. (2010). Large islets, beta-cell proliferation, and a glucokinase mutation. N. Engl. J. Med. *362*, 1348–1350.

Kulkarni, R.N., Jhala, U.S., Winnay, J.N., Krajewski, S., Montminy, M., and Kahn, C.R. (2004). PDX-1 haploinsufficiency limits the compensatory islet hyperplasia that occurs in response to insulin resistance. J. Clin. Invest. *114*, 828–836.

Kwon, G., Marshall, C.A., Pappan, K.L., Remedi, M.S., and McDaniel, M.L. (2004). Signaling elements involved in the metabolic regulation of mTOR by nutrients, incretins, and growth factors in islets. Diabetes *53* (*Suppl 3*), S225–S232.

Magnuson, M.A., She, P., and Shiota, M. (2003). Gene-altered mice and metabolic flux control. J. Biol. Chem. *278*, 32485–32488.

Malaisse, W.J., Sener, A., and Mahy, M. (1974). The stimulus-secretion coupling of glucose-induced insulin release. Sorbitol metabolism in isolated islets. Eur. J. Biochem. *47*, 365–370.

Martens, G.A., and Pipeleers, D. (2009). Glucose, regulator of survival and phenotype of pancreatic beta cells. Vitam. Horm. *80*, 507–539.

Matschinsky, F.M., Magnuson, M.A., Zelent, D., Jetton, T.L., Doliba, N., Han, Y., Taub, R., and Grimsby, J. (2006). The network of glucokinase-expressing cells in glucose homeostasis and the potential of glucokinase activators for diabetes therapy. Diabetes *55*, 1–12.

Meier, J.J., Butler, A.E., Saisho, Y., Monchamp, T., Galasso, R., Bhushan, A., Rizza, R.A., and Butler, P.C. (2008). Beta-cell replication is the primary mechanism subserving the postnatal expansion of beta-cell mass in humans. Diabetes *57*, 1584–1594.

Michael, M.D., Kulkarni, R.N., Postic, C., Previs, S.F., Shulman, G.I., Magnuson, M.A., and Kahn, C.R. (2000). Loss of insulin signaling in hepatocytes leads to severe insulin resistance and progressive hepatic dysfunction. Mol. Cell *6*, 87–97.

Molano, R.D., Pileggi, A., Berney, T., Poggioli, R., Zahr, E., Oliver, R., Malek, T.R., Ricordi, C., and Inverardi, L. (2003). Long-term islet allograft survival in nonobese diabetic mice treated with tacrolimus, rapamycin, and anti-interleukin-2 antibody. Transplantation *75*, 1812–1819.

Muoio, D.M., and Newgard, C.B. (2008). Mechanisms of disease: molecular and metabolic mechanisms of insulin resistance and beta-cell failure in type 2 diabetes. Nat. Rev. Mol. Cell Biol. *9*, 193–205.

Nir, T., Melton, D.A., and Dor, Y. (2007). Recovery from diabetes in mice by beta cell regeneration. J. Clin. Invest. *117*, 2553–2561.

Okada, T., Liew, C.W., Hu, J., Hinault, C., Michael, M.D., Krtzfeldt, J., Yin, C., Holzenberger, M., Stoffel, M., and Kulkarni, R.N. (2007). Insulin receptors in beta-cells are critical for islet compensatory growth response to insulin resistance. Proc. Natl. Acad. Sci. USA *104*, 8977–8982.

Palumbo, P., and De Gaetano, A. (2010). An islet population model of the endocrine pancreas. J. Math. Biol. *61*, 171–205.

Paris, M., Bernard-Kargar, C., Berthault, M.F., Bouwens, L., and Ktorza, A. (2003). Specific and combined effects of insulin and glucose on functional pancreatic beta-cell mass *in vivo* in adult rats. Endocrinology *144*, 2717–2727.

Pechhold, K., Koczwara, K., Zhu, X., Harrison, V.S., Walker, G., Lee, J., and Harlan, D.M. (2009). Blood glucose levels regulate pancreatic beta-cell proliferation during experimentally-induced and spontaneous autoimmune diabetes in mice. PLoS ONE *4*, e4827. 10.1371/journal.pone.0004827.

Postic, C., Shiota, M., Niswender, K.D., Jetton, T.L., Chen, Y., Moates, J.M., Shelton, K.D., Lindner, J., Cherrington, A.D., and Magnuson, M.A. (1999). Dual roles for glucokinase in glucose homeostasis as determined by liver and pancreatic beta cell-specific gene knock-outs using Cre recombinase. J. Biol. Chem. *274*, 305–315.

Sachdeva, M.M., and Stoffers, D.A. (2009). Minireview: meeting the demand for insulin: molecular mechanisms of adaptive postnatal beta-cell mass expansion. Mol. Endocrinol. *23*, 747–758.

Sakai, A. (1970). Humoral factor triggering DNA synthesis after partial hepatectomy in the rat. Nature *228*, 1186–1187.

Stanger, B.Z. (2008). The biology of organ size determination. Diabetes Obes. Metab. *10* (*Suppl 4*), 16–22.

Terauchi, Y., Takamoto, I., Kubota, N., Matsui, J., Suzuki, R., Komeda, K., Hara, A., Toyoda, Y., Miwa, I., Aizawa, S., et al. (2007). Glucokinase and IRS-2 are required for compensatory beta cell hyperplasia in response to high-fat diet-induced insulin resistance. J. Clin. Invest. *117*, 246–257.

Tessem, J.S., Jensen, J.N., Pelli, H., Dai, X.M., Zong, X.H., Stanley, E.R., Jensen, J., and Degregori, J. (2008). Critical roles for macrophages in islet angiogenesis and maintenance during pancreatic degeneration. Diabetes *57*, 1605–1617.

Teta, M., Rankin, M.M., Long, S.Y., Stein, G.M., and Kushner, J.A. (2007). Growth and regeneration of adult Beta cells does not involve specialized progenitors. Dev. Cell *12*, 817–826.

Thorel, F., Nepote, V., Avril, I., Kohno, K., Desgraz, R., Chera, S., and Herrera, P.L. (2010). Conversion of adult pancreatic alpha-cells to beta-cells after extreme beta-cell loss. Nature *464*, 1149–1154.

Weinberg, N., Ouziel-Yahalom, L., Knoller, S., Efrat, S., and Dor, Y. (2007). Lineage tracing evidence for *in vitro* dedifferentiation but rare proliferation of mouse pancreatic beta-cells. Diabetes *56*, 1299–1304.

Xu, X., D'Hoker, J., Stange, G., Bonne, S., De Leu, N., Xiao, X., Van de Casteele, M., Mellitzer, G., Ling, Z., Pipeleers, D., et al. (2008). Beta cells can be generated from endogenous progenitors in injured adult mouse pancreas. Cell *132*, 197–207.