

c-Myc directly induces both impaired insulin secretion and loss of β -cell mass, independently of hyperglycemia in vivo

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Abbreviations: GSIS, glucose stimulated insulin synthesis; MOMP, mitochondrial outer membrane permeabilization

c-Myc (Myc) is a mediator of glucotoxicity but could also independently compromise β -cell survival and function. We have shown that after Myc activation in adult β -cells in vivo, apoptosis is preceded by hyperglycemia, suggesting glucotoxicity might contribute to Myc-induced apoptosis. To address this question conditional Myc was activated in β -cells of adult plns-c-MycER^{TAM} mice in vivo in the presence or absence of various glucose-lowering treatments, including exogenous insulin and prior to transplantation with wild-type islets. Changes in blood glucose levels were subsequently correlated with changes in β -cell mass and markers of function/differentiation. Activation of c-Myc resulted in reduced insulin secretion, hyperglycemia and loss of β -cell differentiation, followed by reduction in mass. Glucose-lowering interventions did not prevent loss of β -cells. Therefore, Myc can cause diabetes by direct effects on β -cell apoptosis even in the absence of potentially confounding secondary hyperglycemia. Moreover, as loss of β -cell differentiation/function and hyperglycemia are not prevented by preventing β -cell apoptosis, we conclude that Myc might contribute to the pathogenesis of diabetes by directly coupling cell cycle entry and β -cell failure through two distinct pathways.

Introduction

Death and dysfunction of β -cells contribute to essentially all major forms of diabetes; collectively this is often referred to as β -cell failure. Insulin biosynthesis and secretion are critical for pancreatic β -cell function, but both are impaired under diabetic conditions, and more recently it has become clear that apoptosis of β -cells is a major factor in the onset of type 2 (T2DM)¹ as well as of type 1 diabetes (T1DM). Although the causes of β -cell failure differ in key respects between T1DM and T2DM, there are also some common features, in particular continuing unsuccessful attempts to renew the missing β -cells or to adapt β -cell numbers to demand.^{2,3}

The Myc transcription factor is essential for successful growth and replication of essentially all cell types examined, but intriguingly, it is also associated with apoptosis (reviewed in ref. 4). This dual function of Myc involved in both replication and apoptosis, may be an important link between the need for β -cell mass adaptation/regeneration and apoptosis (reviewed in ref. 5). Activation of Myc induces β -cell apoptosis in vivo.⁶⁻⁹ Myc is well-known to sensitize various cell-types to a wide range of death triggers (e.g.,

hypoxia, DNA damage, depleted survival factors, CD95 death receptor), in-line with the notion that deregulated Myc possesses a highly effective tumor-suppressive mechanism to protect against carcinogenic progression.^{4,9} This is particularly pertinent given the suggestion that replicating β -cells may be particularly vulnerable to apoptosis,¹⁰ and supporting the notion that Myc could also sensitize β -cells to other potential death triggers. Moreover, upregulation of Myc in β -cells may be an inevitable accompaniment to β -cell replication in response to various triggers in human and rodent islets, including hyperglycemia¹¹⁻¹³ and expression of Pax4.¹⁴ Myc is downregulated in human differentiated β -cells, which are essentially quiescent, whereas Myc is expressed in replicating β -cells in development, during tumorigenesis and regeneration.¹⁴ In several different diabetic models, islets exposed to hyperglycemia display profound impairment in glucose-stimulated insulin secretion (GSIS) and loss of β -cell differentiation, in addition to the fact that hyperglycemia can cause increased expression of c-Myc.¹¹⁻¹³ In fact, this has led to the proposal that hyperglycemia-induced de-differentiation of β -cells is a critical factor leading to loss of insulin secretory function in diabetes. However, the means by which such loss of differentiation takes

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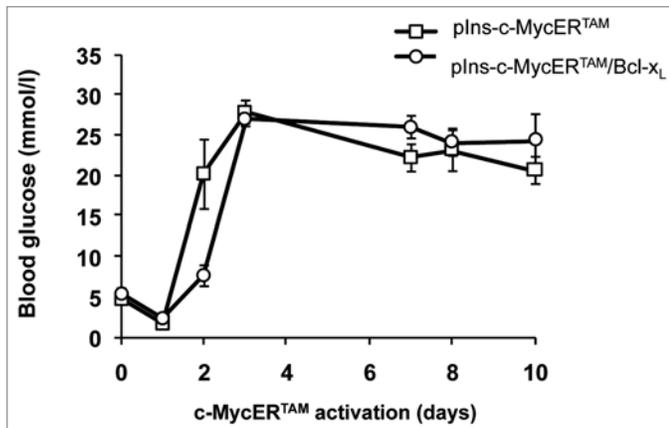


Figure 1. c-MycER^{TAM} activation in β -cells induces diabetes (hyperglycemia) even when apoptosis is prevented by Bcl-x_L. Non-fasted blood glucose levels (mmol/l) were measured from pIns-c-MycER^{TAM} (□) and pIns-c-MycER^{TAM}/Bcl-x_L (○) following activation of c-MycER^{TAM} by 4-OHT. Activation of c-MycER^{TAM} protein induces hyperglycemia in both pIns-c-MycER^{TAM} and pIns-c-MycER^{TAM}/Bcl-x_L by Day 2.

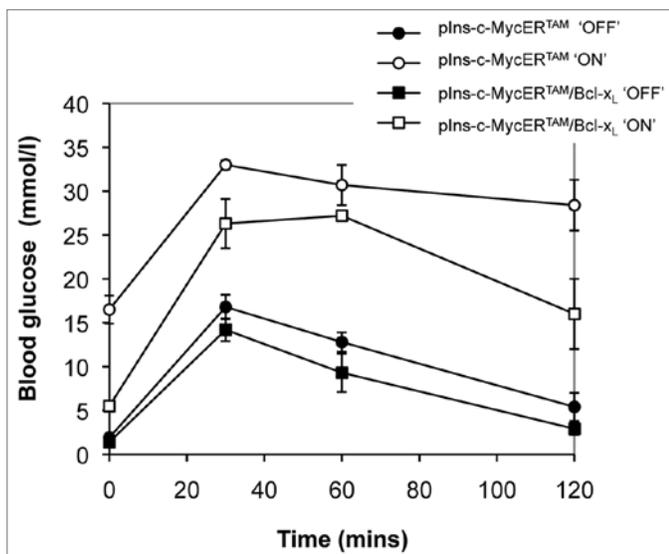


Figure 2. c-MycER^{TAM} activation causes impairment of glucose tolerance in pIns-c-MycER^{TAM} and pIns-c-MycER^{TAM}/Bcl-x_L mice. Mice were given an intraperitoneal glucose injection, during tolerance tests (IPGTT) (2 g/kg body weight). Blood glucose (sampled from tail tip) was monitored in Myc 'OFF' (Day 5) pIns-c-MycER^{TAM} (●), Myc 'ON' (Day 5) pIns-c-MycER^{TAM} (○), Myc 'OFF' (Day 13) pIns-c-MycER^{TAM}/Bcl-x_L (■) and Myc 'ON' (Day 13) pIns-c-MycER^{TAM}/Bcl-x_L mice (□) (n = 3).

place has remained less certain and in particular whether such loss of differentiation was due to raised glucose itself in some way or even directly through expression of Myc. Adenoviral expression of Myc in rat islets in vitro resulted in downregulation of insulin gene expression and impaired GSIS at least in part by interfering with NeuroD activity.¹⁶

We and others have shown that β -cell apoptosis and loss of β -cell differentiation in vivo are observed in transgenic mice

overexpressing Myc in β -cells during embryogenesis⁷ and following activation of Myc in conditional mouse models in adult β -cells.^{6,8} However, certain key issues remain unanswered and are critical to a better understanding of human diabetes, a disease in which β -cells die or malfunction due to various factors during adult life. Firstly, since the onset of hyperglycemia precedes β -cell apoptosis, does β -cell loss result directly and solely from activation of Myc, or is hyperglycemia (glucotoxicity)-contributory? Secondly, if apoptosis of β -cells were prevented, could diabetes secondary to Myc be avoided? To address these issues, we used 'switchable' pIns-c-MycER^{TAM} mice.^{6,17} In these mice, Myc activation in adult β -cells in vivo results in both rapid onset of β -cell proliferation and apoptosis.

We show that hyperglycemia does not contribute to Myc-induced β -cell apoptosis: insulin treatment or islet transplantation in pIns-c-MycER^{TAM} mice, succeeded in controlling glycaemia during Myc activation, but did not prevent nor reduce the loss in islet and β -cell mass. Thus, in the absence of potentially confounding secondary effects of glycaemia, activation of c-Myc still leads to β -cell apoptosis.

Results

c-MycER^{TAM} activation induces impaired glucose homeostasis in pIns-c-MycER^{TAM} mice. As expected, diabetes was induced by c-MycER^{TAM} activation by Day 2. Blood glucose levels increased at least by five-fold by Day 3 of c-MycER^{TAM} activation and remained high at least until Day 10 (Fig. 1, white square n = 3). pIns-c-MycER^{TAM} mice treated with vehicle alone had normal blood glucose levels at an average of 4.4 ± 0.2 mmol/l (data not shown). Diabetes was confirmed by IPGTT (Fig. 2) and area under curve (AUC) values of glucose response, were found to be different between Myc-activated and control pIns-c-MycER^{TAM} mice (t test p = 0.01; circles). Interestingly, activation of c-MycER^{TAM} caused a transient period of paradoxical hypoglycaemia extending through the first 24 hours (Fig. 1; white square).

To confirm that this was not a result of passive insulin leakage due to early initiation of β -cell apoptosis, we showed that similar hypoglycaemia occurs following Myc activation even when apoptosis is completely prevented in pIns-c-MycER^{TAM}/Bcl-x_L mice.⁶ Activation of c-MycER^{TAM} as above, caused pIns-c-MycER^{TAM}/Bcl-x_L mice to display transient hypoglycaemia similarly to pIns-c-MycER^{TAM} (Fig. 1; white circle).

Moreover, as previously shown, we confirm by IPGTT that Myc activation can induce hyperglycemia even in the absence of apoptosis (Fig. 2; squares). Thus, despite complete prevention of β -cell apoptosis, the onset of diabetes in pIns-c-MycER^{TAM}/Bcl-x_L mice occurred after 2 days of c-MycER^{TAM} activation (Fig. 1). Beyond Day 2, blood glucose levels in the double-transgenic mice increased in a similar manner to pIns-c-MycER^{TAM} (pIns-c-MycER^{TAM} vs. pIns-c-MycER^{TAM}/Bcl-x_L; t test p = 0.824) (Fig. 1). Following 10 days of c-MycER^{TAM} activation, hyperglycemia reached an average of 17.9 mmol/l \pm 2.0 (n = 9). When Myc was inactive, pIns-c-MycER^{TAM}/Bcl-x_L mice showed blood glucose levels at 4.4 mmol/l \pm 0.4 (n = 3).

Activation of c-MycER^{TAM} in β -cells causes deregulated insulin release. To investigate the decrease in blood glucose within 24 hrs of c-MycER^{TAM} activation, we measured serum insulin levels in pIns-c-MycER^{TAM}. Within 24 hours after c-MycER^{TAM} activation, serum insulin levels doubled in pIns-c-MycER^{TAM} when compared to untreated mice (t test $p = 0.768$; Fig. 3; Myc 'OFF' vs. MYC 'ON' Day 1) accounting for the transient decrease in blood glucose level on Day 1. However, following 5 days of c-MycER^{TAM} activation, serum insulin levels dropped profoundly by over 6-fold, a change that would account for the onset of hyperglycemia as shown on Figure 1 ($p = 0.0155$).

These results indicate that c-MycER^{TAM} activation in β -cells causes deregulated insulin release, with an early response (within 24 hours) of excessive insulin secretion concomitant with hypoglycaemia, followed by markedly decreasing insulin release from β -cells, resulting in hyperglycemia. Similar data on the decrease of serum insulin levels were seen in pIns-c-MycER^{TAM}/Bcl-x_L mice, where apoptosis is prevented (data not shown).

c-MycER^{TAM} activation promotes loss of expression of β -cell differentiation markers. To investigate how c-MycER^{TAM} activation might promote β -cell dysfunction in relation to the development of diabetes, pancreata from pIns-c-MycER^{TAM} mice treated with vehicle (Myc 'OFF') or 4-OHT (Myc 'ON') for 1 (D1; prior to Myc-induced apoptosis) and 5 days (D5) were analysed immunohistochemically for insulin, glucagon and markers of β -cell differentiation. Immunofluorescent staining for insulin, glucagon, Glut-2 and Pdx-1 was intense in Myc 'OFF' pancreata (Fig. 4; D0). However, the intensity of staining for insulin, Glut-2 and Pdx-1 was reduced within one day of c-MycER^{TAM} activation (Fig. 4; D1). Continuous activation of c-MycER^{TAM} for 5 days resulted in islet involution, accompanied by low expression levels of insulin, Glut-2 and Pdx-1 in the remaining β -cells (Fig. 4; D5). Glucagon expression was predominately localized around the periphery of the islets in Myc 'OFF' and Myc 'ON' Day 1 (Fig. 4; D0 and D1 respectively). However, by Day 5 of c-MycER^{TAM} activation, the majority of remaining cells in the involuted islets were mainly glucagon-positive (Fig. 4; D5).

Lack of β -cell apoptosis in pIns-c-MycER^{TAM}/Bcl-x_L mice. Insulin expression was downregulated following 14 days c-MycER^{TAM} activation (Fig. 5B) in pIns-c-MycER^{TAM}/Bcl-x_L mice compared to MYC 'OFF' (Fig. 5A). Glucagon expression in this islet is distributed in the core of the islet, unlike for Myc 'OFF' which is in the periphery, presumably resulting from β -cells growing out from the original islet. Similarly to pIns-c-MycER^{TAM}, a decrease in the expression levels of Glut-2 and Pdx-1 in pIns-c-MycER^{TAM}/Bcl-x_L mice was observed following c-MycER^{TAM} activation for 14 days (not shown). TUNEL analysis of pIns-c-MycER^{TAM}/Bcl-x_L mice show a lack of β -cell apoptosis following c-MycER^{TAM} activation for 14 days (Fig. 5C) demonstrating downregulation of insulin expression without loss of β -cells. An insulin-negative apoptotic cell can also be observed. DNase treatment of a Myc 'OFF' pancreatic section was included as a TUNEL positive control (Fig. 5D).

Hyperglycemia does not contribute to Myc-induced β -cell apoptosis. Correction of glycaemia in pIns-c-MycER^{TAM} mice by insulin treatment. As hyperglycemia is itself known to promote

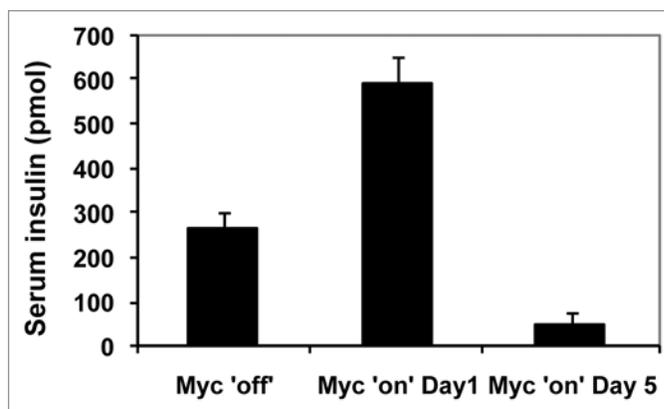


Figure 3. Activation of c-MycER^{TAM} in β -cells causes deregulated insulin release. Serum insulin levels (pmol/l) were monitored in pIns-c-MycER^{TAM} mice, when c-MycER^{TAM} was inactive (Myc 'OFF'; Day 0) or following c-MycER^{TAM} activation for 1 or 5 days (Myc 'ON'; Day 1 or 5, respectively). Data are presented as means of at least 3 replicates \pm SEM. Activation of c-MycER^{TAM} results in the release of over twice the concentration of insulin in the blood following one day's activation when compared to untreated pIns-c-MycER^{TAM}. Insulin concentration is greatly reduced by over five-fold following 5 days' activation of c-MycER^{TAM} in pIns-c-MycER^{TAM}.

β -cell apoptosis,^{1,18,19} we aimed to prevent or limit hyperglycemia to investigate whether c-Myc induced β -cell apoptosis was reduced or even prevented.

To determine whether potential replacement by exogenous insulin could correct high glucose levels and restrict Myc-induced β -cell death, hyperglycemia was corrected in pIns-c-MycER^{TAM} mice by insulin administration. Average blood glucose readings in insulin-treated transgenic mice were close to normal, although some brief periods of hyperglycemia and hypoglycaemia were unavoidable (Table 1). To control blood glucose more efficiently, and thus exclude the possibility that transient hypoglycaemic or high glucose excursions might themselves contribute to Myc-induced apoptosis, a separate group of pIns-c-MycER^{TAM} mice received islet transplants from normal wild type mice prior to Myc activation.

Islet transplantation. pIns-c-MycER^{TAM} mice that received islet grafts (reviewed in ref. 20) from wild-type donor mice, maintained average blood glucose levels below 11.0 mmol/l (Fig. 6; white triangle). Islet transplantation maintained normal blood glucose levels when Myc was 'ON' for 5 days (white circle) compared to mice not receiving grafts ($p = 0.0389$) (white square). There was no significant difference in blood glucose levels when Myc was inactive ($p = 0.082$).

AUC analyses on glucose response curves (IPGTT; Fig. 6B) showed, as expected, that there was a statistically significant difference between Myc 'ON' Day 5 (white square) and control, vehicle-treated, Myc 'OFF' Day 5 animals (black square) ($p = 0.01$). There was also a significant difference between Day 5 Myc 'ON' mice receiving islet transplants (white circle) and those without (white square) ($p = 0.015$). However, there was no difference in IPGTT response due to islet transplantation per se, in the absence of Myc activation ($p = 0.098$).

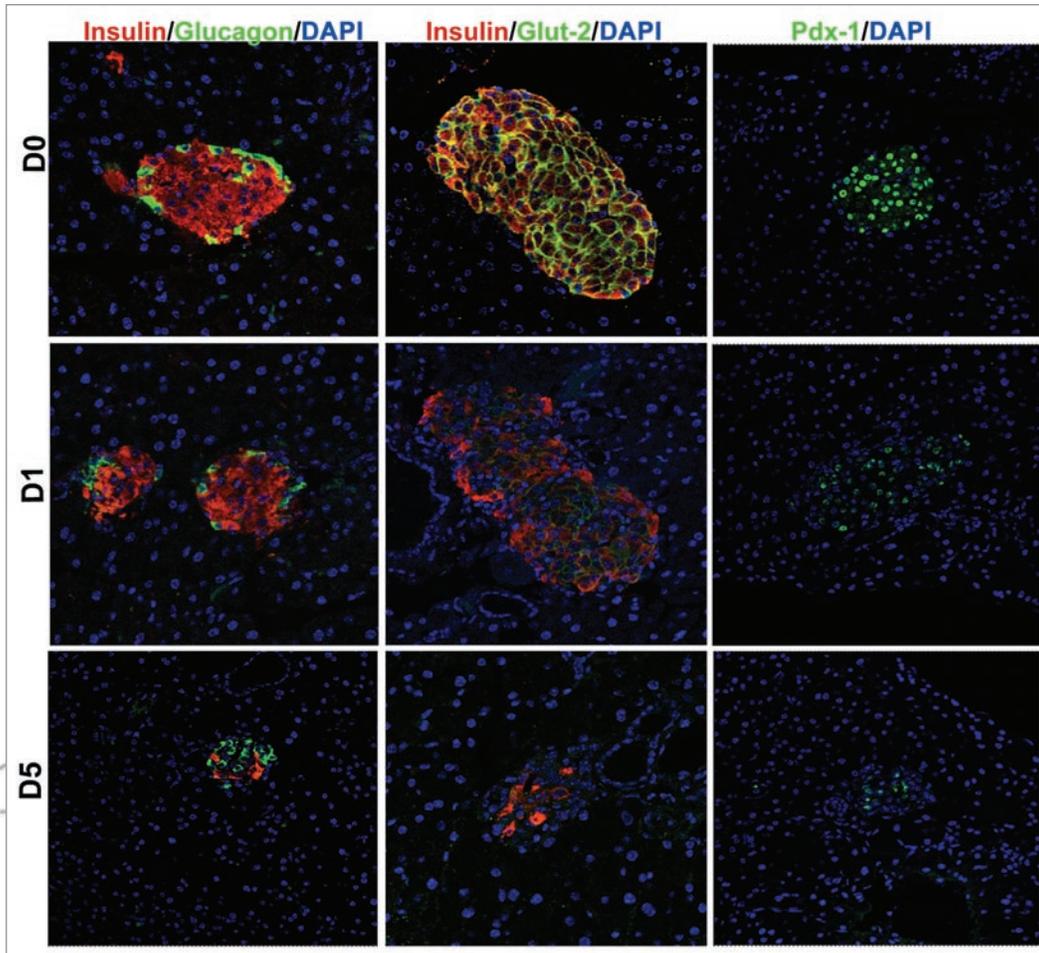


Figure 4. c-MycER^{TAM} activation promotes loss of expression of β -cell differentiation markers in p-Ins-c-MycER^{TAM} mice. Pancreata were examined both before (D0) and following c-MycER^{TAM} activation for 1 or 5 days (D1 or D5 respectively). Protein expression levels are shown for Insulin (red), Glucagon (green), Glut-2 (green), Pdx-1 (green); cell nuclei are stained by DAPI (blue) (x400 obj.).

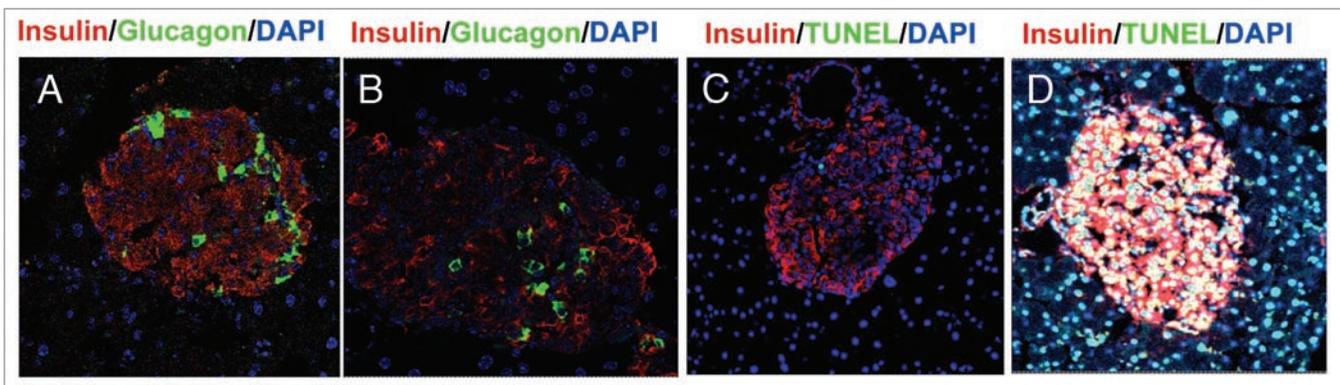


Figure 5. c-MycER^{TAM} activation in plns-c-MycER^{TAM}/Bcl-x_L mice results in a decrease in insulin expression in the absence of β -cell apoptosis. Insulin (red) and Glucagon (green) are shown on (A and B), stained for Days 0 (Myc 'OFF') and Day 14 (Myc 'ON'), respectively. (C) A representative TUNEL-stained islet, 14 days after c-MycER^{TAM} activation, showing no apoptotic β -cells, counter-stained with insulin (red). In most islets, no apoptotic cells were seen, and the occasional ones were insulin-negative, as here. (D) Positive control for apoptotic cells following DNase treatment of a Myc 'OFF' pancreatic section. All sections show DAPI-stained nuclei (blue). Images were taken at either x400 (A and B) and x200 (C and D) magnification.

Loss of β -cell mass even when glycemia is corrected. Morphometric studies show that following 5 days of Myc activation in pIns-c-MycER^{TAM} mice (Myc 'ON'), islet mass and β -cell mass decreased by 80% and 90% respectively compared to Myc 'OFF' control mice (100%) ($p = 0.039$ and $p = 0.0011$, respectively) (Fig. 7). Following either insulin treatment or islet transplantation there was no difference in the extent of loss of mass of either islets ($p = 0.939$ and $p = 0.056$ respectively) or β -cells ($p = 0.727$ and $p = 0.206$, respectively), compared to untreated Myc 'ON' mice. Thus, correction of insulin and glucose levels by both exogenous insulin therapy and with supplementation of endogenous insulin by grafting of wild type islets, does not prevent β -cell apoptosis when Myc is activated.

Discussion

Myc has previously been implicated in β -cell failure in diabetes.^{21,22} β -cell apoptosis is induced in cultured islets exposed to high glucose concentrations, which importantly, is accompanied by increased expression of Myc.²² We and others have also shown that activation of Myc in β -cells in vivo, using a regulated/controlable MycER mouse model can induce β -cell apoptosis that overwhelms proliferation resulting in islet involution and diabetes.^{6,8} However, one key issue here is whether hyperglycemia (glucotoxicity) in fact plays a contributory role to Myc-induced β -cell apoptosis, or does β -cell loss result solely from activation of Myc. Another key issue is whether the onset of diabetes is primarily due to loss of β -cells following Myc activation, or does Myc cause β -cell failure if apoptosis is prevented. These questions are of more than academic interest. Myc has been repeatedly shown to act as a potential mediator of glucotoxicity, a major contributor to β -cell failure in Type 2 diabetes, and several studies suggest that replicating β -cells may be particularly vulnerable to apoptotic insults¹—providing one potential mechanism by which islets adapting to increasing insulin demands may be more than usually vulnerable to environmental stresses.

To address these issues, we have used two mouse strains, the pIns-c-MycER^{TAM} and pIns-c-MycER^{TAM}/Bcl-x_L which both express a switchable form of Myc (c-MycER^{TAM}) in β -cells of adult mice, with the latter also expressing the anti-apoptotic protein Bcl-x_L in β -cells.

We have previously shown and extend those findings here, that activation of c-MycER^{TAM} in the β -cells of pIns-c-MycER^{TAM}/Bcl-x_L induces diabetes similar to that observed in pIns-c-MycER^{TAM}, despite complete prevention of β -cell apoptosis, indicating that c-Myc-induced β -cell apoptosis is not a pre-requisite for the development of diabetes. Rather, it would seem that Myc activation is compromising function and differentiation, supported by the findings that expression of insulin and various factors required for β -cell differentiation/function such as Pdx1 and the glucose

Table 1. Blood glucose levels (mmol/l) during correction of hyperglycaemia using insulin treatment as described in materials and methods

Days of Myc activation	Blood glucose (mmol/l) \pm SEM	
	Myc 'ON' n = 6	Myc 'ON' + insulin n = 3
0	6.0 \pm 0.6	3.6 \pm 0.7
2	16.6 \pm 3.8	5.0 \pm 1.2
3	27.0 \pm 1.9	6.2 \pm 2.3
4	24.7 \pm 1.1	2.7 \pm 1.4
5	24.0 \pm 1.7	2.6 \pm 1.1

The average blood glucose during the fed state, was recorded each day during Myc activation +/- insulin. SEM (\pm) and n numbers are shown for each group of mice.

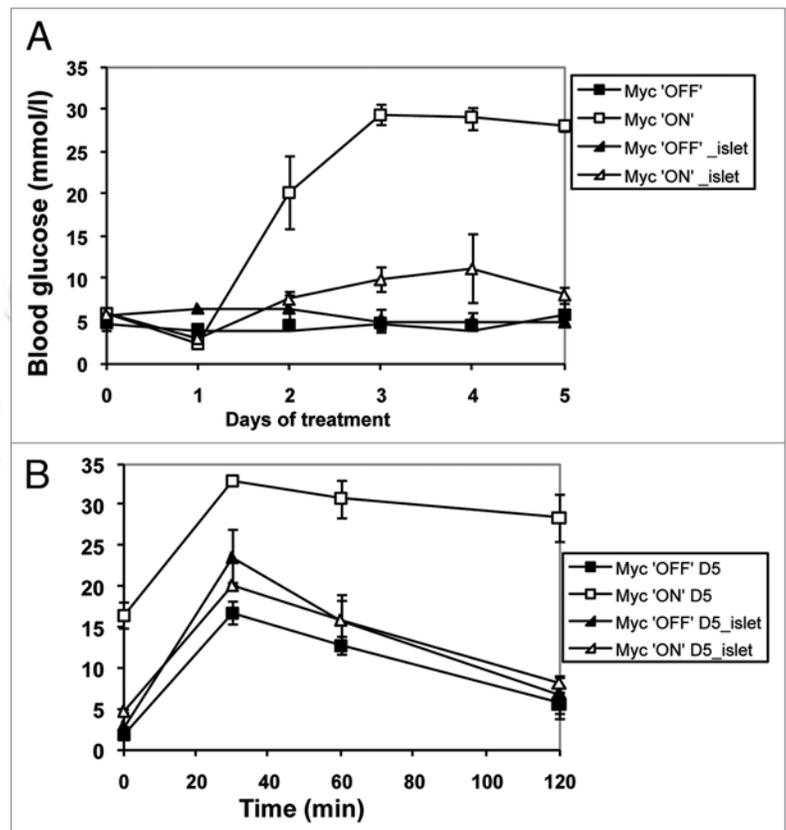


Figure 6. Correction of hyperglycemia via islet transplantation. (A) Blood glucose levels (mmol/l) in p-Ins-c-MycER^{TAM} mice treated with vehicle (n = 3; Myc 'OFF', ■), 4-OHT (n = 3; Myc 'ON', □), vehicle and islet transplantation (n = 2; Myc 'OFF' _islet, ▲) and 4-OHT combined with islet transplantation (n = 2; Myc 'ON' _islet, △). (B) IPGTT results, as shown by blood glucose concentration, after IP glucose challenge in Myc 'OFF' D5; ■, Myc 'ON' D5; □, Myc 'OFF' D5 _islet; ▲ and Myc 'ON' D5 _islet; △.

transporter Glut-2, decreased progressively following activation of c-MycER^{TAM}.

Furthermore, changes observed in islet architecture as shown by localisation of glucagon-positive cells are in agreement with previously published immunohistochemistry data on the same mouse model.⁸

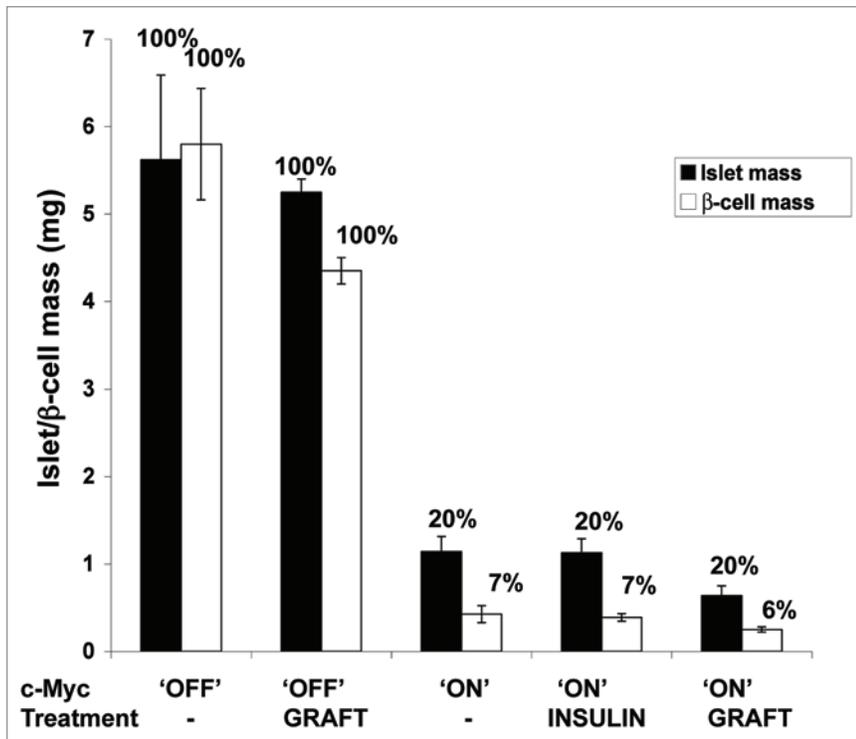


Figure 7. Hyperglycemia does not contribute to Myc-induced β -cell apoptosis. Mean values of islet and β -cell mass in plns-c-MycER^{TAM} treated with vehicle (Myc 'OFF') or 4-OHT (Myc 'ON') alone or with treatment; either exogenous insulin or islet graft transplantation. Numbers show the % in islet and β -cell mass, when 100% represents the levels prior to c-Myc activation (Myc 'OFF'). The following were compared statistically: Myc 'ON' Day 5 vs. Day 5 islet grafts; Day 5 Myc 'ON' vs. Day 5 insulin treatment. Morphometric analysis of islet and β -cell area shows that attempting to control hyperglycemia using insulin or islet grafts in plns-c-MycER^{TAM} (Myc 'ON') does not reduce or prevent the loss in islet and β -cell mass. At least n = 3 for all groups of mice, except for Myc 'OFF'/islet grafts and Myc 'ON'/islet grafts, where n = 2.

More importantly, we confirm our previously published work on the plns-c-MycER^{TAM} mouse system⁶ and expand these findings by investigating the coupling of cell cycle progression and β -cell failure as two independent causes of diabetes. We further explore the potential reversibility of β -cell failure due to hyperglycemia, by using long-acting exogenous insulins or islet grafts.

Our data also show that downregulation of Insulin, Glut-2 and Pdx-1 expression predicts the onset and maintenance of hyperglycemia indicating that the effects of c-Myc in the absence of apoptosis is sufficient to induce diabetes. Previously, loss of β -cell differentiation through downregulation of Glut-2 mRNA was demonstrated in a different model overexpressing c-Myc.⁷ Our present data are on plns-c-MycER^{TAM} and additional data on plns-c-MycER^{TAM}/Bclx₁ (not shown) are in agreement with previous work on the same mouse systems, showing decreased expression levels of Pdx-1 and Glut-2.^{8,23}

Near-normal correction of hyperglycemia by insulin treatment or islet transplantation did not reduce or prevent c-MycER^{TAM}-induced β -cell apoptosis. The loss in islet and β -cell mass in insulin-/islet-transplanted groups of mice, occurred similarly to the group of hyperglycaemic, untreated mice, indicating that β -cell apoptosis results directly and solely from activation

of c-MycER^{TAM} and not from hyperglycemia (glucotoxicity). This view is compatible with a recent study by Pascal et al.²³ showing that activation of c-Myc in cultured islets induces β -cell apoptosis that more closely resembles oxidative stress than exposure to high glucose.

It is known that expression of Myc can sensitize cells to a wide range of apoptotic stimuli by inducing mitochondrial outer membrane permeabilisation (MOMP) and releasing cytochrome *c*, and other proteins, from the mitochondrial inter-membrane space into the cytosol.^{4,5} Myc cooperates with several other pro-apoptotic signals to induce cell death, by downregulating anti-apoptotic BCL-2 family members such as Bcl-x_L, directly or via induction of BH3 only proteins such as BIM, thereby favoring MOMP, particularly in the presence of other signals which activate BAX.²⁵ A recent study showed that Myc induces apoptosis primarily via BAX in β -cells.⁹ In fact, in the absence of Myc the ability to activate BAX and MOMP is severely curtailed.²⁶ Oncogenic Myc, may also trigger apoptosis alone through the ARF-p53 pathway or via DNA damage responses.^{27,28}

Although we achieved near normal correction of hyperglycemia, it is possible that even brief exposure to hyperglycemia, in particular in the insulin treated mice, would be sufficient to induce loss of islet and β -cell mass. Importantly, during our islet transplantation experiment we observed that although blood glucose as low as 8.5 mmol/l was achieved, over 90% of β -cell mass was lost, similarly to hyperglycaemic mice that did not receive transplants. This result is consistent with the hypothesis that c-Myc alone is sufficient to cause β -cell apoptosis. This view is also supported by an earlier study using transplanted pancreata from transgenic mice constitutively overexpressing c-Myc.⁷ Following 6 weeks post-transplantation under the kidney capsule of normal nude mice, no islets were recovered. Given that these mice would have had normal blood glucose, loss of the transplanted islets in their ectopic location was likely at least in part due to sustained Myc activation.

Recent work, suggests that the level of Myc activation may in part determine whether Myc engages just the growth/replication program or also the pro-apoptotic tumor-suppressor one.²⁹ However, we have previously shown that Myc levels are not substantially higher in islets of plns-c-MycER^{TAM} transgenic mice than in normal replicating tissues.⁶ Myc protein activation is likely more persistent than that expected in normal replicating β -cells. Thus, it is possible that more pulsatile Myc expression may be a less powerful inducer of β -cell apoptosis. Certainly, the absence of diabetes may now be excluded as an explanation for the ability of Myc-deregulated β -cells to avoid apoptosis during

progression of insulinomas. As to the importance of Myc-induced apoptosis in diabetes, it will be interesting to see whether future studies knocking down Myc expression in diabetes models, such as the Db/Db mouse, might prevent or reduce the extent of β -cell apoptosis *in vivo*.

We and others have previously demonstrated that activation of c-MycER^{TAM} in β -cells of adult pIns-c-MycER^{TAM} transgenic mice induces β -cell apoptosis that overwhelms proliferation, resulting in islet involution and ultimately diabetes.^{6,8} Interestingly, within 24 hours of c-MycER^{TAM} activation, pIns-c-MycER^{TAM} mice displayed transient hypoglycaemia. Despite low glucose levels, insulin concentration doubled in the serum, suggesting aberrant release of insulin. It is unlikely that hypoglycaemia resulted from unregulated insulin release from apoptosing β -cells, as suggested by Cano et al.⁸ given that hypoglycaemia also occurs almost identically in pIns-c-MycER^{TAM}/Bcl-x_L mice, in which apoptosis is completely prevented due to overexpression of Bcl-x_L (as demonstrated by our current study). In fact it is possible that, the increased serum insulin levels which result in hypoglycaemia, arise through active release of stored insulin from the β -cells. This view is supported by the consistently reduced levels of insulin immunoreactivity observed in islets after 1–2 days following activation of c-MycER^{TAM}.

Furthermore, Laybutt et al.⁷ showed the presence of only a few β granules in the cytoplasm of their transgenic mice, which overexpress c-Myc continuously. The mechanism by which c-Myc induces release of insulin remains to be determined. However, we know that insulin secretion by β -cells is a highly regulated process involving the uptake of glucose with closure of ATP-dependent K-channels, depolarisation of the cell membrane and an elevation in intracellular free Ca²⁺ [Ca²⁺]_i concentration through depolarized-activated Ca²⁺ channels which stimulates the exocytosis of insulin granules.^{30–32} A recent study showed that c-MycER^{TAM} increases islet resting [Ca²⁺]_i in the islets from pIns-c-MycER^{TAM} mice.²⁴ Deficiency in Foxa2 (formerly HNF3 β) may induce excessive insulin release and also impair GSIS.³³ Whether, Foxa2 is involved in Myc action remains to be determined.

Myc can cause β -cell apoptosis in the absence of hyperglycemia, suggesting, that glucotoxicity is not required for Myc-induced β -cell apoptosis *in vivo*. Moreover, even in the absence of apoptosis Myc activation results in a biphasic effect on insulin secretion (data not shown), comprising an initial increase with hypoglycaemia, which rapidly gives way to reduced insulin secretion and hyperglycemia. This suggests two powerful and independent means by which β -cell replication and β -cell failure may potentially be coupled in the pathogenesis of diabetes. This study also raises interesting questions regarding the prevention of β -cell loss in diabetes-correction of glucose levels per se may not suffice to prevent β -cell apoptosis driven by mitogenic proteins such as c-Myc, but rather may require reducing the drive to replicate. Therefore, we conclude that c-Myc is a potentially important mediator of both β -cell apoptosis and impaired insulin secretion and should be considered as a potential therapeutic target in diabetes.

Materials and Methods

Transgenic mice. pIns-c-MycER^{TAM} transgenic mice were generated by cloning a full-length human c-myc cDNA fused to the hormone binding domain of a modified estrogen receptor (c-MycER^{TAM}) downstream of the rat insulin promoter, as described previously.⁶ pIns-c-MycER^{TAM}/Bcl-x_L mice were obtained by crossing both pIns-c-MycER^{TAM} and RIP7-Bcl-x_L transgenic mice (from D. Hanahan, UCSF). Adult female and male mice (MycER, MycER/Bcl-x_L and islet donors), inbred into CBA-C57Bl/6 background and maintained under barrier conditions. The animals were housed with a 12 hour light/dark cycle and access to food and water *ad libitum*. All protocols conformed with the Home Office (UK) laws and regulations, and transgenic mice were registered with the University of Warwick, Biological Sciences Committee for Genetically Modified Experimental Animal Strains.

Activation of c-MycER^{TAM}. Activation of c-MycER^{TAM} in pancreatic β -cells of 10–15 weeks old pIns-c-MycER^{TAM} (with or without insulin treatment/islet transplants) and 6–10-week old pIns-c-MycER^{TAM}/Bcl-x_L transgenic mice were carried out by daily intra-peritoneal (IP) administration of 4-hydroxytamoxifen (4-OHT) or Tamoxifen sonicated in peanut oil (1 mg in 0.1 or 0.2 ml). Control pIns-c-MycER^{TAM} mice (with or without insulin treatment/islet transplants) were injected with vehicle (0.1 or 0.2 ml peanut oil without 4-OHT). Non-fasted blood glucose concentrations were sampled daily from tail bleeds using an Accu-Chek Advantage glucose meter (Roche Diagnostics, Burgess Hill, UK).

Insulin treatment. To maintain normal or near-normal glycaemia, transgenic mice (n = 3) were injected subcutaneously (SC) as and when needed, with either insulin Levemir (Novo Nordisk, Denmark); ~25 mU/g body weight, once daily, or Insulin Lantus (Sanofi Aventis, NJ, USA); ~25 mU/g body weight, twice daily. Blood glucose was monitored as described above, four times daily, including after lights were switched off when feeding begins (8–9 pm) and mornings (8–9 am) when blood glucose levels were most likely to be high.

Islet transplantation. In order to control hyperglycemia in pIns-c-MycER^{TAM}, normal islets were transplanted from wild-type littermates under the kidney capsule. The β -cells in these islets do not express the conditional Myc transgene and were thus unaffected by 4-OHT and were able to maintain normal blood glucose levels despite Myc activation in endogenous β -cells. Pancreatic islets were isolated from adult wild-type pancreata as described previously with slight modification.⁶ In particular, for each pancreas, a 2.5 ml of sterile, ice-cold Hanks Balanced Salt Solution (HBSS), pH 7.4, containing 20 mM HEPES, penicillin (10,000 IU/ml), streptomycin (10,000 μ g/ml) and collagenase (1 mg/ml; Type V; Sigma-Aldrich, Gillingham, UK), was distended into the pancreatic duct. The pancreas was dissected in HBSS and islets were handpicked, kept in groups of less than 150 islets and cultured free-floating for 3–4 days in 10 ml RPMI 1640, pH 7.4, containing 10 mmol/L glucose and supplemented with L-glutamine, penicillin (10,000 IU/ml) streptomycin (10,000 μ g/ml) and 10% (v/v) foetal calf serum at 37°C in

a humidified atmosphere of 5% CO₂. At transplantation, 400 islets were packed in a braking pipette and implanted into the left renal subcapsular space of four mice, as previously described.²⁰ Syngeneic ketamine-xylazine-anaesthetised (75 mg/kg BW i.p.; Sigma-Aldrich) pIns-c-MycER^{TAM} mice were used as recipients. These mice were given a subcutaneous dose of Carprofen (4 mg/kg; from local vet) post transplantation and left for two weeks to recover from the operation before treatment with 4-OHT or peanut oil.

Glucose tolerance test (IPGTT). Following a 16 hour fast, animals were given a single intraperitoneal injection of 30% D-glucose in PBS (2 g/kg body weight). Blood glucose was monitored prior to and at 30, 60 and 120 minutes after glucose administration using an Advantage glucose meter (Roche Diagnostics, Burgess Hill, UK).

Insulin assay. Insulin concentrations in serum were determined using an ELISA according to the manufacturer's instructions (Merckodia, Uppsala, Sweden).

Tissue processing. Animals were culled by cervical dislocation and the pancreata were excised, accurately weighed and immediately fixed in 4% fresh paraformaldehyde/PBS (TAAB, Aldermaston, UK) at room temperature for 2 hours, then transferred to 30% sucrose solution at 4°C overnight. The tissues were embedded in TissueTek OCT medium (Raymond Lamb, Eastbourne, UK), frozen on dry ice and ethanol. Cryo-sections of the pancreata were taken through the fixed tissues in the plane of embedding so that a near complete section of pancreas (head, body and tail) was obtained with each section. Tissues, sectioned at 8–10 μm were mounted on poly-Lysine coated glass slides (VWR International Ltd., Lutterworth, UK), and stored at -80°C until analysed.

Histology and immunohistochemistry. For immunohistochemistry, primary antibodies were anti-guinea pig insulin (1:100; Dako Cytomation, Ely, UK), Pdx-1 (1:1,000) (Abcam, Cambridge, UK), Glucagon (1:500) and Glut-2 (1:500) from Santa Cruz Biotechnology (Insight Biotech, Wembley, UK). Methods were followed as previously described.^{6,19} Briefly, primary antibodies were incubated for 1 hour at room temperature, followed by FITC- and/or Alexa 633-conjugated secondary antibodies (1:200; Dako). For TUNEL apoptotic studies, ApopTag Fluorescein Direct In Situ Apoptosis Detection Kit was used (CHEMICON Int./Millipore, Watford, UK), according to the manufacturer's

protocol. Positive control samples were treated with DNase I to destruct DNA. Finally, mounting was performed using DAPI-containing media (VECTOR Labs Ltd., Peterborough, UK) and the sections were visualized using a Leica confocal microscope and software. All sections were observed and images were captured using x400 objective.

Estimation of islet and β-cell mass. Morphometric analysis was performed using a laser capture microscope (Nikon UK Ltd., Kingston-upon-Thames, UK) at a magnification of x40 (for whole cross-sectional area of pancreatic tissue) or x200 (for islet- and insulin-positive β-cell area). Pancreatic, islet or β-cell area were determined by outlining and measuring the region of interest using the MMI CELLTOOLS software (version 2.0 software; MMI Molecular Machines & Industries, Olympus UK, Southall, UK). For this purpose, series of tissue sections (at least 30 sections apart) were stained with either haematoxylin/eosin (for islet mass) or insulin/haematoxylin (DAB staining; for β-cell mass). The islet and β-cell mass were estimated by calculating the mean percentage islet area and area of the islets immunoreactive for insulin per sectional area of pancreas (six to ten levels per pancreas), respectively. This was expressed as milligram by multiplying the mean percentage islet and β-cell area by the pancreatic weight for the individual animals.

Statistical analysis. SEM means were calculated for all experimental data and where appropriate, two-tailed Student's t test or chi square (χ²) test (Microsoft Excel 2003) were used to compare means of different groups for n = 3 replicates per group of mice, in c-MycER^{TAM} mice. For IPGTT, AUC was estimated using Prism 4.0 (University of Warwick), in addition to SEM, followed by a Student's t test. p values were considered significant when ≤0.01.

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