Phosphatase and tensin homologue (PTEN) deleted on chromosome 10 is a dual-specific phosphatase and a potent antagonist of the phosphoinositide 3-kinase signaling pathway. Although first discovered as a tumor suppressor, emerging evidence supports PTEN as a potential therapeutic target for diabetes. PTEN deletion in β cells leads to increased β cell mass and protection from streptozotocin-induced diabetes. Importantly, PTEN deletion does not lead to tumor formation in β cells. To further assess the potential tumorigenic role of PTEN, we tested the biological role of PTEN in the context of activation of the proto-oncogene c-Myc. We generated and characterized β cell-specific PTEN knock-out mice expressing an inducible c-Myc transgene in β cells. Surprisingly, we found that PTEN loss did not confer protection from the overwhelming apoptosis and diabetes development seen with c-Myc activation. Importantly, despite the combined effect of the loss of a tumor suppressor and activation of an oncogene in β cells, there was no evidence of tumor development with sustained c-Myc activation.

Although PTEN was initially discovered as a tumor suppressor, more recent in vivo studies have highlighted its role in metabolism. Tissue-specific PTEN ablation in peripheral insulin target tissues such as fat, muscle, and liver generally led to improved insulin sensitivity (4–7). Additionally, in pancreatic β cells, PTEN played a role in β cell size determination and islet mass (8, 9). The loss of PTEN in β cells led to enhanced β cell mass without compromise in function. In addition, PTEN loss conferred protection from streptozotocin-induced diabetes (8, 9). Importantly, despite the loss of this tumor suppressor, there was no evidence of tumor formation in β cells.

PTEN deletion and concomitant c-Myc activation do not lead to tumor formation in pancreatic β cells.

**Experimental Procedures**

Mouse Protocol—RIPcre+/Pten+/- mice, expressing the Cre recombinase under the control of the rat insulin promoter, were generated as described previously (9). Myc-EERTAM (Myc+) transgenic mice were generated as described previously (13). Myc+ mice were bred to RIPcre+/Pten+/- mice to generate Myc+RIPcre+/Pten+/- mice, and these mice were intercrossed.
to generate Myc<sup>+/RIP<sub>cre</sub>Pten<sup>+/−</sup></mbox> mice. A small number of Myc<sup>−/−</sup> mice were also produced using this breeding strategy. Control mice for all experiments were Myc<sup>−/−</sup>RIP<sub>cre</sub>Pten<sup>+/−</sup> or Myc<sup>−/−</sup>RIP<sub>cre</sub>Pten<sup>+/+</sup> littermates. Both male and female mice were used for experiments as there were no sex-related differences. Mice were maintained on a mixed background. The activity of the mice was not restricted, and they were maintained on a 12-h light/dark schedule. All mice were fed regular chow (Harlan Laboratories). All protocols were approved by the Ontario Cancer Institute, Animal Care Committee.

**Activation of c-Myc and Blood Glucose Measurements**—The Myc-ER(TAM) transgene was activated by daily intraperitoneal injection of 1 mg of tamoxifen (Sigma) suspended in peanut oil (Sigma) at a final concentration of 10 mg/ml in adult mice (age 2–10 months). Tamoxifen was injected in all experimental mice between 8 and 10 a.m. daily for 1, 6, and 20 days. Blood glucose was measured by an automated glucose monitor (One Touch Ultra; LifeScan Inc., Milpitas, CA) prior to each injection.

**Immunohistochemistry and Immunofluorescent Staining**—Pancreata were isolated from mice at day 0, day 1, day 6, and day 20 after tamoxifen injection. Pancreatic tissue was fixed for 24 h in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (pH 7.4), and sections were immunostained for PTEN (Neomarker), insulin (DAKO), glucagon (Sigma), hematoxylin and eosin (not shown), synaptophysin (Roche Applied Science), laminin (Sigma), β-catenin (BD Biosciences), Ki67 (DAKO), and TUNEL (ID Labs Biotechnology Inc.). Immunofluorescent-stained sections were visualized using a Zeiss inverted fluorescent microscope.

**Western Blotting**—Islets from mice at day 1 of tamoxifen injection as well as from non-injected littermates were isolated, and protein lysates were obtained as described previously (7). Lysates were separated by SDS-PAGE and immunobotted with antibodies for c-Myc (9E10, gift of L. Penn), Akt, phospho-Akt (Ser-473), Bad, phospho-Bad (Ser-136), phospho-Bad (Ser-112), phospho-extracellular signal-regulated kinase (phospho-ERK), ERK, p53 (Cell Signaling), amylin (Bachem), p27, and Bcl-xL (Santa Cruz Biotechnology). Western blot signal densities were analyzed using Image J software. Protein levels were normalized to α-tubulin levels and expressed in arbitrary units relative to littermate control levels.

**Semi-quantitative Reverse Transcription (RT)-PCR**—mRNA was extracted from isolated islets by TRIzol following the manufacturer's protocol (Invitrogen, Toronto, Ontario, Canada) and treated with ribonuclease-free deoxyribonuclease (Invitrogen). RT-PCR amplifications of endogenous mouse c-Myc and β-actin transcripts were performed with a one-step RT-PCR kit (Invitrogen). Primer sequences are available upon request. Densitometric analysis was performed using Image J software. To correct for differences in loading, we corrected densitometric values of c-Myc cDNA with corresponding values of β-actin cDNA and calculated the c-Myc:β-actin ratios.

**Statistical Analysis**—Data are presented as means ± S.E and were analyzed by one-sample t test and independent samples t test. Significant differences were determined using the statistical software SPSS (version 11.0 for Macintosh). p values of < 0.05 were accepted as statistically significant.

**RESULTS**

**PTEN Deletion in Islets Leads to Increased Islet Mass**—Immunohistochemistry for PTEN protein in paraffin-embedded pancreata confirmed deletion of PTEN in islets of Myc<sup>−/−</sup>RIP<sub>cre</sub>Pten<sup>+/−</sup> mice versus control littermates (Fig. 1A). As we and others have previously shown (8, 9), the loss of PTEN in β cells led to increased islet mass when examined under basal conditions without tamoxifen injection (Fig. 1B). Additionally, consistent with its role as a negative regulator of the PI3K/Akt pathway, the loss of PTEN led to increased phospho-Akt and diminished expression of the cell cycle inhibitor p27 as well as increased expression of the antiapoptotic protein Bcl-xL (Fig. 1, A and C). These results indicate that when the c-Myc transgene is not active, the Myc<sup>−/−</sup>RIP<sub>cre</sub>Pten<sup>+/−</sup> mice are similar with respect to islet phenotype as compared with RIP<sub>cre</sub>Pten<sup>+/−</sup> mice without the Myc-ER(TAM) transgene.

The observed differences between p27 and Bcl-xL expression in the Myc<sup>−/−</sup>RIP<sub>cre</sub>Pten<sup>+/−</sup> and control mice without c-Myc activation are abolished after 1 day of c-Myc induction (Fig. 1C). However, there is a persistent increase in phospho-Akt expression in the Myc<sup>−/−</sup>RIP<sub>cre</sub>Pten<sup>+/−</sup> mice as compared with controls even after 1 day of tamoxifen treatment. Furthermore, c-Myc ER protein expression levels were similar in control and Myc<sup>−/−</sup>RIP<sub>cre</sub>Pten<sup>+/−</sup> mice injected for 1 day with tamoxifen, suggesting that the c-Myc transgene was activated to a similar degree in these animals (Fig. 1D). Endogenous mouse c-Myc mRNA transcript levels were similarly low between the islets of Myc<sup>−/−</sup>RIP<sub>cre</sub>Pten<sup>+/−</sup> mice as compared with control mice in the absence of c-Myc activation (Fig. 1E). These results are consistent with the low levels of c-Myc expression that have been observed in association with the low rates of proliferation that occur in β cells under basal conditions (14, 15). After 1 day of tamoxifen injection, levels of endogenous c-Myc in islets were again similarly low between Myc<sup>−/−</sup>RIP<sub>cre</sub>Pten<sup>+/−</sup> and Myc<sup>−/−</sup>RIP<sub>cre</sub>Pten<sup>+/−</sup> mice. These results indicate that endogenous c-Myc does not play a significant role in our transgenic model of c-Myc-activation and that there were no differences between PTEN-deficient and wild-type islets (Fig. 1E).

**PTEN Loss in c-Myc-activated β Cells Does Not Provide Protection against Diabetes**—Mice were injected daily for 20 days with tamoxifen to persistently activate the c-Myc transgene as described previously (13). Both Myc<sup>−/−</sup>RIP<sub>cre</sub>Pten<sup>+/−</sup> and control littermates became hyperglycemic within 4 days of c-Myc activation (Fig. 2A). Furthermore, consistent with the rise in blood glucose, prolonged c-Myc activation led to complete β cell ablation in Myc<sup>−/−</sup>RIP<sub>cre</sub>Pten<sup>+/−</sup> and control littermates (Fig. 2B). Given that Myc<sup>−/−</sup> mice do not have the c-Myc transgene as expected, they did not have any change in blood glucose levels after daily tamoxifen injection (Fig. 2A), and pancreatic islets remained unaffected (Fig. 2B). Thus, with c-Myc activation in β cells by tamoxifen, the concomitant loss of PTEN in β cells does not confer protection from islet involution and diabetes development.
Loss of PTEN Does Not Affect c-Myc-induced Proliferation and Apoptosis—Because the pancreata of Myc\(^+\) RIPcre\(^+\) Pten\(^{−/−}\) mice have enlarged islet area prior to c-Myc activation, we next examined the pancreatic area of Myc\(^+\) RIPcre\(^+\) Pten\(^{−/−}\) mice after c-Myc activation. After 1 day of tamoxifen injection, the Myc\(^+\) RIPcre\(^+\) Pten\(^{−/−}\) mice still had evidence of a greater islet area (Fig. 3A).

Activation of c-Myc in β cells has been shown to lead to an early increase in proliferation (13). Indeed, at day 1 after tamoxifen injection, there was rapid appearance of increased proliferation of β cells, as evidenced by markedly increased Ki67-positive staining (Fig. 3B). This increase in proliferation was similar between Myc\(^+\) RIPcre\(^+\) Pten\(^{−/−}\) and Myc\(^+\) RIPcre\(^+\) Pten\(^{+/+}\) control mice with tamoxifen treatment (Fig. 3B).

As c-Myc in β cells has been shown to have dual roles in both proliferation and apoptosis, we also examined for apoptosis by TUNEL of pancreata. As suggested by the rise in blood glucose observed with 20 days of tamoxifen injection, there was increased TUNEL after 1 day of c-Myc activation to a similar degree in Myc\(^+\) RIPcre\(^+\) Pten\(^{−/−}\) and Myc\(^+\) RIPcre\(^+\) Pten\(^{+/+}\) islets as compared with non-injected mice (Fig. 3C).

These results indicate that with the loss of PTEN in β cells and concomitant c-Myc activation, the β cells do undergo early proliferation. However, the loss of PTEN in β cells is not able to override the predominant apoptotic signal conferred by sustained c-Myc activation in β cells.

c-Myc Activation and PTEN Loss Does Not Lead to Tumor Formation—We have previously shown that the loss of PTEN alone in β cells does not lead to tumorigenesis (9). However, c-Myc is known to be a potent oncogene, and its multiple oncogenic attributes are revealed if apoptosis is suppressed in β cells (13). Thus, we were interested to know whether both the loss of the tumor suppressor PTEN and the activation of the oncogene c-Myc in β cells would lead to islet tumorigenesis.

With the early proliferation seen after 1 day of tamoxifen injection, there was no evidence of disrupted islet architecture as evidenced by preserved β and α cell localization within the islet (Fig. 4A, upper panel). However, after 6 days of sustained c-Myc activation, there was an increase in α cells, as is often observed after β cell destruction (16) (Fig. 4A, lower panel).

β-Catenin is usually localized to the plasma membrane in quiescent cells as part of cell adhesion complexes. However, in rapidly proliferating cells such as tumors, β-catenin is liberated from the cell membrane and migrates to the nucleus, where it...
acts as a transcription factor and mediates cell proliferation (17). In the Myc\textsuperscript{+/-} RIPcre\textsuperscript{+/-} and Myc\textsuperscript{+/-} RIPcre\textsuperscript{+/-} islets, \(\beta\)-catenin was localized at the cell membrane after 1 day of c-Myc activation (18, 19). The inactivation of Bad requires phosphorylation at two distinct sites, serines 112 and 136, which are phosphorylated by the epidermal growth factor receptor/mitogen-activated protein kinase (EGFR/MEK/MAPK) and PI3K/Akt pathways respectively. Prior to c-Myc activation, Bad is phosphorylated at both serine 112 and serine 136. However, upon c-Myc activation, we observed decreased levels of Bad phosphorylation of both serine residues in Myc\textsuperscript{+/-} RIPcre\textsuperscript{+/-} mice relative to control, suggesting that c-Myc apoptosis at least in part is Bad-dependent.

The MAPK pathway regulates the activity of several transcription factors, including p53, which modulates the cell cycle and apoptosis and hence functions as a tumor suppressor (20). MAPK activity, as shown by phosphorylation of ERK, is increased, as is p53 expression upon c-Myc activation in islets of both Myc\textsuperscript{+/-} RIPcre\textsuperscript{+/-} and Myc\textsuperscript{+/-} RIPcre\textsuperscript{+/-} mice. These results would suggest that perhaps PTEN-deficient \(\beta\) cells do not lead to tumor formation.

**c-Myc Induction Leads to Bad Dephosphorylation and Increased MAPK Signaling Activity**—In an attempt to clarify which components of the intrinsic apoptotic pathway were involved in c-Myc-induced apoptosis, we examined the phosphorylation levels of the downstream proapoptotic molecule Bad. The inactivation of Bad requires phosphorylation at two distinct sites, serines 112 and 136, which are phosphorylated by the epidermal growth factor receptor/mitogen-activated protein kinase (EGFR/MEK/MAPK) and PI3K/Akt pathways respectively. Prior to c-Myc activation, Bad is phosphorylated at both serine 112 and serine 136. However, upon c-Myc activation, we observed decreased levels of Bad phosphorylation of both serine residues in Myc\textsuperscript{+/-} RIPcre\textsuperscript{+/-} mice relative to control, suggesting that c-Myc apoptosis at least in part is Bad-dependent.

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PTEN Deletion and c-Myc Activation Does Not Lead to Tumors

Together, we have shown that when PTEN is deleted and c-Myc is activated in β cells, islet disruption and rapid diabetes development ensue due to apoptosis directed by c-Myc activation, which still proceeds despite the loss of PTEN. Importantly, the lack of tumor development suggests that even in the context of oncogenic activation, the loss of PTEN is insufficient to promote tumor formation in β cells.

DISCUSSION

The use of tissue-targeted ablation has shown that PTEN has diverse tissue- and context-specific roles. In the pancreatic β cell, PTEN plays a role in β cell size and islet mass (8, 9). Importantly, the loss of PTEN in β cells does not lead to tumorigenesis, and differentiated β cell function is preserved (9). In addition, within the β cell, the loss of PTEN has been shown to confer protection from β cell apoptosis and diabetes induced by multiple low doses of streptozotocin (8, 9).

c-Myc is a potent onco gene that has a predominantly proapoptotic role in β cells. However, when coupled with Bcl-xL overexpression, c-Myc activation leads to β cell tumorigenesis (13). Given that PTEN is a tumor suppressor, we were interested in examining the effect of the loss of PTEN in β cells with concomitant c-Myc activation. We found that surprisingly, apoptosis due to c-Myc activation still occurred despite the loss of PTEN. Additionally, there was no evidence of islet tumor development in these double mutant mice. Our results suggest that the c-Myc-induced apoptotic signal can overcome the potent prosurvival signal conferred by the loss of PTEN.

The role of PTEN in apoptosis is mediated through the PI3K/Akt pathway via regulation of the antiapoptotic Bcl-2 like factors (such as Bcl-2, Bcl-xL) and proapoptotic factors (such as Bad, Bak). Akt inhibits caspases, which cleave Bcl-2 and Bcl-xL (21) and which are involved in the intrinsic or mitochondrial pathway of apoptosis. Bcl-xL resides in the outer mitochondrial membrane, and increased activation of this pro-

cells may undergo apoptosis using these pathways that are not differentially protected as a consequence of PTEN deletion.
PTEN Deletion and c-Myc Activation Does Not Lead to Tumors

tlein leads to decreased permeabilization of the membrane, blockage of cytochrome c release, and thus, a decrease in apoptosis. Because the loss of PTEN increases signaling through the PI3K pathway, PTEN deletion would be expected to increase Bcl-xL levels and lead to protection from apoptosis. Indeed, we have shown that Bcl-xL expression is increased in PTEN-deficient mice under basal conditions. The Bcl-xL overexpression results by Pelengaris et al. (13) implied that c-Myc-induced apoptosis in β cells is also mediated by the intrinsic apoptotic pathway (13). This makes our results in which we fail to observe tumor development upon c-Myc activation in PTEN-deficient mice all the more surprising given the evidence supporting the role of both PTEN and c-Myc in the same pathway of apoptosis. Our model reveals that physiological prosurvival signals that are unveiled as a consequence of PTEN deletion are clearly different from those at work in the overexpression model of Bcl-xL.

Through its negative regulation of the PI3K/Akt pathway, PTEN also influences the function of proapoptotic proteins such as Bad. Phosphorylation at either of its serine residues is sufficient to bind Bad to 14-3-3, which sequesters it from its effector molecules, thereby preventing it from exercising its proapoptotic function (22). In PTEN-deficient tumor cells, Bad has been implicated as the key molecule responsible for integrating the pathways that suppress apoptosis (23). We, however, have shown decreased levels of Bad phosphorylation in PTEN-deficient islets upon c-Myc activation. This finding suggests that the prosurvival signal conferred by PTEN deletion appears to be insufficient to counteract c-Myc-induced apoptosis. As well, it suggests that c-Myc is exerting its proapoptotic effect, at least in part, by influencing molecules that are involved in Bad phosphorylation.

In addition, the activation of the c-Myc transgene in the Myc+RIPcre+ Pten+/−/ and Myc+RIPcre+ Pten−/−/ mice is associated with increased MAPK activity and p53 expression in islets of both groups. These results are in keeping with a previous study that p53 mediates apoptosis as a safeguard mechanism to prevent cell proliferation induced by c-Myc activation (24), which may further explain the increased apoptosis and the lack of tumorigenesis in these islets.

Accumulating evidence supporting favorable metabolic changes upon PTEN inhibition has led to PTEN as an attractive drug target against type 2 diabetes mellitus (25). Due to the broad tissue distribution of PTEN as well as its role in attenuating numerous PI3K-dependent processes, this approach may pose significant safety risks. However, advances in tissue selectivity for gene targeting may make it possible to inhibit PTEN function specifically in the β cells of diabetic individuals. Not only does the loss of PTEN in β cells not lead to tumorigenesis, but even with a concomitant induction of an oncogene, a situation that should create an environment that is favorable for tumor formation, we observed no tumorigenesis. Thus, in β cells, PTEN deletion seems to maintain and perhaps enhance β cell function without impacting on tumorigenesis, which raises hopes that selective inhibition of PTEN activity may be a promising therapeutic approach to type 2 diabetes.

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