Cell, Vol. 109, 321–334, May 3, 2002, Copyright ©2002 by Cell Press

Suppression of Myc-Induced Apoptosis in β Cells Exposes Multiple Oncogenic Properties of Myc and Triggers Carcinogenic Progression

Stella Pelengaris,[1,2,5] Michael Khan,[1,5] and Gerard I. Evan[2,3,4]
[1]Molecular Medicine Research Center
Department of Biological Sciences
University of Warwick
Coventry CV4 7AL
United Kingdom
[2]Imperial Cancer Research Fund Laboratories
Lincoln's Inn Fields
London WC2A 3PX
United Kingdom
[3]UCSF Cancer Center and Cancer Research Institute
2340 Sutter Street
San Francisco, California 94143

Summary

To explore the role of c-Myc in carcinogenesis, we have developed a reversible transgenic model of pancreatic β cell oncogenesis using a switchable form of the c-Myc protein. Activation of c-Myc in adult, mature β cells induces uniform β cell proliferation but is accompanied by overwhelming apoptosis that rapidly erodes β cell mass. Thus, the oncogenic potential of c-Myc in β cells is masked by apoptosis. Upon suppression of c-Myc-induced β cell apoptosis by coexpression of Bcl-x_L, c-Myc triggers rapid and uniform progression into angiogenic, invasive tumors. Subsequent c-Myc deactivation induces rapid regression associated with vascular degeneration and β cell apoptosis. Our data indicate that highly complex neoplastic lesions can be both induced and maintained in vivo by a simple combination of two interlocking molecular lesions.

Introduction

A wealth of data indicate that cancers are multistage diseases progressing via protracted accumulation of multiple lesions that compromise control of cell proliferation, survival, differentiation, migration, and social interactions with neighboring cells and stroma. Hanahan and Weinberg (2000) construed the axiomatic requirements of cancer cells as the capacity to proliferate irrespective of exogenous mitogens, refractoriness to growth inhibitory signals, resistance to apoptosis, unrestricted proliferative potential (immortality), capacity to recruit a vasculature, and the ability to invade surrounding tissues and eventually metastasize. By implication, tumor progression proceeds by acquisition of lesions that provide the tumor cell with these attributes and which thereby shape the complex phenotype of the tumor cell.

One frequent target for growth deregulation in cancer cells is c-Myc, an essential part of normal cell proliferative machinery (Eisenman, 2001), expressed at low levels throughout the cell cycle of proliferating, mitogen-stimulated somatic cells. In most tumor cells, Myc expression is deregulated, often markedly elevated, and no longer dependent upon mitogenic signaling, although in most cases the underlying cause for such altered expression is unknown. It is also unclear whether it is deregulation or elevated expression that is critical in oncogenic activation of c-Myc.

Paradoxically, c-Myc is also a potent inducer of apoptosis (Evan and Littlewood, 1998), especially in cells depleted of survival factors or subjected to stress—both likely prospects during tumorigenesis. However, the extent to which such apoptosis actually restrains the oncogenic potential of c-Myc remains unclear. Suppression of apoptosis, for example by increased expression of Bcl-2 (Bissonnette et al., 1992; Fanidi et al., 1992; Wagner et al., 1993), excess survival factors (Askew et al., 1991; Evan et al., 1992; Harrington et al., 1994), or genetic inactivation of ARF (Zindy et al., 1998) or p53 (Wagner et al., 1993, 1994) tumor suppressors, exacerbates c-Myc-induced tumorigenesis (Blyth et al., 1995; Eischen et al., 1999, 2001; Jacobs et al., 1999; Lotem and Sachs, 1995; Maestro et al., 1999; Pelengaris et al., 1999; Schmitt et al., 1999; Strasser et al., 1990). However, it is unclear whether they do this by directly antagonizing c-Myc-induced apoptosis or by nonspecifically protecting cells from the apoptotic consequences of genotoxic and other stresses (Eischen et al., 1999; Evan and Vousden, 2001; Sherr, 2000). The difficulties of directly observing c-Myc-induced apoptosis in vivo means that it has never been possible to evaluate the contribution made in any tissue by Myc-induced apoptosis in early, middle, and late progression of Myc-induced malignancies.

Pancreatic β cells comprise the major part of the several hundred Islets of Langerhans, colonies of endocrine cells scattered throughout the exocrine pancreas, and are the sole source of insulin. β cells sustain slow turnover throughout life with an estimated mean β cell lifespan (in the rat) of around 58 days (Bonner-Weir, 2001). Excess apoptosis is implicated in the β cell attrition that accompanies some forms of diabetes, whereas excess β cell propagation is the hallmark of insulinoma (Pavelic et al., 1996).

The best-characterized paradigm of β cell carcinogenesis is the elegant RIP-Tag transgenic model of Hanahan and colleagues. In this model, targeted expression of SV40 T antigens by the rat insulin promoter induces consistent, kinetically reproducible yet sporadic emergence of β cell neoplasms that progress with diminishing frequency through defined stages of increasing "severity," occasionally evolving into full-blown, invasive β cell carcinomas. (Christofori and Hanahan, 1994). RIP-Tag exploits a number of inherent advantages offered by β cells as targets for transgenic tumorigenesis. β cells have an intrinsically low proliferative rate, are exclusively targeted by the insulin promoter, have a well-described and demarked ontogeny,

[4]Correspondence: gevan@cc.ucsf.edu
[5]These authors contributed equally to this work.

and are dispersed throughout the pancreas amongst hundreds of spatially isolated islet colonies. This latter uniquely permits discrimination between direct effects of the transgene, expected to arise synchronously in all islets, and those due to stochastic secondary events, expected to emerge sporadically in isolated islets. Thus, the sporadic and focal tumorigenesis exhibited in the RIP-Tag model indicates that progression arises from stochastic secondary lesions, some of which have been defined (Hodgson et al., 2001).

We have developed a switchable model of Myc carcinogenesis in β cells in which c-Myc function can be rapidly switched on and off. This has allowed us to explore the direct consequences of c-Myc activation and deactivation in mature β cells in vivo and assess the relative importance of Myc-induced proliferation versus apoptosis in inducing and maintaining β cell tumorigenesis. We show that Myc-induced apoptosis is indeed a powerful and effective innate restraint to Myc-induced tumorigenesis, whose suppression exposes multiple neoplastic attributes of Myc and allows it to drive tumor progression without the need for additional neoplastic lesions.

Results

Expression of Switchable c-Myc in Pancreatic β Cells of Adult Transgenic Mice

We expressed a cDNA encoding human c-Myc fused at its C terminus to the hormone binding domain of 4-hydroxytamoxifen (4-OHT)-responsive mutant murine estrogen receptor (c-MycER^{TAM}) (Littlewood et al., 1995) in pancreatic β cells under the control of an insulin promoter (*pIns*) (Figure 1A; Ohashi et al., 1991). Restricted expression of c-MycER^{TAM} protein to the nuclei of β cells was confirmed immunocytochemically using a rabbit polyclonal anti-Myc antibody (pan-Myc) and was blocked by preincubation of antibody with its original Myc peptide immunogen (see Figure 1B). Identical tissue and subcellular localization was observed with polyclonal antibody specific for the ER^{TAM} domain (not shown). We compared the level of c-MycER^{TAM} protein expression in transgenic β cells with that in serum-stimulated Swiss 3T3 (S3T3) cells (Moore et al., 1987) by quantitative immunoblotting with pan-Myc antibody, which sees a conserved epitope present in c-MycER^{TAM} and all Myc proteins of rodents and humans (Figure 1C).

Based on this, we estimate that each transgenic β cell expresses between 4000 and 6000 molecules of c-MycER^{TAM}, comparable with the level of expression in primary serum-stimulated human fibroblasts such as MRC-5 (Moore et al., 1987).

c-Myc Activation Induces Proliferation in Adult Pancreatic β Cells In Vivo, but This Is Rapidly Overwhelmed by Apoptosis

β cells of non-4-OHT-treated transgenic mice exhibited intense insulin immunostaining and no evident cell proliferation (Figure 2A), identical to β cells from adult wild-type mice (not shown). To explore the mitogenic action of c-Myc in adult β cells in vivo, 4-OHT was administered by daily intraperitoneal (IP) injection to *pIns-c-MycER^{TAM}* mice and pancreata from the animals analyzed at various times. Virtually all β cells in every islet were proliferating within 24 hr of c-MycER^{TAM} activation, as indicated by positive nuclear staining for the proliferation marker Ki-67. Proliferation was accompanied by significant, although incomplete, downregulation of insulin expression (Figure 2A). Continuous c-MycER^{TAM} activation induced sustained islet cell proliferation (see below). Thus, activation of c-Myc is sufficient to elicit and maintain multiple rounds of β cell replication in vivo.

To assess the balance between c-Myc-induced proliferation and apoptosis in β cells in vivo, c-MycER^{TAM} was continuously activated in *pIns-c-MycER^{TAM}* mice by daily IP 4-OHT. Analysis of pancreata from representative mice showed that sustained c-Myc activation triggered rapid islet involution. To determine the mechanism of involution, pancreata were examined for the presence of apoptotic cells both histologically, to identify condensed pyknotic nuclei, and by TUNEL staining, to identify fragmented DNA indicative of apoptotic cells. No β cell apoptosis was evident in islets of untreated transgenic mice (Figure 2B) or 4-OHT-treated wild-type animals (data not shown). In contrast, between 4% and 7% of total islet cells appeared apoptotic by 72 hr of c-MycER^{TAM} activation (Figure 2B). Importantly, apoptosis arose to similar extents in each islet (see below) and was restricted to β cells, leaving unaffected glucagon-positive α cells concentrated in the islet periphery (Figure 2B). Almost complete β cell ablation had occurred after 6–10 days of sustained c-MycER^{TAM} activation (Figure 2C). Such dramatic attrition occurred despite the sustained induction of β cell proliferation by activated c-MycER^{TAM} (Figure 3A; note the few residual β cells remain Ki-67 positive and therefore remain in cell cycle), unequivocally demonstrating that the proapoptotic activity of c-Myc predominates over its mitogenic capability in this tissue. Importantly, islet involution was observed to occur with similar kinetics in all islets throughout the pancreas (Figure 3B).

Since pancreatic β cells are the sole source of hormonal insulin required for blood glucose homeostasis, c-Myc-induced suppression of insulin expression and destruction of β cells was mirrored by the acute onset and maintenance of hyperglycemia (Table 1). Hyperglycemia arose with similar kinetics in all mice, male and female. By contrast, both 4-OHT-treated wild-type mice and untreated transgenic control mice maintained normal blood glucose levels throughout the experimental period.

Deactivation of c-MycER^{TAM} Leads to Synchronous Islet Regeneration and Recovery from Diabetes in *pIns-c-MycER^{TAM}* Mice

c-MycER^{TAM} activity is rapidly lost upon withdrawal of 4-OHT both in vitro (Littlewood et al., 1995) and in vivo (Pelengaris et al., 1999), allowing us to assess the effects of c-MycER^{TAM} deactivation on β cell destruction. c-MycER^{TAM} was activated for 6 days, resulting as before in rapid β cell attrition (Figure 3A) accompanied by acute hyperglycemia (Table 1). 4-OHT was then withdrawn and blood and urinary glucose were monitored daily, together with histological analysis of pancreata from representative mice. Within 6–9 days of 4-OHT withdrawal, we observed marked regeneration of pancreatic

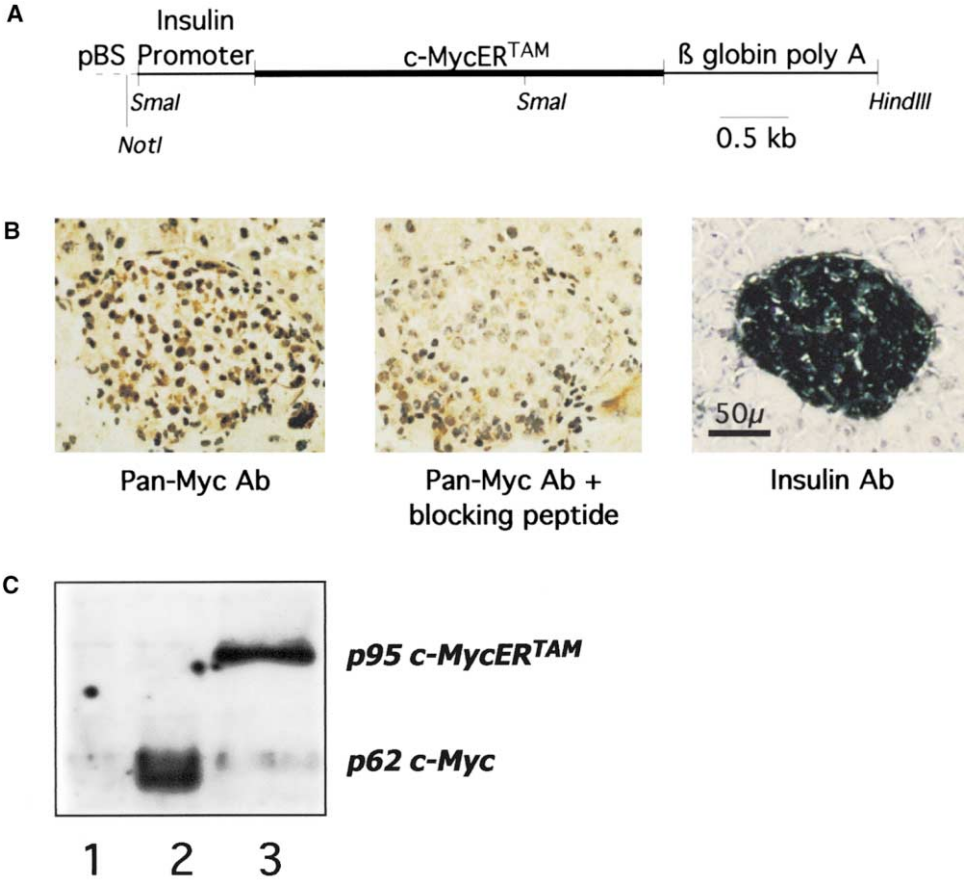

Figure 1. Generation and Characterization of *Insulin-c-MycER^{TAM}* Transgenic Mice

(A) Construction of *pIns-c-MycER^{TAM}* transgene. cDNA encoding human c-*myc* fused to the modified estrogen receptor ligand binding domain (ER^{TAM}) (Littlewood et al., 1995) was cloned, along with β-globin polyA site, downstream of the rat insulin promoter (pIns) (Ohashi et al., 1991). Comparative Southern blotting indicates that the founder line used in all studies carries ~10 copies of the *pIns-c-MycER^{TAM}* transgene.

(B) c-MycER^{TAM} protein expression is restricted to pancreatic islet β cells. Immunocytochemical staining of sequential frozen pancreatic sections from transgenic mice were stained with Pan-Myc antibody (left), Pan-Myc antibody incubated with 10 μg/ml Pan-Myc peptide immunogen (middle), or insulin antibody (right).

(C) Comparison of c-MycER^{TAM} protein levels in pancreatic islets with endogenous c-Myc in serum-stimulated Swiss 3T3 fibroblasts. Lysates were prepared from hand-picked islets isolated from non-4-OHT-treated *pIns-c-MycER^{TAM}* × *RIP-Bclx$_L$* (inactive c-MycER^{TAM}) transgenic mice. Swiss 3T3 were serum deprived for 48 hr (lane 1) and then stimulated with 10% fetal calf serum for 2 hr (lane 2), after which whole-cell lysates were prepared. From each lysate, 10^5 cell equivalents were fractionated on a 10% polyacrylamide gel, electroblotted onto PVDF membrane, and probed with Pan-Myc antibody. In transgenic islets, Pan-Myc antibody identifies the 95 kDa band corresponding to c-MycER^{TAM} protein (lane 3). As expected for a quiescent tissue, no appreciable endogenous 62 kDa c-Myc is expressed. In serum-stimulated S3T3 cells, Pan-Myc antibody identifies the expected 62 kDa c-Myc protein.

islets (Figure 3A) accompanied by normalization of blood glucose (Table 1). Regeneration was accompanied by the appearance of proliferating (Figure 3A, arrows) cells, mainly in pancreatic ducts adjacent to involuted islets.

Rapid and Uniform c-Myc-Induced Tumor Progression upon Suppression of β Cell Apoptosis

Our data imply that apoptosis acts as a formidable barrier to the oncogenic potential of c-Myc in pancreatic β cells. Therefore, to explore the consequences of activation of c-Myc when apoptosis is suppressed, we crossed our *pIns-c-MycER^{TAM}* mice with mice expressing the Bcl-2 homolog Bcl-x$_L$, under direction of the rat insulin promoter (RIP7) (Zhou et al., 2000). Bcl-x$_L$ suppresses the mitochondrial apoptotic pathway, which is the ultimate conduit of both the ARF/p53-dependent (Zindy et al., 1998) and -independent (Juin et al., 1999) c-Myc proapoptotic effector pathways.

In the absence of 4-OHT, *pIns-c-MycER^{TAM}/RIP-Bcl-x$_L$* double transgenic mice exhibited normal islet morphology (Figures 3B and 4A) with no measurable β cell proliferation or apoptosis (data not shown) and normal blood glucose (Table 1). As in *pIns-c-MycER^{TAM}* single transgenic mice, activation of c-MycER^{TAM} in *pIns-c-MycER^{TAM}/RIP-Bcl-x$_L$* double transgenic mice induced rapid and synchronous entry into the cell cycle of virtually all β cells in all islets (not shown). However, in dramatic contrast to *pIns-c-MycER^{TAM}* mice, we observed no c-Myc-induced apoptosis but instead an immediate and progressive expansion of β cells in all islets; within 7 days, histologi-

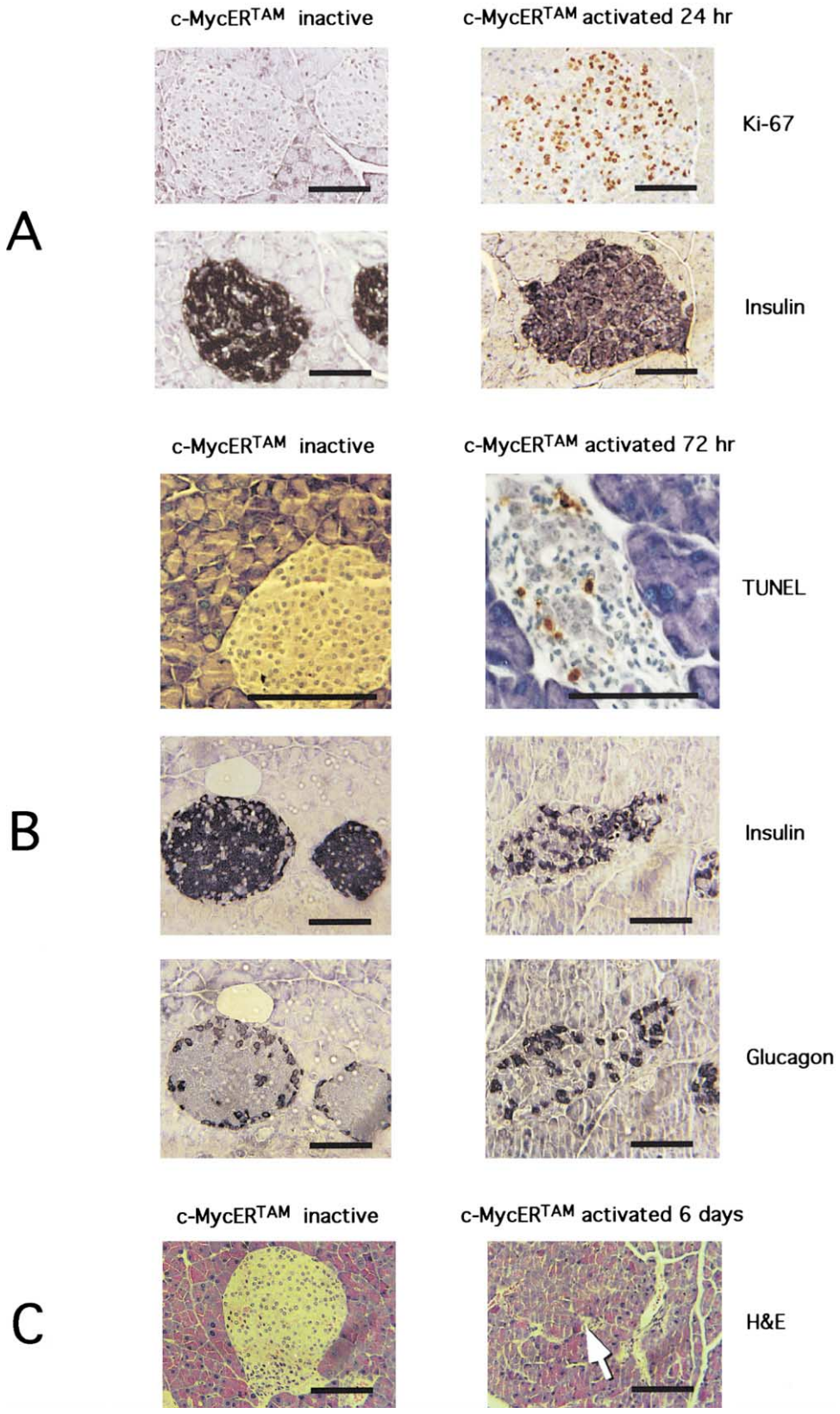

Figure 2. Activation of c-MycER^{TAM} Induces β Cell Proliferation and Apoptosis in Pancreatic β Cells In Vivo

(A) c-MycER^{TAM} activation induces β cell proliferation and insulin downregulation. Paraffin-embedded fixed sections of pancreata, from either untreated *pIns-c-MycER^{TAM}* transgenic or *pIns-c-MycER^{TAM}* mice treated with 4-OHT for 24 hr, were probed with antibodies specific for the proliferation marker Ki-67 or for insulin.

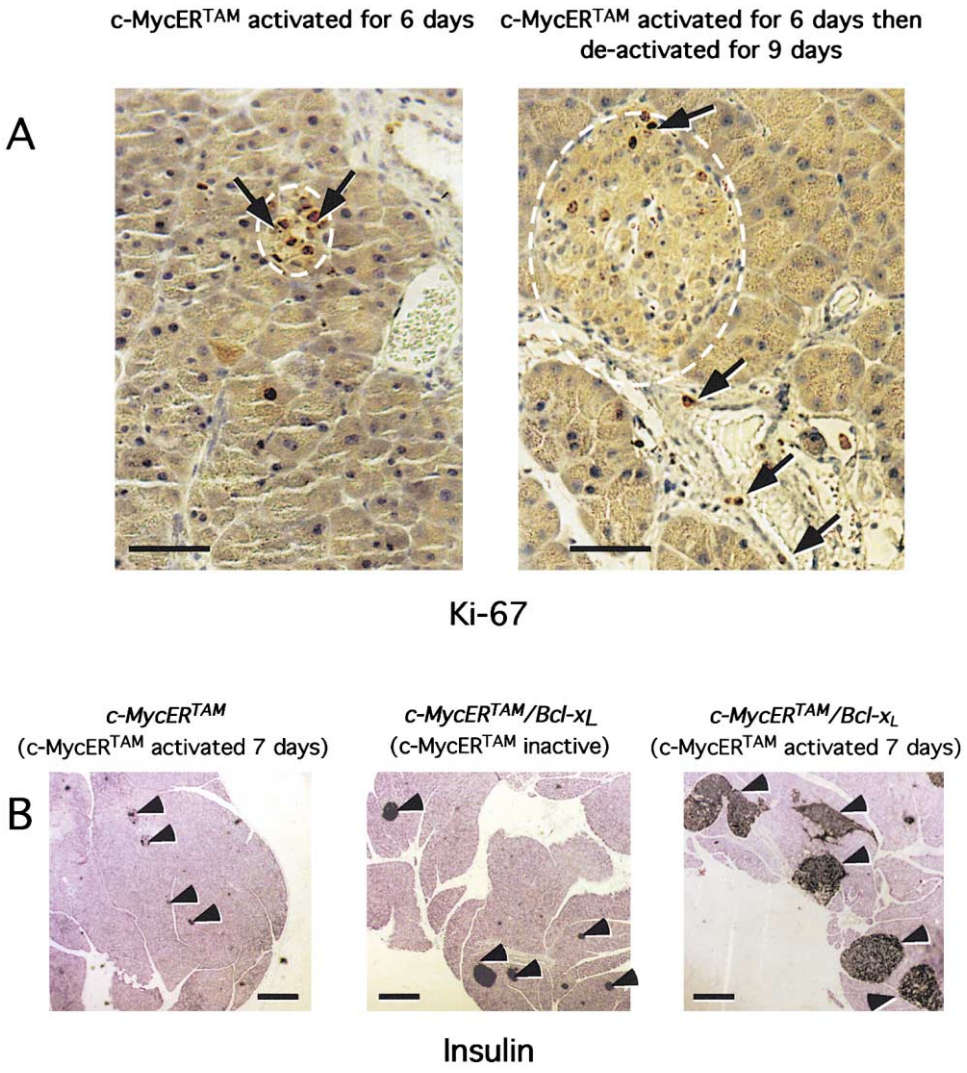

Figure 3. Effects of Myc Activation Arise Synchronously in All Islets and Are Reversible

(A) Ki-67 staining of involuted and regenerated islets. Left: representative involuted islet obtained from *pIns-c-MycER^{TAM}* transgenic mouse pancreas after 6 days continuous activation of c-MycER^{TAM}. Note the few remaining β cells are Ki-67 positive (arrows). Right: after 6 days continuous c-MycER^{TAM} activation, administration of 4-OHT was withdrawn, mice were allowed to recover for 9 days and then sacrificed, and pancreata were fixed, sectioned, and stained. A representative regenerated islet is shown. Note the presence of occasional proliferating (ki67-positive) β cells (arrows) in ducts and regenerating islets. Scale bar equals 50 μm.

(B) Low-power microscopic analysis of effects of Myc activation. *pIns-c-MycER^{TAM}* and *pIns-c-MycER^{TAM}/Bcl-x$_L$* transgenic mice were either untreated or treated for 7 days with 4-OHT to activate c-MycER^{TAM}. Pancreata were isolated, fixed, sectioned, stained for insulin immunoreactivity, and examined under low power. Scale bar equals 200 μm.

cal examination of 4-OHT-treated *pIns-c-MycER^{TAM}/RIP-Bcl-x$_L$* double transgenic pancreata showed grossly hyperplastic islets distributed throughout the pancreas (Figure 3B). Thus, blocking c-Myc-induced apoptosis in β cells by Bcl-x$_L$ expression transforms c-Myc from a net growth suppressor to a growth inducer.

Tumor progression is generally thought to involve accumulation of multiple somatic lesions that confer diverse and novel properties on tumor cells such as loss of differentiated phenotype, angiogenesis, and invasiveness. We therefore asked whether, and with what frequency, c-Myc drove tumor progression in *pIns-c-MycER^{TAM}/RIP-Bcl-x$_L$* β cells. Since Myc inhibits differentiation in multiple cell lineages, we first examined the

(B) c-MycER^{TAM}-induced islet involution involves β cell apoptosis. Sections from pancreata from either untreated *pIns-c-MycER^{TAM}* mice or from *pIns-c-MycER^{TAM}* mice treated with 4-OHT for 72 hr were analyzed for apoptotic DNA by TUNEL or probed with antibodies specific for insulin or glucagon.

(C) Sustained c-MycER^{TAM} activation induces islet involution. Comparison of representative H&E-stained paraffin-embedded sections from *pIns-c-MycER^{TAM}* transgenic pancreata either untreated or treated with 4-OHT for 6 days. Scale bar equals 50 μm.

Table 1. Onset and Recovery of Diabetes (Hyperglycemia) in *pIns-c-mycER^{TAM}* and *pIns-c-MycER^{TAM}/Bcl-x$_L$* Mice

	Duration of 4-OHT Treatment					
	0 Days	6–9 Days	23 Days	42 Days	50 Days	9 Days after 4-OHT Withdrawal
Mouse Genotype	Mean Blood Glucose (*mMol/L*) (SEM)					
Wild-type	11.7 (1.02)	9.3 (0.94)	9.32 (0.41)	ND	ND	9.3 (0.3)
MycER^{TAM}	9.82 (1.05)	30.58 (1.48)	32 (1.3)	ND	ND	8.67 (0.47)
Bcl-x$_L$	11.9 (0.35)	12.3 (0.27)	11.53 (0.55)	8.81 (0.51)	8.87 (1.1)	ND
MycER^{TAM}/Bcl-x$_L$	6.2 (0.91)	24.1 (3.3)	14.42 (1.95)	5.54 (1.35)	2.84 (0.18)	ND

Fasting blood glucose measurements (mMol/l) were taken at specific time points from wild-type mice or transgenic mice following c-MycER^{TAM} activation or subsequent deactivation. All experimental groups consisted of both male and female littermates between 3 and 8 months of age. Activation of c-MycER^{TAM} protein for 6–9 days led to onset of diabetes in *pIns-c-MycER^{TM}* mice, as indicated by elevated blood glucose. Subsequent deactivation of c-MycER^{TAM} led to rapid normalization of blood glucose within 9 days. *pIns-c-MycER^{TM}*/Bcl-x$_L$ double transgenic mice exhibited only transient hyperglycemia, becoming hypoglycemic by 40–50 days of c-Myc activation.

effect of c-Myc activation on β cell differentiation status. Expression of insulin, a hallmark of mature differentiated β cells, was rapidly although incompletely downregulated in β cells following c-MycER^{TAM} activation (Figures 2A and 4A). Such downregulation is presumably responsible for the transient increase in fasting blood glucose levels seen in double transgenic mice (Table 1), while their subsequent recovery is due to the progressively increasing numbers of β cells which, despite their low individual expression, together produce sufficient insulin to reestablish normoglycemia. Thus, activation of c-Myc is sufficient to at least partially revert the differentiated phenotype of mature β cells.

It is proposed that inability to generate requisite vasculature is a major restraint to the unbridled expansion of neoplastic cells (Folkman, 1992). We therefore next assessed the angiogenic status in hyperplastic islets from *pIns-c-MycER^{TAM}/RIP-Bcl-x$_L$* double transgenic mice treated for 7 days with 4-OHT. Histological analysis indicated the presence of an extensive network of blood

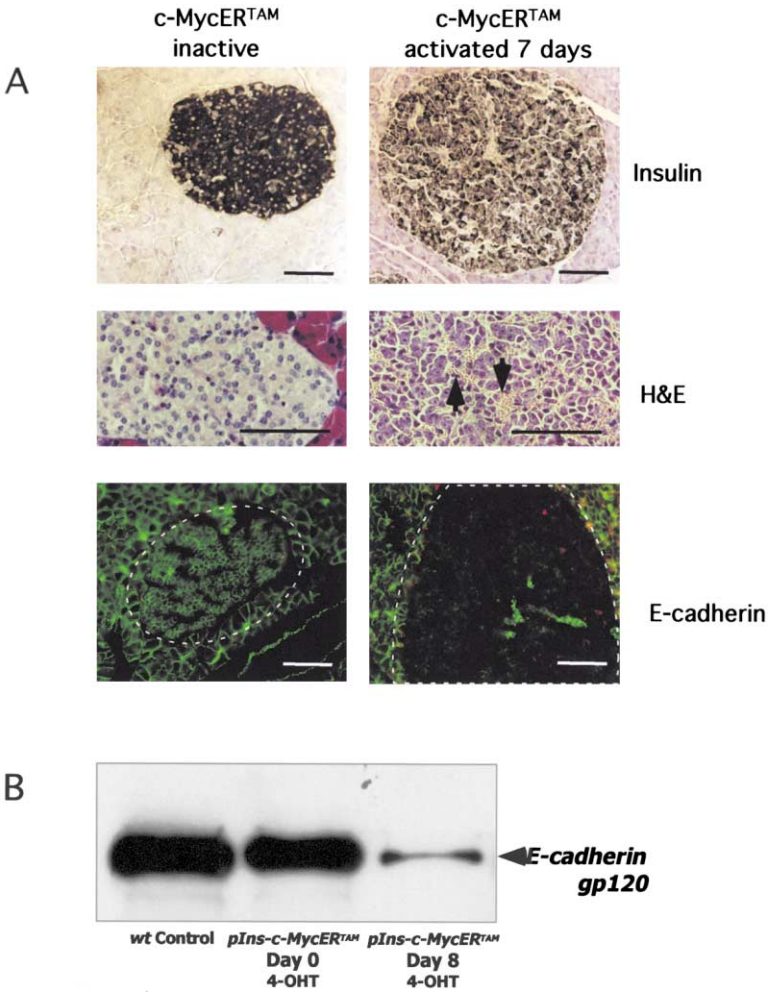

Figure 4. Characterization of c-MycER^{TAM}/Bcl-x$_L$ β Cell Tumors

(A) c-Myc activation triggers rapid β cell hyperplasia, loss of differentiated phenotype, angiogenesis, and downregulation of E cadherin with loss of cell-cell contact. *pIns-c-MycER^{TAM}* × *RIP-Bcl-x$_L$* double transgenic mice were treated with 4-OHT daily for 7 days to drive sustained c-MycER^{TAM} activation. Pancreata were removed, fixed, and stained by H&E and/or insulin or E cadherin antibodies, as appropriate. Dotted white line marks extent of β cell mass. Scale bar equals 50 μm.

(B) Immunoblot analysis of E cadherin expression following c-Myc activation. Islets were isolated from control and 0- and 8-day 4-OHT-treated c-MycER^{TAM}/Bcl-x$_L$ double transgenic mice, equivalent cell numbers were lysed and fractionated by SDS-PAGE, and E cadherin levels were assessed by immunoblotting.

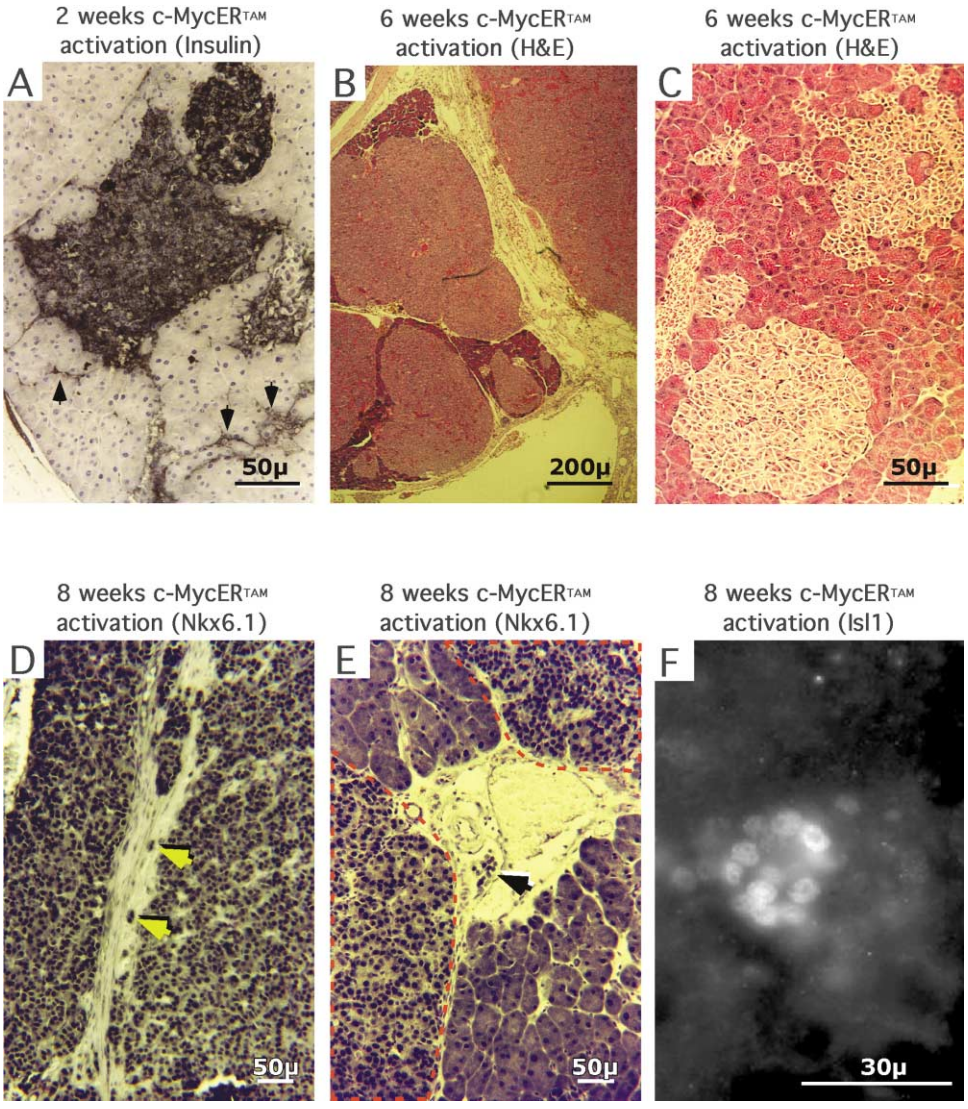

Figure 5. c-MycER^{TAM}/Bcl-x_L β Cell Tumors Are Invasive

c-MycER^{TAM} was continuously activated in c-MycER^{TAM}/Bcl-x_L double transgenic mice for 2–8 weeks, and pancreata from representative mice were fixed, sectioned, and analyzed histologically and immunohistochemically as shown. By 2 weeks of c-Myc activation, locally invasive, insulin-positive β cells were evident in at least 50% of islets per section (A). By 6 weeks, much of the exocrine pancreas had become eroded and replaced by β cell tumors (B and C). Staining at 8 weeks revealed multiple ectopic deposits of Nkx6.1- or Isl-1-staining cells in blood vessels, pancreatic ducts, and draining mesenteric lymph nodes (D–F).

vessels permeating the entirety of every islet (Figure 4A), which was confirmed by staining with the vascular endothelial marker CD31 (not shown). Only modest vascularization was evident in islets from control mice in which c-MycER^{TAM} was inactive. Angiogenesis arose with similar kinetics in each islet, its extent keeping pace with β cell expansion even during prolonged activation of c-MycER^{TAM} (Figure 5B). We conclude that activation of c-Myc elicits an angiogenic response coincident with its induction of β cell hyperplasia.

Perhaps the most lethal attribute of malignant tumor cells is their ability to detach, invade surrounding tissue, and eventually metastasize. The principal intercellular adhesion molecule in epithelia is E cadherin, whose expression is lost in invasive human epithelial cancers (Birchmeier et al., 1995; Christofori and Semb, 1999; Takeichi, 1993). Indeed, loss of E cadherin plays a causal and rate-limiting role in the transition from benign β cell adenoma to malignant carcinoma in the RIP-Tag model (Perl et al., 1998). Islets from either wild-type (not shown) or untreated *pIns-c-MycER^{TAM}/RIP-Bcl-x_L* double transgenic mice (inactive Myc) exhibit intense E cadherin cell surface staining (Figure 4A). By contrast, within 7 days of c-Myc activation, we observed a dramatic reduction in E cadherin immunostaining in islets of *pIns-c-MycER^{TAM}/RIP-Bcl-x_L* double transgenic mice. Immunoblotting of isolated islets confirmed that c-Myc induced progressive loss of E cadherin protein from β cells (Figure 4B). Loss of E cadherin coincided with dramatic withdrawal of β cell-cell contacts throughout all islets of double transgenic mice (most obvious in Figures 4A and 5B).

The rapid and synchronous induction of dedifferentiation, angiogenesis, and loss of cell-cell contact in each of the spatially separated islets in the pancreas implies

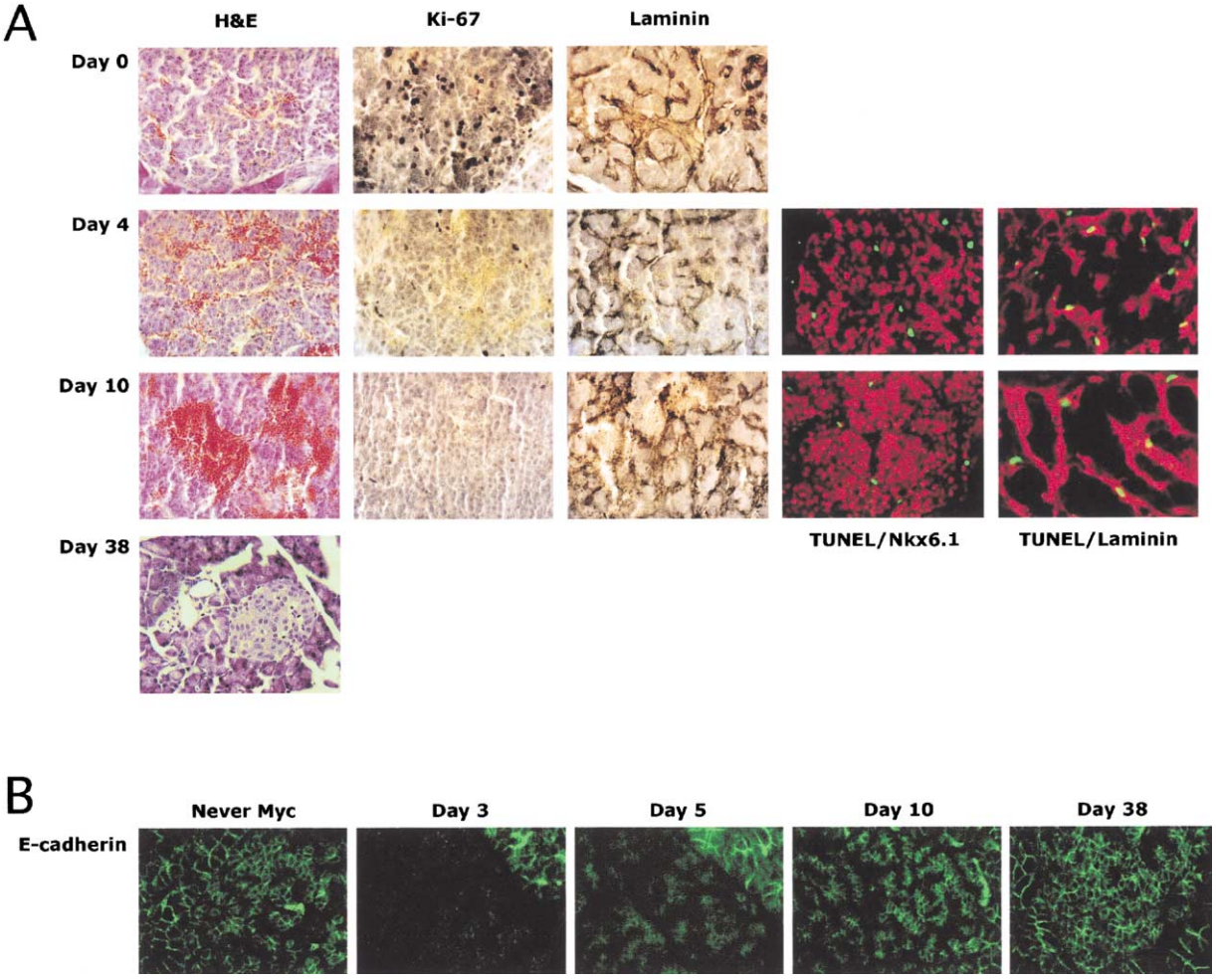

Figure 6. Regression of β Cell Tumors after c-Myc Deactivation

(A) c-Myc deactivation induces β cell growth arrest, vascular collapse, apoptosis of vessels walls and β cells, and eventual tumor involution. *pIns-c-MycER^{TAM}/RIP-Bcl-x$_L$* double transgenic mice were treated IP with 4-OHT daily for 14 days. Administration of 4-OHT was then stopped, and representative mice were sacrificed immediately or after 4, 10, or 38 days. Pancreata were removed, fixed, and examined histologically by H&E staining and immunohistochemically using anti-Ki-67 or anti-laminin antibodies and by Nkx6.1 or laminin (red) together with TUNEL (green).

(B) E cadherin expression is restored rapidly following c-Myc deactivation. *pIns-c-MycER^{TAM}* × *RIP-Bcl-x$_L$* double transgenic mice were treated IP with 4-OHT daily for 14 days. Administration of 4-OHT was then stopped, and representative mice were sacrificed after 3, 5, 10, and 38 days. Sections of their pancreata, together with sections from a double transgenic mouse never treated with 4-OHT (Never Myc), were probed with anti-E cadherin.

that these attributes of more advanced malignancy are direct consequences of c-Myc activation rather than the outcome of sporadic secondary mutations. To explore further this pleiotropic tumorigenic potential of c-Myc, *pIns-c-MycER^{TAM}/RIP-Bcl-x$_L$* double transgenic mice were subjected to sustained c-Myc activation for extended periods up to 12 weeks. Within 2 weeks, local invasion by insulin-positive β cells was evident in at least 50% of islets (e.g., Figure 5A). By 6 weeks of sustained c-Myc activation, pancreata had become filled with multiple, large, intensely vascularized tumors (Figure 5B) with multiple areas of localized β cell invasion (Figure 5C). As before, β cells in these tumors expressed reduced insulin and undetectable E cadherin. Concomitant with widespread development of β cell tumors, all mice developed fasting hypoglycemia (Table 1), a common feature of human insulinoma due to inappropriately excessive insulin secretion, due probably to massively expanded numbers of β cells. Because of c-Myc-induced suppression of insulin expression, we used antibodies specific for the β cell lineage markers Nkx6.1 and Isl-1 to identify and locate metastatic β cells. Ectopic β cells were evident in multiple local blood vessels (Figure 5D), pancreatic ducts (Figure 5E), and draining mesenteric lymph nodes (Figure 5F).

Rapid Regression of β Cell Tumors Following c-Myc Deactivation

To determine whether c-MycER^{TAM} activation was required to maintain β cell neoplasia, c-Myc was continuously activated in 30 *pIns-c-MycER^{TAM}/RIP-Bcl-x$_L$* mice for 14 days to induce β cell tumors. Twelve mice were

sacrificed and all exhibited hypeplastic, angiogenic tumors with local invasion evident in at least 50% of islets in each section. c-Myc was then deactivated by 4-OHT withdrawal, representative mice (n = 4) were sacrificed at 4 and 10 days, and β cell masses were examined histologically together with the status of β cell proliferation, apoptosis, insulin and E-cadherin expression, and angiogenesis. Remaining mice were left for 1 month before sacrifice and analysis. Multiple sections were examined from each mouse, and representative images are shown in Figure 6.

By day 4 of 4-OHT withdrawal, most β cells had left cell cycle, as judged by Ki-67 staining, and insulin expression had returned to control levels in the smaller β cell hyperplasias, although it remained weaker in the larger β cell masses (not shown). We also observed reestablishment of β cell-cell contact, which correlated with the reexpression of E cadherin. Costaining of vascular basal lamina with anti-laminin antibodies together with TUNEL revealed numerous apoptotic cells in vessel walls, particularly in larger β cell masses, while costaining of Nkx 6.1 β cell-specific antibody with TUNEL identified apoptotic β cells (1%–2%). Many of the hyperplastic islets exhibited cavities filled with extravasated erythrocytes, presumably caused by collapse of blood vessels and death of β cells. In addition, small numbers of Ki-67-positive polymorphonuclear leucocytes and macrophages could be identified within islet blood vessels. By 10 days of c-MycER^{TAM} deactivation, all β cells were strongly positive for insulin (not shown) and E cadherin while remaining Ki-67 negative. Staining with laminin antibody indicated dramatic loss of vessel integrity and marked disruption of normal vascular architecture, accompanied by significant numbers of inflammatory cells within and adjacent to the blood vessels. β cell apoptosis was also evident with sizeable erythrocyte-filled cavities present in almost all tumors. Over the ensuing 2 weeks, vasculature continued to degenerate and tumors shrank further, eventually leading to complete apparent reversal of β cell hyperplasia by 38 days (Figure 6).

To explore the consequences of Myc deactivation on ectopic β cells that had invaded ducts, vessels, and lymph nodes, at least 20 mice were treated for 8 weeks (or longer) with continuous 4-OHT. All mice exhibited profound hypoglycemia, indicative of excessive insulin production arising from heavy β cell tumor burden. As in Figure 5, exhaustive analysis involving at least 50 sections from various planes throughout the length of each pancreas from five mice revealed multiple ectopic Nkx6.1-positive cell clusters in local lymph nodes, vessels, and ducts in all animals. c-Myc was then deactivated and blood glucose followed weekly to assess tumor burden. By 4 weeks, all animals recovered and retained normoglycemia. After a further 3 months, two animals were sacrificed and subjected to exhaustive histological analysis of at least 50 sections, each with multiple islets. In both animals, islet morphology, size, and E cadherin staining were normal, and examination of multiple sections failed to reveal any ectopic β cells in vessels, ducts, or mesenteric nodes. Some 80 other animals undergoing similar regression remained healthy and normoglycemic thereafter.

Thus, regression following c-Myc deactivation involved growth arrest, redifferentiation, reexpression of E cadherin, and reattachment of β cells together with involution of supernumerary and ectopic β cells, probably triggered by vascular collapse. These data clearly demonstrate that c-Myc is required not only to induce but also to maintain multiple aspects of neoplastic progression.

Discussion

Expression of Conditional c-Myc Protein in Pancreatic β Cells

To investigate directly the potential role of c-Myc-induced apoptosis in tumor suppression, as well as to mimic more accurately the acute somatic activation of oncogenes in intact tissues that causes adult cancers, we targeted expression of a switchable, 4-OHT-dependent c-MycER^{TAM} protein to pancreatic β cells. Immunohistochemical analysis confirmed uniform expression of c-MycER^{TAM} restricted to pancreatic β cells and at a low level similar to that in serum-stimulated primary human MRC-5 fibroblasts (Moore et al., 1987). Consequently, all the proneoplastic and proapoptotic effects we observe in this in vivo β cell model arise from c-Myc deregulation rather than overexpression.

Activation of c-Myc Alone Is Sufficient to Induce β Cell Proliferation In Vivo

In the absence of 4-OHT, expression of c-MycER^{TAM} exerts no observable effect on islets, as determined by gross islet morphology or size, distribution of α, β, and δ cells within each islet, distribution and number of islets within the whole pancreas, and extent of β cell proliferation or apoptosis. In contrast, activation of c-MycER^{TAM} triggered rapid and synchronous onset of proliferation in essentially all β cells in all islets. Furthermore, sustained c-MycER^{TAM} activation provoked sustained proliferation of β cells through multiple replicative rounds. Importantly, proliferation was not limited to any subpopulation of potential islet stem cells. We conclude that Myc activation is capable of commandeering requisite accessory proliferative machinery needed for adult β cell proliferation in vivo in the absence of overt activation of other mitogenic pathways.

c-Myc Alone Is a Net Growth Suppressor in β Cells

The physiological relevance of Myc-induced apoptosis as a tumor suppressive mechanism in vivo has been unclear. This uncertainty is further compounded by the fact that c-Myc-induced apoptosis is regulated by survival factors that vary both in availability and kind in different tissues. For example, activation of c-Myc in suprabasal keratinocytes in vivo triggers proliferation without apoptosis because of an excess of local survival factors (Pelengaris et al., 1999). In complete contrast, activation of only low levels of deregulated c-Myc in β cells triggered rapid onset of apoptosis, which overwhelmed proliferation and quickly led to attrition of β cells and the onset of diabetes. The overall extent and rate of c-Myc-induced β cell apoptosis was similar in each islet. Such dramatic differences in the effects of c-Myc in skin and β cells in vivo illustrate the fundamentally different responses of cells in differing tissues to growth deregulating lesions, differences that must have a pro-

found influence on the evolutionary pathways by which tumors evolve in each somatic setting. The facile induction of apoptosis by only low levels of c-Myc indicates that apoptosis can, indeed, act as a highly effective tumor suppressive mechanism in β cells. This is most graphically demonstrated by the synchronous and expeditious regeneration of β cells that follows c-Myc deactivation, presumably triggered by endocrine homeostatic influences analogous to those that regulate β cell mass during development and throughout adult life.

Suppression of Apoptosis Transforms c-Myc into a Potent Oncoprotein that Drives Rapid Tumor Progression

To test directly the importance of c-Myc-induced apoptosis in tumor suppression in β cells, we inhibited apoptosis by coexpression of Bcl-x_L. The consequence was dramatic—in place of involution, c-Myc activation induced immediate and progressive β cell expansion in all islets. At least two discrete effector pathways mediate c-Myc-induced apoptosis. One involves the transcriptional induction of the tumor suppressor ARF, which incapacitates the p53 antagonist Mdm-2 and so triggers accumulation of p53 (Zindy et al., 1998). The other involves a p53-independent proapoptotic action through unknown effectors that primes mitochondria for release of proapoptotic factors such as holocytochrome c (Juin et al., 1999). Since Bcl-x_L suppresses the mitochondrial apoptotic pathway, its efficacy at blocking c-Myc-induced apoptosis in β cells intimates that all downstream c-Myc apoptotic effector pathways, both p53-dependent and -independent, are channeled through the mitochondria.

As the β cell tumors expand under the combined influence of c-Myc and Bcl-x_L, they seamlessly, rapidly, and spontaneously acquire attributes of more advanced neoplasia, including dedifferentiation, angiogenesis, tissue disorganization, and invasion. Moreover, such tumor progression arises rapidly and with similar kinetics in each of the hundreds of spatially separated islets within the pancreas. Even local invasion, by necessity a focal process, is sufficiently widespread to be evident in over half of the hyperplastic islets in each tissue plane examined. The uniformity and rapidity of such progression indicates that it is very unlikely to arise through stochastic secondary lesions, which would be expected to arise focally within individual islets after protracted periods, as they do in the RIP-Tag model (Christofori and Hanahan, 1994).

Our conclusion is that c-Myc must directly elicit in each β cell those attributes, such as dedifferentiation, angiogenesis, and invasion, that drive the observed tumor progression. In fact, such properties are consistent with some of the known activities described for c-Myc. For example, c-Myc antagonizes differentiation in multiple cell lineages including β cells, where we show it triggers rapid downregulation of insulin and PDX-1, both de facto markers of β cell differentiation. A potential mechanism for this is suggested by the recent report that c-Myc may directly interfere with the action of the β cell-specifying bHLH transcription factor Beta2/NeuroD by inhibiting its binding to a consensus E box element in the insulin promoter (Kaneto et al., 2002). In addition, however, c-Myc upregulates ID2 which, as a generic quencher of multiple bHLH lineage-specifying transcription factors (Lasorella et al., 2000), has the potential to interfere with multiple differentiation programs.

c-Myc has also been shown to be angiogenic in multiple cells types, both through its repression of the angiogenesis inhibitor Thrombospondin-1 (Janz et al., 2000; Ngo et al., 2000) and its induction of VEGF (Brandvold et al., 2000; Breit et al., 2000; Fotsis et al., 1999; Harris et al., 2000; Pelengaris et al., 1999). Indeed, emerging evidence suggests that many oncogenic lesions, including c-Myc, are innately angiogenic through their abilities to induce expression of the angiogenic transcription factor HIF1α (Semenza, 2002). A direct role for c-Myc in promoting angiogenesis is also strongly supported by our observation that c-Myc deactivation triggers the rapid collapse of β cell tumor vasculature, although the precise mechanism awaits further analysis.

Finally, our own data show that a direct consequence of c-Myc activation is the downregulation of the intercellular adhesion molecule E cadherin in β cells. Since E cadherin downregulation is causally linked to sporadic emergence of invasive β cell carcinomas in the RIP-Tag model (Perl et al., 1998), as well as in most tumors of epithelial origin (Christofori and Semb, 1999), its repression by c-Myc offers a direct potential mechanism by which the oncoprotein promotes β cell invasion.

All In Vivo Effects of c-Myc Are Reversible

A great advantage of the short plasma half-life of 4-OHT is that cessation of 4-OHT administration leads to the rapid deactivation of c-MycER^{TAM} in vivo. In single c-MycER^{TAM} transgenics where c-Myc activation triggers overwhelming β cell apoptosis, subsequent c-MycER^{TAM} deactivation triggers synchronous β cell and islet regeneration. The most likely source of these regenerating β cells is probably some β cell precursor whose insulin negativity spares it from the lethal effects of c-Myc expression and which, from the location of proliferating cells during β cell regeneration, probably resides in the pancreatic ducts. However, the precise lineage and nature of the regenerative cell remains unknown.

In c-MycER^{TAM}/Bcl-x_L double transgenics, c-MycER^{TAM} activation elicits immediate and progressive β cell neoplasia in all islets. However, even after 2–3 weeks of continuous c-Myc activation, over which time multiple angiogenic and locally invasive tumors form, deactivation of c-Myc leads to general regression of tumors back to phenotypically normal, fully functional, insulin-positive, quiescent islets with normal levels of E cadherin. We conclude from this that all the neoplastic paraphernalia we observe in the hyperplastic c-Myc/Bcl-x_L β cell tumors, including dedifferentiation, invasion, and angiogenesis, remain completely dependent upon sustained c-Myc activity for their support and maintenance. Interestingly, regression is also accompanied by substantial infiltration of inflammatory cells that may be responsible for phagocytosis and clearance of apoptotic and/or necrotic debris.

Detailed kinetic analysis suggests that regression is mediated, at least in great part, by rapid collapse of vasculature that triggers death of excess β cells. Such rapid disassembly of vasculature is most consistent with

a direct angiogenic role for c-Myc, as proposed above. However, it is also possible that β cell growth arrest following Myc deactivation allows intrinsic antineoplastic mechanisms such as the immune response to initiate regression. Another possibility is that β cell redifferentiation renders supernumerary β cells prey to such homeostatic forces as normally restrict β cell mass. In this context, it is especially intriguing that regressing β cell tumors return to normal islet size and morphology, illustrating that β cell mass is stringently and adaptively regulated, at least within the pancreas.

c-Myc and Tumor Progression

The best-established and -characterized transgenic model of β cell carcinogenesis is the elegant RIP-Tag system developed by Hanahan and colleagues in which expression of the SV40 early region encoding large and small T antigens is targeted to pancreatic β cells using the rat insulin promoter (Christofori and Hanahan, 1994). RIP-Tag mouse islets appear phenotypically normal at birth, but hyperplastic islets emerge stochastically at around 3–4 weeks of age. Such enlarged islets exhibit hyperproliferation, presumably driven by expression of SV40 T antigens, together with features of dysplasia and carcinoma in situ. Only some 10% of hyperplastic islets eventually switch to an angiogenic phenotype and fewer still develop into solid adenomas, while progression to invasive carcinoma is very rare. Thus, as with many other transgenic oncogene mouse models, tumor progression in RIP-Tag mice is sporadic, protracted, and sequential, consistent with the general view that tumors arise through the stochastic accumulation of multiple mutations, each of which confers its own neoplastic signature on the developing neoplasm.

Our acute c-Myc activation β cell model clearly differs dramatically from the RIP-Tag model. Instead of sporadic malignant progression, acute activation of c-Myc triggers immediate and ubiquitous neoplastic progression in all islets so long as c-Myc-induced apoptosis is suppressed. The speed and uniformity of such malignant progression in so many spatially isolated β cell masses within the pancreas precludes a role for secondary lesions, at least for the development of dedifferentiated, angiogenic, and invasive tumors. Rather, such attributes appear to be direct consequences of c-Myc activation in affected β cells which, by virtue of their deregulated proliferation and suppressed apoptosis, spontaneously acquire the capacity to generate the requisite blood supply and somatic space for their expansion. Since the *RIP-Tag* and *pIns-c-MycER^{TAM}/Bcl-x$_L$* transgenic models differ both in the oncogenes driving tumorigenesis and in the manner and timing of their activation, either or both of these differences may contribute to the dramatic differences in rate, extent, and uniformity of tumor progression in each model. Nonetheless, our data demonstrate unequivocally that complex neoplastic phenomena involving both the affected tumor cells and its interactions with normal tissues can be both induced and maintained by a very limited platform of interlocking pleiotropic mutations that, minimally, drive cell proliferation and incapacitate concomitant apoptosis. It is also clear that growth deregulating lesions such as activated c-Myc do far more than merely drive tumor cell proliferation but, in addition, orchestrate many of the general attributes that expanding somatic cells will require to establish themselves within the soma. It remains to be seen whether such pleiotropic properties are peculiar to c-Myc or are shared by other factors that promote cell expansion, including oncogenic lesions in the Ras and E2F pathways. However, recent studies with other switchable oncogene transgenic models reinforce the notion that incapacitating the driving oncogenic lesion can lead to expeditious regression of tumors induced in many different tissues by c-Myc (Felsher and Bishop, 1999; Pelengaris et al., 1999), Ras (Chin et al., 1999; Fisher et al., 2001), or even T antigens (Berkovich and Efrat, 2001). At least in principle, therefore, the complexity of the tumor phenotype need not be instructed by an equivalent complexity of genetic or epigenetic alteration. Rather, cancers may be underpinned by only a modest number of interdependent, pleiotropic lesions that present themselves as mission-critical targets for effective cancer therapies.

Experimental Procedures

pIns-c-MycER^{TAM} Transgene Construction

A full-length human c-*myc* cDNA fused to the hormone binding domain of a modified estrogen receptor, c-MycER^{TAM}, has been described previously (Littlewood et al., 1995). The insulin promoter construct (Ohashi et al., 1991) was a kind gift from T. Moroy and R.M. Zinkernagel and comprises 0.7 kb of the rat insulin promoter and 1.6 kb of the β-globin polyadenylation sequence (Figure 1A) in a puc18 backbone. c-MycER^{TAM} DNA was excised from pBluescript as an *Eco*RI fragment and blunt-ended with T4 DNA polymerase. The insulin promoter cassette was cut with *BamH1* and blunt-ended with T4 polymerase, and the c-MycER^{TAM} sequence was inserted. Since no unique restriction sites were present in the parent plasmid vector (puc18), the insert was excised from puc18 vector with *Sma*I and *Hind*III to generate two fragments (*Sma*I-*Sma*I and *Sma*I-*Hind*III) that were subsequently subcloned into the *Sma*I-*Hind*III site of pBluescript in a 3-way ligation. The final transgene unit was then excised from pBluescript as a *Not*I-*Hind*III fragment (~4.6 kb). Purified DNA was resuspended for pronuclear injection in sterile injection buffer (10 mM Tris, 0.1 mM EDTA [pH 7.4]) at a concentration of 5 ng/μl.

Generation of *pIns-c-MycER^{TAM}* Transgenic Mice and Estimation of Transgene Copy Number

pIns-c-MycER^{TAM} DNA was injected into male pronuclei of day 1-fertilized (CBA × C57BL/6)F1 embryos. Injected embryos were transferred into day 1-plugged pseudopregnant foster mice, and the litters were screened for presence of the transgene by Southern blotting. Heterozygous founder mice were backcrossed appropriately to establish transgenic lines.

For Southern blot analysis, genomic DNA isolated from mouse tails (3 mm) was digested overnight with *BamH*I and *Eco*RI to excise a fragment of approximately 1.8 kb, fractionated on a 1% agarose gel, transferred to a Hybond-N+ filter (Amersham), and probed with a ^{32}P-radiolabeled 1.2 kb human c-*myc* cDNA. Transgene copy number was estimated by densitometric comparison of the 1.8 kb excised transgene fragment with the endogenous single-copy 2 kb Eμ immunoglobin heavy chain gene excised by *Eco*RI-digestion of genomic DNA.

Genotype Analysis of *pIns-c-MycER^{TAM}* and *RIP7-Bcl-x$_L$* Transgenic Mice

Heterozygous *RIP7-Bcl-x$_L$* transgenic mice were obtained from Dr. Doug Hanahan (UCSF). Litters from both *pIns-c-MycER^{TAM}* and *RIP7-Bcl-x$_L$* transgenic mice and appropriate F1 crosses were routinely genotyped by PCR analysis on genomic DNA isolated from mouse tails.

Histological and Immunohistochemical Analysis of Pancreatic Tissue

Pancreata were excised from mice, and 5 mm pieces of tissue were fixed overnight in neutral-buffered formalin, embedded in paraffin wax, and sectioned (5–10 μm). Frozen sections were prepared from tissue fixed in 4% paraformaldehyde for 4 hr, transferred to cold 30% sucrose in PBS overnight at 4°C, embedded in OCT, and frozen in foil on a bath of dry ice and ethanol. Apoptotic cells were identified in paraffin-embedded sections by TUNEL, using the ApopTag Plus Peroxidase kit (Intergen), and the sections were counterstained with methylene blue.

Primary antibodies were as follows: rabbit polyclonals included Pan-Myc antibodies (Moore et al., 1987); HL7 murine estrogen receptor antibody (kindly provided by Drs. Andreas Sewing and Hartmut Land); Ki-67 (Novacastra); Nkx6.1 antibody (a kind gift from Ole Madsen, NovoNordisk); Isl-1 antibody (a kind gift from Helena Edlund, University of Umea); glucagon antibody (Dako); somatostatin antibody (Dako); laminin (Sigma); guinea pig anti-porcine insulin (Dako); purified rat anti-mouse CD31 (Pharmingen); and rat anti-mouse E cadherin, (Zymed). Specificity of staining with Pan-Myc and HL7 antibodies was demonstrated by preincubation with 2 μg/ml of their cognate peptide immunogen for 30 min at room temperature.

Pan-Myc, HL7, E cadherin, and CD31 antibodies were found to label reliably only frozen tissue sections. Other antibodies were effective when used on both paraffin-embedded and frozen sections, although Ki-67, Nkx6.1, and laminin antibodies required epitope retrieval by microwaving paraffin-embedded sections at 700 W for 2 × 10 min in 0.01 M citrate buffer (pH 6.0) (Vector) followed by immersion in cold water.

Antibodies were diluted in incubation buffer: PBS/0.5% Triton X-100 containing 1:25 dilution of serum from the same species as the secondary antibody. For all antibodies except CD31 and double immunofluorescence staining with E cadherin/Pan-Myc, TUNEL/Nkx6.1, or TUNEL/laminin, appropriate ABC Vectastain Elite kits (Vector) were used according to manufacturers' instructions. Labeled sections were lightly counterstained in hematoxylin. For CD31 immunofluorescence, rabbit anti-rat Ig-conjugated FITC (Vector) was used as secondary antibody. For double E cadherin/Pan-Myc immunofluorescence staining, E cadherin and Pan-Myc antibodies were applied together to sections overnight at 4°C. Sections were then incubated in Texas red-conjugated goat anti-rabbit Ig secondary antibody (Vector) followed by FITC-conjugated rabbit anti-rat secondary antibody. After washing, sections were mounted in Vectashield mounting medium (Vector). To detect cells undergoing apoptosis, double TUNEL/Nkx6.1 and TUNEL/laminin immunofluorescent staining was performed by applying Nkx6.1 or laminin antibodies to frozen sections for 1 hr at room temperature followed by Texas red-conjugated goat anti-rabbit Ig secondary antibody (Vector). TUNEL staining was by ApopTag Fluorescein Direct kit (Intergen).

Isolation of Mouse Pancreatic Islets

Pancreatic islets were isolated from adult transgenic and wild-type mice as described (Lake et al., 1989) with slight modification. Briefly, a 2 ml solution of collagenase (1 mg/ml; Serva) containing DNase (0.1 mg/ml; Pharmacia) in cold minimal essential medium (MEM) was injected into various sites of an exposed pancreas. Warm MEM (10 ml) was then added, and each universal container was placed in a water bath at 37°C for 18 min to allow collagen digestion. The medium was removed and replaced with cold 10 ml MEM to stop digestion, the tubes were cooled on ice, and the tissue was disrupted by vigorously hand shaking the containers for 1 min. Digested material was pooled and poured through a 500 μm mesh into a sterile beaker. The crude islet preparation was washed once with MEM + 10% FCS, pelleted at 200 × g for 1 min at 4°C, washed again, and resuspended in MEM + 10% FCS, and the islets were "hand-picked" under a dissecting microscope to achieve a purity of close to 100%.

Immunoblot Analysis

Isolated pancreatic islets were resuspended in a small volume of cold PBS (50–100 μl), and an aliquot (10–20 μl) was removed for protein quantitation (Bradford assay). Remaining islets were then lysed in an equal volume (50–100 μl) of SDS-PAGE sample buffer (Laemmli, 1970) and denatured by brief boiling, and 5–10 μg total protein was fractionated on a 7.5% PAGE-SDS gel and transferred to a PVDF membrane (NEN Life Science Products) by electroblotting. Filters were incubated in 5% nonfat dried milk (Marvel, Cadbury) in PBS + 0.1% Tween-20 for 1 hr at room temperature and then in appropriate antibody in PBS containing 1% nonfat dried milk and 0.1% Tween-20 overnight at 4°C. Filters were washed in PBS + 0.1% Tween-20 and incubated for 1 hr with peroxidase-conjugated sheep anti-rabbit IgG (for Pan-Myc) or anti-rat IgG (for E cadherin) (Amersham) diluted 1:2000 in the same buffer as the primary antibodies. Filters were washed as before, and immune complexes were detected by ECL-Plus (Amersham).

Culture of Swiss 3T3 Cells

S3T3 cells were grown to 40% confluence in DMEM supplemented with 10% FCS. S3T3 cells were lysed directly on dishes using the same buffers as described above.

Assay of Blood and Urinary Glucose

All animals used in these studies were adults between 3 and 8 months of age. For blood glucose measurement, mice were fasted for 4 hr and then placed into a heat chamber (32°C) for approximately 10 min to induce vasodilation. Blood (0.2 ml) was collected from tail veins, and glucose concentration was determined using a Gluco-Trend 2 kit (Roche). Diastix test strips were used to determine urinary glucose concentrations.

Activation of c-MycER^{TAM} by Administration of 4-Hydroxytamoxifen (4-OHT)

To activate c-MycER^{TAM} protein in pancreatic β-cells of adult transgenic mice, 1 mg of 4-OHT (Sigma) sonicated in peanut oil (1 mg/0.2 ml) was administered daily by IP injection. Control wild-type and *RIP7-Bcl-x$_L$* also received daily IP injections of 4-OHT as appropriate. Untreated *pIns-c-MycER^{TAM}* mouse controls received 0.2 ml IP peanut oil without 4-OHT daily.

Acknowledgments

We are especially grateful to Trevor Littlewood for advice and technical assistance and Roger James for help with isolation of mouse pancreatic islets. We thank Drs. Doug Hanahan for providing *RIP7-Bcl-x$_L$* mice and Tarik Moroy for the insulin promoter construct. Our thanks to the animal units at Imperial Cancer Research Fund, University of Warwick, UK and University of California at San Francisco Cancer Center, to Ian Rosewell for assistance with transgene microinjection, to Drs. Helena Edlund, Ole Madsen, and Palle Serup for valuable antibodies, to Dr. Lamorna Brown Swigart for help and advice with everything, and finally to Profs. H. Dalton and P. Bell, to Drs. P. O'Hare and L. Boothe, and to D. Hanahan, J. Bluestone, I. Rulifson, and our other UCSF colleagues for invaluable help and discussion. S.P. thanks the Samuel Scott of Yews Trust and Coventry General Charities, and G.E. thanks Gerson and Barbara Bakar and the Daiichi Cancer Research Program for their continued support.

This manuscript is dedicated by G.E. to the memories of Max Perutz and César Milstein: their intellect, dedication, kindness, and courtesy are a continued inspiration.

Received: December 14, 2001
Revised: April 15, 2002

References

Askew, D., Ashmun, R., Simmons, B., and Cleveland, J. (1991). Constitutive c-*myc* expression in IL-3-dependent myeloid cell line suppresses cycle arrest and accelerates apoptosis. Oncogene *6*, 1915–1922.

Berkovich, I., and Efrat, S. (2001). Inducible and reversible beta-cell autoimmunity and hyperplasia in transgenic mice expressing a conditional oncogene. Diabetes *50*, 2260–2267.

Birchmeier, W., Hulsken, J., and Behrens, J. (1995). E-cadherin as an invasion suppressor. Ciba Found. Symp *189*, 124–141, 174–176.

Bissonnette, R., Echeverri, F., Mahboubi, A., and Green, D. (1992). Apoptotic cell death induced by c-*myc* is inhibited by *bcl*-2. Nature *359*, 552–554.

Blyth, K., Terry, A., O'Hara, M., Baxter, E.W., Campbell, M., Stewart, M., Donehower, L.A., Onions, D.E., Neil, J.C., and Cameron, E.R. (1995). Synergy between a human c-myc transgene and p53 null genotype in murine thymic lymphomas: contrasting effects of homozygous and heterozygous p53 loss. Oncogene *10*, 1717–1723.

Bonner-Weir, S. (2001). beta-cell turnover: its assessment and implications. Diabetes *50* (*Suppl 1*), S20–S24.

Brandvold, K.A., Neiman, P., and Ruddell, A. (2000). Angiogenesis is an early event in the generation of myc-induced lymphomas. Oncogene *19*, 2780–2785.

Breit, S., Ashman, K., Wilting, J., Rossler, J., Hatzi, E., Fotsis, T., and Schweigerer, L. (2000). The N-myc oncogene in human neuroblastoma cells: down-regulation of an angiogenesis inhibitor identified as activin A. Cancer Res. *60*, 4596–4601.

Chin, L., Tam, A., Pomerantz, J., Wong, M., Holash, J., Bardeesy, N., Shen, Q., O'Hagan, R., Pantginis, J., Zhou, H., et al. (1999). Essential role for oncogenic Ras in tumour maintenance. Nature *400*, 468–472.

Christofori, G., and Hanahan, D. (1994). Molecular dissection of multi-stage tumorigenesis in transgenic mice. Semin. Cancer Biol. *5*, 3–12.

Christofori, G., and Semb, H. (1999). The role of the cell-adhesion molecule E-cadherin as a tumour-suppressor gene. Trends Biochem. Sci. *24*, 73–76.

Eischen, C.M., Weber, J.D., Roussel, M.F., Sherr, C.J., and Cleveland, J.L. (1999). Disruption of the ARF-Mdm2-p53 tumor suppressor pathway in Myc-induced lymphomagenesis. Genes Dev. *13*, 2658–2669.

Eischen, C.M., Woo, D., Roussel, M.F., and Cleveland, J.L. (2001). Apoptosis triggered by Myc-induced suppression of Bcl-X(L) or Bcl-2 is bypassed during lymphomagenesis. Mol. Cell. Biol. *21*, 5063–5070.

Eisenman, R.N. (2001). Deconstructing myc. Genes Dev. *15*, 2023–2030.

Evan, G., and Littlewood, T. (1998). A matter of life and cell death. Science *281*, 1317–1322.

Evan, G.I., and Vousden, K.H. (2001). Proliferation, cell cycle and apoptosis in cancer. Nature *411*, 342–348.

Evan, G., Wyllie, A., Gilbert, C., Littlewood, T., Land, H., Brooks, M., Waters, C., Penn, L., and Hancock, D. (1992). Induction of apoptosis in fibroblasts by c-*myc* protein. Cell *63*, 119–125.

Fanidi, A., Harrington, E., and Evan, G. (1992). Cooperative interaction between c-*myc* and *bcl*-2 proto-oncogenes. Nature *359*, 554–556.

Felsher, D.W., and Bishop, J.M. (1999). Reversible tumorigenesis by myc in hematopoietic lineages. Mol. Cell *4*, 199–207.

Fisher, G.H., Wellen, S.L., Klimstra, D., Lenczowski, J.M., Tichelaar, J.W., Lizak, M.J., Whitsett, J.A., Koretsky, A., and Varmus, H.E. (2001). Induction and apoptotic regression of lung adenocarcinomas by regulation of a K-Ras transgene in the presence and absence of tumor suppressor genes. Genes Dev. *15*, 3249–3262.

Folkman, J. (1992). The role of angiogenesis in tumor growth. Semin. Cancer Biol. *3*, 65–71.

Fotsis, T., Breit, S., Lutz, W., Rossler, J., Hatzi, E., Schwab, M., and Schweigerer, L. (1999). Down-regulation of endothelial cell growth inhibitors by enhanced MYCN oncogene expression in human neuroblastoma cells. Eur. J. Biochem. *263*, 757–764.

Hanahan, D., and Weinberg, R.A. (2000). The hallmarks of cancer. Cell *100*, 57–70.

Harrington, E.A., Bennett, M.R., Fanidi, A., and Evan, G.I. (1994). c-Myc-induced apoptosis in fibroblasts is inhibited by specific cytokines. EMBO J. *13*, 3286–3295.

Harris, V.K., Coticchia, C.M., List, H.J., Wellstein, A., and Riegel, A.T. (2000). Mitogen-induced expression of the fibroblast growth factor-binding protein is transcriptionally repressed through a noncanonical E-box element. J. Biol. Chem. *275*, 28539–28548.

Hodgson, G., Hager, J.H., Volik, S., Hariono, S., Wernick, M., Moore, D., Albertson, D.G., Pinkel, D., Collins, C., Hanahan, D., and Gray, J.W. (2001). Genome scanning with array CGH delineates regional alterations in mouse islet carcinomas. Nat. Genet. *29*, 459–464.

Jacobs, J.J., Scheijen, B., Voncken, J.W., Kieboom, K., Berns, A., and van Lohuizen, M. (1999). Bmi-1 collaborates with c-Myc in tumorigenesis by inhibiting c-Myc-induced apoptosis via INK4a/ARF. Genes Dev. *13*, 2678–2690.

Janz, A., Sevignani, C., Kenyon, K., Ngo, C.V., and Thomas-Tikhonenko, A. (2000). Activation of the myc oncoprotein leads to increased turnover of thrombospondin-1 mRNA. Nucleic Acids Res. *28*, 2268–2275.

Juin, P., Hueber, A.O., Littlewood, T., and Evan, G. (1999). c-Myc-induced sensitization to apoptosis is mediated through cytochrome c release. Genes Dev. *13*, 1367–1381.

Kaneto, H., Sharma, A., Suzuma, K., Laybutt, D.R., Xu, G., Bonner-Weir, S., and Weir, G.C. (2002). Induction of c-Myc expression suppresses insulin gene transcription by inhibiting NeuroD/BETA2-mediated transcriptional activation. J. Biol. Chem. *277*, 12998–13006.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680–685.

Lake, S., Chamberlain, J., Walczak, K., Bell, P., and James, R. (1989). A test gradient system for optimizing density gradient isolation of pancreatic islets. Transplantation *48*, 354–358.

Lasorella, A., Noseda, M., Beyna, M., and Iavarone, A. (2000). Id2 is a retinoblastoma protein target and mediates signalling by Myc oncoproteins. Nature *407*, 592–598.

Littlewood, T., Hancock, D., Danielian, P., Parker, M., and Evan, G. (1995). A modified oestrogen receptor ligand-binding domain as an improved switch for the regulation of heterologous proteins. Nucleic Acids Res. *23*, 1686–1690.

Lotem, J., and Sachs, L. (1995). A mutant p53 antagonizes the deregulated c-myc-mediated enhancement of apoptosis and decrease in leukemogenicity. Proc. Natl. Acad. Sci. USA *92*, 9672–9676.

Maestro, R., Dei Tos, A.P., Hamamori, Y., Krasnokutsky, S., Sartorelli, V., Kedes, L., Doglioni, C., Beach, D.H., and Hannon, G.J. (1999). Twist is a potential oncogene that inhibits apoptosis. Genes Dev. *13*, 2207–2217.

Moore, J.P., Hancock, D.C., Littlewood, T.D., and Evan, G.I. (1987). A sensitive and quantitative enzyme-linked immunosorbence assay for the c-*myc* and N-*myc* oncoproteins. Oncogene Res. *2*, 65–80.

Ngo, C.V., Gee, M., Akhtar, N., Yu, D., Volpert, O., Auerbach, R., and Thomas-Tikhonenko, A. (2000). An in vivo function for the transforming Myc protein: elicitation of the angiogenic phenotype. Cell Growth Differ. *11*, 201–210.

Ohashi, P.S., Oehen, S., Buerki, K., Pircher, H., Ohashi, C.T., Odermatt, B., Malissen, B., Zinkernagel, R.M., and Hengartner, H. (1991). Ablation of "tolerance" and induction of diabetes by virus infection in viral antigen transgenic mice. Cell *65*, 305–317.

Pavelic, K., Hrascan, R., Kapitanovic, S., Vranes, Z., Cabrijan, T., Spaventi, S., Korsic, M., Krizanac, S., Li, Y.Q., Stambrook, P., et al. (1996). Molecular genetics of malignant insulinoma. Anticancer Res. *16*, 1707–1717.

Pelengaris, S., Littlewood, T., Khan, M., Elia, G., and Evan, G. (1999). Reversible activation of c-Myc in skin: induction of a complex neoplastic phenotype by a single oncogenic lesion. Mol. Cell *3*, 565–577.

Perl, A.K., Wilgenbus, P., Dahl, U., Semb, H., and Christofori, G. (1998). A causal role for E-cadherin in the transition from adenoma to carcinoma. Nature *392*, 190–193.

Schmitt, C.A., McCurrach, M.E., de Stanchina, E., Wallace-Brodeur, R.R., and Lowe, S.W. (1999). INK4a/ARF mutations accelerate lymphomagenesis and promote chemoresistance by disabling p53. Genes Dev. *13*, 2670–2677.

Semenza, G.L. (2002). Involvement of hypoxia-inducible factor 1 in human cancer. Intern. Med. *41*, 79–83.

Sherr, C.J. (2000). The Pezcoller lecture: cancer cell cycles revisited. Cancer Res. *60*, 3689–3695.

Strasser, A., Harris, A.W., Bath, M.L., and Cory, S. (1990). Novel

primitive lymphoid tumours induced in transgenic mice by cooperation between *myc* and *bcl*-2. Nature *348*, 331–333.

Takeichi, M. (1993). Cadherins in cancer: implications for invasion and metastasis. Curr. Opin. Cell Biol. *5*, 806–811.

Wagner, A.J., Small, M.B., and Hay, N. (1993). Myc-mediated apoptosis is blocked by ectopic expression of *bcl*-2. Mol. Cell. Biol. *13*, 2432–2440.

Wagner, A.J., Kokontis, J.M., and Hay, N. (1994). Myc-mediated apoptosis requires wild-type p53 in a manner independent of cell cycle arrest and the ability of p53 to induce p21waf1/cip1. Genes Dev. *8*, 2817–2830.

Zhou, Y.P., Pena, J.C., Roe, M.W., Mittal, A., Levisetti, M., Baldwin, A.C., Pugh, W., Ostrega, D., Ahmed, N., Bindokas, V.P., et al. (2000). Overexpression of Bcl-x(L) in beta-cells prevents cell death but impairs mitochondrial signal for insulin secretion. Am. J. Physiol. Endocrinol. Metab. *278*, E340–E351.

Zindy, F., Eischen, C.M., Randle, D.H., Kamijo, T., Cleveland, J.L., Sherr, C.J., and Roussel, M.F. (1998). Myc signaling via the ARF tumor suppressor regulates p53-dependent apoptosis and immortalization. Genes Dev. *12*, 2424–2433.