

Tissue-Specific Deletion of the Retinoblastoma Protein in the Pancreatic β -Cell Has Limited Effects on β -Cell Replication, Mass, and Function

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Animal studies show that $G_{1/S}$ regulatory molecules (D-cyclins, cdk-4, p18, p21, p27) are critical for normal regulation of β -cell proliferation, mass, and function. The retinoblastoma protein, pRb, is positioned at the very end of a cascade of these regulatory proteins and is considered the final checkpoint molecule that maintains β -cell cycle arrest. Logically, removal of pRb from the β -cell should result in unrestrained β -cell replication, increased β -cell mass, and insulin-mediated hypoglycemia. Because global loss of both pRb alleles is embryonic lethal, this hypothesis has not been tested in β -cells. We developed two types of conditional knockout (CKO) mice in which both alleles of the pRb gene were inactivated specifically in β -cells. Surprisingly, although the pRb gene was efficiently recombined in β -cells of both CKO models, changes in β -cell mass, β -cell replication rates, insulin concentrations, and blood glucose levels were limited or absent. Other pRb family members, p107 and p130, were not substantially upregulated. In contrast to dogma, the pRb protein is not essential to maintain cell cycle arrest in the pancreatic β -cell. This may reflect fundamental inaccuracies in models of β -cell cycle control or complementation for pRb by undefined proteins. *Diabetes* 56:57–64, 2007

Whereas it is clear that pancreatic β -cells can and do replicate, it is also clear that they replicate extremely slowly (1–9). Thus, the large majority (>97–99%) of β -cells at any given time are in $G_{1/0}$ arrest (1–9). The cellular mechanisms that prevent passage of the majority of β -cells through the $G_{1/S}$ transition are beginning to be elucidated (see Fig. 1S in the online appendix [available at <http://diabetes.diabetesjournals.org>]). For example, upstream inhibitors of the $G_{1/S}$ cascade, including p18, p21, p27, p53, and p57, have all been shown to be involved in maintaining $G_{1/0}$ arrest in β -cells (1,10–15). The importance of the $G_{1/S}$ control point is further illustrated by the observation that

SV40 large T-antigen (TAg), which inhibits the function of p53 and the retinoblastoma protein (pRb), markedly accelerates cell cycle progression in the β -cells of the rat insulin promoter (RIP)-TAg transgenic mouse (16,17). Implicit in all of these findings is that all of the upstream cell cycle inhibitors ultimately feed into the pRb pathway. Thus, it is reasonable to assume that the pRb protein is the ultimate “brake” or “checkpoint control” protein that restrains cell cycle progression through $G_{1/S}$ in the pancreatic β -cell and thereby enforces $G_{1/0}$ arrest (1,18,19).

pRb is a 105-kDa protein that possesses 16 different phosphorylation sites, principally on serines and threonines (for recent reviews of pRb structure and function, see refs. 1,18,20–23). Nonphosphorylated pRb binds to the E2F family of transcription factors, which in turn bind to promoters of a variety of genes required for cell cycle progression, such as those involved in DNA synthesis, t-RNA synthesis, ribosomal synthesis, and generalized protein synthesis. pRb-E2F interactions generally serve to repress these genes. pRb also binds to, and recruits histone deacetylase to, E2F-containing promoters, allowing for a second epigenetic mechanism for enforcing cell cycle arrest. Finally, pRb binds to a number of basic helix-loop-helix transcription factors and, by binding to these tissue-specific transcription factors, is believed to contribute to the maintenance of the fully differentiated phenotype of a variety of cell types.

As suggested above, pRb can be phosphorylated, and this occurs principally via two kinase complexes composed variably of cdk-4 or cdk-6 (interacting with one or more of the three D-cyclins) or of cdk-2 (interacting with cyclin E or cyclin A) (1,18,20–23) (Fig. 1S). Phosphorylation of pRb to phospho-pRb functionally inactivates pRb, a result of conformational changes that impair E2F family members from binding pRb, with the consequent release from transcriptional repression and with resultant cell cycle progression. Indeed, cdk-4 or cyclin D₂ loss results in β -cell failure (24–27), and overexpression of cdk-4 or cyclin D₁ results in marked increases in β -cell proliferation (8,24,28).

Surprisingly, given its widely accepted central role in β -cell cycle control, pRb has not been intensively studied in the pancreatic islet. We have recently demonstrated that pRb is indeed present in the mouse, rat, and human islet and that it can be phosphorylated by overexpression of cyclin D₁ alone or in combination with cdk-4 (8,12). As one would predict from other tissues, pRb phosphorylation (inactivation) by cdk-4/cyclin D₁ in the islet is associated with release of $G_{1/0}$ arrest and new DNA synthesis in

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BrdU, bromodeoxyuridine; RIP, rat insulin promoter.

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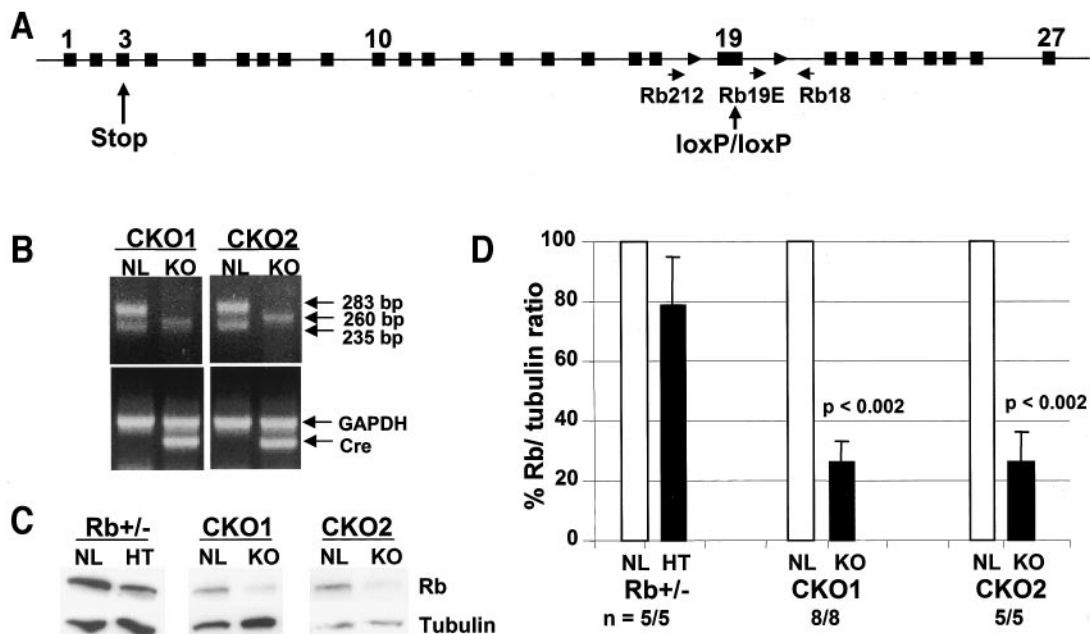


FIG. 1. β -Cell-targeted disruption of the pRb gene. **A** shows a schematic diagram of the 27 exons of the murine pRb gene and the exon targets of the two types of gene disruption. As indicated by the “stop” below exon 3, in the Jacks model (31), exon 3 is altered to contain a premature stop codon. As shown by the two black arrowheads on either side of exon 19, in the Berns model (32), exon 19 is flanked by two loxP sites that permit Cre-mediated excision of exon 19. The PCR primers used to detect the loxP-containing allele and the recombinated allele are shown as arrows, denoted “Rb212,” “Rb19E,” and “Rb18,” as described in RESEARCH DESIGN AND METHODS and reported by Marino et al. (32). **B** shows the results of PCR for pRb in islets derived from the CKO1 and CKO2 mice and their normal littermates. NL, normal littermates; KO, conditional knockouts. The 283-bp PCR product results from Rb19E-Rb18 amplification of the loxP-containing allele and indicates the presence of the allele containing the loxP sites. The 260-bp PCR product results from amplification of the recombinated or disrupted allele between primers Rb18 and Rb212 and represents the recombinated allele. The 235-bp product results from amplification of the native or wild-type Rb19E-Rb18 PCR product that lacks the loxP sites. The lower two panels indicate uniform amplification of the GAPDH housekeeping standard and the RIP-Cre transgene in the CKO islets. **C** shows a Western immunoblot for pRb in isolated pancreatic islets derived from heterozygous animals with the Jacks construct and from the two types of mice with conditional deletion of pRb. HT, heterozygous pRb-null mice. **D** shows the means of densitometric analysis of five to eight immunoblots from islets of each of the three genotypes above, as well as controls.

β -cells, as demonstrated by both tritiated thymidine as well as bromodeoxyuridine (BrdU) incorporation (8).

Given that pRb is present in the islet and that experimentally induced pRb phosphorylation (inactivation) leads to cell cycle progression in the pancreatic β -cell, it seems axiomatic that loss of pRb, the key $G_{1/S}$ checkpoint protein, would lead to marked acceleration of β -cell replication. Surprisingly, this question has not been asked previously. Homozygous loss of pRb results in early (embryonic day 12–14, depending on the genetic background) embryonic lethality (29,30), before islet development occurs. Loss of one allele of pRb is consistent with relatively normal survival until \sim 6 months of age, when a variety of tumors develop, resulting in premature death of pRb^{+/-} mice (29,30). Interestingly, despite the presumed central role of pRb in β -cell cycle control, none of the tumors that occur in pRb^{+/-} mice are derived from the pancreatic islet (29,30). Finally, pRb^{+/-} mice have not been carefully studied with regard to β -cell replication or glucose homeostasis.

Here, using three different mouse genetic approaches, we report that in contrast to widely accepted dogma, pRb is not essential for normal pancreatic β -cell growth and development. Mice lacking pRb in the islet display normal islet number, proliferation, and function and near-normal islet mass. These findings suggest that either the widely held model for $G_{1/S}$ cell cycle control is incorrect or that other molecules are able to adapt and compensate for pRb loss. Finally, these observations underscore how little we currently know regarding fundamental cell cycle control in the pancreatic β -cell.

RESEARCH DESIGN AND METHODS

Mouse models. Mice that are heterozygous knockouts for pRb were purchased from the National Cancer Institute (Frederick, MD). These mice were prepared by Jacks et al. (31) and contain a premature stop codon in exon 3 of the 27 pRb exons (Fig. 1A). These mice were on a C57Bl6 background and are referred to hereafter as “pRb^{+/-} mice.” Mice in which exon 19 of the pRb gene (which encodes a critical site in the small pocket of pRb) was flanked by loxP sites (32) (Fig. 1A) were generously provided by Drs. Anton Berns and Gustavo Leone and are referred to as “pRb^{lox/lox} mice.” Both the Jacks exon 3 and Berns exon 19 pRb knockouts, when homozygous, have previously been reported to result in embryonic lethality. RIP-Cre mice (33) were generously provided by Dr. Mark Magnuson, Vanderbilt University, and had previously been bred onto a CD-1 background (34). The pRb^{+/-} heterozygous mice were propagated by crossing with wild-type C57Bl6 mice. Conditional excision of one or both copies of pRb allele was accomplished using two different strategies. For strategy 1, RIP-Cre mice were crossed with pRb^{+/-} mice to generate RIP-Cre/pRb^{+/-} mice. These mice were crossed with pRb^{lox/lox} mice to generate RIP-Cre/pRb^{-lox} mice, referred to hereafter as strategy 1 conditional knockout (CKO1) mice, and pRb^{lox/+} mice were used as wild-type controls. In strategy 2, RIP-Cre mice were bred to pRb^{lox/lox} mice, and the resultant offspring of interest, RIP-Cre/pRb^{lox/lox} mice, are referred to hereafter as strategy 2 conditional knockout (CKO2) mice. Either pRb^{lox/+} or pRb^{lox/lox} mice were used as wild-type controls. All of the CKO1 and CKO2 mice were on a mixed CD-1/C57Bl6 background. Genotyping was performed using tail DNA as described previously (12,34–36). All studies were performed on animals between 2 and 3 months of age, and males and females were comparably represented. All of these studies were approved in advance by, and performed in compliance with, the University of Pittsburgh Institutional Animal Care and Use Committee.

Immunoblotting and PCR for pRb, p107, and p130. Islets were isolated and extracts prepared as described previously (8,12,37). For PCR of pRb in isolated islets, DNA was prepared from fresh snap-frozen isolated islets, and PCR for pRb genomic DNA was performed using primers shown in the legend of Fig. 1, previously described by Marino et al. (32). The Rb212 sequence was

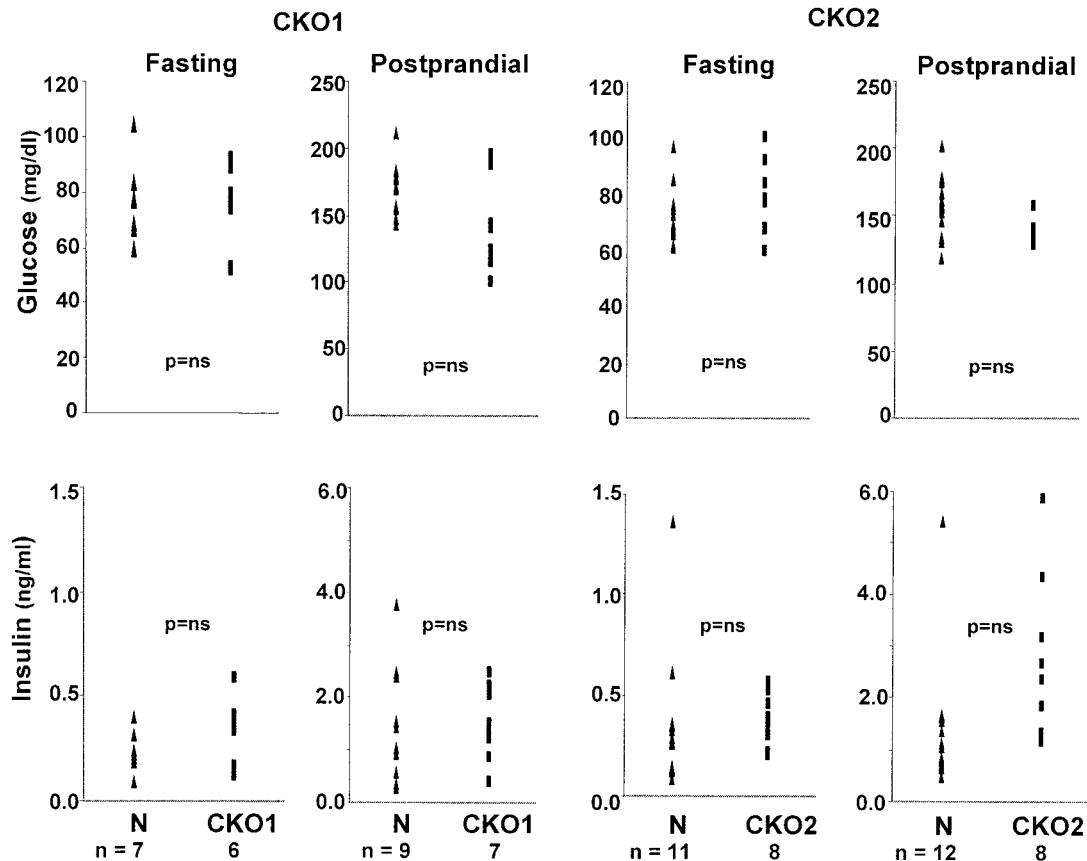


FIG. 2. Paired glucose and insulin values in the CKO1 and CKO2 mice. Blood was obtained by retro-orbital bleed from CKO1 and CKO2 mice for circulating glucose and insulin. There were no quantitatively or statistically important differences between the CKO and control mice, with the possible exception of the insulin values in the CKO2 mice, which were mildly, but not significantly, higher than the control. $P > 0.05$.

5'-GAA AGG AAA GTC AGG GAC ATT GGG-3', the Rb19E sequence was 5'-CTC AAG AGC TCA GAC TCA TGG-3', and the Rb18 sequence was 5'-GGC GTG TGC CAT CAA TG-3'. Immunoblotting for pRb was performed using a primary antibody from Pharmingen (San Jose, CA), p107 and p130 antisera from Santa Cruz (Santa Cruz, CA), and appropriate secondary antisera, as we have described in detail previously (12).

Glucose and insulin homeostasis and intraperitoneal glucose tolerance testing. Blood glucose and insulin, both fasting and postprandial, and intraperitoneal glucose tolerance testing were performed as detailed previously (12,34–37).

Islet histology, quantitative islet histomorphometry, and in vivo β -cell proliferation. Pancreata were excised, fixed in Bouin's solution, embedded in paraffin, sectioned, and stained with insulin and BrdU as described in detail previously (12,34–37). Quantitative islet histomorphometry and BrdU incorporation rates were performed as described (12,34–37). Proliferating cell nuclear antigen immunohistochemistry was performed using a rabbit polyclonal primary antibody from Santa Cruz Biotechnology (Santa Cruz, CA). Propidium iodide staining and quantitation of pyknotic, insulin-positive nuclei, and GLUT2 immunohistochemistry were performed as described previously (34–37).

Statistical analysis. Statistical analysis was performed using paired and unpaired two-tailed Student's *t* tests as appropriate. P values < 0.05 were considered significant.

RESULTS

pRb expression is near normal in the pRb heterozygotes but markedly reduced in both CKO models. Three different pRb mouse genotypes were used: the pRb heterozygous knockout mouse and two different β -cell-specific CKO mice. To determine the efficiency of recombination in the two CKO models, and the level of pRb expression in islets from the heterozygous pRb^{+/-} mice, isolated islets were examined for the presence and quan-

tity of pRb DNA and protein. As can be seen in Fig. 1B, DNA prepared from islets of CKO1 and CKO2 mice demonstrated almost complete excision of the floxed pRb allele as evidenced by the replacement of the 283 loxP-loxP PCR fragment with the 260-bp fragment. Figure 1C and D examine pRb protein by immunoblot and demonstrate that the pRb^{+/-} islets have an ~20% reduction of pRb compared with islets from control littermates. In contrast, the two CKO models display a marked (~75%) reduction in pRb in their islets compared with islets from normal littermates. These experiments were repeated and confirmed on five to eight separate islet preparations from each of the two types of CKO islets and controls. This 75% reduction corresponds nicely to the 77% of β -cells normally present in the mouse islet (38). Moreover, despite the different conditional knockout strategies, the amount of pRb protein in islets from the two CKO strategies was comparable. Collectively, these observations suggest that recombination was highly efficient in β -cells of both types of CKO mice.

Glucose homeostasis approximates are normal in mice of the three pRb genotypes. We had hypothesized that marked islet hyperplasia with resultant hypoglycemia would occur with pRb gene disruption. Figure 2S of the online appendix displays the fasting and postprandial (nonfasting) blood glucose values in 2- to 3-month-old mice of the three genotypes compared with their normal littermates. As can be seen in Figure 2S, whereas there were some statistical differences among the groups, fasting and postprandial glucose values were generally normal

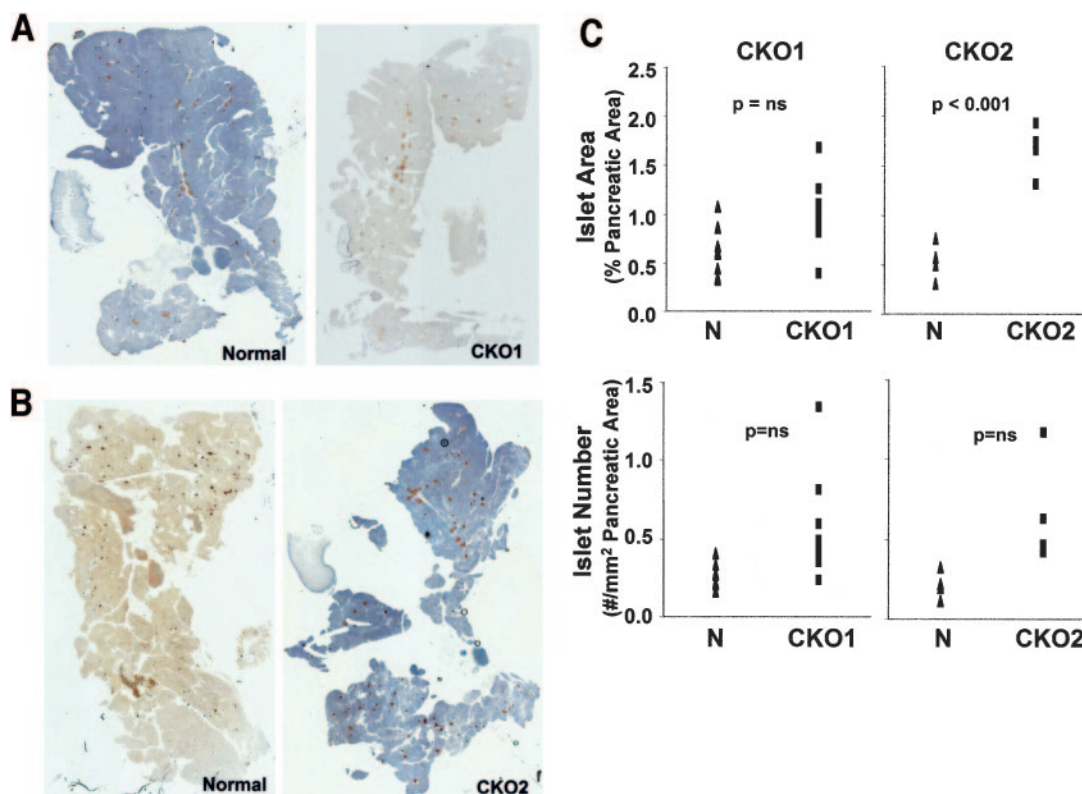


FIG. 3. Islet histomorphometry in the three types of mice. *A* and *B*: Insulin-stained sections of whole pancreases from CKO1 and CKO2 mice and their normal littermates. *C*: Quantitative histomorphometry of islet mass and islet number. Pancreas weights in the three groups were not significantly different: means \pm SE values were 302 ± 13 , 346 ± 24 , and 301 ± 29 mg in control, CKO1, and CKO2 pancreata, respectively; NS.

or near normal in mice of each of three genotypes. To directly assess insulin production by the pRb-deficient islets, we measured simultaneous insulin and glucose values under fasting and postprandial (nonfasting) conditions in the CKO1 and CKO2 mice. As can be seen in Fig. 2, both fasting and postprandial insulin and glucose concentrations were comparable in CKO versus normal littermates, although there may have been a statistically nonsignificant trend toward an increase in circulating insulin in the CKO2 mice. To search further for differences in glucose homeostasis, intraperitoneal glucose tolerance testing was performed. As can be seen in Fig. 3S in the online appendix, there was no improvement in glucose tolerance among the three pRb-deficient types of mice compared with their normal littermates. Indeed, the CKO2 animals were actually mildly glucose intolerant compared with their control littermates.

pRb-deficient mice display only a modest increase in islet mass. Whereas the pRb^{+/-} mice have previously reported to have “normal” pancreases (29,30), the phenotype and histology have not previously been detailed, and no information on the islet histology or histomorphometry in islets deficient for both pRb alleles has been described. Inspection of insulin-stained sections of pancreata from the pRb^{+/-} mice reveal no obvious difference from normal (data not shown).

We had hypothesized that bi-allelic pRb loss would result in dramatic increases in β -cell mass and number. Surprisingly, this effect, if present, was modest. As can be seen in Fig. 3*A* and *B*, islets from CKO1 and CKO2 mice appear generally normal in size, number, and distribution. Quantitative pancreatic histomorphometry (Fig. 3*C*) does reveal a 1.5- to 3-fold increase in both islet mass and islet

number in both the CKO1 and CKO2 mice compared with their normal littermates, but this reached significance only for the CKO2 mice.

pRb loss does not lead to changes in β -cell replication rates, cell death rates, or cell size. We also anticipated that release from pRb-mediated cell cycle arrest would result in striking increases in β -cell replication rates. As can be seen in Fig. 4*A*, β -cell replication rates as assessed using BrdU labeling in the two CKO models reveals that, although there may be a small quantitative trend toward an increase in replication rates, particularly in CKO1 mice, this did not approach statistical significance and is certainly not of the magnitude anticipated. These findings are complimented by proliferating cell nuclear antigen immunohistochemistry (Fig. 4*B*), which confirms that β -cell replication rates are not different between CKO and control mice.

β -Cell death rates were very low and not different among the normal controls (means \pm SE, $0.30 \pm 0.06\%$ of β -cells) versus CKO1 ($0.24 \pm 0.09\%$) versus CKO2 ($0.25 \pm 0.05\%$, $n = 5$ each, NS).

To assess β -cell size, GLUT2 immunohistochemistry was performed. As shown in Fig. 4*SA* and *B* in the online appendix, there were no apparent differences in cell size in the control versus CKO1 or CKO2 β -cells.

Endogenous p107 and p130 are not significantly increased in the islet in response to pRb loss. The observation that loss of pRb is not associated with significant increases in β -cell replication rates is consistent with a scenario in which pRb is an important enforcer of cell cycle arrest in β -cells under normal conditions, but that additional inhibitors of cell cycle progression may be able to compensate for, or substitute for the loss of, pRb. pRb

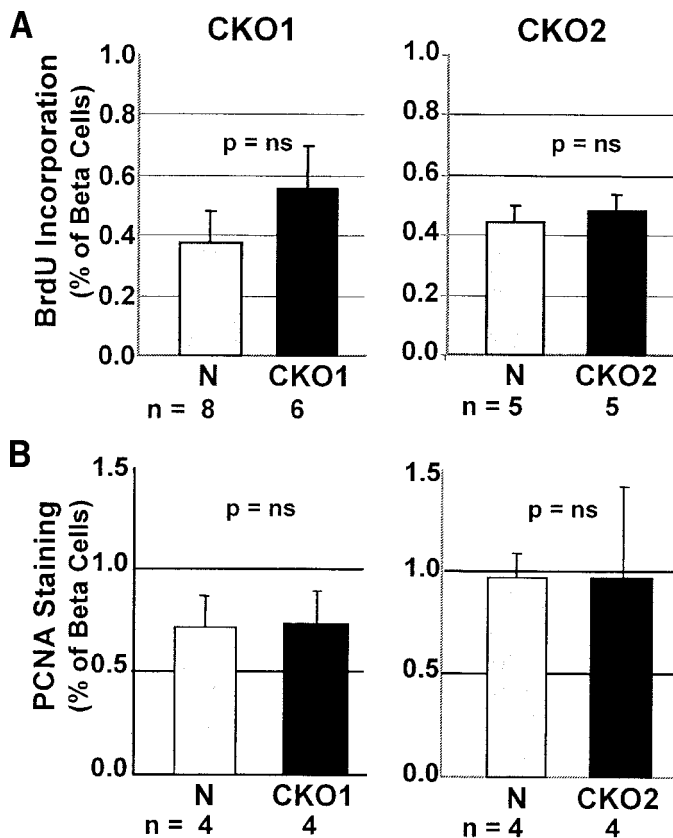


FIG. 4. β -Cell replication rates in the two types of CKO mice. The panels in *A* display β -cell replication as assessed using BrdU incorporation into insulin-positive cells, and the panels in *B* display β -cell replication as assessed using proliferating cell nuclear antigen (PCNA) immunohistochemistry in β -cells. The numbers below the bars indicate the numbers of animals studied. Increases in β -cell replication were not apparent in either of the CKO mouse types.

is one member of the “pocket protein” family, which includes p107 and p130 (Fig. 1S). p107 and p130 are thus obvious candidates for such a complementary role (1,18,39–43). Figure 5*A* and *B* demonstrate that, whereas p107 is reproducibly increased by a factor of ~50–60% in $pRb^{+/-}$ and CKO1 islets, and only ~20% in CKO2 islets, none of these changes are statistically significant. As can be seen in Fig. 5*C* and *D*, the other “pocket protein,” p130, is not appreciably changed in islets from the three types of mice.

DISCUSSION

pRb , the first tumor suppressor gene to be identified, was discovered because of its role in children with retinoblastoma (44). Children with hereditary loss of one allele develop retinoblastomas in early childhood and osteosarcomas later in life, both as a result of loss or mutation in the second pRb allele—observations that led Knudson (44) to generate his “two-hit” model of oncogenesis. In addition to osteosarcoma and retinoblastoma, additional mutations upstream in the pRb pathway (D- and E-cyclins, the INK family, the CIP family, p53, etc.) comprise the most common mutations in human cancer of all types. Moreover, these observations in combination with the observation that biallelic loss of pRb in mice results in embryonic lethality (1,18,31,32) have led to general acceptance of a model in which pRb is the principal “brake” on the $G_{1/S}$ transition in general, and its loss is associated with unrestrained cell cycle progression.

In the β -cell, the observations that upstream activators of the pRb pathway (cdk-4, D-cyclins) are associated with pRb phosphorylation and accelerated cell cycle progression (8,24,28), whereas loss of these molecules (cdk-4, D-cyclins) is associated with β -cell cycle arrest (24–28), are consonant with the pRb model described above. Conversely, the observations that loss or inactivation of cell cycle inhibitors such as p18, p21, p27, p53, or p57 is associated with β -cell cycle progression (12–15), whereas overexpression of p27 is associated with cell cycle slowing or arrest (14), has also been interpreted as supporting a key role for pRb in the control of β -cell replication.

These studies described herein provide strong support for the idea that this model is incorrect, at least as it applies to the β -cell. That is, these studies make it clear that pRb is not an essential cell cycle “brake” in the β -cell, or if it is a “brake” in β -cells, its function can be duplicated by other proteins.

To draw the firm conclusion from these studies that pRb is not essential for normal restraint of β -cell replication, it is necessary to document that pRb has in fact been effectively deleted from at least a minority of β -cells. It is not possible to address this immunohistochemically, with the current lack of high-quality antisera directed against murine pRb . However, that pRb was markedly reduced seems a firm conclusion, since we observed a dramatic reduction in intact floxed pRb DNA in CKO islets. Moreover, this was independently confirmed at the protein level, where islets isolated from both types of CKO mice displayed a reproducible 75% reduction in pRb protein. Indeed, this is an underestimate of pRb in β -cells, since only some 77% of murine islet cells are β -cells (38). Thus, the 23% remaining cells (α -cells, δ -cells, PP cells, exocrine cells, ductal cells, and endothelial cells) all would be expected to retain pRb in the presence of a RIP-driven Cre recombinase. One can therefore reasonably conclude that the 25% of pRb visualized by Western blotting is likely derived at least in part from these remaining 23% of non- β -cells, as well as a small minority of β -cells in which recombination did not occur, and that a large proportion of β -cells lack both pRb alleles. Thus, it seems reasonable to conclude that pRb recombination was efficient and nearly complete in β -cells from both CKO1 and CKO2 mice.

It is critical to emphasize that if pRb were absolutely essential for maintaining cell cycle arrest of β -cells, then loss of both alleles of pRb in even a single β -cell would result in markedly accelerated β -cell replication and clonal expansion of the daughter cells. Thus, even if recombination were very inefficient, affecting only 1–5% of β -cells, by the 3 months of age studied here, one would have expected to find, at a minimum, multiple hyperplastic hyperproliferative nodules in many or most islets.

Mice heterozygous for pRb had no metabolic, histological, or proliferative phenotype despite a rigorous search. Harvey et al. (29) and Williams et al. (30) have previously indicated that the pancreas was “normal” in $pRb^{+/-}$ mice, but this is the first rigorous demonstration that loss of a single pRb allele has no adverse effect on the β -cell. Given prior experience, these results were expected.

It was not expected that loss of both pRb alleles would have little or no histological or metabolic phenotype. While it is true that CKO mice did display quantitatively small changes in glucose and insulin, a 1.5- to 3-fold increase in β -cell mass, and a possible trend toward an increase in BrdU incorporation rates, we had expected a

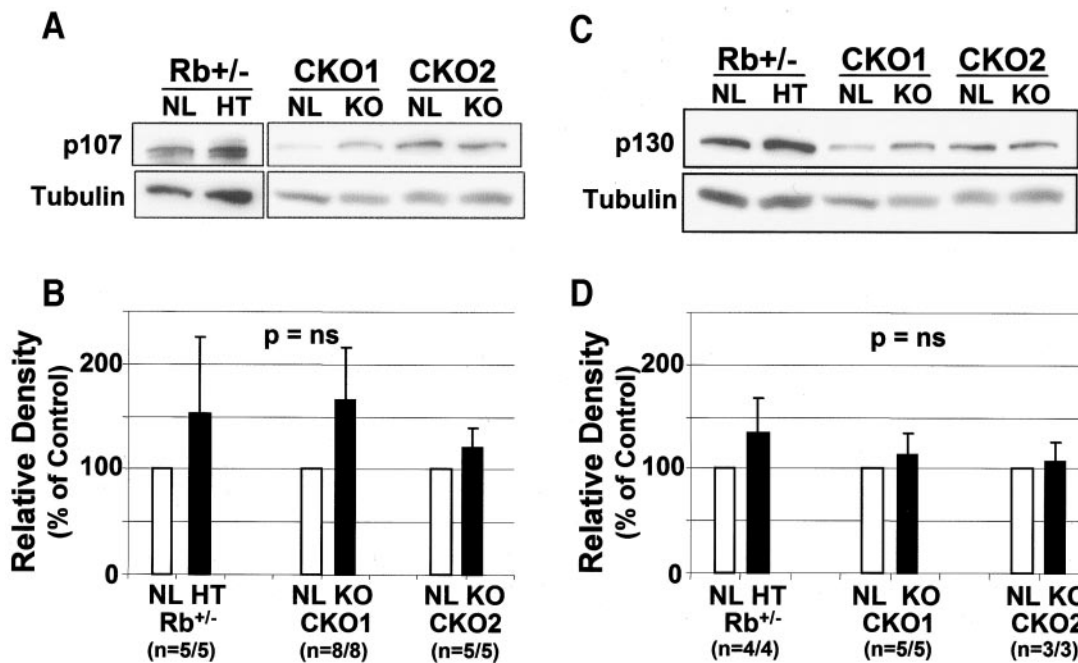


FIG. 5. p107 and p130 in pRb-deficient islets. *A* and *B* show a representative Western blot and quantitative densitometry for p107. *C* and *D* show the same for p130. See the text for details. HT, heterozygous pRb-null mice; KO, conditional knockouts; NL, normal littermates.

far more striking phenotype. For example, in mice deficient for menin, which is upstream of the pRb checkpoint, β -cell mass is some 10–50 times greater than normal, and β -cell replication rates are markedly elevated (13). Similarly, in mice doubly heterozygous for loss of the insulin receptor and insulin receptor substrate 1, β -cell mass is some 30 times greater than normal (45). In RIP-TAG mice, in which T-antigen is presumed to disrupt the pRb pathway, β -cell replication rates and β -cell mass are dramatically increased (16). Even simple rodent models of diet-induced obesity and insulin resistance display far greater increases in β -cell mass and proliferation (14,46). Thus, while one could argue that pRb loss may have increased β -cell proliferation, one would have to concede that such an increment was surprisingly small.

Could mild hypoglycemia in the CKO mice have prevented an increase in β -cell mass in the pRb-deficient mice? This seems an unlikely explanation, for the hypoglycemia was very mild. More importantly, even severe hypoglycemia does not prevent increases in β -cell proliferation and islet mass in menin-deficient or T-antigen-overexpressing islets (13,16).

If the retention of cell cycle arrest in pRb CKO mice is not attributable to hypoglycemia or inefficient recombination for pRb in the majority of β -cells, one can only conclude that the current paradigm for pRb in cell cycle regulation is incorrect, or does not apply to the β -cell, or that a normal role for pRb is replaced or subserved by another protein. Each of these explanations is likely to be true. Regarding the possibility that the model is incorrect, Leone and colleagues (47) have recently demonstrated that, whereas biallelic loss of pRb results in early embryonic lethality, embryonic death results from placental defects. If the placenta is rescued using genetic techniques, pRb-null embryos are carried to late gestation and are grossly normal in size and organ development (47). They do have erythropoietic and central nervous system developmental defects (47), but the key point is that most cells and lineages can progress normally through develop-

ment in the absence of functional pRb. The work described here would suggest that this applies to the pancreatic β -cell as well.

Regarding the possibility that another protein or proteins might compliment for pRb, this seems to be likely as well, since, as shown in Fig. 4, pRb loss is associated with normal β -cell proliferation rates. What might the complimentary proteins be? The other two members of the “pocket protein” family, p107 and p130, would seem obvious candidates (1,18,39–43) (Fig. 1S). Sage et al. (48,49) have shown that replacement of any one of the three pocket proteins in murine embryonic fibroblasts deficient for all three can restore normal restraint of cell cycle progression. Moreover, Sage et al. (49) have demonstrated that with chronic pRb loss in murine embryonic fibroblasts, p107, but not p130, increases, and knockdown of p107 using lentiviral p107 siRNA resulted in a resumption of cellular proliferation.

In the current study, we did not observe strong support for this possibility: p107 increased nonsignificantly in CKO1 islets and not at all in CKO2 mice. p130 did not change in either model. It remains formally possible that although the levels of total p107 or p130 may not have changed in a statistically significant manner in whole-islet extracts, these proteins may have shifted their location from cytoplasmic to nuclear, where they would be expected to restrain cell cycle progression. It is also formally possible that these proteins could have increased in a small subpopulation of β -cells particularly susceptible to proliferation but that such a change was not appreciated on immunoblots of whole-islet extracts. While immunohistological approaches may provide circumstantial support for these possibilities, in the end, these hypotheses are best tested using mouse genetic models in which multiple pocket protein family members are deleted in β -cells (39–42). Of course, it is equally possible that other completely unknown proteins could also compliment for pRb loss.

We also sought evidence that other members of the $G_{1/S}$

control checkpoint were affected by the loss of pRb. Immunoblots of islet extracts from control and CKO mice of both genotypes were assessed for p21, p27, cyclin A, cyclin E, and E2F1. No changes in these five proteins were observed (not shown). We interpret this to mean that these proteins are unaffected by pRb loss, that the molecules that compensate or compliment for pRb loss (e.g., p107, p130) also maintain normality of these other $G_{1/S}$ components, or that important differences in these molecules may have occurred with respect to nuclear-cytosolic trafficking, or protein-protein partnering, but these were not apparent as assessed by simple immunoblots. Clearly, future studies need to be directed at the cellular mechanisms for compensation for pRb loss.

Finally, these studies used RIP-Cre mice. The RIP-Cre construct has been demonstrated to cause metabolic effects of its own (50), so one must consider whether some of the observations here were confounded by the presence of the RIP-Cre transgene. This would seem unlikely, since in the phenotype we sought, markedly enhanced β -cell replication, marked increases in β -cell mass, and insulin-mediated hypoglycemia are not features associated with RIP-Cre mis-expression. Moreover, the metabolic consequences of RIP-Cre mis-expression (generally glucose intolerance and reductions in insulin production) were not an important part of the phenotype observed, although it is possible that this could account, at least in part, for the mild glucose intolerance of the CKO2 mice (Fig. 3S).

These studies demonstrate that while the “pRb pathway” is unquestionably important for β -cell development and function, the precise role of pRb per se in the β -cell remains undefined. It is possible that pRb is not importantly involved in the control of β -cell replication, i.e., that the pRb model of cell cycle control is incorrect. It is also possible, and we believe likely, that pRb is important under normal circumstances but that its loss is complimented by another protein or proteins, the nature of which remains to be defined. These observations underscore the point while the pRb pathway is likely central to the control of β -cell replication, development of a clear coherent comprehensive model will be required before cell cycle control can be harnessed for therapeutic purposes for the treatment of diabetes.

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