Postnatal Expansion of the Pancreatic β-Cell Mass Is Dependent on Survivin

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OBJECTIVE—Diabetes results from a deficiency of functional β-cells due to both an increase in β-cell death and an inhibition of β-cell replication. The molecular mechanisms responsible for these effects in susceptible individuals are mostly unknown. The objective of this study was to determine whether a gene critical for cell division and cell survival in cancer cells, *survivin*, might also be important for β-cells.

RESEARCH DESIGN AND METHODS—We generated mice harboring a conditional deletion of *survivin* in pancreatic endocrine cells using mice with a *Pax-6*-Cre transgene promoter construct driving tissue-specific expression of Cre-recombinase in these cells. We performed metabolic studies and immunohistochemical analyses to determine the effects of a mono- and biallelic deletion of *survivin*.

RESULTS—Selective deletion of *survivin* in pancreatic endocrine cells in the mouse had no discernible effects during embryogenesis but was associated with striking decreases in β-cell number after birth, leading to hyperglycemia and early-onset diabetes by 4 weeks of age. Serum insulin levels were significantly decreased in animals lacking endocrine cell survivin, with relative stability of other hormones. Exogenous expression of survivin in mature β-cells lacking endogenous survivin completely rescued the hyperglycemic phenotype and the decrease in β-cell mass, confirming the specificity of the survivin effect in these cells.

CONCLUSIONS—Our findings implicate survivin in the maintenance of β-cell mass through both replication and anti-apoptotic mechanisms. Given the widespread involvement of survivin in cancer, a novel role for survivin may well be exploited in β-cell regulation in diseased states, such as diabetes. ***Diabetes* 57:2718–2727, 2008**

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Production and maintenance of the pancreatic β-cell mass is a highly regulated process driven by mechanisms that differ in developing compared with adult animals. These mechanisms include, but are not limited to, β-cell replication, β-cell hypertrophy, β-cell differentiation (neogenesis), and β-cell apoptosis (1–3), each having variable importance depending on the age of the animal and changes in the metabolic demands of the body. During early embryogenesis in the mouse, most β-cells are generated through endocrine cell differentiation, a process that depends on key transcription factors, including Pdx1, Isl1, Nkx2.2, Nkx6.1, and the Maf proteins (4,5). Differentiated β-cells first appear around embryonic day (E) 13 at the start of a wave of the secondary transition. During late embryogenesis (E18.5) and immediately after birth, a transient burst of replication of these β-cells occurs (1,6,7) with a consequent marked increase in β-cell growth (2,8). New β-cells continue to form in the adult animal, primarily from the replication of mature β-cells (9,10). The mechanism by which fully differentiated, mature β-cells can reenter the cell cycle without undergoing a process of programmed cell death is unclear.

An attractive candidate regulator of β-cell replication and survival after birth is *survivin*, a cancer gene implicated in both the control of cell division and the regulation of apoptosis in cancer cells but with unknown functions in most normal cells (11–13). Survivin was originally discovered as a homolog to the inhibitor of apoptosis proteins in cancer cells (14). These proteins block the function of caspase proteins in the mitochondria-dependent cell death pathway (15), protecting cells from succumbing to a cascade of cellular and molecular events that characterize apoptosis. Survivin is a potent inhibitor of cell death in diverse malignant tumor cell types (11) and in some normal cells, including hepatocytes (16) and bone marrow stem cells (17). In addition, survivin also plays a role in cell division in some normal cells during embryogenesis and in cancer cells. Biallelic deletion of *survivin* in embryonic stem cells leads to embryonic lethality between E4 and E6 (18), with cells lacking survivin having abnormal, enlarged nuclei. This phenotype parallels that of other genetic models targeting proteins known as chromosomal passenger proteins (CPPs) (19). Survivin forms a complex with other CPPs and plays an active role in recruiting aurora kinase B to the kinetochore to ensure the proper regulation of cytokinesis (20). A clear understanding of how survivin regulates both cell division and apoptosis is not known. Some evidence suggests that the protein exists in multiple subcellular pools (21) and can interact with

different partner proteins (22–24), including its own splice forms (25), to perform these different functions.

Survivin is expressed in a range of normal embryonic tissues and in a restricted number of highly proliferative adult tissues (26), including bone marrow–derived stem cells (17) and neural progenitor cells (27). Its expression during both normal development and cancer suggests that it is a critical molecule for maintaining cellular homeostasis and that its aberrant regulation can contribute to either disease initiation or progression. Recently, exogenous expression of survivin in a streptozotocin-induced model of diabetes demonstrated protection of pancreatic β-cells from programmed cell death (28). This provides some evidence that it may play a role as an apoptotic inhibitor in β-cells in the setting of diabetes. Based on the bifunctional role of survivin in cancer and in stem cells, we hypothesized that it may play a role in the replication and/or survival of mature pancreatic β-cells after birth.

RESEARCH DESIGN AND METHODS

Pax6-Cre mice (also called *Le-Cre*) were crossed with B6;129S-Gtrosa26^{tm1sor} mice to evaluate Cre activity (29). Mice harboring a conditionally targeted mouse *survivin* gene flanked by two *loxP* sites (*survivin*$^{lox/lox}$) have been described previously (16,27), as have *Pax6-Cre* mice (30). *Pax6-Cre* mice were crossed with *survivin*$^{lox/lox}$ mice to generate *Pax6-Cre;survivin*$^{lox/lox}$ mice. For the rescue experiment, two lines of mice harboring a mouse *survivin* transgene under control of an rat insulin promoter (RIP) (28) were mated with *survivin*$^{lox/lox}$ mice. Offspring with the genotype of *Pax6-Cre;RIP-SVV*$^{+}$; *survivin*$^{lox/lox}$ were compared with those of with *Pax6-Cre*; *RIP-SVV*$^{-}$; *survivin*$^{lox/lox}$ mice. All experimental procedures were performed with the approval of the Institutional Animal Care and Use Committee. Islet isolation was as previously described (31).

Morphometry and islet mass. Serial pancreatic sections were collected every 100 μm and stained with hematoxylin-eosin (H-E). Images were taken with a Leica microscope equipped with a SPOT RT KE digital camera (Diagnostic Instruments). Islet cell areas for the control and survivin knockout were measured by point counting morphometry (see the supplemental methods available in an online appendix at http://dx.doi.org/10.2337/db08-0170).

Immunohistochemistry. Pancreata were fixed in either 10% neutral-buffered formalin or Histochoice (Ameresco), dehydrated through a graded series of ethanol, and embedded in paraffin wax. Sections were cut and stained with H-E. For fluorescent immunostaining, fresh frozen sections were cut and fixed in 4% paraformaldehyde. The primary antibodies used were survivin (TIAP; rabbit polyclonal, 1:200; Chemicon), glucagon (mouse monoclonal, 1:1,000; Sigma), somatostatin (rabbit polyclonal anti-srif28 IHC 8004, 1:1,000; Peninsula), active caspase 3 (rabbit, 1:20; Abcam), insulin (guinea pig, 1:200; Linco), MafB (rabbit, 1:100; Bethyl Laboratories), Nkx2.2 (mouse, 1:100; Developmental Studies Hybridoma Bank), mouse Nkx6.1 (rabbit, 1:500; provided by Dr. P. Serup), Pax6 (rabbit, 1:500; Covance), β-catenin (mouse, 1:100; BD Biosciences), and Isl1 (mouse, 1:100; Developmental Studies Hybridoma Bank). Rabbit anti-MafA antibody was described previously (32). Apoptosis was assayed with the FragELTM kit (Calbiochem), according to the manufacturer's instructions.

Metabolic studies. Serum blood glucoses were measured with One Touch InDuo Glucometer and test strips (Lifescan, Milpitas, CA). For the glucose challenge, mice were fasted then injected intraperitoneally with dextrose at 2 g/kg body wt. Blood glucose levels were subsequently measured at indicated times. Insulin challenge was performed in the fed state. A dose of 0.75 unit/kg body wt was injected intraperitoneally. Blood glucose levels were measured every 15 min for 90 min after injection. A minimum of four animals per group per time point was used for each of these measurements.

RESULTS

Survivin is dispensable for endocrine cell differentiation during embryogenesis. To gain insight into the regulatory potential of survivin in pancreatic β-cells, we determined its expression pattern in the mouse pancreas during normal embryonic development and after birth. Survivin protein was readily detected throughout the pancreatic epithelium, including endocrine cells during the secondary transition (E15.5), as shown by the cytoplasmic staining of survivin in cells that express β-catenin, insulin, and Isl1 (Fig. 1*A*). Survivin expression gradually became restricted to endocrine (Isl1^{+}) cells in late embryogenesis (E19.5) and postnatally (postnatal day [P] 14) (Fig. 1*A*). By P21, one can detect several Isl1^{+} cells that do not express survivin, suggesting a further restriction of survivin expression to a subpopulation(s) of endocrine cells (Fig. 1*A*). Colocalization of survivin with insulin was also observed at these time points, with a relative lack of survivin staining within cells that stained positive for glucagon and somatostatin (Fig. 1*A*, *panel 2*), suggesting that survivin expression becomes restricted to β-cells by P21. Expression of survivin in mature, differentiated β-cells after birth was unexpected, because activation of this gene was previously reported only in undifferentiated and highly proliferating cells in the adult animal (26). Therefore, we confirmed the expression of survivin in these cells by isolating islets from normal mice at 1 and 2 weeks after birth and performing RT-PCR for *survivin*, *insulin*, and *glyceraldehyde-3-phosphate dehydrogenase* (supplementary Fig. 1*A*. The pattern of survivin expression within the pancreas is similar to the reported pattern of key transcription factors that contribute to β-cell development during embryogenesis and after birth, including Nkx6.1 and Pdx1 (5,33). It is possible that such factors may restrict survivin expression to β-cells during the postnatal period.

To examine the functional role of survivin in all pancreatic endocrine cells both during embryogenesis and after birth, we chose a *Pax6-Cre* transgene construct (also known as *Le-Cre*) that drives tissue-specific expression of Cre-recombinase in the cells expressing Pax6 (30). The onset of endogenous *Pax6* expression normally occurs at E9.5 in mouse endocrine progenitors and persists in mature endocrine cells throughout development and postnatally, thus providing us with a tool to examine the role of survivin in all endocrine cells during fetal development and postnatally (30,34). The construct incorporated a 6.5-kb genomic region of the mouse *Pax6* promoter that has been previously shown to initiate transcription of *Pax6* in the lens and in developing pancreatic endocrine cells but not in other pancreatic cells or in the central nervous system (34). We further characterized the expression pattern of Cre-recombinase within the pancreas of *Pax6-Cre* mice after birth by mating them with Rosa26 Cre-reporter mice [*Gt*(*ROSA*)*26Sor*^{tm1Sor}], collecting pancreatic tissues, and staining sections with X-Gal. β-Galactosidase was expressed in all endocrine-producing cells but not in exocrine cells, confirming the specificity of the *Pax6* promoter within the pancreas after birth (supplementary Fig. 1*B*).

To determine the potential effects of survivin on endocrine cell differentiation during embryogenesis and its effects on cell survival after birth, *Pax6-Cre* mice were bred to mice carrying the mouse *survivin* gene flanked by two *loxP* sites, generating *Pax6-Cre;survivin*$^{lox/lox}$ mice, deleting *survivin* in Pax6-expressing cells. Deletion of *survivin* within the pancreatic endocrine cells of affected mice was confirmed both by staining pancreas tissue from control and mutant animals with an antibody to survivin (Fig. 1*B*, *panel 1*) and by performing PCR for the deleted and nondeleted *survivin* alleles on isolated islet cell DNA (supplementary Fig. 1*C*). Quantification of survivin and insulin protein in the knockout animals showed that only ~6% of all insulin^{+} cells expressed survivin. By compari-

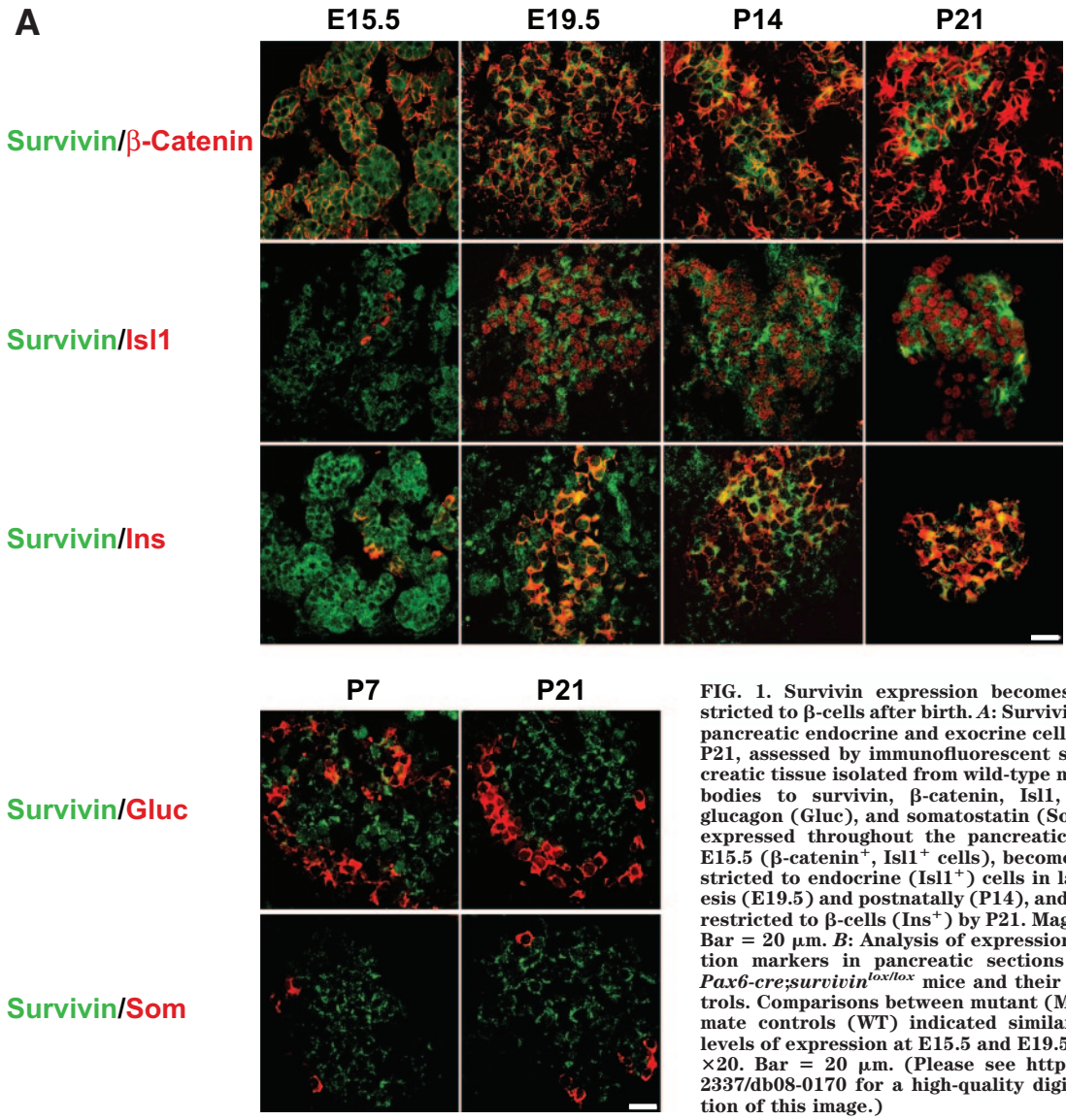

FIG. 1. Survivin expression becomes gradually restricted to β-cells after birth. *A*: Survivin expression in pancreatic endocrine and exocrine cells from E15.5 to P21, assessed by immunofluorescent staining of pancreatic tissue isolated from wild-type mice, using antibodies to survivin, β-catenin, Isl1, insulin (Ins), glucagon (Gluc), and somatostatin (Som). Survivin is expressed throughout the pancreatic epithelium at E15.5 (β-catenin$^+$, Isl1$^+$ cells), becomes gradually restricted to endocrine (Isl1$^+$) cells in late embryogenesis (E19.5) and postnatally (P14), and then is further restricted to β-cells (Ins$^+$) by P21. Magnification ×20. Bar = 20 μm. *B*: Analysis of expression of differentiation markers in pancreatic sections isolated from *Pax6-cre;survivin$^{lox/lox}$* mice and their littermate controls. Comparisons between mutant (MUT) and littermate controls (WT) indicated similar patterns and levels of expression at E15.5 and E19.5. Magnification ×20. Bar = 20 μm. (Please see http://dx.doi.org/10.2337/db08-0170 for a high-quality digital representation of this image.)

son, all insulin-expressing cells in control animals expressed survivin; therefore, we estimated a ~94% efficiency of recombination in this model. To demonstrate the specificity of the *survivin* deletion to pancreatic endocrine tissue of *Pax6-Cre;survivin$^{lox/lox}$* animals, we performed PCR for the deleted and nondeleted *survivin* alleles in tissues outside the pancreas, including liver, muscle, fat, and brain (supplementary Fig. 1*D*), and showed that the *survivin* gene was largely intact in these other tissues. A minor amount of a second product was detected in fat that may represent the deleted allele. This could indicate that there is some recombination in fat; however, no prior evidence of Pax6 expression in adipose tissue has been reported.

Examination of the expression pattern of key proteins in endocrine cell differentiation during embryogenesis showed that mice lacking endocrine-survivin had essentially the same expression pattern of these factors (Pdx1, Isl1, Nkx2.2, and the Maf proteins) in endocrine precursors as did their littermate controls (Fig. 1*B*, *panels 2* and *3*). Based on these data, survivin may be dispensable for the proliferation of pancreatic progenitors and the differentiation of pancreatic endocrine cells during embryogenesis. The lack of identifiable effects on cell number during this time period was somewhat surprising because conventional deletion of *survivin* in embryonic stem cells resulted in early embryonic lethality (18) and because deletion of *survivin* at E9.5 in neural stem cells led to significant embryonic neural stem cell loss and death immediately after birth (27). By contrast, here, all *Pax6-Cre;survivin$^{lox/lox}$* mice were born in the expected Mendelian ratios, suggesting no embryonic lethality, and had birth weights and sizes similar to those of littermate controls (not shown), suggesting no significant metabolic effects in utero.

Survivin is required for mature β-cell function after birth. To determine the potential physiological effects of survivin loss within the endocrine cells after birth, we performed serial metabolic studies on mice lacking endocrine-survivin (*Pax6-Cre;survivin$^{lox/lox}$* mice). During the first 3 weeks after birth, *survivin*-deficient animals had random glucose levels that were similar to their littermate controls with intact *survivin* genes (Fig. 2*A*). At 4 weeks of life, however, the *Pax6-Cre;survivin$^{lox/lox}$* mice developed hyperglycemia (Fig. 2*A*) and a reduced glucose tolerance, as determined by injection with 2 g/kg dextrose after a 5-h fast (Fig. 2*B*), findings consistent with early-onset diabetes. The glucose abnormalities in these mice

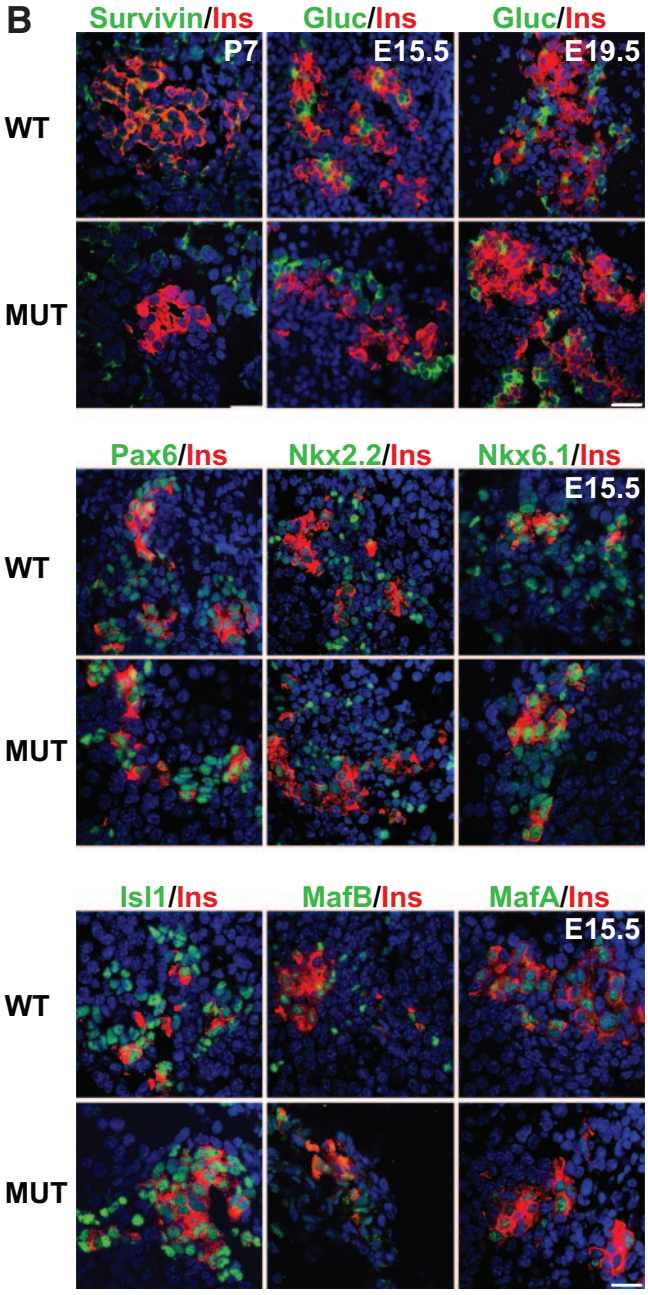

FIG. 1. Continued.

became more striking as the animals aged (Fig. 2*A*). At 4 weeks of age, *Pax6-Cre*;*survivin*$^{lox/lox}$ mice responded similarly to littermate control animals when treated with the same doses (0.75 unit/kg) of exogenous insulin (Fig. 2*C*), consistent with a primary lack of insulin availability as the cause of the hyperglycemia due to a loss of survivin. To further understand this process, we quantified the serum insulin of the mice over time. Survivin-deficient mice had very low to undetectable (below assay threshold) insulin levels from 3 to 13 weeks of life (Fig. 2*D*; supplementary Table 1), suggesting either a failure of insulin production or secretion. Mice with a one-allele loss of endocrine-*survivin* (*Pax6-Cre*;*survivin*$^{lox/+}$ mice) had 12-h fasting glucose levels that were comparable with control animals at 4 and 5 weeks of age (supplementary Fig. 2*A*). At 17 weeks of age, however, these heterozygotes began to develop some signs of glucose intolerance, as shown by their higher serum glucose levels after dextrose administration (supplementary Fig. 2*B*).

Pax6-Cre;*survivin*$^{lox/lox}$ mice that remained untreated and therefore exposed to high serum glucose levels for several months after birth became relatively resistant to exogenous insulin, as would be expected (supplementary Fig. 3*A*). Many of these older *survivin*-deficient mice also developed other metabolic hallmarks of human diabetes, including metabolic acidosis, hyperkalemia, polyuria, and ketonuria (supplementary Fig. 3*B*; supplementary Table 2). In addition, they developed hypertriglyceridemia, hypoproteinemia (supplementary Fig. 3*C*; supplementary Table 2), and pathological evidence of fatty livers, most likely due to the secondary effects of prolonged glucose and lipid toxicity (supplementary Fig. 3*D*), findings reminiscent of untreated human diabetes. These mice ultimately showed poor weight gain and signs of dehydration and had shortened life spans of 4–7 months.

To determine the cause of the metabolic abnormalities resulting from the low serum insulin levels, we examined pancreatic sections from the mice for pathological abnormalities over time. The onset of hyperglycemia in mice lacking *survivin* within endocrine cells was associated with a significant decrease in the number of insulin-producing cells after 4 weeks of age as measured by immunohistochemical staining for insulin (Fig. 3*A*) and by islet mass (Fig. 4*A*), suggesting that there was a lack of insulin production due to an inappropriate decrease in β-cell number. By contrast, a decrease in the number or function of α- and δ-cells, as determined by immunohistochemical staining for glucagon and somatostatin (Fig. 3*A*) and by measuring serum glucagon levels (Fig. 3*B*), was not observed during the early postnatal period. The findings of hyperglycemia, insulin deficiency, and a lack of insulin-producing β-cells in the face of a relative preservation of α- and δ-cells suggest that survivin plays an essential role in the regulation of β-cell number early after birth, preferentially affecting these cells over other endocrine subtypes. Given the known mechanisms of survivin function in cancer cells, both as an inhibitor of apoptosis and a regulator of cell division, survivin could have either one or both functions in pancreatic β-cells.

Survivin regulates β-cell mass after birth. To establish the effect of a *survivin* deletion on the onset and extent of β-cell expansion, we compared the total mass of β-cells as a function of time in *Pax6-Cre*;*survivin*$^{lox/lox}$ versus littermate controls containing both *survivin* alleles. Although the β-cell masses of the control and mutant animals were similar at birth, beginning at 2 weeks of age they became significantly smaller in animals lacking *survivin* within these cells (0.17 vs. 0.41 mg in littermate controls [$n = 5$], $P < 0.001$; Fig. 4*A*), reaching a 10-fold reduction at 8 weeks of life (0.12 vs. 1.29 mg [$n = 4$], $P < 0.001$; Fig. 4*A*). Serial examination of pancreatic tissue collected from control and mutant animals at P0 and at 1, 2, and 3 weeks of age showed that the *survivin*-deficient cells had many enlarged, dysmorphic nuclei, characterized by an increase in nuclear size that was not apparent in the littermate control cells (mean nuclear size, 37.3 vs. 30.9 μm^2 at 2 weeks; 39.7 vs. 31.5 μm^2 at 3 weeks [$n = 5$], $P < 0.001$; Fig. 4*B* and *C*). This phenotype is similar to that described after the disruption of survivin in cultured cancer cells (35,36) and after homozygous deletion of *survivin* in murine embryonic cells during early embryogenesis (18). The morphological defects observed in the β-cells lacking survivin are consistent with

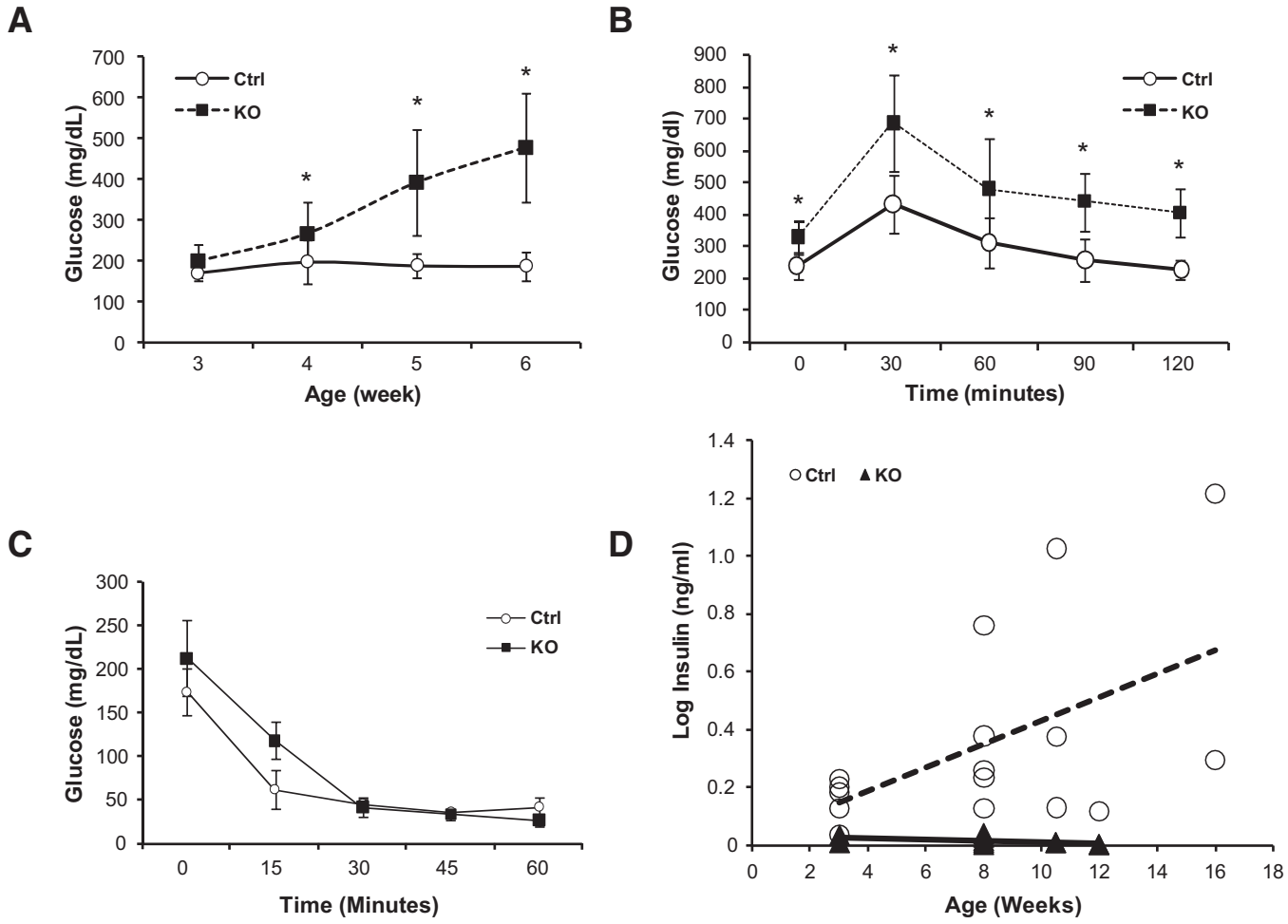

FIG. 2. Loss of survivin results in insulin-deficient diabetes. ***A*****: Mean (±SD) random glucose levels in littermate control (*survivin*$^{lox/+}$ or *survivin*$^{lox/lox}$) mice (○, $n = 8$ at each time point) and homozygous *survivin*-deficient (*Pax6-Cre;survivin*$^{lox/lox}$) mice (■, $n = 8$ at each time point) at various ages after birth. *Significance at $P < 0.01$.** ***B*****: Glucose tolerance test (mean ± SD) in 6-week-old male littermates. Control (○, $n = 7$) and homozygous *survivin*-deficient (■, $n = 7$) mice were fasted for 5 h then injected with dextrose (2 g/kg body wt) intraperitoneally. *Significance at $P < 0.05$.** ***C*****: Insulin challenge test in 4-week-old littermates. Four-week-old control (○, $n = 3$) and homozygous *survivin*-deficient (■, $n = 4$) mice were injected with 0.75 unit/kg insulin (fed state). No statistically significant differences were found at any time point.** ***D*****: Random serum insulin levels measured by enzyme-linked immunosorbent assay in age-matched animals at the indicated times. ○, insulin levels in individual control animals ($n = 16$); ▲, levels in individual *Pax-6Cre;survivin*$^{lox/lox}$ animals ($n = 12$). See supplementary Table 1 for individual values.**

defective cell division, suggesting that survivin may play a role in β-cell replication.

Survivin regulates cell division and protects cells against cell death. To test the hypothesis that survivin regulates cell division and/or cell death in pancreatic β-cells during a time period shortly after birth, we examined tissue sections isolated from 2-week-old animals for the expression of proliferating cell nuclear antigen (PCNA), a marker of cell cycle proliferation, and for transferase-mediated dUTP nick-end labeling (TUNEL), a marker for apoptosis. We observed a 50% decrease in PCNA staining in the *survivin*-deficient β-cells (Fig. 5*A*) but no significant change in the number of TUNEL^{+} cells (not shown). To increase the sensitivity for the detection of cell death, we isolated islet cells from the pancreata of 1- to 2-week-old animals and subjected them to functional caspase 3 activity assays. With this methodology, we did observe a twofold increase in caspase 3 activity in the *survivin*-deficient cells (Fig. 5*B*), suggesting an effect on a caspase 3–dependent cell death pathway. To further characterize the cell cycle abnormalities, we performed flow cytometry analyses on the same isolated islets. This revealed an excess of cells with >4N modal chromosome numbers in the *survivin*-deficient islets (14 vs. 9%; Fig. 5*C*) and an accumulation of *survivin*-deficient islets in late S/G2 (36 vs. 23%; Fig. 5*C*), suggesting a delay in cell cycle progression. To attempt to gain further insight into potential cell cycle proteins regulated by survivin in β-cells, we performed quantitative PCR on RNA from the isolated islets for genes involved in cell cycle progression. These analyses revealed a significant (average threefold) increase in expression of the cell cycle inhibitor *p21*^{WAF1} and a twofold decrease in expression in *cyclin E* in the *survivin*-deficient cells (Fig. 5*D*). No significant changes in the expression levels of *Cyclin A*, *B1*, *B2*, *C*, *D1*, *F*, *p27*, *Cdk2*, or *Cdk4* were seen (Fig. 5*D*). Taken together, our findings support the hypothesis that survivin regulates cell cycle progression in pancreatic β-cells. These effects could be mediated through repression of p21^{WAF1}, which might occur as a consequence of survivin-dependent repression of p53 protein (37,38).

Survivin is functionally specific for mature pancreatic β-cells. To establish survivin as a specific regulator of β-cell mass distinct from other endocrine cell subtypes, we mated the *Pax6-Cre;survivin*$^{lox/lox}$ mice with transgenic mice expressing the survivin protein under control

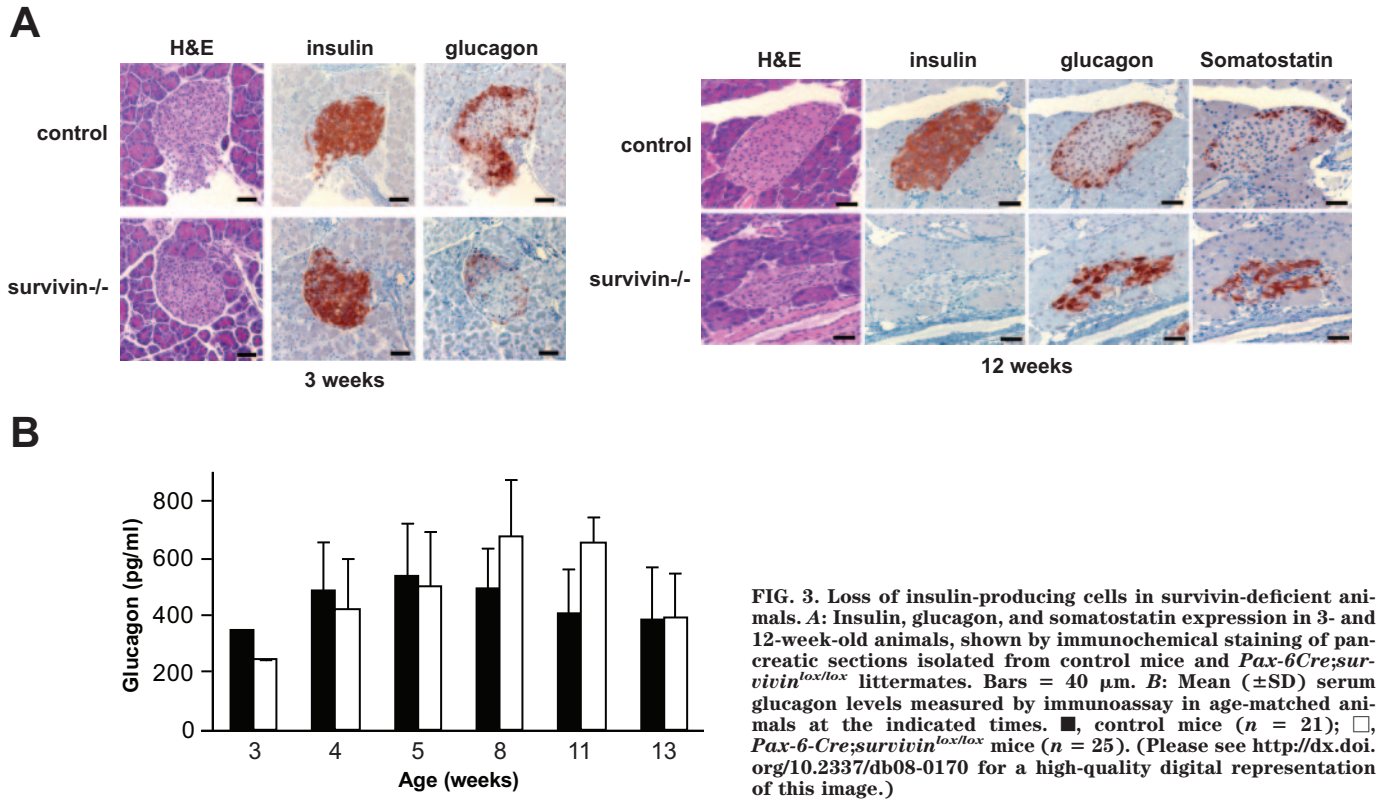

FIG. 3. Loss of insulin-producing cells in survivin-deficient animals. *A*: Insulin, glucagon, and somatostatin expression in 3- and 12-week-old animals, shown by immunochemical staining of pancreatic sections isolated from control mice and *Pax-6Cre;survivin*$^{lox/lox}$ littermates. Bars = 40 μm. *B*: Mean (±SD) serum glucagon levels measured by immunoassay in age-matched animals at the indicated times. ■, control mice (n = 21); □, *Pax-6-Cre;survivin*$^{lox/lox}$ mice (n = 25). (Please see http://dx.doi.org/10.2337/db08-0170 for a high-quality digital representation of this image.)

of the RIP (yielding *Pax-6-Cre;survivin*$^{lox/lox}$*;RIP-SVV* mice). We then followed the triple transgenic mice from birth to 8 weeks of age, measuring weekly random serum glucose concentrations in the *Pax-6-Cre;survivin*$^{lox/lox}$*; RIP-SVV* mice and comparing these with random glucose levels in the *RIP-SVV*, *Pax-6-Cre;survivin*$^{lox/lox}$, and *survivin*$^{lox/lox}$ mice (Fig. 6*A*). RIP-driven transgenic expression of survivin completely rescued the diabetic phenotype of the *Pax6-Cre;survivin*$^{lox/lox}$ mice. It also restored normal growth (Fig. 6*B*, 12 weeks) and mass of the islets (Fig. 6*C*). This finding supports a major role for survivin in maintaining β-cell number and function in normal animals after birth.

DISCUSSION

In this work, we show that survivin, a protein involved in both replication and apoptosis in cancer cells, plays an important role in the maintenance of mature pancreatic β-cells after birth. Toward the end of embryonic development, survivin expression becomes restricted to β-cells within the endocrine pancreas. Genetic disruption of *survivin* in all pancreatic endocrine cell types results in a selective decrease in the pancreatic β-cell mass early after birth, beginning at 2 weeks of life. Animals lacking survivin within their pancreatic endocrine cells become hyperglycemic and are unable to produce sufficient amounts of insulin by 4 weeks of life but maintain production of other endocrine hormones, including glucagon and somatostatin. Interestingly, blood glucose levels in the *survivin*-deficient animals were not significantly increased at 3 weeks of age, although insulin levels were at least twofold below normal. A likely explanation for the relative normoglycemia at 3 weeks of life is that the animals were maintained on a low-carbohydrate (maternal milk) diet, and, thus, the requirements for insulin were low. Once the animals were weaned to a high-carbohydrate (standard chow) diet between 3 and 4 weeks, however, the requirement for insulin increased, resulting in significant hyperglycemia in the absence of endogenous insulin. Because of the hepatic and renal toxicity resulting from massive hyperglycemia over time, the animals lacking survivin had shortened life spans. This dramatic phenotype was completely reversed by exogenously expressing the mouse survivin protein in mature pancreatic β-cells of the *survivin*-deficient animals. The molecular mechanism underlying the loss of pancreatic β-cells in the *survivin*-deficient animals, although limited because of the in vivo nature of this study, is supportive of a defect in both cell cycle progression and an apoptotic pathway.

Some intriguing questions generated from this work are *1*) why does survivin more selectively affect β-cells over other endocrine cell types, and *2*) why are there no observable effects of a loss of survivin during embryogenesis? One answer to the cell-selective effect may be that the expression levels of survivin in the β-cells are much higher than those in the other endocrine subtypes and that a critical level of survivin is necessary to confer its function. This hypothesis is consistent with the data here and also with the effects observed in cancer cells; high levels of survivin found in malignant human tumor cells promote tumor cell survival, whereas low survivin levels inherent to benign tumors confer no survival advantage (13). Prior evidence for a requirement for β-cell–specific cell cycle proteins, like survivin, for precise regulation of proliferation comes from whole mouse knockout models of other proteins, such as CDK4, that give rise to a selective β-cell phenotype, without affecting additional endocrine or exocrine cell types (39,40). With regard to the timing of survivin loss during early embryogenesis and its effect on postnatal β-cells, this could be due to a specific necessity for its activation during the rapid proliferative phase between the end of embryogenesis and the first 2

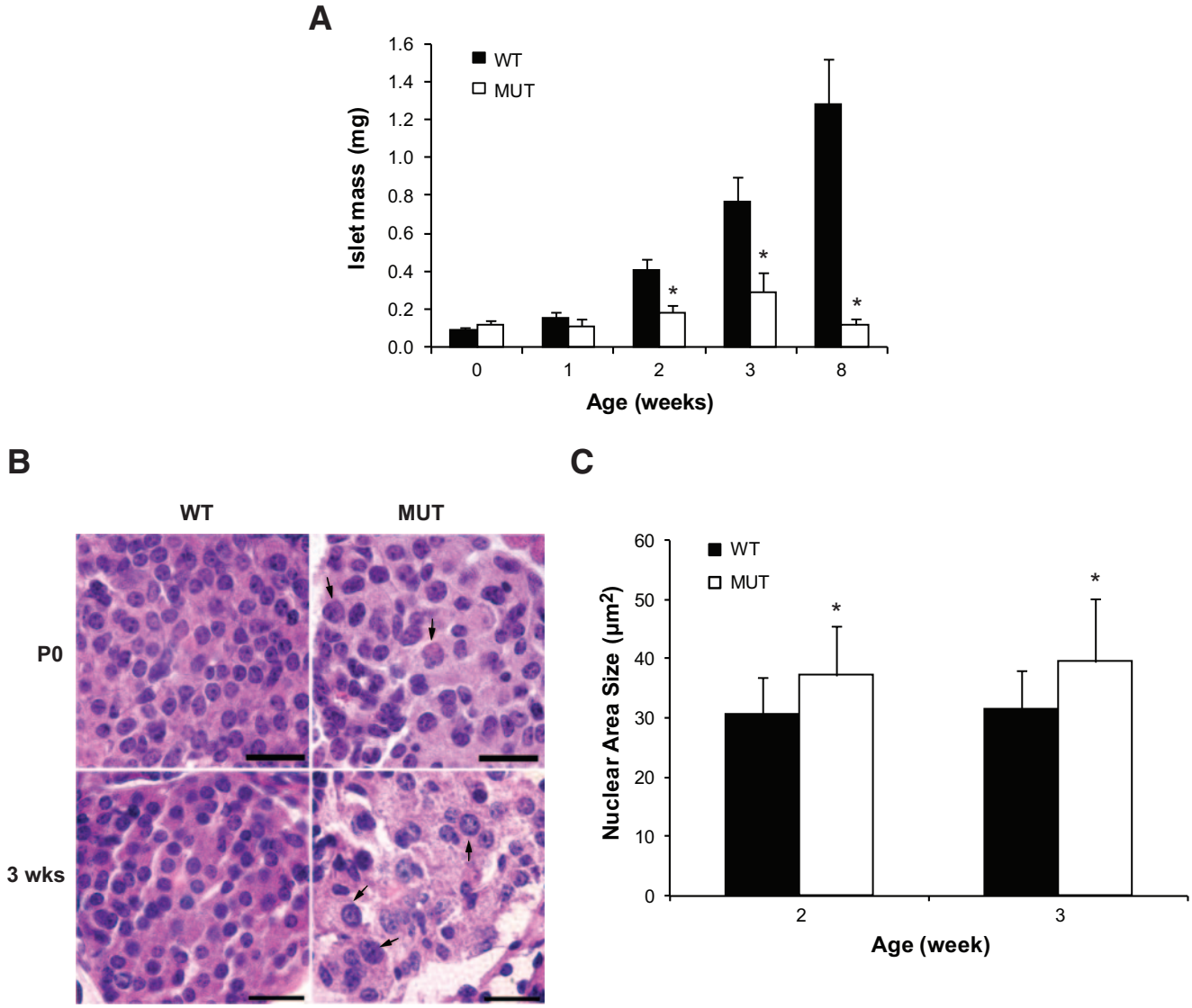

FIG. 4. Loss of islet cell mass and nuclear abnormalities in survivin-deficient mice. *A*: Expansion of the endocrine cell mass after birth. Pancreatic tissue was isolated from age-matched animals at the indicated times. The mean (± SD) islet cell mass of control (■, n = 8 [P0], 3 [P7], 5 [P14], 4 [P21], and 4 [8W]) and *survivin*-deficient (□, n = 7 [P0], 3 [P7], 5 [P14], 4 [P21], and 4 [8W]) tissues was calculated by point morphometry. *Significant ($P < 0.001$) difference. *B*: β-Cell size differences in affected animals. Pancreatic tissue isolated from animals at the indicated times was stained with H-E and visualized by light microscopy. Mutant β-cells with enlarged nuclei are indicated by arrows. Bars = 20 μm. *C*: Nuclear size measurements from *B*. ■, control tissue (n = 5); □, *survivin*-deficient tissue (n = 5). *Significance at $P < 0.001$. (Please see http://dx.doi.org/10.2337/db08-0170 for a high-quality digital representation of this image.)

weeks of life (41,42). To answer these questions more definitively, signaling pathways that control the regulation of survivin protein during embryogenesis and after birth will need to be explored.

In the mammalian cell cycle, D-type cyclins bind to and activate the cyclin-dependent kinase protein CDK4 during G1, and, together, they coordinate the transition from G1 to S phase via phosphorylation of Rb protein and release of the transcriptional activator E2F1 (43–45). Previously, we have shown that E2F1 binds to and activates the *survivin* promoter in mouse embryonic fibroblasts and is responsible, in part, for its cell-cycle dependency (46). The spontaneous development of insulin-deficient diabetes after deletion of *survivin* within the endocrine pancreas is strikingly similar to the phenotype observed after disruption of *Cdk4* in ES cells (39,40). *Cdk4*-null mice develop diabetes manifested by hyperglycemia, polyuria, and low serum insulin levels within the first 2 months of life (39,40). Sections of pancreas tissue from these mice, like those from the survivin mutant mice, show a decrease in islet cell mass with a selective decrease in the expression of insulin and a relative preservation of glucagon, somatostatin, and pancreatic polypeptide. In addition, pancreas tissue from the *Cdk4*-null mice show evidence of both a decrease in β-cell proliferation and an increase in apoptosis (40), the latter likely induced after activation of a cell cycle checkpoint in response to a lack of cell cycle progression (47). Thus, survivin is much like CDK4 in that it can selectively regulate β-cell growth during the postnatal period and, when inactivated, causes a slowing of cell cycle progression and an increase in apoptosis. Given the similar phenotypes of the *Cdk4* and *survivin* knockout animals, the E2F1-mediated regulation of *survivin* transcription, and the activation of E2F1 by the CDK4/CyclinD complex, survivin fits well within the molecular pathway of these proteins and likely functions to assist them in modulating β-cell replication after birth. Further work showing that transgenic expression of survivin can rescue

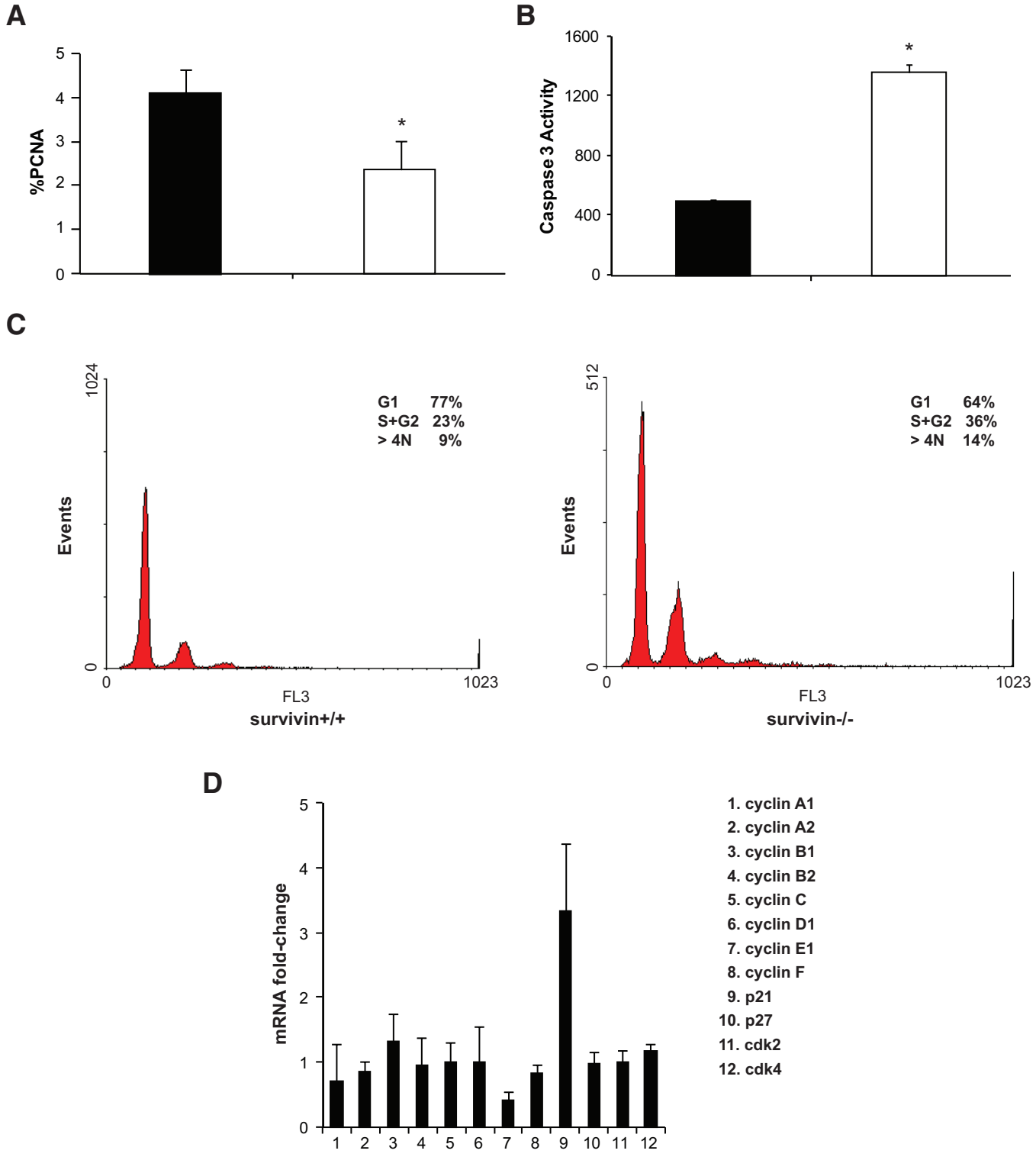

FIG. 5. Defects in replication and apoptosis pathways. *A*: PCNA staining of pancreatic tissues indicates a 50% decrease in the proliferation of islet cells isolated from *survivin*-deficient animals (□, $n = 5$) compared with their littermate controls (■, $n = 5$). Data are mean (±SD) percentages of positive cells. *Significance at $P < 0.05$. *B*: Caspase 3 activity assays performed on islets isolated from 2-week-old mice indicate a two- to threefold increase in caspase 3 activation in islet cells isolated from *survivin*-deficient animals (□) compared with their littermate controls (■). Data are mean (±SD) percentages of positive cells. *Significance at $P < 0.05$. *C*: Aberrant cell cycle progression in islet cells lacking survivin. Islet cells isolated from 2-week-old *Pax6-cre*;*survivin*$^{lox/lox}$ mice and their littermate controls were fixed, stained with propidium iodide, and analyzed by flow cytometry. Comparisons between *survivin*-deficient animals and their controls are shown. *D*: Fold changes in the expression of the indicated genes as determined by quantitative PCR of isolated islets at 1–2 weeks of age. ■, *Pax6-cre*;*survivin*$^{lox/lox}$ cells in comparison with controls. Data are mean (±SD).

the *Cdk4*-null mouse diabetic phenotype, currently underway, would confirm this hypothesis.

Although limited by the in vivo nature of this study, by the technical difficulty of isolating sufficient numbers of islet cells from young (1- to 2-week-old) survivin mutant mice for protein analysis, and by the extremely low in vitro

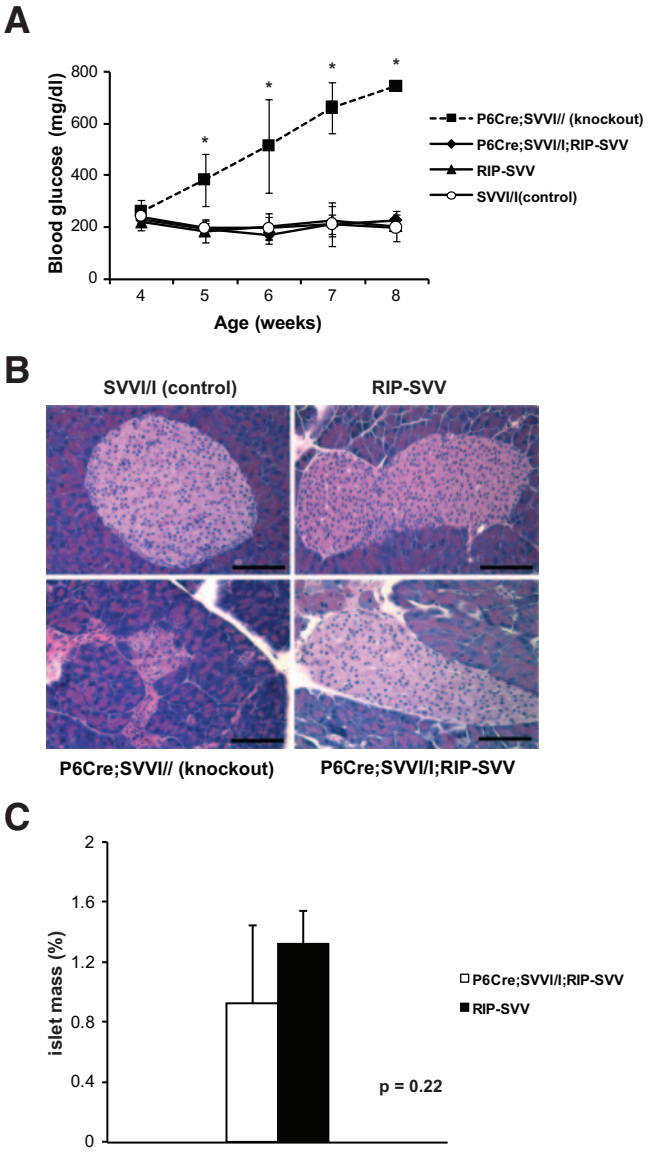

FIG. 6. Survivin, under control of the RIP, rescues the diabetic phenotype and islet cell mass of the *Pax6-Cre;survivin$^{lox/lox}$* mice. *A*: Mean (± SD) random glucose levels in *Pax6-Cre;survivin$^{lox/lox}$* ($n = 7$) mice compared with *Pax6-Cre;survivin$^{lox/lox}$;RIP-SVV* ($n = 6$), *RIP-SVV* ($n = 5$), and *survivin$^{lox/lox}$* (SVVl/l) mice ($n = 6$) from 4 to 8 weeks after birth. *Significance at $P < 0.05$. *B*: Representative islet cell morphology of the mice in *A* at 12 weeks of age. Bar = 40 μm. *C*: Islet cell mass in *RIP-SVV* (■, $n = 4$) and *Pax6-Cre;RIP-SVV;survivin$^{lox/lox}$* (□, $n = 4$) mice. No statistically significant differences were found. (Please see http://dx.doi.org/10.2337/db08-0170 for a high-quality digital representation of this image.)

replication rates of primary islet cells (0.06–0.15% per day) (48), by isolating some islets from the mutant mice, we were able to use quantitative PCR to identify the cell cycle regulator $p21^{WAF1}$ as consistently induced in islet cells that lacked survivin. p21 protein, within a family of proteins that also includes p27 and p57, can function to inhibit (49–51) cyclin-dependent kinases, leading to a decrease in the rate of cell cycle progression. p21 is expressed in both human and mouse β-cells and is induced in response to β-cell injury (52) and growth factors (53). Functionally, islet cells lacking p21 grow faster than those with an intact p21 protein (53). Interestingly, p21 is not induced by metabolic signals, leading to a suggestion that it specifically acts to inhibit mitogenic signals in β-cells (54). If p21 is a true downstream target of survivin in β-cells, then upregulation of survivin during the developmental period of β-cell growth may result in a decrease in p21 activity, releasing the cells to proceed through the cell cycle.

Although dispensable for the differentiation of endocrine cells during embryonic development, survivin fits well within the framework of known mediators of cell cycle progression in mature β-cells, including the D-type cyclins, the cyclin-dependent kinases, the E2F/RB family of proteins, and the moderators of these molecules, the cyclin-dependent kinase inhibitors (9,54,55). Our data strongly support a critical role for survivin in the maintenance of the β-cell mass in the early postnatal period. Understanding β-cell mass regulation is critical to understanding the pathogenesis of diabetes and to islet cell transplantation protocols. Our findings suggest that current efforts to regenerate and expand the β-cell mass (56) will need to consider the novel role of survivin during this process.

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