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 if 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 bi-allelic 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.
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 body’s metabolic demands. 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 E13 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 (IAPs) 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 ES 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). Indeed, 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-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**
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Animals. Pax6-Cre mice (also called Le-Cre) were crossed with B6;129S-Gtrosa26tm1sor mice to evaluate Cre-activity (29). Mice harboring a conditionally targeted mouse survivin gene flanked by two loxP sites (survivinlox/lox) have been described previously (16; 27) as have Pax6-Cre mice, (30). Pax6-Cre mice were crossed with survivinlox/lox mice to generate Pax6-Cre;survivinlox/lox mice. For the rescue experiment, two lines of mice harboring a mouse survivin transgene under control of an RIP promoter (28) were mated with survivinlox/lox mice. Offspring with the genotype of Pax6-Cre; RIP-SVV+;survivinlox/lox were compared with those of with Pax6-Cre; RIP-SVV-;survivinlox/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 H&E. Images were taken with a LEICA microscope equipped with a SPOT RT KE digital camera (Diagnostic Instruments Inc.). Islet cell areas for the control and survivin knockout were measured by Point Counting Morphometry (see Supplemental Methods).

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% PFA. The primary antibodies used were: Survivin (TIAP, rabbit polyclonal, 1:200, Chemicon.), glucagon (mouse monoclonal, 1:1000, Sigma), somatostatin (rabbit polyclonal anti-srif28 IHC 8004, 1:1000, 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 bioscience) and Isl1 (mouse, 1:100, Developmental Studies Hybridoma Bank). Rabbit anti-MafA antibody was described previously (Nishimura et al. 2006). 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 Inc. Milpitas, CA, USA). For the glucose challenge, mice were fasted then injected intraperitoneally with dextrose at 2 g/kg body weight. Blood glucose levels were subsequently measured at indicated times. Insulin challenge was performed in the fed state. A dose of 0.75 units/kg of body weight was injected intraperitoneally. Blood glucose levels were measured every 15 min for 90 minutes post injection. A minimum of 4 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. 1A). Survivin expression gradually became restricted to endocrine (Isl1 positive) cells in late embryogenesis (E19.5) and postnatally (P14) (Fig. 1A). By P21, one can detect several Isl1 positive cells that do not express
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survivin, suggesting a further restriction of survivin expression to a sub-population(s) of endocrine cells (Fig. 1A). Co-localization 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. 1A, 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, as activation of this gene was previously reported only in undifferentiated and highly-proliferating cells in the adult animal (26). We therefore 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 gapdh (Supplemental Fig. 1A). 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; 32). 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; 33). 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 (33). 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<sup>tm1Sor</sup>), 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 (Supplemental Fig. 1B).

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<sup>lox/lox</sup> 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. 1B, panel 1) and by performing PCR for the deleted and non-deleted survivin alleles on isolated islet cell DNA (Supplemental Fig. 1C). Quantification of survivin and insulin protein in the knockout animals showed that only ~6% of all insulin-positive cells expressed survivin. By comparison, 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<sup>lox/lox</sup> animals, we performed PCR for the deleted and non-deleted survivin alleles in tissues outside the pancreas, including liver, muscle, fat, and brain (Supplemental Fig. 1D) 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
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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. 1B, 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 since conventional deletion of survivin in embryonic stem cells resulted in early embryonic lethality (18) and deletion of survivin at E9.5 in neural stem cells led to significant embryonic neural stem cell loss and death immediately post-birth (27). By contrast, here all Pax6-Cre;survivinlox/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 physiologic effects of survivin loss within the endocrine cells after birth, we performed serial metabolic studies on mice lacking endocrine-survivin (Pax6-Cre;survivinlox/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 2A). At 4 weeks of age however, the Pax6-Cre;survivinlox/lox mice developed hyperglycemia (Fig. 2A) and a reduced glucose tolerance, as determined by injection with 2 g/kg dextrose after a 5-hour fast (Fig. 2B); findings consistent with early-onset diabetes. The glucose abnormalities in these mice became more striking as the animals aged (Fig. 2A). At 4 weeks of age, Pax6-Cre;survivinlox/lox mice responded similar to littermate control animals when treated with the same doses (0.75u/kg) of exogenous insulin (Fig. 2C), 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. 2D and Supplemental Table 1), suggesting either a failure of insulin production or secretion. Mice with a one allele loss of endocrine-survivin (Pax6-Cre;survivinlox/+ mice) had 12-hour fasting glucose levels that were comparable to control animals at 4 and 5 weeks of age (Supplemental Fig. 2A). At 17 weeks of age however these heterozygotes began to develop some signs of glucose intolerance, as shown by their higher serum glucose levels following dextrose administration (Supplemental Fig. 2B).

Pax6-Cre;survivinlox/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 (Supplemental Fig. 3A). Many of these older survivin-deficient mice also developed other metabolic hallmarks of human diabetes including metabolic acidosis, hyperkalemia, polyuria and ketonuria (Supplemental Fig. 3B and Supplemental Table 2). In addition, they developed hypertriglyceridemia and hypoproteinemia (Supplemental Fig. 3C and Supplemental Table 2) as well as pathologic evidence of fatty livers, most likely due to the secondary effects of prolonged glucose and lipid toxicity (Supplemental Fig. 3D), findings reminiscent of untreated human diabetes. These mice ultimately showed poor weight gain and signs of dehydration and had shortened lifespans of 4 to 7 months.
Survivin regulates β-cell mass after birth. To determine the cause of the metabolic abnormalities resulting from the low serum insulin levels, we examined pancreatic sections from the mice for pathologic 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. 3A) and by islet mass (Fig. 4A), 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. 3A) and by measuring serum glucagon levels (Fig. 3B), 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 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 TUNEL, a marker for apoptosis. We observed a 50% decrease in PCNA staining in the survivin-deficient β-cells (Fig. 5A) but no significant change in the number of TUNEL-positive cells (not shown). To increase the sensitivity for the detection of cell death we isolated islet cells from the pancreases of 1-2 week-old animals and subjected them to functional caspase 3 activity assays. With this methodology, we did observe a 2-fold increase in caspase 3 activity in the survivin-deficient cells (Fig. 5B), 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%...
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Survivin regulates β-cell mass vs. 9%, Fig. 5C) and an accumulation of survivin-deficient islets in late S/G2 (36% vs. 23%, Fig. 5C), 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 3-fold) increase in expression of the cell cycle inhibitor p21WAF1 and a 2-fold decrease in expression in cyclin E in the survivin-deficient cells (Fig. 5D). No significant changes in the expression levels of Cyclin A, B1, B2, C, D1, F, p27, Cdk2, or Cdk4 were seen (Fig. 5D). 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 (36; 37).

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;survivinlox/lox mice with transgenic mice expressing the survivin protein under control of the rat insulin promoter (yielding Pax-6-Cre;survivinlox/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;survivinlox/lox; RIP-SVV mice and comparing these with random glucose levels in the RIP-SVV, Pax-6-Cre;survivinlox/lox, and survivinlox/lox mice (Fig. 6A). RIP-driven transgenic expression of survivin completely rescued the diabetic phenotype of the Pax6-Cre;survivinlox/lox mice. It also restored normal growth (Fig. 6B, 12 weeks) and mass of the islets (Fig. 6C). 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 cells were not significantly increased at 3 weeks of age, though insulin levels were at least two-fold 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. Due to the hepatic and renal toxicity resulting from massive hyperglycemia over time, the animals lacking survivin had shortened lifespans. 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, while limited due to the in vivo nature of this study, is supportive of a defect in both cell cycle progression and an apoptotic pathway.
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Some intriguing questions generated from this work are i) why does survivin more selectively affect β-cells over other endocrine cell types and ii) 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, while 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 (38; 39). 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 weeks of life (40; 41). 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 (42-44). 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 (27). The spontaneous development of insulin-deficient diabetes following deletion of survivin within the endocrine pancreas is strikingly similar to the phenotype observed following disruption of Cdk4 in ES cells (38; 39). Cdk4 null mice develop diabetes manifested by hyperglycemia, polyuria, and low serum insulin levels within the first 2 months of life (38; 39). 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 (39), the latter likely induced following activation of a cell cycle checkpoint in response to a lack of cell cycle progression (45). 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 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 (1-2 week old) survivin mutant mice for protein analysis and by the extremely low in vitro replication rates of primary islet cells (0.06% to 0.15% per day)(46), by isolating some islets from the mutant mice we were able to use quantitative PCR to identify the
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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 (47-49) 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 (50) and growth factors (51). Functionally, islet cells lacking $p21$ grow faster than those with an intact $p21$ protein (51). Interestingly, $p21$ is not induced by metabolic signals, leading to a suggestion that it specifically acts to inhibit mitogenic signals in β-cells (52). 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; 52; 53). 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 (54) will need to consider the novel role of survivin during this process.

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Survivin regulates β-cell mass
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Figure 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), then further restricted to β-cells (Ins+) by P21. Magnification = 20X. Bar = 20 μm. (B) Analysis of expression of differentiation markers in pancreatic sections isolated from Pax6-cre;survivinlox/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 = 20X. Bar = 20 μm.
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Figure 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 (open circles, n>= 8 at each time point) and homozygous survivin-deficient (Pax6-Cre;survivin$^{lox/lox}$) mice (closed squares, n>= 8 at each time point) at various ages after birth. Asterisks indicate significance at P < 0.01. (B)
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Glucose tolerance test (mean ± SD) in 6-week-old male littermates. Control (open circles, n = 7) and homozygous survivin-deficient (closed squares, n = 7) mice were fasted for 5 hours then injected with dextrose (2 g/kg body weight) intraperitoneally. Asterisks indicate significance at P < 0.05. (C) Insulin challenge test in 4-week old littermates. 4-week old control (open circles, n = 3) and homozygous survivin-deficient (closed squares, n = 4) mice were injected with 0.75 units/kg of insulin (fed state). No statistically significant differences were found at any time point. (D) Random serum insulin levels measured by ELISA in age-matched animals at the indicated times. Open circles denote insulin levels in individual control animals (n = 16), while triangles denote levels in individual Pax-6Cre;survivinlox/lox animals (n = 12). See Supplemental Table 1 for individual values.
Figure 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 immunohistochemical staining of pancreatic sections isolated from control mice and Pax-6Cre;survivin<sup>lox/lox</sup> littermates. Bars = 40 μm. (B) Mean (± SD) serum glucagon levels measured by immunoassay in age-matched
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Figure 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 [black bars, n = 8 (P0), 3 (P7), 5 (P14), 4 (P21), 4 (8W)] and survivin-deficient [white bars, n = 7 (P0), 3 (P7), 5
Survivin regulates β-cell mass

(P14), 4 (P21), 4 (8W)] tissues was calculated by point morphometry. Asterisks denote a 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 hematoxylin and eosin and visualized by light microscopy. Mutant β-cells with enlarged nuclei are indicated by arrows. Bars = 20 μm. (C) Nuclear size measurements from (B). Black bars represent control tissue (n = 5) and white bars survivin-deficient tissue (n = 5). Asterisk denotes significance at p < 0.001.
Figure 5. Defects in replication and apoptosis pathways. (A) Proliferating cell nuclear antigen
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staining (PCNA) of pancreatic tissues, indicates a 50% decrease in the proliferation of islet cells isolated from survivin-deficient animals (white bar, n = 5), compared with their littermate controls (black bar, n = 5). The data are mean (± SD) percentages of positive cells. Asterisk denotes significance at p < 0.05. (B) Caspase 3 activity assays performed on islets isolated from 2-week-old mice indicate a 2- to 3-fold increase in caspase 3 activation in islet cells isolated from survivin-deficient animals (white bars), compared with their littermate controls (black bars). The data are mean (± SD) percentages of positive cells. Asterisk denotes significance at p < 0.05. (C) Aberrant cell cycle progression in islet cells lacking survivin. Islet cells isolated from 2-week-old Pax6-cre;survivinlox/lox mice and their littermate controls were fixed, stained with propidium iodide, and analyzed by flow cytometry. Comparison 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. Black bars represent Pax6-cre;survivinlox/lox cells in comparison with controls. The data are mean (± SD).
Survivin regulates β-cell mass

A

![Graph showing blood glucose levels over age (weeks).](image)

B

![Images of mouse pancreata.](image)

C

![Bar graph showing islet mass.](image)

Figure 6
Figure 6. Survivin, under control of the rat insulin promoter (RIP), rescues the diabetic phenotype and islet cell mass of the Pax6-Cre; survivin<sup>lox/lox</sup> mice. (A) Mean (± SD) random glucose levels in Pax6-Cre; survivin<sup>lox/lox</sup> (n = 7) mice compared with Pax6-Cre; survivin<sup>lox/lox</sup>; RIP-SVV (n = 6), RIP-SVV (n = 5), and survivin<sup>lox/lox</sup> (SVVl/l) mice (n = 6) from 4 to 8 weeks after birth. Asterisks indicate 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 (black bar, n = 4) and Pax6-Cre; RIP-SVV; survivin<sup>lox/lox</sup> (white bar, n = 4) mice. No statistically significant differences were found.