Cyclin D1 can stimulate proliferation by driving cells from the G1 into the S-phase of the mammalian cell cycle. Previous animal studies have implicated the G1-S transition as a key regulatory checkpoint governing the proliferation of pancreatic islet cells. We expressed cyclin D1 in the β-cells of mice and islet hyperplasia developed in a time-dependent manner. The hyperplastic β-cells exhibited higher rates of proliferation. However, blood glucose levels in fasting as well as nonfasting conditions remained normal. Furthermore, glucose tolerance tests demonstrated nearly normal responses, and diabetes did not develop in any of the animals. No islet tumors were observed, even among animals >2 years of age. Under our experimental conditions, the proliferative stimulus provided by cyclin D1 is not tumorigenic, does not result in diabetes, and does not result in hypoglycemia. Cyclin D1 may thus be considered a potential candidate to augment the β-cell population ex vivo as a prelude to islet transplantation for diabetes. Diabetes 54:712–719, 2005

New β-cells can arise from fully differentiated β-cells through the process of replication, and growth factors including parathyroid hormone–related peptide, hepatocyte growth factor, glucagon-like peptide-1/exendin-4, prolactin, growth hormone, IGF-I, and IGF-II have demonstrated important roles (1–6). Understanding the complex molecular mechanisms that regulate growth and proliferation of the β-cell has tremendous implications for the design of strategies aimed at β-cell replacement therapy in diabetes.

The replication rate of β-cells in the mature pancreatic islet is low and estimated to be <3% in rodents (7). Replication of islet cells is balanced by apoptosis. Interestingly, total β-cell mass in the rodent has been demonstrated to gradually increase as animals age (8), and this is partially attributable to ongoing β-cell replication. Several genetic mouse models have provided unique insights into some of the molecular signals that may control islet cell replication. Specifically, it has been demonstrated that genetic manipulations of the cell cycle machinery uniquely alter the proliferation of pancreatic islet cells. No pancreatic islet phenotype was observed in mice deficient in either the Rb or p53 gene, but compound Rb<sup>−/−</sup>, p53<sup>−/−</sup> knockout animals developed striking islet cell hyperplasia as well as islet cell tumors (9,10). One critical point at which the Rb and p53 pathways overlap is the G1 checkpoint of the cell cycle; Rb inhibits the E2F transcription factor that is necessary for progression of the cell cycle into S-phase (rev. in 11), and p53 also functions to arrest cells at the G1 checkpoint, in part through the induction of p21 (rev. in 12). These findings provided suggestive evidence that pancreatic islet cell growth may be tightly regulated at the G1 checkpoint.

The hypothesis that the G1 checkpoint may be a key regulator of pancreatic β-cell proliferation was strengthened by in vivo studies of cyclin-dependent kinase (cdk)-4, an enzyme that directly regulates the G1 checkpoint through its phosphorylation of Rb. When cdk-4 was deleted in the germline of mice, there were no widespread developmental defects (13,14). Rather, the primary phenotypes were infertility due to reduced cell numbers in the pituitary gland and diabetes from a marked reduction in β-cells (15). When the constitutively active cdk-4<sup>R24C</sup> allele was “knocked in” to the germline, dramatic β-cell hyperplasia was observed. These studies illustrated the pivotal role of cdk-4, a specific regulator of the G1 checkpoint, in β-cell proliferation.

Another critical regulator of the G1 checkpoint is cyclin D1, a specific catalyst for cdk-4 (rev. in 16). Overexpression of cyclin D1 has been demonstrated in benign proliferations of several human endocrine tissues including parathyroid adenomas (17) and pancreatic islet cell adenomas (18), suggesting a role in the proliferation of islet cells in humans. In some highly differentiated cell types, such as neurons, overexpression of cyclin D1 induces apoptosis (19), but in other endocrine tissues, such as the parathyroid gland, there is enhanced proliferation (20). Recently, in vitro studies of cultured human and rat islets demonstrated that adenoviral transfer of cyclin D1 induced the replication of β-cells (21). We therefore sought to determine whether expression of cyclin D1 would stimulate islet cell proliferation in vivo. Our studies revealed that targeted expression of cyclin D1 in pancreatic β-cells of mice resulted in marked islet hyperplasia. Nota-
bly, this occurred without the formation of islet cell tumors. Despite this hyperplasia, there were only minimal perturbations in blood glucose homeostasis. These hyperplastic β-cells expressing cyclin D1 did not exhibit dramatic derangements in insulin metabolism and secretion. Thus, cyclin D1 may have potential as a novel therapy to stimulate β-cell replication.

RESEARCH DESIGN AND METHODS

Generation of transgenic mice. Expression of the human cyclin D1 gene was targeted to the pancreatic β-cell using the well-characterized rat insulin promoter. The mouse mammary tumor virus (MMTV) promoter was excised from the MMTV-cyclin D1 cDNA construct containing the 1.4-kb human cyclin D1 cDNA upstream of the SV40 polyadenylation sequence, as previously described (22). A 450-bp rat insulin 1 promoter fragment that included 370 bp of proximal promoter sequence and exon 1 (23) was PCR amplified, and 5’ XhoI and 3’ EcoRV sites were introduced. The fragment was digested with XhoI and EcoRV and subcloned upstream of the human cyclin D1 cDNA. The transgenic construct was confirmed by direct DNA sequencing. A 3.5-kb fragment containing the rat insulin promoter human cyclin D1 cDNA and SV40 polyadenylation sequence (Ins-D1) was excised, purified, and microinjected into BDF2F1 embryos, which were then implanted into pseudopregnant foster mothers. Potential founders were analyzed by Southern blotting of EcoRI-digested tail DNA with a random-primed full-length 1.4-kb human cyclin D1 cDNA probe. Founder animals were identified and bred into the C57BL/6 strain. The subsequent found offspring were genotyped with PCR using primers specific for the transgene (primer sequences: forward, 5’ TCTCACAACCCTAAGTGAC and reverse, 5’ GGAAGTGTTCAATGAAATCG) and then confirmed with Southern blotting. This protocol was approved by the institutional subcommittee on research animal care.

RNA analysis. Expression of the cyclin D1 transgene in the pancreas was assessed through Northern blotting of total RNA isolated from whole pancreas using the TRIZOL reagent (Gibco). After separation on a 1% formaldehydeagarose gel, 10 μg total RNA was transferred onto a nylon membrane and hybridized with a random primer 32P-labeled human cyclin D1 cDNA probe. The blot was stripped and rehybridized with a control probe for GAPDH.

RESULTS

Analysis of Ins-D1 transgenic mice at 2–4 months. Mice expressing cyclin D1 in pancreatic β-cells were generated using the rat insulin promoter fragment containing the tissue-specific FAR, FLAT, and NIR elements that have been previously demonstrated to direct expression of a transgene specifically to the pancreatic β-cells (23). Analysis of potential founders by Southern blotting of tail DNA identified seven animals that carried the transgene (6103, 6104, 6111, 6118, 6121, and 6128), the transgene was transmitted in the germline. Transgenic expression of cyclin D1 in the pancreas was confirmed by Northern blotting of RNA isolated from whole pancreata (data not shown). Immunohistochemistry for cyclin D1 protein confirmed β-cell–specific expression of the transgene, and representative images of nuclear cyclin D1 staining in pancreatic islet cells are illustrated in Fig. 1A and B. No cyclin D1 staining was observed in islet cells from paired wild-type littermates.

The ratio of transgenic mice to wild-type littermates was the expected 1:1. The male-to-female ratio was also 1:1, as expected, and the transgenic animals were fertile.

During the first 4 months of life, overall growth and survival were similar in transgenic and wild-type littermates. In addition, pancreatic weights were similar (transgenic 0.20 ± 0.04 g, n = 8, and wild type 0.19 ± 0.04 g, n = 5; P = NS). Histologically, there was no apparent increase in the number of islets in transgenic animals. In addition, the islet cells did not display any obvious evidence for hyperplasia, hypertrophy, or atrophy (Figs. 1C and D). Mitotic figures are rare in islet cells, and there was no change in islet cells that overexpressed cyclin D1. Insulin staining was preserved, and there was a normal distribution of β-cells within the islets (data not shown). Of note, one slightly enlarged islet was noted in the pancreas from a single transgenic animal. Apart from the overall size of that single islet, however, there were no other histological abnormalities.

To determine whether islet cells that overexpress cyclin...
D1 functioned physiologically in the regulation of blood glucose levels, fasting blood sugars were measured. There was no significant difference between transgenic and wild-type animals in the four lines tested, indicating that expression of cyclin D1 did not perturb the β-cell's ability to accurately sense blood glucose levels and precisely secrete appropriate amounts of insulin. Because no distinctive alterations in the growth or function of islet cells were discernible at this early time point, the animals were allowed to age. Two lines (6118 and 6128) with the strongest levels of transgene expression (Fig. 2) were selected for this extended analysis.

Analysis of Ins-D1 transgenic mice at 10–27 months. Animals in the 6118 and 6128 lines were next analyzed between 10–12 months of age and were observed up to 27 months of age. The growth of transgenic animals over this extended time period was similar to paired wild-type littermates. Total body weights were comparable between wild-type and transgenic mice in both the 6118 and 6128 lines. At 4–6 months, the weight of the transgenic animals was 32.5 ± 8.6 g compared with 28.7 ± 7.1 g (P = 0.07) for the wild-type animals. At 12 months, the average weights for the transgenic and wild-type animals were 37.8 ± 9.2 and 35.5 ± 7.4 g (P = 0.36), respectively, and at 18–22 months, the weights were 41.0 ± 10.3 and 38.5 ± 8.1 g (P = 0.28), respectively.

FIG. 1. Immunohistochemical localization of cyclin D1 protein in pancreata from 2-month-old animals. A representative image from a wild-type female (A) and a paired transgenic female (B). Nuclear cyclin D1 staining is observed in many of the islet cells of transgenic animal, but no staining is observed in the wild-type islet. Magnification = 500×. H+E-stained sections of 3-month-old pancreata. A representative low-power image from a wild-type female (C) and a paired transgenic female (D) from the 6128 line. There is no significant difference in the number or size of islets between the two animals. TG, transgenic; WT, wild-type. Magnification = 5×.

FIG. 2. RT-PCR for cyclin D1 transgene expression. One microgram of pancreatic RNA from wild-type and transgenic mice of the 6118 and 6128 strains was reverse transcribed and then amplified with PCR with specific primers for the human cyclin D1 transgene (40 cycles) and 18s RNA (35 cycles). Lanes 1–7, wild-type (WT) animals; lanes 8–19, transgenic (TG) animals. The mean ages of the 6118 and 6128 transgenic animals were 26.9 and 21.8 months, respectively. The mean ages of the wildtype 6118 and 6128 animals were 25.7 and 22.5 months, respectively.
months, the weights were 41.3 ± 11.3 and 37.6 ± 8.6 g (P = 0.09), respectively. There was a slight increase in body weight of the male and female transgenic animals at each time point, but these differences were not statistically significant. Transgenic expression of cyclin D1 remained robust throughout this time period (Fig. 2). In addition, no significant alterations in the expression of the endogenous cyclin D1, D2, or D3 genes were observed. Quantitative RT-PCR revealed that levels of murine cyclin D1, D2, and D3 mRNA in the transgenic pancreata of both lines were 1.0-, 1.2-, and 1.3-fold, respectively, the mRNA levels in wild-type pancreata.

Between 10 and 27 months, animals were killed for analysis of pancreatic islet histology. In total, 18 transgenic animals between the ages of 10 and 18 months and 18 transgenic animals between the ages of 19 and 27 months were necropsied. No islet tumors were identified, even among animals that lived to 27 months. Rather, dramatic islet hyperplasia was observed in the transgenic animals in both the 6118 and 6128 lines. Furthermore, the morphology of some of the hyperplastic islets was slightly irregular. Representative images are displayed in Fig. 3. Despite the marked hyperplasia of the islet compartment, the total weight of the pancreata was comparable in these wild-type and transgenic animals (6118 line: transgenic 179 ± 46 mg and wild type 188 ± 34 mg (P = NS); 6128 line: transgenic 218 ± 83 mg and wild type 227 ± 59 mg (P = NS). To quantify the extent of hyperplasia, the two-dimensional islet surface area was calculated by analyzing longitudinal cross-sections of whole pancreas. Morphometric studies revealed that β-cell mass was increased in both transgenic lines greater than threefold (Table 1). There was some variation in the islet mass in the transgenic animals. A few of the highest islet masses were seen in 10- to 12-month-old animals, but there was no definitive relationship between islet mass and age.

This islet hyperplasia could be potentially explained by alterations in either replication or apoptosis rates. To determine the level of β-cell replication, immunostaining

**TABLE 1**

Total islet cell mass (in milligrams) in wild-type and transgenic animals

<table>
<thead>
<tr>
<th></th>
<th>Wild type</th>
<th>Transgenic</th>
<th>P</th>
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<tbody>
<tr>
<td>6118</td>
<td>3.1 ± 1.2 (n = 3)</td>
<td>10.4 ± 7.6 (n = 10)</td>
<td>0.015</td>
</tr>
<tr>
<td>6128</td>
<td>4.2 ± 2.4 (n = 5)</td>
<td>14.8 ± 11.1 (n = 10)</td>
<td>0.014</td>
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Data are means ± SE. n = number of animals analyzed.
for PCNA was performed on male mice >12 months of age. The number of cells that stained positive for PCNA was significantly increased in the animals carrying the cyclin D1 transgene (Fig. 4A). In the 6118 line, 2.0% of islet cells in cyclin D1-transgenic mice were PCNA positive, whereas only 0.38% of wild-type islet cells were PCNA positive ($P = 0.002$). Similarly, 3.12% of islet cells from transgenic mice in the 6128 line were PCNA positive compared with 0.38% in paired wild-type animals ($P = 0.001$). The cellular death rates were determined by the TUNEL assay. Only rarely were TUNEL-positive cells identified, and the rates were comparable between transgenic islets and wild-type islets. In the 6118 and 6128 lines combined, 0.03% of transgenic islet cells were TUNEL positive, and 0.05% of wild-type islet cells were TUNEL positive ($P = 0.23$). Thus, it appears that the islet cell hyperplasia is accounted for primarily by increased rates of proliferation.

Insulin staining was preserved in β-cells expressing cyclin D1, indicating that these transgenic β-cells were capable of producing intact hormone (Fig. 4B). Thus, these transgenic β-cells retained a key feature of their intrinsic differentiated phenotype. In wild-type islets, glucagon staining localized to scattered cells in the periphery, consistent with the known distribution of α-cells. In the transgenic islets, the distribution of glucagon-producing cells was slightly altered. α-Cells were distributed more diffusely throughout the islet (Fig. 4B), indicating that the spatial organization of the islet cell subtypes was modified in these hyperplastic islets.

To define whether this islet hyperplasia resulted functionally in hyperinsulinemia and hypoglycemia, fasting
blood sugars and serum insulin levels were measured. The data are displayed in Fig. 5. Age-matched (18–22 months) male mice were analyzed. The fasting blood glucose levels were nearly identical in transgenic and paired wild-type mice in both the 6118 and 6128 lines (Fig. 5A). In addition, the mean fasting serum insulin levels fell within the normal range (2.0 ng/ml) in the transgenic animals (Fig. 5B). There was a slight trend toward higher insulin levels in the 6118 but not in the 6128 transgenic animals, but this difference was not statistically significant. Of note, there were a few animals with markedly elevated serum insulin levels. However, there was no clear-cut correlation with fasting hypoglycemia in these animals, suggesting that compensatory mechanisms served to prevent hypoglycemia and maintain euglycemia.

Although there was marked β-cell hyperplasia, the amount of insulin released into the bloodstream appeared to be appropriate to the levels of blood glucose. One potential explanation is that release of insulin into the circulation was impaired. This would be predicted to result in a diabetic phenotype. Blood sugars were therefore ascertained in the nonfasting or fed state (Fig. 5A). No significant differences were observed, suggesting that release of insulin was appropriate to maintain glucose homeostasis with feeding. To further elucidate the dynamic response of these hyperplastic β-cells to acute fluctuations in blood glucose levels, glucose tolerance tests were performed in age-matched male mice of both lines (Fig. 5C). At 30 min, the rise in blood glucose was slightly blunted in transgenic animals in both lines, but this difference was not statistically significant. At 60 min, the blood glucose levels had fallen and were identical. Thus, the response to a glucose challenge was normal in the transgenic animals. This suggested that despite the increased proliferation of β-cells and pancreatic concentration of insulin induced by cyclin D1, blood glucose homeostasis remained nearly physiologic. The comparable growth curves between the transgenic and wild-type animals further indicated that glucose homeostasis was not seriously disrupted in animals carrying the cyclin D1 transgene.

DISCUSSION
Defining the mechanisms that regulate the growth and proliferation of pancreatic β-cells is an essential prerequisite for the design of rational strategies to replace this population in diabetes. Previous animal models have im-
plicated a unique role for regulators of the G1-S transition point of the cell cycle. We tested the hypothesis that expression of cyclin D1, a distinct regulator of this checkpoint, may also stimulate islet cell proliferation. Our studies demonstrated that cyclin D1 can induce proliferation of β-cells in vivo without the formation of cancer and that in spite of this marked β-cell hyperplasia, there was no hypoglycemia observed.

Human conditions that result in the pathological proliferation of β-cells include nesidioblastosis and insulinoma. Their most dangerous and characteristic feature is profound hyperinsulinemia and hypoglycemia. The specific molecular alterations that lead to this dysregulation of insulin secretion and blood glucose homeostasis are not well characterized. The demonstration that overexpression of cyclin D1 in β-cells does not result in hyperinsulinemia and hypoglycemia suggests that the proliferative stimulus provided by cyclin D1 is unique. Specifically, the proliferation induced by cyclin D1 increases β-cell number but does not appear to alter the ability of the β-cell to sense and respond to changes in blood glucose levels. However, there are alternative explanations. For example, chronic hyperinsulinemia may result in insulin resistance, and euglycemia may thus be achieved because of this insulin resistance.

In another model of ectopic cyclin D1 expression, hyperplastic parathyroid cells demonstrated the ability to respond to shifts in serum calcium levels, but the “set point” was altered so that a state of chronic hyperparathyroidism and hypercalcemia developed (20). Consistent with this model, pancreatic β-cells overexpressing cyclin D1 displayed increased proliferation, while maintaining a well-differentiated phenotype. Although chronic hyperglycemia was not observed, peak blood glucose levels following a glucose load were blunted, implying that there was enhanced release of insulin. This may reflect a subtle alteration in the cellular mechanisms that sense glucose and secrete insulin in cyclin D1–expressing cells, or it may simply represent increased insulin secretion due to the increased overall β-cell mass.

Regulators of the G1 checkpoint of the cell cycle appear to be particularly important in the control of islet cell proliferation. In addition to the previously described role of cdk-4 (13), we have now demonstrated the in vivo function of cyclin D1. Although both cdk-4 and cyclin D1 have been shown to control progression through the G1 checkpoint in islet cells in vitro (21), there are distinctive differences in the phenotype observed when they are genetically altered in mice. Specifically, mice expressing the constitutively active cdk-4\(^{R24C}\) display marked islet cell hyperplasia by 3 months of age, and some ultimately develop islet cell tumors. In contrast, the hyperplasia observed in cyclin D1 transgenic islet cells is not observed until 12 months of age. The longevity of these animals to as great as 27 months establishes that this hyperplasia induced by cyclin D1 is not associated with any significant morbidity or mortality. This discrepancy in timing may reflect differential sensitivity to regulators of G1, depending on the age of the animal. Susceptibility to exogenous cyclin D1 may be present only at older ages, when the G1 checkpoint may be more permissive for proliferative signals. Consistent with this hypothesis is the absence of any developmental defects in the islets of our transgenic animals expressing cyclin D1 or in cyclin D1–deficient animals (26). It should also be noted that in addition to its regulation of the G1 checkpoint, cyclin D1 has been demonstrated to control the transition between G0 and G1 (27). This property may be particularly relevant, as a large subset of pancreatic β-cells are believed to be in G0 arrest (28).

In addition to its role in controlling the cell cycle, cyclin D1 can independently function as a regulator of gene transcription (29). Recently, cyclin D1 has been shown to repress the islet-specific transcription factor β2/NeuroD (30). β2/NeuroD plays a pivotal role in the development of the endocrine pancreas (31). The absence of any developmental phenotype in our transgenic mice overexpressing cyclin D1 suggests that its interaction with β2/NeuroD may not be pivotal in vivo. The possibility that cyclin D1 may activate insulin gene transcription through a cdk-independent mechanism cannot be ruled out.

Collectively, these findings imply a potential role for cyclin D1 in the in vitro expansion of β-cells before transplantation for diabetes. In addition to demonstrating the ability of cyclin D1 to enhance the proliferation of cultured primary islet cells, it will be critical to confirm that cyclin D1 is not tumorigenic in this setting. Multiple transgenic mouse models, including this one, have consistently demonstrated that expression of cyclin D1 alone results in benign hyperplasia but not malignancy (20,32,33). However, notable exceptions are the expression of cyclin D1 in murine mammary tissue or in hepatocytes (22,34). It would be essential to identify the determinants that may cause cyclin D1 to be tumorigenic in vivo. For example, there may be organ-specific differences that render endocrine tissues more resistant to transformation, or there may be dose-dependent effects such that expression of cyclin D1 below a threshold level may be insufficient for tumorigenesis.

In summary, we have demonstrated that overexpression of the cell cycle regulator cyclin D1 can enhance the proliferation of pancreatic β-cells in vivo. This effect is sustained and does not result in the formation of malignant tumors. Rather, there is an increased β-cell mass that preserves the ability to closely regulate blood glucose levels. Future studies will test the viability of this approach to expand the population of islet cells as a prelude to transplantation.

ACKNOWLEDGMENTS

D.C.C. was supported in part by a grant from the Juvenile Diabetes Foundation.

We are grateful to Susan Bonner-Weir for critical review of the manuscript and helpful discussions throughout. We are also grateful to Timothy Wang, David Rhoads, Melissa Thomas, and Ulysses Balis for helpful advice; to Andrew Arnold for the gift of human cyclin D1 cDNA; and to Lawrence Zukerberg for the gift of the cyclin D1 polyclonal antibody.

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