Loss of Cyclin-dependent Kinase 2 in the Pancreas Links Primary β-Cell Dysfunction to Progressive Depletion of β-Cell Mass and Diabetes*

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The failure of pancreatic islet β-cells is a major contributor to the etiology of type 2 diabetes. β-Cell dysfunction and declining β-cell mass are two mechanisms that contribute to this failure, although it is unclear whether they are molecularly linked. Here, we show that the cell cycle regulator, cyclin-dependent kinase 2 (CDK2), couples primary β-cell dysfunction to the progressive deterioration of β-cell mass in diabetes. Mice with pancreas-specific deletion of Cdki2 are glucose-intolerant, primarily due to defects in glucose-stimulated insulin secretion. Accompanying this loss of secretion are defects in β-cell metabolism and perturbed mitochondrial structure. Persistent insulin secretion defects culminate in progressive deficits in β-cell proliferation, reduced β-cell mass, and diabetes. These outcomes may be mediated directly by the loss of CDK2, which binds to and phosphorylates the transcription factor FOXO1 in a glucose-dependent manner. Further, we identified a requirement for CDK2 in the compensatory increases in β-cell mass that occur in response to age- and diet-induced stress. Thus, CDK2 serves as an important nexus linking primary β-cell dysfunction to progressive β-cell mass deterioration in diabetes.

Diabetes is strongly associated with a failure of pancreatic islet β-cells, although the mechanisms of β-cell failure remain unclear (1, 2). β-Cell dysfunction is considered a likely mechanism whereby β-cells are incapable of compensating for rising plasma sugar levels with a concomitant increase in insulin secretion (3). Further, ample evidence supports the notion that reduced β-cell mass is a contributor to insulin insufficiency (3, 4). Understanding the link between β-cell function and β-cell mass and the compensatory mechanisms that are elicited in response to rising blood sugar will aid in designing the next generation of therapeutic agents to restore β-cell function while preserving β-cell mass in diabetes.

Two common themes emerge from the many genome-wide association studies of type 2 diabetes: (i) there is an enriched set of loci that mainly affect β-cells, and (ii) there seem to be an overabundance of cell cycle genes involved (5–7). The cell cycle pathway integrates inputs from diverse signaling networks to regulate cell growth, proliferation, differentiation, and apoptosis (8, 9). Cyclin-dependent kinases (CDKs) are the gatekeepers of the cell cycle pathway, and their association with cognate regulatory proteins, the cyclins, promotes cell cycle progression in mammalian cells. CDKs phosphorylate downstream substrates, chiefly the retinoblastoma (RB) family proteins, on specific serine/threonine phosphorylation sites, resulting in the release of transcription factors that in turn modulate the expression of target gene promoters. Interestingly, the CDKN2A gene that codes for the CDK inhibitor p16Ink4a is identified in all genome-wide association studies of diabetes (5–7). p16Ink4a expression is increased in aged islets and correlates strongly with age-dependent reduction in β-cell proliferation and regeneration potential (10). p16Ink4a inhibits the activities of multiple CDKs, including CDK4, CDK6, and, indirectly, CDK2 (11–13), and as such, p16Ink4a is able to regulate cell proliferation, differentiation, and senescence via multiple signaling pathways.

We previously showed that CDK4 deficiency causes β-cell hypoplasia and insulin-deficient diabetes, whereas CDK4 activation enhances β-cell mass (14, 15), regeneration potential (16), early pancreas development, and commitment to the endocrine lineage by inducing Ngn3 transcription (17) and sta...
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FIGURE 1. CDK2 expression in pancreatic islets. Immunofluorescence analyses indicates that the majority of CDK2-positive cells (red) are insulin+ β-cells (white). Rare glucagon+ α-cells (green) expressing CDK2 (indicated by arrows) are seen. The merged image shows the relative distribution of CDK2 in the islet.

Similar findings were observed in mice derived from crosses using either of the Pdxd1-Cre strains.

Examination of embryonic day 12.5 (E12.5) pancreas by immunofluorescence using antibodies to the key pancreas transcription factors Pdx1, Nkx2.2, and Nkx6.1 and the epithelial cell-specific marker E-cadherin revealed morphologically normal staining in the Cdk2-pKO embryos, compared withagematched wild-type Cdk2-/-Con embryos (Fig. 3, A–F). Thus, CDK2 loss does not appear to affect early pancreas development. Cdk2-/-pKO mice were born at the predicted Mendelian frequency and appeared normal in size and weight compared with age- and sex-matched control littermates. To better understand the very early effects of CDK2 deletion in the pancreas, we examined control and Cdk2-/-pKO mice at post-natal days 2–10. Body weights, fed glucose levels, pancreas histology, α-cell and β-cell immunostaining patterns, α-cell and β-cell mass, indices of β-cell proliferation, and level of expression of α-cell- and β-cell-specific markers were all found to be similar in Cdk2-/-pKO mice compared with age- and sex-matched control mice (Fig. 4), indicating normal early pancreas development. Glucose tolerance and insulin secretion were also similar at 1 month in mice of both genotypes (data not shown).

We next monitored the effects of CDK2 loss on glycemia in adult Cdk2-/-pKO mice. Fed glucose levels were similar in mice from both genotypes, and no gender-specific differences were detected (Fig. 5, A and B). Fasting glucose levels in Cdk2-/-pKO mice trended toward high normal ranges after 6 months of age, with male fasting glucose levels being significantly elevated at 8 and 12 months of age (Fig. 5, C and D). Insulin and glucagon staining appeared normal in pancreases from 2-month-old Cdk2-/-pKO mice, despite the lack of CDK2 expression (Fig. 6A). Consistent with this, α- and β-cell mass (Fig. 6B) and pancreatic insulin content (Fig. 6C) were also similar in Cdk2-/-pKO and control pancreas at 2 months of age. However, despite maintaining normal β-cell mass and insulin content, 2-month-old Cdk2-/-pKO mice exhibited impaired glucose tolerance (Fig. 7A). Moreover, in vivo insulin secretion was defective during the glucose tolerance test (Fig. 7B). Western blotting analyses confirmed a lack of CDK2 expression in Cdk2-/-pKO islets (Fig. 7C) and glucose-stimulated insulin secretion was severely blunted in these Cdk2-/-pKO islets ex vivo (Fig. 7D). Analyses were performed in Cdk2-/-pKO mice of both genders, and similar observations were obtained with respect to β-cell secretory defects and glucose intolerance (data not shown). We confirmed these findings in Cdk2-/-pKO mice generated using a different strain of Pdx1-Cre transgenic mice (22) (data not shown). Furthermore, no changes in glucagon secretion were seen in 2-month-old Cdk2-/-pKO mice, compared with age- and sex-matched control mice (data not shown).

Consistent with these data, incubating primary cultures of wild-type mouse islets (Fig. 8A) or non-diabetic human islets (Fig. 8, B and C) and insulin-secreting MIN6 β-cells (Fig. 8, D and E) with a CDK2 inhibitor (CDK2i; SU9516) (24) or shRNA knocking down CDK2 expression (Fig. 8F) suppressed glucose-stimulated insulin secretion while preserving insulin content. Taken together, these results suggest that the loss of CDK2 significantly compromised β-cell insulin secretory function.

CDK2 Loss Results in Pancreatic Islet β-Cell Dysfunction—CDK2 is preferentially expressed in the endocrine pancreas with no detectable expression in the exocrine pancreas (Fig. 1). The majority of CDK2+ cells were insulin+ β-cells, and it was rare to observe glucagon+ α-cells expressing CDK2. Germ line whole-body Cdk2 knock-out (Cdk2-/-) mice are viable but infertile due to defects in germ cell development (20, 21). CDK2 is essential for the completion of prophase 1 during germ cell meiosis (21), and CDK2 loss affects the timing of S-phase, consistent with a role in mitosis (20). Although these studies elucidated the role of CDK2 in germ cell development, the importance of CDK2 in glucose homeostasis was not investigated. Here we show that whole-body germ line Cdk2-/- mice exhibit elevated fasting and fed-state hyperglycemia (Fig. 2, A and B). Further, Cdk2-/- mice exhibit severely impaired glucose tolerance (Fig. 2C), and glucose-stimulated insulin levels in plasma were reduced in Cdk2-/- mice (Fig. 2D). Cdk2-/- islets exhibited reduced glucose-stimulated insulin secretion ex vivo (Fig. 2, E and F), indicative of β-cell dysfunction. Similar observations were seen in both male and female Cdk2-/- mice, eliminating any gender-specific effects of CDK2 loss. Together, these results are supportive of an important role for CDK2 in β-cell function.

To specifically examine the role of CDK2 within the pancreas, we generated mice with pancreas-specific CDK2 deletion (Cdk2-/-pKO mice) by breeding Cdk2lox/lox (Cdk2df/df) mice with two different mouse strains expressing CRE recombinase under the control of the Pdx1 promoter, a transcription factor expressed in both the pancreas and the duodenum (22, 23).
Impaired Metabolism in $\beta$-Cells Lacking CDK2—Levels of insulin transcripts were unchanged in Cdk2-pKO mice at postnatal day 2 (Fig. 4G) or at 2 months of age (Fig. 9). To further study the effect of CDK2 deletion, we analyzed RNA transcripts of a set of genes known to participate in $\beta$-cell metabolism and insulin secretion and found that these were significantly reduced by CDK2 deletion. These included glucokinase ($GCK$), uncoupling protein 2, SNAP25, Kir6.2, and Rab27A (Fig. 9), suggesting that both proximal and distal steps in the $\beta$-cell secretory pathway are probably affected by CDK2 deletion. To examine the underpinnings of the insulin secretory defect caused by CDK2 deficiency, we acquired EM images of the pancreatic islets from control and Cdk2-pKO mice (Fig. 10A). Swollen mitochondria appeared clustered near the center of the $\beta$-cells from Cdk2-pKO islets, which we quantified as increased mitochondrial volume (proxied by mitochondrial surface area) in Cdk2-pKO $\beta$-cells relative to controls (Fig. 10B). There was no significant change in mitochondrial number ($21.9 \pm 0.7$ versus $19.9 \pm 0.8$ mitochondria/10 $\mu$m$^2$ in control and Cdk2-pKO $\beta$-cells, respectively; $p = 0.085$). To examine metabolic function, we monitored the ratio of endogenous NAD(P)H to flavin fluorescence in response to glucose (25). As can be seen in Fig. 10C, Cdk2-pKO islets exhibited a reduced baseline, a slower rise time, and a reduced maximal NAD(P)H/flavin ratio in response to a glucose challenge. Thus, defects in mitochondrial morphology and function contribute to the secretory defect in Cdk2-deficient islets, consistent with a central role of the mitochondria in the triggering and amplifying pathways of insulin secretion (26).

CDK2 Phosphorylates FOXO1 in $\beta$-Cells in a Glucose-dependent Manner—$\beta$-Cell function is regulated by insulin receptor signaling, resulting in the phosphorylation of several down-
stream transcription factors (27). Although no changes in phosphorylation of the transcription factor AKT were observed in Cdk2-pKO islets (Fig. 11, A and B), significantly reduced glucose-dependent phosphorylation of the transcription factor FOXO1 was observed in these islets at the serine 256 residue (Fig. 11, C and D). We also observed reduced phosphorylation of FOXO1Ser256 in MIN6 cells following incubation with the CDK2 inhibitor (Fig. 11E). Consistent with the hypothesis that FOXO1 inactivation was linked to β-cell failure, the expression levels of two FOXO1 target genes (28), MafA and NeuroD, were significantly down-regulated in the Cdk2-pKO islets (Fig. 11F).

Co-immunoprecipitation experiments using glucose-stimulated MIN6 cells revealed a physical association between CDK2 and FOXO1Ser256 proteins (Fig. 11G). The CDK2-FOXO1Ser256 interaction was not observed in the absence of glucose, despite a high level of total FOXO1 protein being present, suggesting that the association of CDK2 with phosphorylated FOXO1Ser256 was glucose-dependent. To test whether CDK2 directly transfers a phosphate to FOXO1, we assayed kinase activity by immunoprecipitating endogenous CDK2 and exogenous HA-tagged CDK2. The data obtained suggested that efficient CDK2-mediated phosphorylation of FOXO1 (Fig. 11H) and histone H1 (a known CDK2 substrate) occurred (data not shown), supporting the hypothesis that FOXO1 is a substrate of CDK2 in β-cells. Moreover, a CDK2-dependent, glucose-stimulated phosphorylation of FOXO1Ser256 was also seen in normal human islets, and FOXO1Ser256 phosphorylation was reduced by the CDK2 inhibitor in this case (Fig. 11I). Importantly, compared with islets from non-diabetic human donors, reduced CDK2 protein was observed in human islets from type 2 diabetic donors under normal glucose conditions (Fig. 11J). Lower CDK2 levels were mostly associated with reduced levels of FOXO1Ser256 phosphorylation, despite there being abundant total FOXO1 protein present (Fig. 11J).
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Due to Defects in ß-Cell Proliferation and Reduced ß-Cell Mass—To test whether metabolic stress might unmask additional roles of CDK2 with respect to ß-cell mass and function,

FIGURE 5. Analyses of fed and fasting glycemia. Shown are levels of fed glucose in Cdk2-Con and Cdk2-pKO male (A) and female (B) mice (n = 6/group) and levels of fasting glucose in Cdk2-Con and Cdk2-pKO male (C) and female (D) mice (n = 6/group). *, p < 0.05, Student’s t test. Error bars, S.D.

FIGURE 6. CDK2 loss does not affect young adult pancreas development. A, immunofluorescence of insulin (white), CDK2 (red), and glucagon (green) expression in the pancreas of 2-month-old Cdk2-Con and Cdk2-pKO mice. B, morphometry analysis of α- and ß-cell mass in the pancreas of 2-month-old Cdk2-Con (open bars) and Cdk2-pKO (closed bars) male mice (n = 8 mice/genotype; 2 sections/mouse). C, pancreatic insulin content was measured by ELISA from 2-month-old male Cdk2-WT (Con, open bar, n = 3) and Cdk2-pKO (pKO, closed bar, n = 3) mice. Total pancreatic insulin content was normalized to total pancreatic protein. Error bars, S.D.

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FIGURE 7. ß-Cell dysfunction upon CDK2 deletion. Shown are GTT (A) and serum insulin levels (B) during the GTT in 2-month-old male Cdk2-Con (diamonds) and Cdk2-pKO (squares) mice (n = 7 in each genotype). C, Western blotting analyses to compare CDK2 protein expression in islets from Cdk2-WT (Con) and Cdk2-pKO mice. ß-Tubulin protein levels are shown as loading control. D, glucose-stimulated insulin secretion under low (3.3 mM) and high (25 mM) glucose condition in Cdk2-Con (open bars) and Cdk2-pKO (closed bars) 2-month-old male mice islets (n = 15 islets/pancreas harvested from five mice of each genotype). Insulin secretion is normalized by total cellular insulin content of islets. The data comprises results derived from three independent experiments. Statistical analysis was performed with Student’s t test; *, p < 0.05; **, p < 0.001. Error bars, S.D.
control and Cdk2-pKO mice were challenged with a modest high fat diet (HFD) for 8 weeks, a relatively short time compared with the more usual protocol of 14–16 weeks of HFD feeding. We found that this HFD challenge resulted in elevated fasting glucose and significant glucose intolerance in Cdk2-pKO mice compared with littermate Cdk2-WT control mice (Fig. 12A). Furthermore, we observed mild insulin resistance in the Cdk2-pKO mice compared with controls, albeit this effect was only seen during the initial 30 min of insulin injection (Fig. 12B). In contrast, no defect in insulin sensitivity was observed in Cdk2-pKO mice fed a regular diet (data not shown). In addition, the HFD regimen significantly blunted insulin secretion in Cdk2-pKO mice under both low and high glucose conditions (Fig. 12C). Further, treating non-diabetic human islets with the CDK2 inhibitor, with or without the addition of free fatty acids to the culture medium, resulted in significantly reduced insulin secretion (Fig. 12D). These results, taken together, suggest that the loss of CDK2 increases the susceptibility to HFD-induced glucose intolerance, insulin resistance, and β-cell dysfunction.

Interestingly, we also observed abnormal islet architecture in the HFD-fed Cdk2-pKO mouse pancreas, with many disorganized insulin-positive β-cells (Fig. 12E). Consistent with these changes, there was a significant reduction in β-cell mass under these conditions (Fig. 12C). Further, treating non-diabetic human islets with the CDK2 inhibitor, with or without the addition of free fatty acids to the culture medium, resulted in significantly reduced insulin secretion (Fig. 12D). These results, taken together, suggest that the loss of CDK2 increases the susceptibility to HFD-induced glucose intolerance, insulin resistance, and β-cell dysfunction.

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mass probably contribute to the severe deterioration in glucose tolerance of HFD-fed Cdk2-pKO mice.

**Progressive Age-dependent Decline in β-Cell Proliferation and β-Cell Mass Due to CDK2 Deficiency**—We hypothesized that the primary insulin secretory defect and glucose intolerance, despite normal β-cell mass, at 2 months of age might compromise β-cell compensatory mechanisms, resulting in reduced β-cell mass and diabetes in older Cdk2-pKO mice. Indeed, a progressive worsening of glucose tolerance was observed in 8-month-old Cdk2-pKO mice (Fig. 13A). Interestingly, whereas young Cdk2-pKO mice had normal fasting glucose (Fig. 7A), older adult Cdk2-pKO mice became hyper-
glycemic with elevated fasting glucose levels compared with age-matched Cdk2-Con mice (Fig. 13, B and C; also see Fig. 5, C and D). Moreover, compared with age- and sex-matched control mice, Cdk2-pKO mice displayed significantly reduced β-cell mass with advancing age (Fig. 13, D and E). Examination of Cdk2-pKO islets from 2-, 5-, 8-, and 14-month-old mice fed a normal chow diet revealed no difference in the levels of TUNEL+ and insulin− co-positive cells (data not shown). These data are indicative of no difference in rates of β-cell death in the Cdk2-pKO islets compared with age- and sex-matched controls, thus ruling out apoptosis as a mechanism responsible for the β-cell mass reduction. In contrast, deficits in β-cell proliferation were seen in older Cdk2-pKO mice (Fig. 13, F and G). It is unclear at this time whether this finding reflects (i) the role of CDK2 in β-cell compensation in response to the prevailing insulin secretory defect or (ii) an independent role of CDK2 in β-cell proliferation in older adult islets. In either case, these findings are indicative of an age-associated and progressive

FIGURE 12. CDK2 loss promotes high fat diet-induced diabetes due to defective compensatory β-cell proliferation. A and B, glucose tolerance test (A) and insulin tolerance test (B) (percentage of baseline) in 5-month-old male Cdk2-Con (diamonds) and Cdk2-pKO (squares) mice fed an HFD for 8 weeks (n = 5/genotype). HFD feeding was initiated when mice were 3 months old. C, glucose-stimulated insulin secretion assayed in Cdk2-Con (open bars) and Cdk2-pKO (closed bars) HFD-fed islets cultured in low (3.3 mM) and high (25 mM) glucose. Islets derived from male mice (n = 2/genotype) were used in three independent experiments, all done in triplicates. D, insulin secretion in islets from non-diabetic human donors (n = 2; done in duplicates) challenged by 25 mM glucose after 48-h treatment with DMSO vehicle (open bars) or CDK2i (closed bars), without (−) or with (+) a 24-h incubation with free fatty acids (FFA). Data represent two independent experiments using islets harvested from two non-diabetic human cadaver donors. E, representative image of insulin immunofluorescence (red) in pancreatic islets from HFD-fed Cdk2-Con and Cdk2-pKO mice (n = 3/genotype). F, morphometry of β-cell mass in HFD fed Cdk2-Con (open bars) and Cdk2-pKO (closed bars) mice (n = 3/genotype). G and H, co-immunofluorescence analyses of insulin (red) and BrdU (green) (G) and β-cell proliferation percentage (H) showing the number of BrdU-incorporating β-cells expressing insulin in HFD-fed male Cdk2-Con (open bars) and Cdk2-pKO (closed bars) mice (n = 3 mice/genotype with an average of 125 islets examined per genotype and at least 3000 β-cells counted for determination of BrdU positivity). All data represent the mean ± S.E. (error bars) of independent experiments. *, p < 0.05; **, p < 0.001; Student’s t test.
worsening of the metabolic phenotype in older Cdk2-pKO mice, in conjunction with deterioration of β-cell proliferation and β-cell mass due to loss of CDK2 in the pancreas.

Discussion

Although a combination of β-cell dysfunction and reduced β-cell mass is believed to accelerate type 2 diabetes progression, it is unclear (i) whether the two processes are molecularly linked and (ii) whether a defect in one arm impacts the other in some sequence. Here, we show that the cell cycle kinase, CDK2, links β-cell function and β-cell mass regulation in healthy mice and during diabetes. Loss of CDK2 initially compromises adult β-cell function with defects in insulin secretion due, at least in part, to disturbances in the metabolic response of the β-cell to glucose. Interestingly, the primary β-cell dysfunction and persistent defects in glucose-stimulated insulin secretion unmask a secondary β-cell mass deficiency that is due to reduced β-cell proliferation in the face of overnutrition or with advancing age. CDK2 thus molecularly links a primary defect in β-cell function to a secondary and progressive loss of β-cell mass during diabetes. We propose that one molecular effector of this linkage is FOXO1, which associates with and is phosphorylated by CDK2 in a glucose-dependent manner. Importantly, our findings show that CDK2, like FOXO1, presides over both the regulation of β-cell mass and β-cell function in the context of diabetes.

It is striking that the CDK2-mediated effects on β-cell function that we observed appeared to be independent of changes in β-cell mass, at least initially. Instead, CDK2 loss seems to reduce insulin secretion in addition to grossly impairing normal glucose metabolism. Because the redox measurements performed in our study largely reflect mitochondrial processes (25), we cannot state whether this defect reflects glycolytic or mitochondrial dysfunction; however, we did observe corresponding defects in mitochondrial structure. There is some evidence that the control of β-cell mass and β-cell function, specifically insulin secretion, are linked to key transcription factors, such as FOXO1, that can serve as signaling nodes (29). We find that CDK2 binds to and phosphorylates FOXO1 at the Ser256 residue in a glucose-dependent manner. This observation is intriguing, keeping in mind the established role of FOXO1 in metabolic homeostasis, specifically as it pertains to the β-cell (30). FOXO1 regulates β-cell proliferation and prevents β-cell failure by inducing the key β-cell transcription factors NeuroD and MafA (28), and we observe reduced expression of both factors in Cdk2-pKO islets. It is unclear at this time whether phosphorylation by CDK2 is associated with changes in the subcellular location of FOXO1. Glucose-induced phosphorylation of FOXO1-Ser256 is known to require PI3K signaling (31). However, we observed equivalent levels of phospho-AKT in Cdk2-pKO and control islets, suggesting that perhaps CDK2 plays a role downstream of PI3K. It has been shown that CDK2 targets FOXO1 at Ser249 as part of an apoptotic response to DNA damage (32), although we did not observe (i) FOXO1Ser249 phosphorylation or (ii) changes in TUNEL + apoptosis rates in Cdk2-pKO islets or under conditions of CDK2 inhibition (data not shown).

CDK2 inhibition precluded glucose-dependent FOXO1Ser256 phosphorylation in non-diabetic human islets, and reduced CDK2 levels in type 2 diabetic islets were associated with
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Reduced FOXO1Ser256. These results are consistent with the findings of Kulkarni and co-workers (33) that human type 2 diabetic islets have severely reduced CDK2 transcript levels, and islets from mice lacking β-cell insulin receptors also lack CDK2 protein. Further, the analysis of gene expression in laser capture microdissected β-cell-enriched tissue samples from type 2 diabetic cadaver pancreas shows a reduction in CDK2 and FOXO1 transcripts (34). Ablation of FOXO1 in the β-cell promotes β-cell dedifferentiation and consequent β-cell failure, resulting in hyperglycemia and reduced β-cell mass (35). Thus, we propose that CDK2, like FOXO1, regulates both β-cell function and β-cell mass in the context of diabetes pathogenesis. Whether, as with FOXO1, the loss of CDK2 signaling contributes to the β-cell dedifferentiation phenotype in T2D remains to be determined.

Interestingly, we observed reduced β-cell mass in the Cdk2-pKO mice under two conditions: in response to a high fat diet challenge and at advancing age. Under both conditions, we observed further deterioration of glucose tolerance, leading to diabetes. Mice with normal CDK2 are able to evoke compensatory β-cell proliferation in response to metabolic stress and can preserve their β-cell mass, whereas Cdk2-pKO mice fail to do so, resulting in reduced β-cell mass. This is consistent with a study by Rahier et al. (36), who observed a significant reduction in β-cell mass in type 2 diabetes subjects that was correlated to the duration of diabetes. Moreover, it is striking that the decline in β-cell mass that we observed in the metabolically stressed or aged Cdk2-pKO mice happened despite there being normal levels of CDK4, a cell cycle kinase that has a prominent role in β-cell mass regulation (14). Based on this, we infer that β-cell mass compensation in the face of β-cell dysfunction, HFD-induced metabolic stress, and advancing age might be under the purview of CDK2.

We used Pdx1-Cre transgenic mice to target CDK2 in this study. The Pdx1 gene is expressed in prepancreatic endoderm starting at E8.5 in mice. Later in development and in adult pancreas, PDX1 expression becomes more abundant in β cells, with lower level expression detected in acinar cells and other endocrine cells. However, besides marking pancreatic cells, Pdx1 driver alleles cause recombination in the duodenum, stomach, bile duct, inner ear, and regions of the hypothalamus (37–40). We have not investigated whether Pdx1-Cre causes deletion of CDK2 in other cells and as such have not investigated effects of CDK2 loss in other organs. Specifically, we cannot rule out the effects of a possible loss of CDK2 in nutrient-sensing neurons of the hypothalamus contributing to the phenotype described in this study.

The effects of CDK2 loss that we observed in the Cdk2-pKO pancreas were first detectable around 2 months of age. It is possible that related CDKs or other kinases that can regulate insulin secretion successfully compensate for the loss of CDK2 in islets before about 2 months of age. We have previously demonstrated that CDK4 plays an important role in the development, proliferation, and regeneration potential of β-cells, for example (14, 16–18). Further experiments will reveal whether CDK4 or other kinases involved in glucose-stimulated insulin secretion indeed can compensate for the loss of CDK2 in young islets but become progressively unable to do so in older and metabolically challenged islets. It is plausible that the reduced β-cell mass and defective β-cell proliferation that we observed in the older Cdk2-pKO mice reflect the role of CDK2 as an age-dependent regulatory process, wherein the role of CDK2, like that of p16Ink4a, is more prominent in the expansion of the adult β-cell compartment. Expression of p16Ink4a increases in the β-cells of older animals and is associated with β-cell senescence (10) and improved β-cell function (41). Further, a loss of CDK2 in MEFs is similarly associated with cellular senescence (42). It is possible that an age-dependent decline in CDK2 expression and activity contributes to the diabetes pathophysiology typically seen in aging. In that regard, the loss of CDK2, similar to an increase in p16Ink4a expression, would help force β-cells out of the cell cycle and prevent compensatory increases in β-cell proliferation, thereby further exacerbating glucose intolerance and the subsequent development of frank diabetes. If so, CDK2 agonists may prove useful to restore β-cell function and reinitiate β-cell proliferation in the face of metabolic stresses to counter β-cell failure in diabetes.

Experimental Procedures

Mice—Global Cdk2-KO mice were described previously (20). Cdk2<sup>+/−</sup> mice (43) were crossed with two independent strains of Pdx1-Cre mice (22, 23) to generate two lines of pancreas-specific Cdk2-knock-out mice (Cdk2-pKO mice). The Pdx1-Cre line, B6.FVB-Tg(Jdpf1-cre)1Tuv/Nci (23), was obtained from the NCI-Mouse Models repository. The Pdx1-Cre strain, STOCK Tg(Pdx1-cre)89.1Dam/Mmucd (22), was obtained from the Mutant Mouse Regional Resource Centers. CDK2 expression and CRE-mediated recombination were confirmed by Western blotting and immunohistochemistry. For the embryonic study, timed mating regimens were performed with E0.5 as the day of discovery of a vaginal plug.

For β-cell proliferation, experiments were performed on age-matched male mice. BrdU was injected (50 mg/kg body weight) intraperitoneally for 6 h in most experiments. Considering that turnover of β-cells is very slow in old mice (44), we used a modified protocol in older mice to study β-cell proliferation rates. Thus, 14-month-old mice were treated with BrdU (2 mg/ml)-containing drinking water for 4 weeks, after which the BrdU-labeled insulin<sup>+</sup> cells were counted, and the data are presented as the percentage of BrdU<sup>+</sup>/insulin<sup>+</sup> cells. For high fat feeding studies, Cdk2 wild type (Cdk2-Con) and Cdk2-pKO male mice were fed either regular diet or a high fat diet (fat content by calories 60%; Research Diets Inc., New Brunswick, NJ) for 8 weeks. Body weights were recorded weekly, and glucose and insulin tolerance tests were performed during the last week of the feeding period. Animal protocols were approved by the National Institutes of Health Animal Care and Use Committee.

Islet Isolation and Cell Culture—Islets were isolated after pancreases were digested by liberase R1 (0.2 mg/ml; Roche Applied Science) and purified by Histopaque gradient, followed by culture in RPMI with 10% FBS before experiments. MIN6 cells were cultured in DMEM plus 10% FBS. Cells or islets were treated with either DMSO or Cdk2 inhibitor (SU9516 (Calbiochem) at a final concentration of 5 μM) or with Cdk2-shRNA lentiviruses, followed by culture in medium without glucose or
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TABLE 1
Human pancreatic islet donor information
Islets were provided by the Integrated Islet Distribution Program, City of Hope. NA, information not available from the Integrated Islet Distribution Program.

| Parameter                  | Non-diabetic subjects | Type 2 diabetic subjects |
|----------------------------|------------------------|--------------------------|
|                           | YKJ446                 | YIY429                   | 1064                     | YL0444                 | ZAX388                   |
| Source for islets          | University of Pennsylvania | University of Miami      | University of Illinois   | University of Pennsylvania | University of Miami       |
| Age (years)                | 49                     | 52                       | 48                      | 57                     | 55                       |
| Gender                     | Female                 | Male                     | Male                    | Female                 | NA                       |
| Height (cm)                | 167.4                  | 170.2                    | 182.90                  | 165.1                  | NA                       |
| Weight (kg)                | 87.7                   | 97.5                     | 104.4                   | 60                     | NA                       |
| Body mass index (kg/m²)    | 31.3                   | 33.7                     | 31.2                    | 22                     | 28                       |
| Ethnicity/race             | White                  | White                    | White                   | White                  | NA                       |
| Cause of death             | Cerebrovascular/stroke | Cerebrovascular/stroke   | Cerebrovascular/stroke  | NA                     | NA                       |

with 16.7 or 25 mM glucose for determination of insulin secretion. Information on the Cdk2-shRNA sequence was published elsewhere (45).

Human Islet Studies—Islets from previously non-diabetic or type 2 diabetic human cadaver donor pancreases were obtained via the NIDDK-sponsored Integrated Islet Distribution Program (Duarte, CA). Details regarding the human islet batches from donors used in this study are provided in Table 1. Within 24 h of isolation, islets were transferred to fresh islet culture medium. Islets were used for stimulation with CDK2 inhibitor with or without the addition of free fatty acid (FFA).

FFA Preparation, Cell Treatment—FFA/BSA solutions were prepared as described previously (46). For experiments, 0.5 mM FFA (P9767, Sigma) complexed with 1% BSA was used. Nondiabetic human islets were treated with DMSO or CDK2 inhibitor for 48 h, followed by incubation with FFA for an additional 24 h and assayed for glucose-stimulated insulin secretion by ELISA.

Immunostaining—Mouse tissues were harvested, paraffin-sectioned, and immunostained overnight at 4 °C with the following primary antibodies: rabbit anti-PDX1 (Millipore), mouse anti-NKX2.2 (Developmental Studies Hybridoma Bank, University of Iowa), mouse anti-NKX6.1 (Developmental Studies Hybridoma Bank), mouse anti-E-cadherin (BD Biosciences), rabbit anti-CDK2 (Santa Cruz Biotechnology, Inc.), guinea pig anti-insulin (Dako), rabbit anti-glucagon (Dako), and mouse anti-BrDu (Dako). For estimation of β-cell proliferation, mice were injected with BrdU (50 mg/kg of body weight; Sigma-Aldrich) 6 h before being euthanized, and pancreases were harvested and fixed in 4% paraformaldehyde. The relative β- and α-cell area were obtained by immunohistochemistry for insulin and glucagon. Slides were imaged on an LSM 510 confocal microscope (Zeiss) and scanned by ScanScope (Aperio, CA). To estimate the total β-cell mass, the weight of the pancreas was determined, and the percentage of β- or α-cell area with regard to total pancreas area was determined. The absolute mass of the pancreatic β- and α-cells was estimated from the equation, β- or α-cell area/total area = β- or α-cell mass/total pancreas weight (g). Otherwise, we presented the β-cell mass percentage as percentage of β-cell area/total pancreas area. For BrdU incorporation analyses, a minimum of n = 3 age- and sex-matched mice per genotype were used. Averages of 125 islets were examined per genotype with at least 3000 β-cells counted for BrdU positivity to derive the results presented (β-cell proliferation percentage).

Live Cell Imaging of Islet Metabolism—NAD(P)H and flavin fluorescence were excited at 365 and 430 nm, respectively (25), using a TILL Polychrome V monochrometer adjusted for a 15-nm bandwidth (full width at half-maximum) and 10% light output and a ×10 0.3 numeric aperture objective mounted in an Olympus IX-71 inverted microscope. The filter cube contained a 465HSP excitation filter (Omega), 458LP dichroic mirror (Semrock), and D500/100m emission filter (Chroma). Images were collected by a QuantEM512 EM-CCD camera every 5 s under the control of MetaFluor software (Molecular Devices).

Electron Microscopy—we used Cdk2-WT and Cdk2-pKO mice (2-month-old males, n = 3 for each genotype) to harvest pancreatic islets for electron microscopy (EM). Islets preincubated in 3.3 mM glucose were fixed in 2.5% glutaraldehyde, 0.1 M NaH₂PO₄/Na₂HPO₄ phosphate buffer (pH 7.2) and washed with PBS. These islets were post-fixed with 1% OsO₄ and embedded in Epon812. Ultrathin sections were mounted and stained with uranyl acetate/lead citrate (JEM-1010, Jeol). Ten islet EM sections from each mouse were scanned, and multiple images covering the entire section were acquired for analyses. The sections were randomly scanned at 15–20 different spots per treated group at ×3000. Granule density and mitochondrial area were quantified from the EM images using the Integrated Morphometry Analysis toolkit in MetaMorph (Molecular Devices).

Glucose Tolerance Test and Insulin Tolerance Test—An intraperitoneal glucose tolerance test was carried out after overnight fasting by intraperitoneal injection of 2 g/kg glucose. Blood glucose levels were determined with a glucometer (Buyer) before (0 min) and 10, 15, 30, 60, and 120 min after glucose injection. Serum or plasma insulin and glucagon levels during the intraperitoneal glucose tolerance test were measured with mouse ultrasinulin and glucagon ELISA kits (Alpco). Insulin tolerance tests were performed on regular diet- and HFD-fed mice by intraperitoneal injection of insulin (0.75 units/kg) followed by measurement of blood glucose level at the indicated times.

Glucose-stimulated Insulin Secretion and Insulin Content Measurement—After isolation, 15 similar sized islets from each group were hand-picked under a stereoscope and used for glucose-stimulated insulin secretion. The islets were cultured in Krebs-Ringer bicarbonate buffer (KRBB) without glucose for 30 min and stimulated with KRBB containing 2 or 3.3 mM (low) or 16.7 or 25 mM (high) glucose for 30 min. After glucose stimulation, the buffer was collected to assay levels of glucose-stimu-
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lated insulin release by insulin ELISA. MIN6 cells and islets harvested at the end of each experiment were then sonicated in 70% acid ethanol, and total cellular insulin content was measured by ELISA (Alpco). Insulin secretion values are presented after normalizing to the total cellular insulin content. The y axis represents secreted insulin divided by cellular insulin content in the indicated number of islets. Pancreatic insulin content was measured using total pancreas obtained from 2-month-old male mice (n = 3 of each genotype). The whole pancreas was isolated, homogenized in acid alcohol, and extracted overnight at 4 °C. The solution was centrifuged to remove unhomogenized tissue and neutralized. Insulin content was determined by ELISA. Total pancreatic insulin content was normalized by total pancreatic protein.

Western Blotting and Immunoprecipitation—Protein extracts were prepared from freshly isolated islets, MIN6 cells, and non-diabetic and type II human diabetic islets in lysis buffer. MIN6 cell lysates were subjected to immunoprecipitation with rabbit IgG or rabbit anti-CDK2, followed by immunoblotting with pFOXO1 (Ser256). Antibodies used were pAKT, AKT, pFOXO1 (Ser256), and FOXO1 (Cell Signaling); CDK2 (Santa Cruz Biotechnology); and α-tubulin (Sigma).

In Vitro Kinase Assay—Immunoprecipitations were performed using rabbit IgG-, rabbit anti-CDK2-, or monoclonal anti-HA-labeled cell lysates from MIN6 cells and CDK2-overexpressing MIN6 cells after high glucose challenge. Complexes were adsorbed onto protein G-agarose beads and washed three times in lysis buffer and once in kinase buffer. Beads were resuspended in 20 μl of kinase buffer containing 1 μCi of [γ-32P]ATP. Commercially purified histone H1 (Roche Applied Science) and FOXO1 (Millipore) were incubated with IgG and CDK2 immunoprecipitated from whole-cell extracts equivalent to total protein. Reactions were incubated at room temperature for 30 min, and the kinase reaction was stopped by the addition of 2X sample buffer. Samples were analyzed by SDS-PAGE, followed by autoradiography.

Real-time RT-PCR—Total RNA was prepared from embryonic (E12.5) or post-natal (P2–P10) pancreas or from islets harvested from Cdk2- Con and Cdk2-pKO mice using the RNAqueous®-Micro total RNA isolation kit (Ambion) or the RNeasy minikit (Qiagen). PCRs were performed and analyzed using the Applied Biosystems Fast 7500 real-time PCR system and SDS software. Relative changes were calculated by the comparative ΔΔCt method, in which 18S rRNA was used for normalization. Reactions were performed in triplicate, and relative amounts of cDNA were normalized to 18S rRNA.

Statistical Analysis—All values were expressed as means ± S.E. or mean ± S.D. Student’s t test was employed for two-group and multiple comparisons, respectively. p values of <0.05 were considered to represent statistically significant differences.

Author Contributions—S. Y. K. conducted most of the experiments and analyzed the results. J.-H. L. conducted experiments on β-cell mass estimation and other functional studies, and M. J. M. and L. S. S. performed β-cell metabolism experiments and drafted their results. P. K. and X. B. provided the Cdk2KO and Cdk2Flox mice and helped with the shRNA studies. O. G. assisted on mouse phenotyping. S. G. R. conceived the idea for the project and wrote the paper.

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