β cell-specific deficiency of the stimulatory G protein α-subunit $G_s\alpha$ leads to reduced β cell mass and insulin-deficient diabetes

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The G protein α-subunit $G_s\alpha$ is required for hormone-stimulated cAMP generation. In pancreatic β cells, $G_s\alpha$ mediates the signaling of glucagon-like peptide 1 and other incretin hormones, which are implicated as important regulators of β cell survival and insulin release. Studies have suggested that $G_s\alpha$/cAMP mediates these actions by stimulating insulin receptor substrate 2 (IRS2) expression. Mice with β cell-specific $G_s\alpha$ deficiency (βGsKO) were generated by mating $G_s\alpha$-floxed mice to rat insulin II promoter-cre recombinase mice. βGsKO mice had poor survival and postnatal growth with low serum insulin-like growth factor 1 levels. βGsKO mice also developed severe hyperglycemia and glucose intolerance with severe hypoinsulinemia and reduced islet insulin content and glucose-stimulated insulin release. βGsKO mice had markedly reduced average islet size and β cell mass, which was partially explained by reduced β cell size. In addition, βGsKO mice had significantly reduced β cell proliferation and increased β cell apoptosis and markedly reduced expression of the cell cycle protein cyclin D2. The effects on β cell mass and proliferation, but not apoptosis, were present from birth. Unexpectedly expression of *Irs2* and the downstream gene *Pdx1* were unaffected. These results show that $G_s\alpha$/cAMP pathways are critical regulators of β cell function and proliferation that can work through IRS2-independent mechanisms.

Both type 1 and type 2 diabetes are associated with β cell dysfunction, with reduced glucose-stimulated insulin secretion and reduced β cell mass because of reduced β cell proliferation and survival (1). β cell proliferation is important for maintaining adequate β cell mass under normal and stress conditions, and β cell mass appears to depend on proteins involved in cell cycle regulation (2). In particular, both cyclin D2 and cyclin-dependent kinase 4 (cdk4), which are expressed at high levels in β cells and are critical for G_1/S phase transition, are required to maintain normal β cell mass (3–7).

Several signaling pathways have been implicated in the regulation of insulin production and secretion and β cell proliferation and survival. One important signaling component is the heterotrimeric G protein α-subunit $G_s\alpha$ that mediates receptor-stimulated intracellular cAMP production (8). In β cells, $G_s\alpha$ mediates the effects of the gut-derived incretin hormones glucagon-like peptide 1 (GLP1) and glucose-dependent insulinotropic polypeptide to increase glucose-stimulated insulin secretion, β cell proliferation, and survival (9, 10). Because of these beneficial effects, the GLP1 receptor agonist exendin 4 has recently been approved as a therapeutic agent for diabetes. One study suggested that the proliferative and antiapoptotic effects of cAMP in β cells were mediated via induction of the insulin receptor substrate 2 gene (IRS2) by cAMP-response element binding protein (CREB) (11). However, recent studies suggest that GLP1-stimulated proliferation may be due to transactivation of EGF receptors by its receptor (12, 13), and therefore it is independent of $G_s\alpha$ signaling.

In this study, we examined the role of $G_s\alpha$ in β cell function *in vivo* by generating mice with β cell-specific $G_s\alpha$ deficiency (βGsKO). βGsKO mice developed insulin-deficient diabetes with reduced glucose-stimulated insulin secretion and β cell mass with a primary defect in β cell proliferation from birth. However, we found no decrease in expression of either IRS2 or a downstream gene induced by IRS2, suggesting that $G_s\alpha$ is an important primary regulator of β cell proliferation that works through IRS2-independent pathways.

Results

Generation and Characterization of βGsKO Mice. βGsKO ($E1^{fl/fl}$:RIP2-cre^+) mice were generated by mating $E1^{fl/fl}$ females with $E1^{fl/+}$:RIP2-$cre^{+/-}$ males, and their phenotypes were compared with cre^- control and $E1^{fl/+}$:cre^+ littermates. Coimmunostaining with insulin and $G_s\alpha$ antibodies showed robust $G_s\alpha$ expression in control islets with significant costaining of $G_s\alpha$ and insulin (Figs. 1*A* and 3*I*). In contrast, $G_s\alpha$ was virtually absent in islets from βGsKO mice with very few cells costaining for both insulin and $G_s\alpha$, consistent with loss of $G_s\alpha$ expression from the vast majority of β cells. $G_s\alpha$ mRNA expression was reduced by ≈40% in our islet preparations (see Fig. 3*G*). Several factors contribute to this apparent partial reduction of $G_s\alpha$ mRNA. First, RIP2-cre has been reported to be expressed in only ≈80–90% of β cells (14, 15). Moreover, it has been estimated that β cells account for only 75% of islet endocrine cells (16) and a lower percentage of total islet cells. Finally, islet preparations also contain other nonislet cell contaminants. Immunoblots showed $G_s\alpha$ protein expression to be unaffected in brown adipose tissue, liver, and hypothalamus but to be reduced by ≈20% in pituitaries from βGsKO mice (data not shown), consistent with RIP2-cre expression patterns reported in refs. 14 and 17.

Both male and female βGsKO mice had a very high rate of early postnatal lethality, with 49% of expected survival (99/680 total offspring) at weaning (3.5 weeks) and 30% of expected survival by adulthood. Hyperglycemia was noted in many mice as early as the first week postpartum, but some mice were hypoglycemic when measured in the preterminal state. $E1^{fl/+}$:cre^+ mice had 90% expected survival at weaning. Analysis of mice at postnatal day (P)1 and P7 confirmed that a β cell defect is present in βGsKO mice from birth (see below).

βGsKO Mice Have a Postnatal Growth Defect. Both male and female βGsKO mice had significantly reduced growth after 2–3 weeks, whereas $E1^{fl/+}$:cre^+ mice grew normally (Fig. 1 *B* and *C*). The poor growth of βGsKO mice was associated with reduced body length and only a slight decrease in body mass index (g/cm^2) (Fig. 1 *C* and *D*) with no change in the relative proportions of fat and

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The authors declare no conflict of interest.

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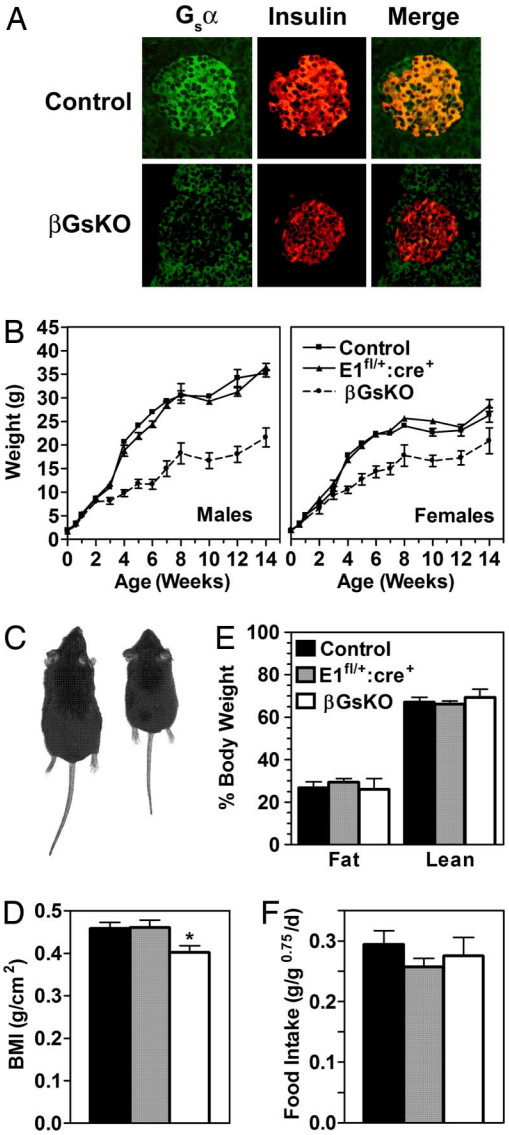

Fig. 1. Growth, body composition, and food intake in βGsKO mice and controls. (*A*) Immunofluorescence staining of islets from control (*Upper*) and βGsKO (*Lower*) mice, using antibodies for $G_s\alpha$ (green), insulin (red), or both (merge). (*B*) Growth curves of male (*Left*) and female (*Right*) control (■), $E1^{fl/+}$:cre^+ (▲), and βGsKO (●; dashed line) mice. (*C*) Photograph of 10-week-old control (*Left*) and βGsKO (*Right*) mice. (*D*) Body mass index (weight in grams/nasoanal length in cm^2) of male control (solid bar), $E1^{fl/+}$:cre^+ (gray bar), and βGsKO (open bar) mice ($n = 6–8$ per group; *, $P < 0.05$ vs. control). (*E*) Fat and lean mass in same groups of mice expressed as the percentage of body weight. (*F*) Food intake per day normalized to body weight ($n = 6$ per group).

lean mass (Fig. 1*E*). Although βGsKO mice had lower food intake in absolute amounts, there was no difference in food intake when normalized to body mass (Fig. 1*F*).

To determine whether pituitary hypofunction contributed to the growth defect, we measured serum hormone levels in adult mice (Table 1). There were no significant differences in serum TSH or corticosterone levels between the three groups, although corticosterone levels tended to be higher in βGsKO mice, possibly as a consequence of stress. Serum insulin-like growth factor 1 (IGF1) levels were significantly decreased in βGsKO mice compared with control and $E1^{fl/+}$:cre^+ mice, and this may be an important contributing factor to the poor linear growth in these mice. Insulin has been shown to regulate the expression of

Table 1. Serum chemistries in 3- to 4-month-old male mice

	Mice		
Serum	Controls	$E1^{fl/+}$:cre^+	βGsKO
Fasting glucose, mg/dl	88 ± 9	95 ± 8	206 ± 40*
Random glucose, mg/dl	241 ± 12	206 ± 26	291 ± 22*
Random insulin, ng/ml	3.94 ±0.71	5.71 ±2.10	0.36 ±0.20*
Cholesterol, mg/dl	149 ± 6	143 ± 11	117 ± 3*
Triglycerides, mg/dl	176 ± 20	163 ± 14	217 ± 26*
Creatinine, mg/dl	0.39 ±0.02	0.38 ±0.03	0.32 ±0.02*
Urea nitrogen, mg/dl	17 ± 1	21 ± 3	32 ± 2*
Glucagon, pg/ml	99 ± 6	89 ± 4	71 ± 6*
GLP1, pM	5.09 ±0.14	5.53 ±0.14	5.72 ± 0.28
IGF1, ng/ml	3.00 ±0.01	2.91 ±0.08	1.19 ±0.30*
TSH, ng/ml	2.44 ±0.13	3.32 ±0.59	3.18 ± 0.30
Corticosterone, ng/ml	83 ± 27	89 ± 37	152 ± 48

$n = 4–25$; *, $P < 0.05$ vs. controls.

both IGF1 and its binding proteins, and poor growth in insulin-deficient diabetics has been ascribed to low IGF1 levels (18–23). Therefore, the poor growth and low IGF1 levels observed in βGsKO mice may be secondary to hypoinsulinemia, although reduced growth hormone secretion from the pituitary is another potential contributing factor.

***βGsKO* Mice Develop Insulin-Deficient Diabetes.** Adult (3- to 4-month-old) βGsKO had very high fasting and random serum glucose levels and very low serum insulin levels compared with $E1^{fl/+}$:cre^+ and control littermates (Table 1). The severe hyperglycemia appeared to lead to dehydration, because urea nitrogen and the urea nitrogen/creatinine ratio were also significantly increased. In addition, βGsKO mice had lower cholesterol and higher triglyceride levels than $E1^{fl/+}$:cre^+ and control littermates (Table 1). βGsKO had mildly reduced glucagon levels, whereas GLP-1 levels were unaffected. βGsKO also had markedly impaired glucose tolerance (Fig. 2*A*). Glucose tolerance was also slightly impaired in $E1^{fl/+}$:cre^+ mice, but $E1^{fl/+}$:cre^+ and $E1^{+/+}$:cre^+ mice had similar impairments in glucose tolerance (data not shown), indicating that the glucose intolerance of $E1^{fl/+}$:cre^+ mice was due to Cre expression in β cells, as previously observed (24), rather than $G_s\alpha$ deficiency.

Glucose intolerance in βGsKO mice was not associated with insulin resistance, and in fact these mice had markedly increased *in vivo* insulin sensitivity based on their markedly greater acute hypoglycemic response to insulin (Fig. 2*B*). Increased insulin sensitivity was likely secondary to reduced down-regulation of insulin receptor pathways because of chronically low circulating insulin levels. Rather, glucose intolerance in βGsKO mice was associated with virtual absence of insulin release (both first- and second-phase) in response to a high dose of glucose even in the presence of higher glucose levels (Fig. 2 *C* and *D*). Glucose tolerance and insulin release were less severely impaired in $E1^{fl/+}$:cre^+ mice in the first 30 min after glucose injection, but, by 120 min, glucose and insulin levels were similar in $E1^{fl/+}$:cre^+ and control mice. Reduced insulin release is at least partially explained by islet insulin content being only ≈35% of normal in βGsKO mice when normalized to islet protein (Fig. 2*E*). Reduced insulin protein content in islets was confirmed by immunohistochemistry (Figs. 1*A* and 3*A*). When correcting for reduced islet mass (see below), total insulin capacity of βGsKO mice was only ≈25% of controls. Insulin (*Ins2*) mRNA expression as determined by quantitative RT-PCR was also significantly reduced in islets (Fig. 2*E*).

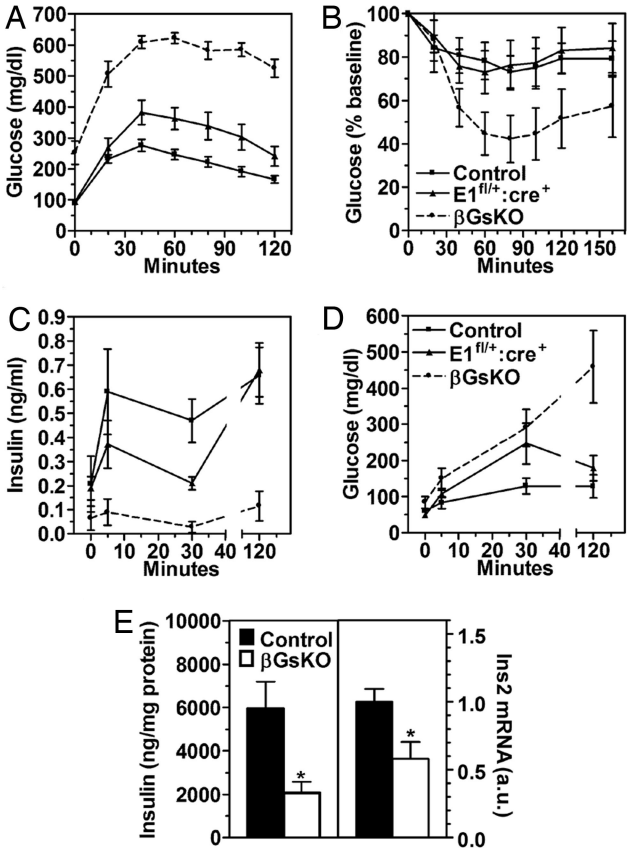

Fig. 2. Glucose tolerance, insulin sensitivity, and glucose-stimulated insulin secretion in βGsKO mice and controls. (*A* and *B*) Glucose tolerance tests (*A*) (n = 5–9 per group) and insulin tolerance tests (*B*) (n = 6–7 per group; results expressed as the percentage of baseline) performed on overnight fasted control (■), $E1^{fl/+}$:cre^+ (▲), and βGsKO (●; dashed line) mice. (*C* and *D*) Glucose (3 mg/g) was administered i.p. to overnight fasted mice, and plasma insulin (*C*) and glucose (*D*) were measured at indicated time points (n = 5–8 per group). (*E*) Islet insulin content normalized to total protein and insulin (*Ins2*) mRNA levels as determined by quantitative real-time RT-PCR in control (solid bar) and βGsKO mice (open bar) mice (n = 4–7 per group; *, $P < 0.05$ vs. control).

βGsKO Mice Have Reduced Islet Cell Mass. Islets from βGsKO mice showed a normal cellular arrangement, with the majority of cells being β cells that were surrounded by glucagon-staining α cells in the periphery (Fig. 3*A*). However, the islets were significantly smaller, with a mean area that was <50% of those in controls (Fig. 3*B*). Total islet mass was reduced by 32% relative to total pancreatic mass (Fig. 3*C*) and was similarly reduced relative to total body mass (data not shown). Although there was a small, but significant, reduction in mean islet cell size (Fig. 3*D*), the major factor accounting for reduced islet size was reduced cell number. Islet cell proliferation, as measured by BrdU labeling, was reduced by 65% relative to controls (Fig. 3*E*). Conversely, the rate of β cell apoptosis, measured as the frequency of $TUNEL^+$ insulin-staining cells, was 2-fold higher in βGsKO mice (Fig. 3*F*). Therefore, adult βGsKO mice have reduced islet cell mass associated with reduced islet cell proliferation and increased apoptosis.

βGsKO Mice Have Normal IRS1 and PDX1 Expression but Reduced Cyclin D2 Expression in β Cells. To examine molecular mechanisms underlying the reduced islet cell mass, we examined the expression of genes implicated in cell cycle progression and β cell proliferation by real-time RT-PCR (Fig. 3*G*). Although studies have suggested that incretin hormones and cAMP increase β cell proliferation and survival by induction of the IRS2 gene (*Irs2*) (11), we found no change in expression of the *Irs2* or *Irs1* genes in islets from βGsKO mice. We also found no change in expression of *Akt2* or pancreatic and duodenal homeobox 1 (*Pdx1*), two other genes that are stimulated by IRS2 and implicated in β cell growth (25–27). Consistent with these mRNA results, immunohistochemistry showed no reduction of IRS2 or PDX1 protein in islets (Fig. 3*H*) even though $G_s\alpha$ expression was virtually absent (Figs. 1*A* and 3*I*).

Recent studies show that the cyclin D/cdk4 complex is an important regulator of postnatal β cell proliferation and that cyclin D2 plays a dominant role among the cyclins in β cell growth regulation (3, 4, 7). We found no change in the levels of either cyclin D1 (*Ccnd1*) or *Cdk4* mRNA in βGsKO islets, but we found an increase in *Cdk2* expression (Fig. 3*G*). However, cyclin D2 (*Ccnd2*) mRNA expression was significantly reduced in βGsKO islets. Although reduced by only 40%, it is important to note that this reduction is similar to that observed for $G_s\alpha$ expression in the same samples. Immunohistochemistry showed the majority of cells costaining for both $G_s\alpha$ and cyclin D2 in control islets, whereas there was virtually no staining for either $G_s\alpha$ or cyclin D2 in βGsKO islets (Fig. 3*I*).

βGsKO Mice Have a β Cell Defect from Birth. To determine the time of onset of the β cell defect and to confirm that the β cell defect is not secondary to metabolic abnormalities, such as hyperglycemia, we examined β cell function on the day of birth (P1) and at 7 days postpartum (P7). On P1, βGsKO (but not $E1^{fl/+}$:cre^+) mice showed evidence for β cell dysfunction, with reduced serum insulin levels (Fig. 4*A*) and β cell mass as a percentage of pancreas area (Fig. 4*B*), even though there were no differences in serum glucose levels among the three groups at this age (Fig. 4*A*). Although greatly reduced, the differences for serum insulin were not statistically significant because of the low numbers of mice that we could examine (n = 3). By P7, those βGsKO mice that survived began to develop hyperglycemia (Fig. 3*A*). At this age, the insulin levels remained lower in βGsKO mice and analysis of a small number of mice showed persistence of a ≈40% reduction of β cell mass (data not shown). At P1, when mice were still normoglycemic, β cell proliferation as determined by Ki67 staining was markedly reduced in βGsKO compared with control and $E1^{fl/+}$:cre^+ mice, and this difference in β cell proliferation in βGsKO mice was still present at P7 (Fig. 4*C*). Apoptosis rates as assessed by TUNEL assay were very low at P1 and P7, and we found no differences in apoptosis among the three groups at P1 or P7 as assessed by staining for cleaved caspase 3 (data not shown). At P1, both islet (Fig. 4*D*) and overall pancreatic architecture were similar in βGsKO and control mice, although βGsKO islets were smaller and had fewer β cells. Overall, these results show that βGsKO mice have low β cell mass and a severe β cell proliferative defect from birth before the development of hyperglycemia. These changes were not secondary to maternal effects, because the mothers were all cre^- and therefore had normal $G_s\alpha$ expression and normal phenotype.

Discussion

Our findings confirm in an *in vivo* model that $G_s\alpha$ pathways are required for normal insulin production, glucose-stimulated insulin secretion, and β cell proliferation. βGsKO mice had reduced serum insulin and β cell mass starting from birth even before the onset of hyperglycemia. These findings show that the defect in β cell proliferation and in maintenance of normal β cell mass in these mice occurs very early (most likely in the prenatal period) and is not secondary to metabolic abnormalities, such as hyperglycemia. The early defect in β cell function most likely explains why many βGsKO mice developed hyperglycemia and early lethality. We observed increased β cell apoptosis in βGsKO mice at 3–4 months of age but not in the early postnatal period.

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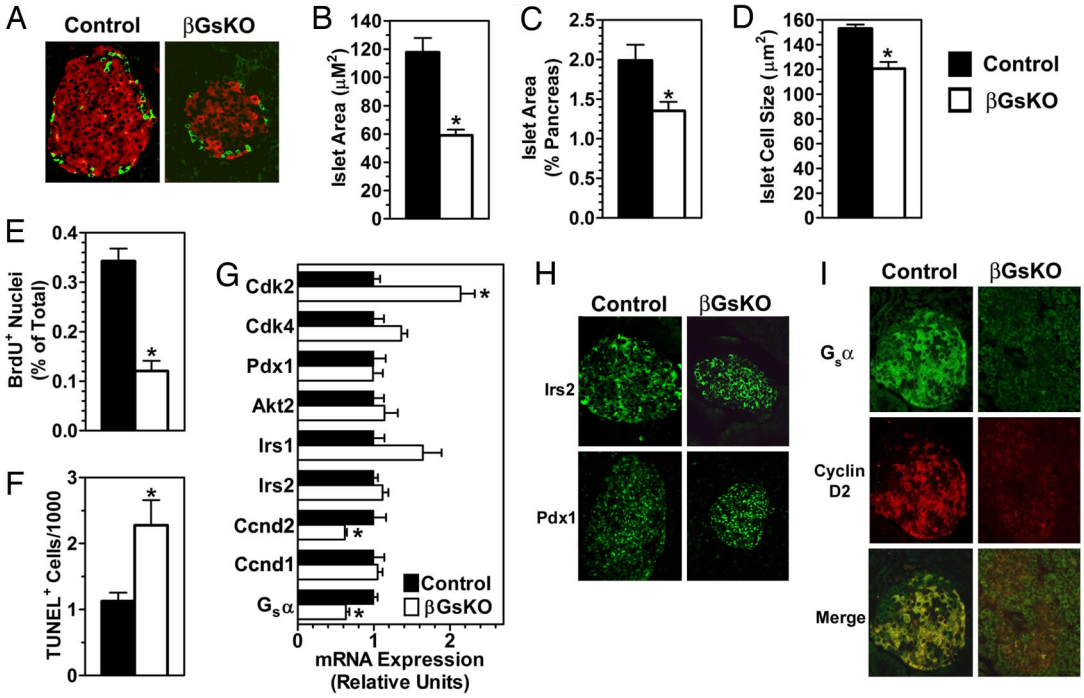

Fig. 3. β cell mass, proliferation, and apoptosis in control and βGsKO mice. (*A*) Immunofluorescence staining of islets from a control (*Left*) and βGsKO mouse (*Right*), using antibodies for glucagon (green) and insulin (red). (*B–D*) Mean islet area ($n = 3$ mice per group) (*B*), islet area expressed as the percentage of total pancreas area ($n = 3$ mice per group) (*C*), and mean islet cell size ($n = 14–18$ per group) (*D*) in control (filled bars) and βGsKO (open bars) mice. (*E*) Frequency of BrdU$^+$-staining nuclei in islets as the percentage of total islet nuclei ($n = 3$ mice per group). (*F*) Frequency of insulin-staining cells in islets that were also TUNEL$^+$ ($n = 4–8$ mice per group). (*G*) Expression of various genes in control and βGsKO mice as determined by quantitative real-time RT-PCR ($n = 4–8$ mice per group). (*H*) Immunofluorescence staining for IRS2 (*Top*) and PDX1 (*Lower*) of islets from a control (*Left*) and a βGsKO mouse (*Right*). (*I*) Immunofluorescence staining of islets from a control (*Left*) and a βGsKO mouse (*Right*) mouse for $G_s\alpha$ (green), cyclin D2 (red), or both (merge). *, $P < 0.05$.

Although it is possible that we could not measure a difference in apoptosis in the early postnatal period because apoptosis rates were low, our findings suggest that the increased β cell apoptosis observed in adult βGsKO mice may be secondary to other metabolic or hormonal abnormalities that develop in these mice. In addition, insulin mRNA and protein levels were significantly reduced in β cells of βGsKO mice. Both first- and second-phase insulin responses to i.p. glucose were virtually absent in βGsKO mice, consistent with these mice having a severe defect in glucose-stimulated insulin release that is only partially explained by these mice having reduced β cell mass and insulin reserve.

In addition to effects on β cell function, βGsKO mice had significantly reduced linear growth and low IGF1 levels. Insulin induces IGF1 expression and poor growth in diabetics with low IGF1 levels is reversed by IGF1 replacement (18–23). Therefore, hypoinsulinemia may account for the low IGF1 levels and poor growth in βGsKO mice. Although it has been reported that RIP2-cre can lead to gene recombination in the hypothalamus and pituitary (14, 17), we did not see loss of $G_s\alpha$ expression in the hypothalamus in βGsKO mice and hypothalamic $G_s\alpha$ deficiency does not lead to the poor growth and β cell defects observed in βGsKO mice (M.C. and L.S.W., unpublished results). Although $G_s\alpha$ expression was only reduced by 20% in the pituitary, we cannot rule out the possibility that reduced growth hormone secretion also contributes to the low IGF1 levels and poor growth observed in βGsKO mice. However, islet-specific GsKO mice generated by using *Pdx1*-cre, which is not expressed in the central nervous system, also develop severe diabetes and very poor growth (T.X., unpublished results).

IGF1 has been implicated as an important stimulatory factor for β cell growth and function. However, recent findings in tissue-specific IGF1 and IGF1 receptor knockout mice have called into question the role of IGF1 in β cell growth, although it may be required for optimal glucose-stimulated insulin secretion (28). Mice with β cell-specific knockout of both the insulin and IGF1 receptors developed β cell defects and diabetes, suggesting that loss of both signaling pathways may produce a synergistic effect (29). However, unlike βGsKO mice, these mice had a marked reduction in islet PDX1 expression, suggesting that the β cell defect in these two mouse models occur via independent mechanisms. Although we cannot rule out the possibility that low IGF1 levels contribute to the β cell defect in βGsKO mice, the presence of severe diabetes in islet GsKO generated by using *Pdx1*-cre (T.X., unpublished results) provides further evidence that β cell dysfunction in βGsKO mice results from a primary defect in β cells.

The mechanisms mediating the insulinotropic and growth promoting effects of cAMP in β cells are not fully understood. cAMP is known to directly bind to and stimulate both protein kinase A (PKA)- and cAMP-regulated guanine nucleotide exchange factors (cAMP-GEFs or Epacs). One mechanism reported to be important in mediating the effects of cAMP on β cell growth and survival is induction of the *Irs2* gene by the transcription factor CREB, which is activated by PKA phosphorylation (11). This leads to stimulation of Akt kinase, which phosphorylates the forkhead transcription factor FOXO1, leading to its exclusion from the nucleus. Because FOXO1 normally inhibits expression of *Pdx1*, an islet-specific transcription factor that promotes β cell function (30), induction of the *Irs2* pathway by $G_s\alpha$ signaling would be predicted to result in increased *Pdx1* expression. Consistent with this hypothesis, *Pdx1* was shown to be required for the insulinotropic effects and β cell growth and survival effects of GLP1 (25). However, we found no difference in the expression of either *Irs2* or *Pdx1* at the mRNA or protein levels in βGsKO islets even though these mice had marked changes in β cell hormonal function, growth, and survival. Our

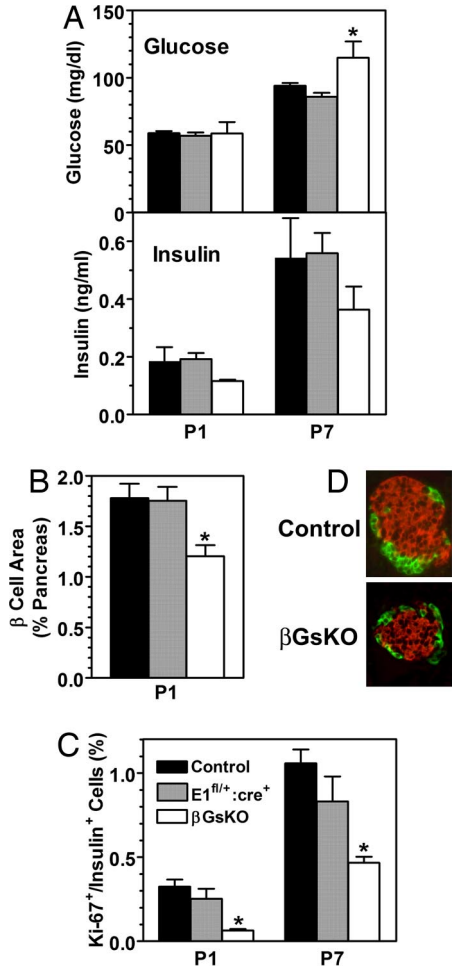

Fig. 4. βGsKO mice have a β cell defect from birth. (*A*) Serum glucose (*Upper*) and insulin (*Lower*) levels at P1 and P7 in male control (solid bar), $E1^{fl/+}$:cre^{+} (gray bar), and βGsKO (open bar) mice ($n = 3$–34 per group). (*B*) β cell (insulin^{+}) area expressed as the percentage of total pancreas area in males at P1 ($n = 3$–5 per group). (*C*) β cell proliferation as determined as the percentage of insulin^{+} cells costaining with Ki67 at P1 and P7 ($n = 2$–4 per group). (*D*) Immunofluorescence staining of islets from a control (*Upper*) and a βGsKO mouse (*Lower*) for glucagon (green) and insulin (red). *, $P < 0.05$.

results suggest that loss of $G_s\alpha$ signaling leads to β cell dysfunction through other molecular mechanisms and that GLP1 may stimulate *Pdx1* expression through $G_s\alpha$-independent mechanisms.

Although we observed no effect in *Irs2* or *Pdx1* expression, we did observe a significant decrease in cyclin D2 mRNA and protein expression. The question of whether loss of $G_s\alpha$/cAMP signaling directly leads to cyclin D2 deficiency in β cells requires further study, although recent studies in both lymphocytes and ovarian granulosa cells demonstrated that cAMP induces the *Ccnd2* gene promoter via binding of PKA-phosphorylated CREB to a CREB binding site (31, 32). Cyclin D2 and its associated cyclin-dependent kinase cdk4 have both been shown to be critical regulators of β cell proliferation and β cell mass (3–7, 33). However, it is unclear to what extent cyclin D2 deficiency contributes to the β cell defect resulting from $G_s\alpha$ deficiency, because cyclin D2 knockout mice have been shown to affect β cell mass and proliferation within the early postnatal period in one genetic background (7) but to only affect β cells in later life in another genetic background (4). Therefore, it is likely that other mechanisms contribute to the β cell defect that results from $G_s\alpha$ deficiency.

Materials and Methods

Generation of βGsKO Mice. Mice with loxP sites surrounding $G_s\alpha$ exon 1 ($E1^{fl/fl}$) (34) were maintained on Black Swiss genetic background. Rat insulin II promoter (RIP2)-cre mice (15) were obtained from The Jackson Laboratory and crossed with Black Swiss WT mice for three or more generations before the studies. Female $E1^{fl/fl}$ mice were mated to male $E1^{fl/+}$:$cre^{+/-}$ mice to generate βGsKO mice ($E1^{fl/fl}$:cre^{+}), mice with heterozygous $G_s\alpha$ deletion ($E1^{fl/+}$:cre^{+}), and control cre^{-} littermates. Mice were maintained on standard pellet diet (NIH-07, 5% fat by weight) and 12:12-h light/dark cycle. Except where noted, studies were performed in 3- to 4-month-old mice and were approved by the NIDDK Animal Care and Use Committee. Immunoblots of tissue extracts, using a $G_s\alpha$-specific antibody, were performed as described in ref. 34. Mice were genotyped by PCR as described in ref. 35.

Body Composition and Food Intake. Body composition was measured by using the Minispec mq10 NMR analyzer (Bruker). Food intake was measured over 12 days in individually caged mice.

Blood Chemistry and Hormone Assays. Blood was obtained by retroorbital bleed. Serum chemistries were measured by the National Institutes of Health Clinical Chemistry Laboratory. Serum insulin, glucagon, GLP1 (Linco), IGF1 (R&D Systems), TSH (rat TSH assay; Alpco Diagnostics), and corticosterone (Immunodiagnostic Systems) were measured by ELISA or RIA.

Glucose and Insulin-Tolerance Tests and Insulin Secretion. Glucose and insulin-tolerance tests were performed as described in ref. 34 in overnight-fasted mice after i.p. injection of glucose (2 mg/g) or insulin (Humulin; 0.75 milli international units/g), respectively. To test glucose-stimulated insulin release, glucose (3 mg/g) was administered i.p. to overnight fasted mice, and tail vein blood was collected in heparinized capillary tubes at indicated time points. Plasma insulin was measured by ultrasensitive rat insulin ELISA (Crystal Chem), and glucose was measured by glucometer.

Islet isolation and Insulin Content Measurement. Islets were isolated by collagenase digestion and hand-picked under stereoscopy. Fifteen to 20 islets were extracted with 95% ethanol and 0.1 M HCl (200 μl). Insulin content was determined by ultrasensitive rat insulin ELISA and normalized to protein, which was measured by a BCA protein assay kit (Pierce).

Immunostaining and Islet Morphometry. Pancreata were fixed in 10% neutral formalin and paraffin-embedded, and sections were H&E-stained. Three whole sections (>300 μm apart) were photographed, and areas of all visible islets and the whole section were determined by using ImageJ software. Average islet cell size was calculated by total islet area (>20 mature islets per group) divided by number of cell nuclei. For immunostaining, 5-μm sections of paraffin blocks were rehydrated with xylene followed by decreasing concentrations of ethanol; microwaved in 0.01 M sodium citrate, pH 6.0, for 20 min; and permeabilized with 1% Triton X-100 in PBS. Sections were then incubated with mouse antiinsulin (Invitrogen), rabbit antiglucagon, mouse anticyclin D2 (Lab Vision), rat anti-pancreatic and duodenal homeobox 1 (PDX1), rat anti-IRS2 (Santa Cruz Biotechnologies), or rabbit anti-$G_s\alpha$ (36) antibodies, followed by secondary antibodies that were labeled with rhodamine or Cy2 (Jackson ImmunoResearch). Sections were counterstained with DAPI (Invitrogen).

β Cell Proliferation and Apoptosis Studies. For proliferation studies, 28-day-old mice ($n = 7$ per group) were injected with BrdU

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(Invitrogen, 100 μg/g) 6 h before killing. Pancreata were formalin-fixed and paraffin-embedded, and four sections (>300 μm apart) were stained with a BrdU staining kit (Invitrogen) and lightly counterstained with H&E. Islets (>300 fields) were imaged with an Zeiss Axioskop microscope to count $BrdU^+$ cells and total nuclei within islets. For younger mice, islets were counterstained with antiinsulin and rabbit anti-Ki67 (Vector) antibodies. For apoptosis studies, 3-month-old mice ($n = 4$ per group) were killed, and three pancreatic sections per animal were stained by the TUNEL method (Roche). Sections were also immunostained for insulin and counterstained with DAPI. $TUNEL^+$ insulin-staining β cells were counted and divided by the total number of β cells, which was estimated by dividing the area of insulin staining by the average cell size. In younger mice, apoptosis was measured by using a rabbit anti-cleaved caspase 3 (Asp-175) antibody (Cell Signaling).

Quantitative PCR. Islet RNA samples were prepared by using the RNeasy kit (Qiagen), and quantitative real-time PCR was performed in an Mx3000P (Stratagene) as described in ref. 37, using SYBR Green as detector and normalizing to β-actin. Primer sequences are available upon request.

Statistical Analysis. Data are expressed as mean ± SEM. Statistical significance was determined by using unpaired Student's t test (two-tailed) or one-way ANOVA with Tukey's post hoc test with differences considered significant at $P < 0.05$.

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