

# Pancreas-specific $G_s\alpha$ deficiency has divergent effects on pancreatic $\alpha$ - and $\beta$ -cell proliferation

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## Abstract

The ubiquitously expressed G protein  $\alpha$ -subunit  $G_s\alpha$  mediates the intracellular cAMP response to glucagon-like peptide 1 (GLP1) and other incretin hormones in pancreatic islet cells. We have shown previously that mice with  $\beta$ -cell-specific  $G_s\alpha$  deficiency ( $\beta G_s\alpha$  KO) develop severe early-onset insulin-deficient diabetes with a severe defect in  $\beta$ -cell proliferation. We have now generated mice with  $G_s\alpha$  deficiency throughout the whole pancreas by mating  $G_s\alpha$ -floxed mice with Pdx1-cre transgenic mice (PGsKO). PGsKO mice also developed severe insulin-deficient diabetes at a young age, confirming the important role of  $G_s\alpha$  signaling in  $\beta$ -cell growth and function. Unlike in  $\beta G_s\alpha$  KO mice, islets in PGsKO mice had a relatively greater proportion of  $\alpha$ -cells, which were spread throughout the interior of the islet. Similar findings were observed in mice

with pancreatic islet cell-specific  $G_s\alpha$  deficiency using a neurogenin 3 promoter-cre recombinase transgenic mouse line. Studies in the  $\alpha$ -cell line  $\alpha TC1$  confirmed that reduced cAMP signaling increased cell proliferation while increasing cAMP produced the opposite effect. Therefore, it appears that  $G_s\alpha$ /cAMP signaling has opposite effects on pancreatic  $\alpha$ - and  $\beta$ -cell proliferation, and that impaired GLP1 action in  $\alpha$ - and  $\beta$ -cells via  $G_s\alpha$  signaling may be an important contributor to the reciprocal effects on insulin and glucagon observed in type 2 diabetics. In addition, PGsKO mice show morphological changes in exocrine pancreas and evidence for malnutrition and dehydration, indicating an important role for  $G_s\alpha$  in the exocrine pancreas as well.

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## Introduction

Both type 1 and type 2 diabetes have been associated with pancreatic  $\beta$ -cell dysfunction, with reduced glucose-stimulated insulin secretion and decreased  $\beta$ -cell mass due to impaired  $\beta$ -cell proliferation and survival (Butler *et al.* 2003). In addition, type 2 diabetics also have increased glucagon (GCG) secretion from pancreatic  $\alpha$ -cells and inappropriately increased serum levels of GCG (Goke 2008). Glucagon-like peptide 1 (GLP1) and other incretin hormones promote glucose-stimulated insulin secretion and  $\beta$ -cell growth, and inhibit GCG secretion from  $\alpha$ -cells by activating the G protein  $\alpha$ -subunit  $G_s\alpha$ , which stimulates intracellular cAMP production (Weinstein *et al.* 2001, Brubaker & Drucker 2004, Preitner *et al.* 2004, Ahren 2009). Because of these beneficial effects, the GLP1 receptor (*Glp1r*) agonist exendin 4 has recently been approved as a therapeutic agent for diabetes.

We have previously shown in mice with  $\beta$ -cell-specific  $G_s\alpha$  deficiency ( $\beta G_s\alpha$  KO mice) that  $G_s\alpha$ /cAMP signaling is critical for normal  $\beta$ -cell function and growth (Xie *et al.* 2007). Although GLP1 tends to inhibit GCG secretion

*in vivo*, these effects may not be due to direct effects of GLP1 on GCG secretion from  $\alpha$ -cells as several studies have shown that  $G_s\alpha$ /cAMP signaling stimulates both proglucagon gene expression (Drucker *et al.* 1991, Islam *et al.* 2009) and GCG release (Hermansen 1985, Ding *et al.* 1997, Gromada *et al.* 1997, Ma *et al.* 2005) in isolated  $\alpha$ -cells. Little is known about the role of GLP1 or  $G_s\alpha$ /cAMP signaling on the regulation of pancreatic  $\alpha$ -cell proliferation.

In this present study, we generated mice with  $G_s\alpha$  deficiency throughout the endocrine and exocrine pancreas (PGsKO mice) by mating  $G_s\alpha$ -floxed mice with Pdx1-cre mice in order to directly examine the *in vivo* role of  $G_s\alpha$ /cAMP signaling in both pancreatic  $\alpha$ - and  $\beta$ -cells. These mice had the same  $\beta$ -cell defect with early-onset insulin-deficient diabetes as that seen in  $\beta G_s\alpha$  KO mice. In addition, PGsKO had relatively increased numbers of  $\alpha$ -cells, and a similar finding was observed in a similar mouse model that was generated using neurogenin 3 (*Ngn3*) promoter-cre mice. Studies in the  $\alpha$ -cell line  $\alpha TC1$  showed that  $G_s\alpha$ /cAMP signaling inhibits  $\alpha$ -cell proliferation, an effect opposite to that known to occur in  $\beta$ -cells.

Therefore, reduced GLP1 actions on both  $\alpha$ - and  $\beta$ -cells via  $G_s\alpha$  signaling may be an important contributor to the reciprocal effects on insulin and GCG observed in type 2 diabetics. In addition, we show evidence that  $G_s\alpha$  is also important in pancreatic exocrine function.

## Materials and Methods

### Mice

Mice with loxP sites surrounding  $G_s\alpha$  exon 1 ( $E1^{\Delta/\Delta}$ ; Chen *et al.* 2005) were maintained on Black Swiss genetic background.  $Pdx1$ -cre<sup>+</sup> mice (Lammert *et al.* 2003) and  $Ngn3$  promoter-cre<sup>+</sup> mice (Schonhoff *et al.* 2004) were crossed with Black Swiss WT mice for three or more generations before the studies in order to obtain a purer genetic background. Female  $E1^{\Delta/\Delta}$  mice were mated with male  $E1^{\Delta/+};Pdx1$ -cre<sup>+/-</sup> mice to generate pancreatic  $G_s\alpha$  knockout mice ( $PGsKO$ ;  $E1^{\Delta/\Delta};cre^+$ ), mice with heterozygous pancreatic  $G_s\alpha$  deletion ( $E1^{\Delta/+};cre^+$ ), and control cre<sup>-</sup> littermates, or were mated with  $E1^{\Delta/+};Ngn3$ -cre<sup>+/-</sup> mice to generate mice with pancreatic islet-specific  $G_s\alpha$  deficiency. Mice were maintained on standard pellet diet (NIH-07, 5% fat by weight) and 12 h light:12 h darkness 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. Mice were genotyped by PCR as previously described (Sakamoto *et al.* 2005).

### Body composition and food intake

Body composition was measured using the Minispec mq10 NMR analyzer (Bruker Optics Inc., Woodlands, TX, USA). Food intake was measured over 21 days in individually caged mice.

### Serum chemistry

Blood was obtained by retro-orbital bleed. Serum insulin (Crystal Chem, Downers Grove, IL, USA), GCG (R&D Systems, Minneapolis, MN, USA), insulin-like growth factor 1 (IGF1; Alpco Diagnostics, Salem, NH, USA), and the circulating active forms of GLP1 (Millipore, Billerica, MA, USA) were measured by ELISA or RIA. The remainder of the chemistries was measured in the NIH Clinical Chemistry Laboratory.

### Glucose and insulin tolerance tests and insulin secretion

Glucose and insulin tolerance tests were performed in overnight fasted mice after i.p. injection of glucose (2 mg/g) or insulin (Humulin; 0.75 mIU/g) respectively. Plasma glucose was measured by glucometer.

### Immunostaining and islet morphometry

Pancreata were fixed in 4% paraformaldehyde and paraffin-embedded, and sections were H&E stained. For immunostaining, 5- $\mu$ m sections of paraffin blocks were deparaffinized and 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 anti-insulin (Invitrogen), rabbit anti-GCG, rat anti-somatostatin (Lab Vision, Fremont, CA, USA), rabbit anti-prohormone convertase 1/3 (PC1/3), rabbit anti-PC2 (Millipore), or rabbit anti- $G_s\alpha$  (Simonds *et al.* 1989) antibodies, followed by secondary antibodies that were labeled with rhodamine or Cy2 (Jackson ImmunoResearch, West Grove, PA, USA). Sections were counterstained with DAPI (Invitrogen). For  $\alpha$ -cell proliferation studies, pancreatic sections of 7-day-old mice ( $n=3$ /group) were stained with anti-GCG and rabbit anti-Ki67 (Vector, Burlingame, CA, USA) antibodies.

### Cell proliferation and GCG secretion in culture

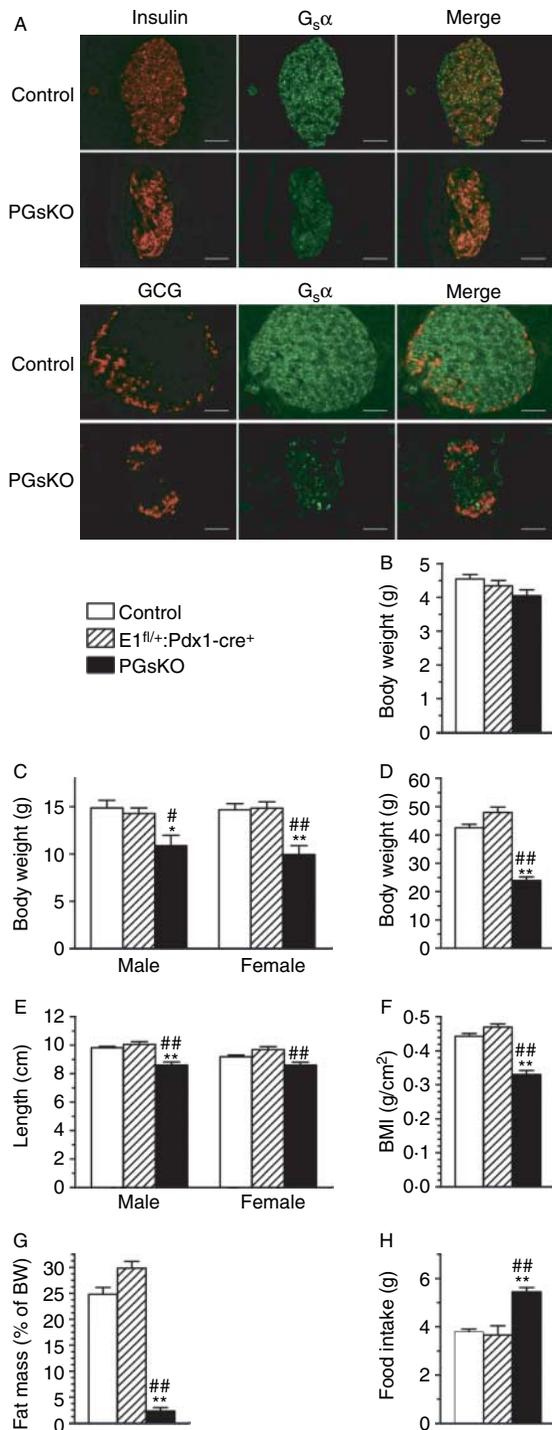
A pancreatic  $\alpha$ -cell line ( $\alpha$ TC1.9; Powers *et al.* 1990) was purchased from ATCC (Manassas, VA, USA; catalog # CRL-2350) and grown in 37 °C under 10% CO<sub>2</sub> in DMEM modified to contain 0.3% glucose, 15 mM HEPES, 0.1 mM nonessential amino acids, 0.02% BSA, and 10% fetal bovine serum. For RNAi  $G_s\alpha$  knockdown assay,  $G_s\alpha$ -specific siRNA and negative control siRNA were purchased from Ambion (Austin, TX, USA; catalog #16706, Gene ID46627), and lipofectamine 2000 (Invitrogen) was used for transfection. To test the effect of increased intracellular cAMP on  $\alpha$ TC1 growth, cells were seeded into 24-well plates at  $2 \times 10^5$  cell/well and incubated overnight before medium containing either forskolin (FSK; 20 mM) and isobutylmethylxanthine (IBMX; 100 mM), or vehicle alone was added and then refreshed every 24 h. Treated and control cells were stained with 0.1% thiazolyl blue tetrazolium bromide (Sigma) in PBS at 37 °C for 30 min. Stained cells were incubated with isopropanol to elute the stain, and absorbances were read at OD560. To test the effects on GCG secretion, media were changed at 24 h after transfection or treatment, and replaced with new media containing 16.7 or 5.6 mM glucose. The cells were then incubated for 4 h, and the GCG concentrations in the culture media were measured by ELISA (R&D Systems).

### Quantitative RT-PCR

Islets were isolated and RNA samples were prepared as previously described (Xie *et al.* 2007), and quantitative real-time PCR was performed using SYBR-Green based detection in an Mx3000P (Stratagene, La Jolla, CA, USA). Gene expressions were normalized by geometric averaging of the levels of  $\beta$ -actin, *Hprt*, and *Gapdh* mRNAs. Primer sequences are available upon request.

## Statistical analysis

Data are expressed as mean  $\pm$  S.E.M. Statistical significance was determined by unpaired Student's *t*-test (two-tailed) or one-way ANOVA with Tukey's *post hoc* test with differences considered significant at  $P < 0.05$ .



## Results

## PGsKO mice have reduced body mass and adiposity

PGsKO (E1<sup>fl/fl</sup>:Pdx1-cre<sup>+</sup>) mice with pancreatic G<sub>s</sub>α deficiency were generated by mating of E1<sup>fl/fl</sup> females with E1<sup>fl/+</sup>:Pdx1-cre<sup>+/-</sup> males, and their phenotypes were compared with cre<sup>-</sup> control and E1<sup>fl/+</sup>:Pdx1-cre<sup>+</sup> littermates. Coimmunostaining of pancreatic sections with a G<sub>s</sub>α antibody and either an insulin or GCG antibody showed robust G<sub>s</sub>α expression in both α- and β-cells of control islets which was markedly reduced in PGsKO islets (Fig. 1, panel A). This is in contrast to βGsKO mice, in which G<sub>s</sub>α deficiency was confined to β-cells (Xie *et al.* 2007). Compared with islets, G<sub>s</sub>α expression in the surrounding exocrine pancreas was much lower even in control mice (Fig. 1, panel A). PGsKO mice showed no change in G<sub>s</sub>α expression in the pituitary or hypothalamus (data not shown). Unlike βGsKO mice, which had significant lethality throughout the first few months of postnatal life (Xie *et al.* 2007), PGsKO mice showed minimal effects on postnatal survival (102 and 89% of expected survival at 7 days and 4 weeks of age respectively;  $n = 82$  and 282 total offspring respectively).

PGsKO mice had a severe postnatal growth defect. Body weights of control, E1<sup>fl/+</sup>:Pdx1-cre<sup>+</sup>, and PGsKO mice were similar on postnatal day 7 (Fig. 1, panel B) but by 3.5 weeks both male and female PGsKO had markedly reduced body weight compared with either E1<sup>fl/+</sup>:Pdx1-cre<sup>+</sup> mice or controls (Fig. 1, panel C), and this difference became even greater by 3 months of age (Fig. 1, panel D). Although adult PGsKO mice had slightly reduced body length compared with E1<sup>fl/+</sup>:Pdx1-cre<sup>+</sup> mice and controls (Fig. 1, panel E), their overall body mass index (body weight/body length<sup>2</sup>) was significantly reduced (Fig. 1, panel F). Body composition analysis showed PGsKO mice to have markedly reduced fat mass (Fig. 1, panel G). While impaired growth in βGsKO mice was associated with reduced circulating levels of IGF1 (Xie *et al.* 2007), IGF1 levels were similar in PGsKO, E1<sup>fl/+</sup>:Pdx1-cre<sup>+</sup>, and control mice (Table 1). In spite of their impaired growth, these mice had significantly increased food intake (Fig. 1, panel H). One possible explanation for the poor growth in PGsKO mice may be malabsorption due to pancreatic exocrine dysfunction, as PGsKO mice showed markedly abnormal histology of the exocrine pancreas

**Figure 1** PGsKO mice have lower body weight in spite of being hyperphagic. (A) Immunostaining of a control and PGsKO islet for either insulin (red) or G<sub>s</sub>α (green) (top panels) or for either GCG (red) or G<sub>s</sub>α (green) (bottom panels). In both cases, the merged images are shown on the right. Body weight of control, E1<sup>fl/+</sup>:Pdx1-cre<sup>+</sup>, and PGsKO mice at (B) 7 days (males only,  $n = 21$ –36/group) (C) 3.5 weeks (males and females,  $n = 3$ –8/group), and (D) 3 months (males only,  $n = 6$ –10/group) after birth. (E) Body length (males and females,  $n = 3$ –10/group), (F) body mass index (males,  $n = 4$ –10/group), (G) % fat mass (males,  $n = 5$ –13/group), and (H) food intake (males,  $n = 4$ –8/group) in 3-month-old mice. Data are mean  $\pm$  S.E.M. (\* $P < 0.05$  or \*\* $P < 0.01$  versus controls. # $P < 0.05$  or ## $P < 0.01$  versus E1<sup>fl/+</sup>:Pdx1-cre<sup>+</sup>). Bars equal 50  $\mu$ m.

**Table 1** Serum chemistries in adult mice<sup>a</sup>

	Control	E1 <sup>fl/+</sup> ; Pdx-cre <sup>+</sup>	PGsKO
Glucose (mg/dl)	184 ± 8	238 ± 11**	602 ± 2*** <sup>†</sup>
Cholesterol (mg/dl)	129 ± 6	160 ± 14	206 ± 15*** <sup>†</sup>
Triglycerides (mg/dl)	277 ± 60	320 ± 49	523 ± 73*
Sodium (mEq/l)	153 ± 1	152 ± 3	147 ± 4
Potassium (mEq/l)	5.9 ± 0.2	5.8 ± 0.3	6.6 ± 0.3
Chloride (mEq/l)	117 ± 1	113 ± 2	109 ± 4
Creatinine (mg/dl)	0.40 ± 0.03	0.56 ± 0.09	0.90 ± 0.13**
BUN (mg/dl)	24 ± 2	22 ± 1	49 ± 16
Albumin (g/dl)	1.52 ± 0.04	1.48 ± 0.02	1.22 ± 0.07*** <sup>†</sup>
IGF1 (ng/ml)	641 ± 39	682 ± 25	673 ± 19
GCG (pg/ml)	155 ± 30	160 ± 46	185 ± 61
Insulin (ng/ml)	1.23 ± 0.16	1.02 ± 0.14	0.35 ± 0.08*** <sup>†</sup>
GLP1 (active) pM	6.63 ± 0.09	6.42 ± 0.23	6.40 ± 0.14

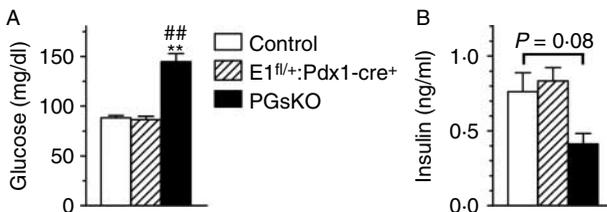
\*\**P* < 0.01 versus control; <sup>†</sup>*P* < 0.05 or <sup>†</sup>*P* < 0.01 versus E1<sup>fl/+</sup>:Pdx1-cre<sup>+</sup> by ANOVA.

<sup>a</sup>Measured in 3–4-month-old males, *n* = 3–12/group.

(see below) presumably as a result of *G<sub>s</sub>α* deficiency in these regions as well as the endocrine pancreas. Consistent with severe malnutrition, serum albumin levels were significantly decreased in PGsKO mice (Table 1).

#### PGsKO mice develop early-onset insulin-deficient diabetes

Even by day 7 *post partum*, PGsKO mice were very hyperglycemic as compared with E1<sup>fl/+</sup>:Pdx1-cre<sup>+</sup> and control mice (Fig. 2, panel A). Serum insulin levels on day 5 were ~50% lower in PGsKO as compared with the other two groups (*P* = 0.08 versus controls; Fig. 2, panel B). In adult mice (3–4 months of age), glucose levels were slightly higher in E1<sup>fl/+</sup>:Pdx1-cre<sup>+</sup> mice as compared with controls, while they were greater than threefold higher in PGsKO mice (Table 1). Insulin levels were markedly reduced in adult PGsKO mice as compared with controls and E1<sup>fl/+</sup>:Pdx1-cre<sup>+</sup> mice (Table 1). Adult male and female PGsKO mice had markedly impaired glucose tolerance compared with E1<sup>fl/+</sup>:Pdx1-cre<sup>+</sup> and control mice (Fig. 3, panels A and B), while insulin tolerance tests showed similar levels of insulin sensitivity between the three groups (Fig. 3, panels C and D). In addition, adult



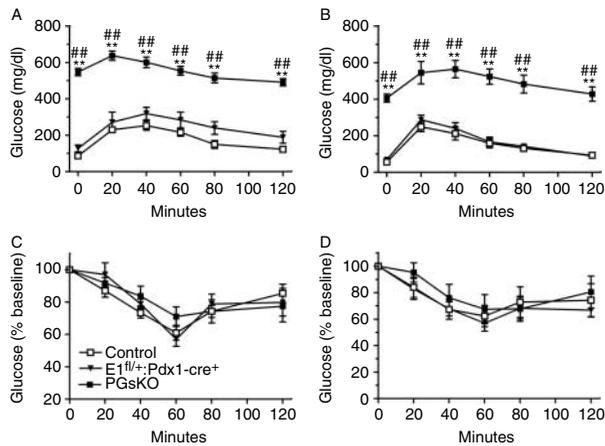
**Figure 2** Mice develop insulin deficiency and hyperglycemia at a young age. (A) Serum glucose (*n* = 21–38/group) and (B) insulin (*n* = 3–7/group) in control, E1<sup>fl/+</sup>:Pdx1-cre<sup>+</sup>, and PGsKO mice at day 7 and 5 *post partum* respectively. Data are mean ± S.E.M. (\*\**P* < 0.01 versus controls; ##*P* < 0.01 versus E1<sup>fl/+</sup>:Pdx1-cre<sup>+</sup>).

PGsKO mice had increased serum triglyceride and cholesterol levels as compared with the other two groups of mice (Table 1). Serum IGF1, GLP1 (active forms), and GCG levels were similar between the three groups (Table 1). While electrolytes were normal, serum creatinine was significantly increased and blood urea nitrogen (BUN) levels were more than double in PGsKO mice, although this was not statistically significant (Table 1), indicating that PGsKO mice were dehydrated, most likely secondary to severe hyperglycemia. Therefore, PGsKO mice mimic βGsKO mice in terms of developing very early-onset insulin-deficient diabetes, although βGsKO mice had low IGF1 levels (Xie *et al.* 2007) while IGF1 remained unaffected in PGsKO mice.

#### PGsKO mice have reduced islet size and β-cell mass with increased numbers of α-cells

Total pancreas weights of PGsKO mice were greater than controls (Fig. 4, panel A). When pancreas weights were normalized to total body weights, they were ~75% greater in PGsKO mice as compared with E1<sup>fl/+</sup>:Pdx1-cre<sup>+</sup> or control mice (Fig. 4, panel A). This was primarily due to marked enlargement of the pancreatic exocrine ducts in PGsKO mice, which were also more eosinophilic as compared with those in controls on H&E staining (Fig. 4, panel B). These changes are probably the result of *G<sub>s</sub>α* deficiency in exocrine pancreas, and suggest that PGsKO mice may have pancreatic exocrine insufficiency and malabsorption.

H&E staining of adult pancreas showed that PGsKO mice, like βGsKO mice (Xie *et al.* 2007), had markedly smaller islets than controls with irregular shapes and increased cellularity (Fig. 4, panel B). Coimmunostaining of islets with antibodies for insulin and GCG showed that at 5 days of age, PGsKO islets were only slightly smaller than controls with a similar distribution of α- and β-cells (Fig. 4, panel C). By 4 weeks, PGsKO islets had a greater ratio of α- to β-cells than controls, although the α-cells remained on the periphery. At 14 weeks, PGsKO islets were much smaller than in controls, and had less β-cells with reduced intensity of insulin staining, similar to what was observed in βGsKO mice (Xie *et al.* 2007). However, unlike what was observed in βGsKO mice (Xie *et al.* 2007), PGsKO mice had increased numbers of α-cells with a very significant number of α-cells mixed with β-cells throughout the islet (Fig. 4, panel C). A similar pattern was also observed in E1<sup>fl/fl</sup> mice:Ngn3-cre<sup>+</sup> (Schonhoff *et al.* 2004), another model with *G<sub>s</sub>α* deficiency throughout the pancreatic islet (Fig. 4, panel D). Coimmunostaining of PGsKO islets with insulin and somatostatin antibodies showed reduced numbers of β-cells, and a slight increase in somatostatin-staining δ-cells with some intermingling of δ-cells with β-cells within the interior of the islet (Fig. 4, panel E). Immunostaining of duodenal sections for serotonin, GLP1, cholecystokinin, or ghrelin showed no obvious change in the number or distribution of endocrine cells in the duodena of PGsKO mice (data not shown).



**Figure 3** PGsKO mice are glucose intolerant but have normal insulin sensitivity. Glucose tolerance tests performed in (A) male ( $n=4-5/\text{group}$ ) and (B) female 3-month-old control ( $\square$ ),  $E1^{fl/+};Pdx1\text{-cre}^+$  ( $\blacktriangledown$ ), and PGsKO mice ( $\blacksquare$ ) ( $n=4-7/\text{group}$ ). Insulin tolerance tests performed in (C) male ( $n=6-8/\text{group}$ ) and (D) female mice ( $n=4-6/\text{group}$ ). Data are mean  $\pm$  S.E.M. (\*\* $P<0.01$  versus controls; \*\* $P<0.01$  versus  $E1^{fl/+};Pdx1\text{-cre}^+$ ).

The ductal enlargement observed in PGsKO mice suggests that the relative greater proportion of islet  $\alpha$ -cells may be due to increased  $\alpha$ -cell neogenesis. During embryonic development, fetal  $\alpha$ -cells transiently express the prohormone convertase PC1/3 which converts proglucagon to GLP1 (Wilson *et al.* 2002), whereas adult  $\alpha$ -cells express PC2 which converts proglucagon to GCG. Both PC1/3 and PC2 are expressed in islet  $\beta$ -cells, and are required for proinsulin processing. To determine whether  $\alpha$ -cell neogenesis is increased in PGsKO mice, we looked for the presence of  $\alpha$ -cells expressing PC1/3 by coimmunostaining islets for GCG and either PC1/3 or PC2 (Fig. 5, panels A and B respectively). We found that control and PGsKO islets had similar distributions of PC1/3 and PC2, with the absence of PC1/3 and the presence of PC2 within GCG-producing  $\alpha$ -cells, and the presence of both PC1/3 and PC2 in the nonglucagon staining cells, which are primarily  $\beta$ -cells. Therefore, we found no evidence for increased  $\alpha$ -cell neogenesis in PGsKO mice.

Gene expression studies in control and PGsKO islets by quantitative RT-PCR (Fig. 4, panel F) showed a significant reduction in  $G_s\alpha$  mRNA in PGsKO islets, as expected. Insulin (*Ins2*) mRNA was also markedly reduced, consistent with the marked reduction in  $\beta$ -cells. Expression of the proglucagon gene (*Gcg*) which generates GCG was similar in control and PGsKO islets. Given that PGsKO islets have a greater proportion of  $\alpha$ -cells, this suggests that expression levels per  $\alpha$ -cell may be decreased, although we were unable to detect a change in *Gcg* mRNA levels in cultured  $\alpha$  ( $\alpha$ TC1)-cells transfected with  $G_s\alpha$  RNAi (data not shown). *Glp1r* gene expression was also very low in PGsKO islets. This is probably mostly due to the reduced proportion of  $\beta$ -cells, as

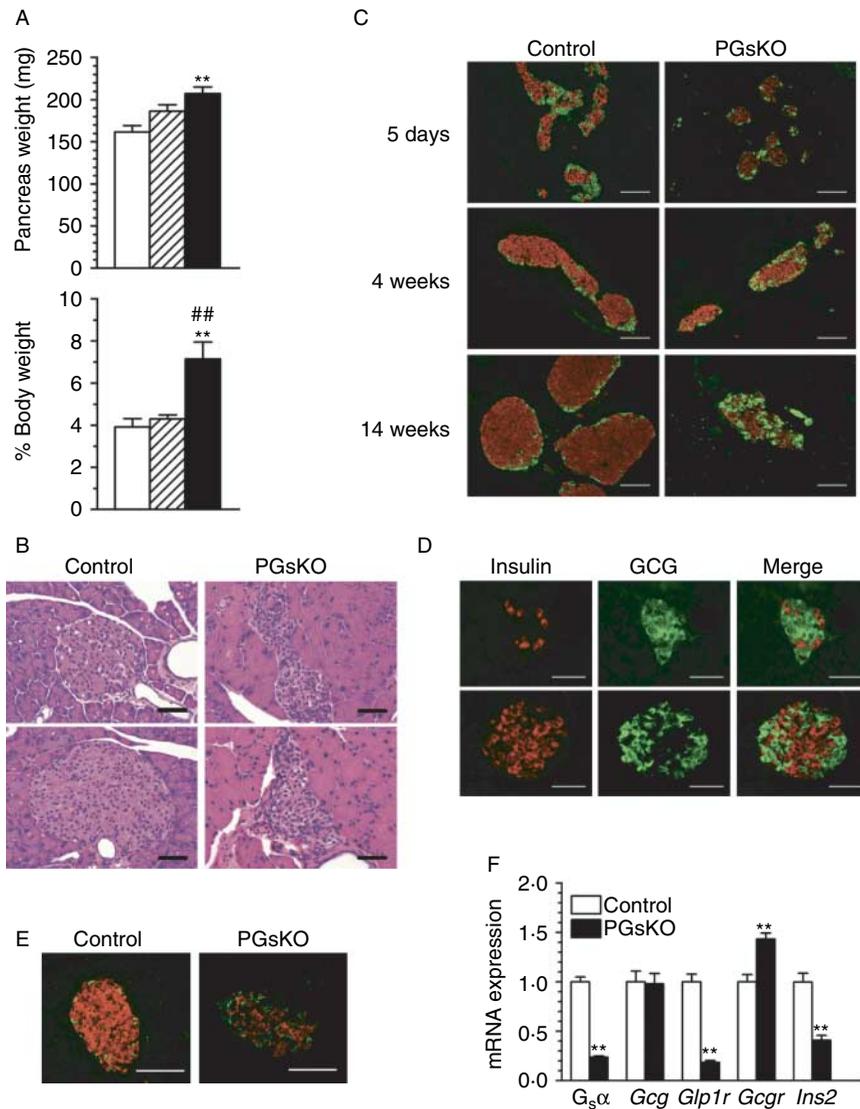
these receptors were recently shown to be exclusively expressed within  $\beta$ -cells in mouse islets (Tornehave *et al.* 2008). In addition, *Glp1r* expression in islets has been shown to be inhibited by hyperglycemia, but to be unaffected by  $G_s\alpha/cAMP$  signaling (Abrahamsen & Nishimura 1995). Finally, GCG receptor (*Gcgr*) expression was up-regulated in PGsKO islets. This is consistent with prior studies showing that  $G_s\alpha/cAMP$  signaling downregulates while high extracellular glucose levels upregulate this gene in pancreatic islets (Abrahamsen & Nishimura 1995) and cultured hepatocytes (Abrahamsen *et al.* 1995).

#### *G<sub>s</sub>α deficiency leads to increased proliferation of pancreatic α-cells*

We have previously shown that  $G_s\alpha$  deficiency in  $\beta$ -cells leads to markedly reduced  $\beta$ -cell proliferation and reduced  $\beta$ -cell mass (Xie *et al.* 2007), and our findings in PGsKO mice are consistent with these prior observations. However, in PGsKO mice, which also had  $G_s\alpha$  deficiency in  $\alpha$ -cells, costaining of islets from 7-day-old mice with antibodies to GCG and the proliferative marker Ki67 showed no evidence for a proliferative defect in  $\alpha$ -cells, and in fact, the proportion of proliferating  $\alpha$ -cells in PGsKO mice tended to be slightly higher than in controls, although the differences were not statistically significant (Fig. 6, panels A and B).

We further examined the effects of  $G_s\alpha$  signaling on proliferation in the  $\alpha$ -cell line  $\alpha$ TC1.  $\alpha$ TC1 cells were treated with either a control (scrambled) RNAi or a  $G_s\alpha$  RNAi which reduced  $G_s\alpha$  expression by  $\sim 70\%$  as determined by quantitative RT-PCR (data not shown). Cells treated with the  $G_s\alpha$  RNAi had a markedly higher proliferative rate than the control cells with the  $G_s\alpha$  RNAi-treated cells doubling at 72 h while the control cell number only increased by  $\sim 30\%$  during the same time period (Fig. 6, panel C). We also performed the opposite experiment, in which  $\alpha$ TC1 cells were treated with the adenylyl cyclase stimulator FSK and the cAMP phosphodiesterase inhibitor IBMX, which lead to increased intracellular cAMP levels (FSK-IBMX). In contrast to the cells treated with  $G_s\alpha$  RNAi, proliferation of FSK-IBMX-treated cells was significantly lower than controls (Fig. 6, panel D). These *in vitro* results confirm that  $G_s\alpha$  signaling pathways have an anti-proliferative effect in  $\alpha$ -cells, which is opposite to their known proliferative effect in  $\beta$ -cells.

We next examined the effect of perturbing  $G_s\alpha/cAMP$  signaling on GCG secretion in  $\alpha$ TC1 cells. Cells were transfected with either control or  $G_s\alpha$  RNAi, and GCG secretion into the media was measured from 24 to 28 h later at both 5.6 and 16.7 mM glucose to avoid the potential confounding effects of differing glucose concentrations on GCG secretion. While GCG secretion was increased after  $G_s\alpha$  RNAi transfections at both glucose concentrations (Fig. 6, panel E), the percent increase was similar to the percent increase in  $\alpha$ TC1 cell numbers at 24 h after  $G_s\alpha$  RNAi treatment (Fig. 6, panel C), and therefore, this does not represent an increase in the amount of GCG secreted

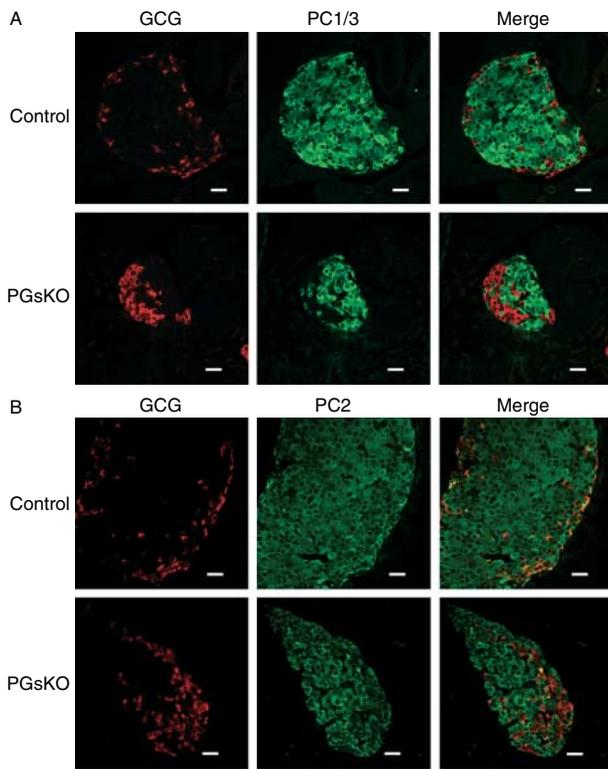


**Figure 4** Pancreatic exocrine and islet pathology in PGsKO mice. (A) Pancreatic weights in mg (top panel;  $n=7-15$ /group) and as % body weight (lower panel;  $n=4-7$ /group) in adult male control (open bars),  $E1^{fl/+};Pdx1-cre^+$  (striped bars), and PGsKO mice (solid bars). (B) H&E-stained sections (two each) of an islet and surrounding exocrine pancreas from a control and a PGsKO mouse. (C) Immunostaining of pancreatic sections from 5-day-old, 4-week-old, and 14-week-old control and PGsKO mice for insulin (red) and GCG (green). (D) Two sections showing islets from  $E1^{fl/+};Ngn3-cre^+$  mice that were immunostained for insulin (red) or GCG (green) with the merged images on the right. (E) Sections of a control and a PGsKO islet immunostained for insulin (red) and somatostatin (green). (F) mRNA expression levels of the  $G_s\alpha$ , glucagon (*Gcg*), GLP1 receptor (*Glp1r*), glucagon receptor (*Gcgr*), and insulin (*Ins2*) genes in islets isolated from control and PGsKO mice ( $n=7$ /group). Data are mean  $\pm$  s.e.m. (\*\* $P<0.01$  versus controls; ## $P<0.01$  versus  $E1^{fl/+};Pdx1-cre^+$ ). Bars equal 50  $\mu$ m.

per cell. Interestingly,  $\alpha$ TC1 cells treated with FSK-IBMX showed a more marked twofold increase in GCG secretion at 24–28 h in the presence of 5.6 mM glucose (Fig. 6, panel F), despite the fact that there was no increase in cell numbers (Fig. 6, panel D), indicating that excess  $G_s\alpha$ /cAMP signaling directly promotes GCG secretion in  $\alpha$ -cells.

## Discussion

Previously, we generated mice with  $\beta$ -cell-specific  $G_s\alpha$  deficiency ( $\beta$ GsKO mice) using Rip2-cre, and demonstrated that  $G_s\alpha$ /cAMP pathways are critical regulators of  $\beta$ -cell function and promote  $\beta$ -cell proliferation (Xie *et al.* 2007).

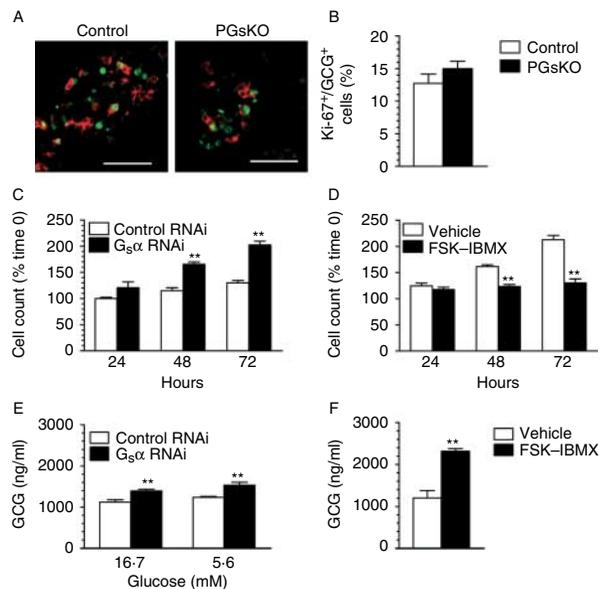


**Figure 5** Prohormone convertase 1/3 (PC1/3) is absent in  $\alpha$ -cells from control and PGsKO mice. Immunostaining of a control and PGsKO islet for (A) either GCG (red) or PC1/3 (green) or for (B) either GCG (red) or PC2 (green). In both cases, the merged images are shown on the right. Bars equal 20  $\mu$ m.

However, the  $\beta$ GsKO model is limited by the fact that G<sub>s</sub>α deficiency occurs in other tissues such as the hypothalamus and pituitary due to the expression of the *Rip2* gene in these tissues. In this work, we extended the study of G<sub>s</sub>α function in pancreatic cells by deleting G<sub>s</sub>α from all major types of pancreatic endocrine and exocrine cells using Pdx1-cre transgenic mice (Gu *et al.* 2002, Jorgensen *et al.* 2007). The utilization of Pdx1-cre mice avoids the confounding effects of G<sub>s</sub>α deficiency in the central nervous system present in  $\beta$ GsKO mice as *Pdx1* is not expressed in the central nervous system. Similar to  $\beta$ GsKO mice, PGsKO mice developed early-onset hypoinsulinemia and hyperglycemia with reduced  $\beta$ -cell mass. However, GCG levels in PGsKO mice remained similar to those in control mice, indicating that G<sub>s</sub>α deficiency likely does not lead to a similar effect on  $\alpha$ -cell function and hormone production as it does in  $\beta$ -cells.

The production and secretion of GCG can be regulated by many factors including nutrients, hormones, and neurotransmitters. Although the net effect of GLP1 on GCG secretion is inhibitory (Ahren 2009), several studies have shown that G<sub>s</sub>α/cAMP-stimulating agents increase both proglucagon gene expression (Drucker *et al.* 1991, Islam *et al.* 2009) and GCG release (Hermansen 1985, Ding *et al.* 1997, Gromada *et al.* 1997, Ma *et al.* 2005) in pancreatic  $\alpha$ -cells. We also found a

stimulatory effect of increased cAMP on GCG release in  $\alpha$ TC1 cells, although the effect of lowering cAMP on GCG release was not significant. Overall, these observations would predict that loss of G<sub>s</sub>α expression in  $\alpha$ -cells of PGsKO mice should lead to a reduction in serum GCG levels. Another factor that might be expected to reduce GCG levels in PGsKO mice is hyperglycemia (Dumonteil *et al.* 2000), as elevated glucose levels do not appear to affect GCG expression levels but do inhibit GCG secretion from  $\alpha$ -cells (Gromada *et al.* 2007). In fact, this may help to explain the moderate reduction in GCG levels in  $\beta$ GsKO mice (Xie *et al.* 2007). However, GCG levels and islet GCG mRNA levels were maintained in PGsKO mice, despite the fact that they have more severe hyperglycemia than  $\beta$ GsKO mice. Low insulin levels would be expected to increase GCG levels in PGsKO mice as insulin has an inhibitory effect on  $\alpha$ -cell activity (Kawamori *et al.* 2009). However, insulin levels were similarly reduced in  $\beta$ GsKO mice as in PGsKO mice (Xie *et al.* 2007).



**Figure 6** G<sub>s</sub>α deficiency leads to increased pancreatic  $\alpha$ -cell proliferation. (A) Staining of a control and PGsKO islet for GCG (red) and Ki-67 (green). (B) Percentage of GCG-staining cells that are also Ki-67+ in islets examined from three control and three PGsKO mice. (C) Proliferation of  $\alpha$ TC1 cells after transfection with either control (scrambled) or G<sub>s</sub>α RNAi ( $n=6$ /group). (D) Proliferation of  $\alpha$ TC1 cells after treatment with either forskolin plus IBMX (FSK-IBMX) or with vehicle alone ( $n=12$ /group). For panels C and D, cell counts are expressed as % of cell number at time 0. (E) GCG secreted into media of  $\alpha$ TC1 cells from hours 24–28 after transfection with either control (scrambled) or G<sub>s</sub>α RNAi in the presence of 16.7 or 5.6 mM glucose ( $n=6$ /group). (F) GCG secreted into media of  $\alpha$ TC1 cells from hours 24–28 after treatment with either forskolin plus IBMX (FSK-IBMX) or with vehicle alone in the presence of 5.6 mM glucose ( $n=6$ /group). Data are mean  $\pm$  S.E.M. (\*\* $P<0.01$  versus control or vehicle). Bars equal 50  $\mu$ m.

Maintenance of normal GCG levels and islet GCG mRNA levels in PGsKO mice in spite of the presence of hyperglycemia and loss of G<sub>s</sub>α expression in α-cells may be partially explained by an increment of the α-cell population. While it is difficult to accurately measure the extent of total α-cell mass alteration in PGsKO mice as all pancreatic endocrine and exocrine cells were morphologically abnormal and therefore could not be used as reference controls for each other, there are morphological characteristics to suggest that α-cells are overrepresented in PGsKO mice compared with other pancreatic cell types. In normal mouse islets, β-cells are the most prominent cell population, and are located in the interior of the islet while α-cells are less numerous and located at the periphery. In comparison with islets from βGsKO mice which maintained the normal architecture (Xie *et al.* 2007), PGsKO islets were small and deformed with a significantly greater number of α-cells within the interior of the islet and a greater α- to β-cell ratio. The increment in α- to β-cell ratio happened relatively early in postnatal life, occurring within the first 4 weeks after birth. These findings are consistent with our results in the αTC1 cell line showing that G<sub>s</sub>α signaling pathways have an antiproliferative effect in α-cells, which would suggest that α-cells in PGsKO mice may have growth advantage due to loss of G<sub>s</sub>α expression. However, using Ki-67/GCG costaining, we were unable to document a clear increase in α-cell proliferation in PGsKO mice, at least at day 7 *post partum*. We also found no evidence for the presence of fetal α-cells in PGsKO islets, suggesting that increased neogenesis was not an important factor. Hyperglycemia and hyperlipidemia also likely contribute to an increased α/β-cell ratio through β-cell toxicity (Poitout & Robertson 2008), although these factors were also present in βGsKO mice (Xie *et al.* 2007).

The mechanisms underlying the antiproliferative and proproliferative effects of G<sub>s</sub>α/cAMP signaling in α- and β-cells respectively are unknown. One possible mechanism may be the stimulatory effect of G<sub>s</sub>α/cAMP signaling on *Pdx1* expression (Jhala *et al.* 2003), as both *in vivo* and *in vitro* studies in β-cells have shown that loss of *Pdx1* expression leads to conversion of insulin-secreting cells to GCG-secreting cells (Ahlgren *et al.* 1998, Lottmann *et al.* 2001, Wang *et al.* 2001). Conversely, ectopic expression of *Pdx1* in cultured α-cells inhibits GCG gene expression (Ritz-Laser *et al.* 2003). However, *Pdx1* is not expressed in mature α-cells, so *Pdx1*-independent mechanisms may be responsible for the antiproliferative effect of G<sub>s</sub>α on α-cells that we observed *in vitro*. Moreover, β-cell-specific G<sub>s</sub>α deficiency did not lead to reduced *Pdx1* expression in βGsKO mice (Xie *et al.* 2007). Further studies will be required to determine whether *Pdx1* plays a role in postnatal α-cell neogenesis, and contributes to enlarged α-cell mass. Recent studies have also shown islet-specific microRNA miR-375 to be a powerful regulator of insulin secretion and pancreatic α- and β-cell mass (Poy *et al.* 2004, 2009). miR-375-null mice develop hyperglycemia with decreased β-cell mass and increased α-cell mass, similar to

PGsKO mice, while overexpression of miR-375 in β-cells leads to a β-cell defect with diabetes (Poy *et al.* 2009).

In conclusion, our studies in βGsKO and PGsKO mice as well as cultured α-cells show that G<sub>s</sub>α/cAMP pathways play important and distinct roles in pancreatic α- and β-cell growth and function. While many studies, including those in βGsKO mice, suggest that these pathways stimulate hormone production in both types of cells, our results show that there are opposite effects of G<sub>s</sub>α/cAMP on cell proliferation (proproliferative in β-cells and antiproliferative in α-cells). In both βGsKO and PGsKO mice, the primary defect leading to hyperglycemia is hypoinsulinemia due to a primary β-cell defect. However, compared with βGsKO mice, PGsKO mice had higher GCG levels and α-cell mass relative to β-cell mass, leading to a higher serum GCG/insulin ratio which may contribute to the more severe hyperglycemia in PGsKO mice. This is similar to what is typically observed in type 2 diabetes mellitus and opposite to the effects of GLP1 administration (Ahren 2009). As G<sub>s</sub>α mediates the signals of GLP1, impaired GLP1 action in α- and β-cells may be an important factor in the observed changes in the islets of type 2 diabetics (Goke 2008). In addition to its role in the endocrine pancreas, morphology in PGsKO mice also indicates an important role in normal maturation of pancreatic exocrine cells.

#### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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