The FOXP1, FOXP2 and FOXP4 transcription factors are required for islet alpha cell proliferation and function in mice

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Abstract

Aims/hypothesis Several forkhead box (FOX) transcription factor family members have important roles in controlling pancreatic cell fates and maintaining beta cell mass and function, including FOXA1, FOXA2 and FOXM1. In this study we have examined the importance of FOXP1, FOXP2 and FOXP4 of the FOXP subfamily in islet cell development and function.

Methods Mice harbouring floxed alleles for *Foxp1*, *Foxp2* and *Foxp4* were crossed with pan-endocrine *Pax6*-Cre transgenic mice to generate single and compound *Foxp* mutant mice. Mice were monitored for changes in glucose tolerance by IPGTT, serum insulin and glucagon levels by radioimmunoassay, and endocrine cell development and proliferation by immunohistochemistry. Gene expression and glucose-stimulated hormone secretion experiments were performed with isolated islets.

Results Only the triple-compound *Foxp1/2/4* conditional knockout (cKO) mutant had an overt islet phenotype, manifested physiologically by hypoglycaemia and hypoglucagonaemia. This resulted from the reduction in glucagon-secreting alpha cell mass and function. The proliferation of alpha cells was profoundly reduced in *Foxp1/2/4* cKO islets through the effects on mediators of replication (i.e. decreased *Ccna2*, *Ccnb1* and *Ccnd2* activators, and increased *Cdkn1a* inhibitor). Adult islet *Foxp1/2/4* cKO beta cells secrete insulin normally while the remaining alpha cells have impaired glucagon secretion.

Conclusions/interpretation Collectively, these findings reveal an important role for the FOXP1, 2, and 4 proteins in governing postnatal alpha cell expansion and function.

Keywords Alpha cell · Function · Hypoglycaemia · Proliferation

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Abbreviations

4W	4-week-old
8W	8-week-old
ARX	Aristaless related homeobox
CDK	Cyclin-dependent kinase
cKO	Conditional knockout
FOX	Forkhead box
MAFA	v-maf Musculoaponeurotic fibrosarcoma oncogene family, protein A [avian]
MAFB	v-maf Musculoaponeurotic fibrosarcoma oncogene family, protein B [avian]
PDX1	Pancreatic and duodenal homeobox 1
STZ	Streptozotocin
TF	Transcription factor

Introduction

The mammalian pancreas is partitioned into two functional compartments: the exocrine pancreas, comprising acinar and ductal cells; and the endocrine pancreas, which has an essential role in maintaining blood glucose homeostasis via secretion of glucose regulatory hormones into the circulation. The endocrine pancreas is comprised of micro-organs, known as the islets of Langerhans, containing five distinct cell populations: beta, alpha, delta, epsilon and pancreatic polypeptide cells that secrete the hormones insulin, glucagon, somatostatin, ghrelin and pancreatic polypeptide, respectively. Type 1 diabetes arises from insulin deficiency resulting from autoimmune-mediated destruction of beta cells, whereas type 2 diabetes is characterised by insulin resistance in peripheral tissues, insulin deficiency and loss of beta cell identity [1]. Although lesser studied, increasing evidence supports the importance of alpha cell dysregulation in the aetiology of both type 1 and type 2 diabetes [2]. Glucagon counteracts insulin action in peripheral tissues to increase hepatic glucose production, thus raising blood glucose levels. Consequently, alpha cell dysfunction confounds glycaemic control in both type 1 and type 2 diabetic patients.

Current efforts are aimed at elucidating mechanisms of endocrine cell lineage determination and expansion as a means of improving protocols for beta cell replacement therapies. In this regard, islet-enriched transcription factors (TFs) have been demonstrated to play an important role in reprogramming terminally differentiated cells and embryonic stem cells (ESCs) into beta-like cells (e.g. v-maf musculoaponeurotic fibrosarcoma oncogene family, protein A [avian] [MAFA], pancreatic and duodenal homeobox 1 [PDX1] and neurogenin 3 [NGN3]) [3–5]. Furthermore, the observation that functional beta cells can arise from differentiated alpha cells has fuelled significant interest in the mechanisms controlling alpha cell development and growth [6]. Interestingly, human alpha cells retain bivalent epigenetic marks on mature

beta cell determination genes, *MAFA* and *PDX1*, suggesting that they are poised for conversion [7]. Success in generating therapeutic beta cells and understanding normal alpha cell development and function will rely on increased knowledge of the fundamental transcriptional processes underlying pancreatic cell specification and islet cell activity.

Many different TF classes have been found to be critical for pancreatic cell development and function [8]. For example, members of the forkhead box (FOX) superfamily of TFs play a critical role in these processes. FOXA2 is an essential regulator that specifies early pancreatic cell fates, activates a master regulator of pancreas development, *Pdx1*, and is required for euglycaemia in neonatal mice [9, 10]. Furthermore, the related FOXM1 controls expression of cell cycle factors and augments beta cell mass under conditions of metabolic stress, including pregnancy and partial pancreatectomy [11, 12]. The function of the FOXP subfamily of regulators is not described in the mammalian pancreas, but FOXP1, FOXP2, FOXP4 were previously reported to be expressed in *Xenopus laevis* pancreas and eye fields during development [13]. These proteins have overlapping and important roles during mouse central nervous system, lung and heart development [14–16]. In contrast, closely related FOXP3 is critical for CD4$^+$ regulatory T cell production in mouse and humans [17], but is not expressed in the mammalian pancreas (Human Protein Atlas, www.proteinatlas.org, accessed 15 January 2015, [18]).

To investigate the impact of FOXP1, FOXP2 and FOXP4 on pancreatic endocrine cell formation and function, we generated conditional knockout (cKO) variants using pan-endocrine *Pax6*-Cre transgenic mice. Interestingly, only the triple-compound *Foxp1/2/4* mutant (*Foxp1/2/4* cKO) manifested changes in glucose homeostasis. These mutant mice were born in expected ratios, but developed postnatal hypoglycaemia and hypoglucagonaemia. *Foxp1/2/4* cKO mice had profoundly reduced numbers of alpha cells (~85% reduction) and decreased alpha, delta and beta cell proliferation, with no observed increase in apoptosis. The proliferative defect is mediated through effects on the expression of cell cycle activators (*Ccna2*, *Ccnb1* and *Ccnd2*) and the cyclin-dependent kinase (CDK) inhibitor, *Cdkn1a*. Furthermore, only glucose-stimulated glucagon secretion, and not insulin secretion, was defective in adult *Foxp1/2/4* cKO islets. We conclude that FOXP1, FOXP2 and FOXP4 are essential for islet alpha cell proliferation and function. This work may impact developing strategies to expand alpha cells for transdifferentiation into therapeutic beta cells as well as understanding the aetiology of alpha cell dysfunction in type 1 and type 2 diabetes.

Methods

Animals The *Foxp1*$^{f/f}$ [19, 20], *Foxp2*$^{f/f}$ [21], *Foxp4*$^{f/f}$ [19, 20] and *Pax6*-Cre [22] mice were maintained on a mixed

(sv129 and C57BL/6) genetic background. Notably, the *Pax6*-Cre transgene is active in developing eye and islet cells, and does not contain the human growth hormone sequences augmenting islet beta cell mass and insulin content (termed Le-Cre in Ashery-Padan et al [22]; personal communication: Ashery-Padan, Tel-Aviv University Tel Aviv, Israel). *Foxp1/2/4* cKO (*Pax6*-Cre, *Foxp1*^{f/f}, *Foxp2*^{f/f}, *Foxp4*^{f/f}) and control littermate mice (*Foxp1*^{f/f}, *Foxp2*^{f/f} and *Foxp4*^{f/f}) were generated by mating *Pax6*-Cre with *Foxp1*^{f/f}, *Foxp2*^{f/f} and *Foxp4*^{f/f} mice. The morning of vaginal plug discovery was considered embryonic day (e)0.5. Besides the islet phenotype described herein, various FOXP TF mutants had defects in eye development (electronic supplementary material [ESM] Table 1 and data not shown). The Vanderbilt University Institutional Animal Use and Care Committee approved all animal experiments. Mice were housed in a conventional facility with a 12 h night/day cycle and had free access to food/water.

Immunohistochemical analyses Staged embryonic and postnatal littermate control and *Foxp1/2/4* cKO pancreatic tissues were fixed in 4% (vol./vol.) paraformaldehyde, paraffin embedded, and cut to 6 μm. The Gift of Hope organ procurement organisation (Itsaca, IL, USA) generously provided the de-identified normal and type 2 diabetic cadaver pancreases, and their use was approved by the institutional review board (normal: reference number H94, 59 years old, BMI 25.4 kg/m^2; type 2 diabetes: reference number H78, 59 years old, BMI 21.2 kg/m^2, 14 year duration of diabetes; reference number H58, 51 years old, BMI 34 kg/m^2, 15 year duration of diabetes). Sections were blocked with 5% (vol./vol.) normal donkey serum in 1% (wt/vol.) BSA/PBS and incubated with primary antibodies overnight at 4°C. Cyanine dye (Cy)2-, Cy3-, or Cy5-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, 1:2,000) were used for fluorescent detection. Peroxidase staining was performed using the DAB substrate kit (Vector Labs, Burlingame, CA) and counterstained with eosin. Images were collected on a Zeiss Axioimager M2 (Jena, Germany) or an Aperio ScanScope (Leica, Buffalo Grove, IL, USA) whole slide scanner. The following primary antibodies were used: insulin-guinea pig (Dako, Carpinteria, CA, USA; A056401-2, 1:1,000); glucagon-mouse (Sigma, St Louis, MO, USA; G2654, 1:2,000); somatostatin-goat (Santa Cruz, Dallas, TX, USA; sc-7819, 1:1,000); Ki67-mouse (BD Pharmingen, San Jose, CA, USA; 550609, 1:1,000); v-maf musculoaponeurotic fibrosarcoma oncogene family, protein B [avian] (MAFB) (Bethyl, Montgomery, TX, USA; IHC-00351,1:1,000); PDX1-goat (kindly provided by C. Wright, Vanderbilt University, 1:20,000); FOXP1 (1:1,000) and FOXP4 (1:1,000) antibodies (E. Morrisey).

Hormone cell quantification Six sections (~240 μm apart) from 4-week-old (4W) control and *Foxp1/2/4* cKO (*n*=3)

mice were analysed for hormone$^+$ mass using DAB substrate and counterstained with eosin. The percentage of alpha, beta and delta cell area (relative to whole tissue area) was multiplied by the pancreatic mass to obtain hormone mass. For Ki67$^+$ proliferative cell counting, the total number of hormone$^+$ and Ki67$^+$ co-positive cells were counted and divided by the total number of hormone$^+$ cells.

Islet isolation and RNA preparation Postnatal day (p)10 *Foxp1/2/4* cKO and control littermates (*n*=4) were used for pancreatic islet isolations. Islet RNA was isolated using TRIzol (Invitrogen) and treated with DNAse1. RNA quality was analysed by the Vanderbilt Technology for Advanced Genomics Core Facility using an Agilent 2100 Bioanalyzer. The cDNA made using the iScript cDNA synthesis kit (Bio-Rad, 170-8890, Hercules, CA, USA) was subjected to quantitative (q)PCR analysis in a LightCycler 480 II Real-Time PCR System. Gene expression changes between *Foxp1/2/4* cKO and control islet RNA was normalised to *Gapdh* mRNA levels and fold changes calculated using the $\Delta\Delta C_t$ method. Primers sequences are available upon request.

Islet glucagon and insulin secretion assays Islets from 4W *Foxp1/2/4* cKO and control mice were incubated overnight in islet culture media at 37°C (RPMI1640, 10% [vol./vol.] FBS, penicillin/streptomycin, 11 mmol/l D-glucose). The next day, islets were incubated for 1 h at 37°C in a baseline solution (1.25 mmol/l CaCl$_2$, 0.6 mmol/l MgS0$_4$:7H$_2$O, 0.6 mmol/l KH$_2$PO$_4$, 2.4 mmol/l KCl, 64.0 mmol/l NaCl, 20 mmol/l HEPES pH 7.9, 5 mmol/l NaHCO$_3$, 2.8 mmol/l D-glucose). The secretion of insulin (25 islets per reaction for control and *Foxp1/2/4* cKO) and glucagon (25 control islets and 150 *Foxp1/2/4* cKO islets) was measured after a 45 min incubation at 37°C in stimulation media (baseline: 2.8 mmol/l or 16.7 mmol/l glucose for insulin secretion or 1.0 mmol/l glucose, 10 mmol/l L-arginine for glucagon). Secretion supernatant fraction was collected and hormone content measured from islets lysed in 1.5% HCl, 70% ethanol (vol./vol.). An RIA was used to determine insulin content in the Vanderbilt Hormone Assay and Analytical Services Core, and an ELISA (RayBiotech, Norcross, GA, USA; EIA-GLU) for glucagon. Data are presented as the percentage of secreted hormone over total hormone content (% islet content).

IPGTT and plasma serum collection The 4W mice (*n*≧8) were fasted for 6 h and blood glucose levels were determined using a FreeStyle glucometer. A preparation of 2 mg dextrose/g body weight in sterile PBS was injected intraperitoneally. Blood glucose levels were measured at 15, 30, 60 and 120 min post-injection. Serum was isolated and subjected to glucagon and insulin analysis following a 16 h fast.

Statistical analysis All data are expressed as mean±standard error of the mean (SEM) and were tested for statistical significance using the Student's two-tail t test.

Results

Endocrine-specific removal of FOXP1, FOXP2 and FOXP4 results in hypoglycaemia and hypoglucagonaemia FOX proteins have important roles in tissue-specific gene programming in several developing organs including the brain, liver, heart, lung, gut and pancreas. It was previously reported that FOXP1, FOXP2 and FOXP4 are expressed in the mucosal and epithelial layers of the developing intestine by e12.5 in mice [13, 23]. We investigated the spatio–temporal pancreatic expression of FOXP1 and FOXP4 proteins by immunofluorescence. Both FOXP1 and FOXP4 are broadly expressed throughout the PDX1$^+$ pancreatic endoderm at e12.5 and e15.5 (ESM Fig. 1a, b). Furthermore, FOXP1 and FOXP4 are ubiquitously expressed throughout the endocrine compartment by 3 weeks of age (ESM Fig. 1c) and are present in beta, alpha and delta cells (ESM Fig. 2a–d). FOXP3 was not investigated in this study as there is no evidence for expression in the mammalian pancreas (Human Protein Atlas, www.proteinatlas.org accessed 15 January 2015, [18]), while satisfactory FOXP2 staining could not be obtained using commercially available antibodies.

To evaluate the contributions of *Foxp1*, *Foxp2* and *Foxp4* to endocrine pancreas development and function, we generated single and compound conditional knockouts using pan-endocrine-driven *Pax6-Cre* mice. Recombination of floxed *Foxp1* and *Foxp4* alleles was achieved in hormone-producing cells by e15.5 as demonstrated by loss of immuno-reactive nuclei in *Foxp1/2/4* cKO hormone$^+$ cells (ESM Fig. 3a, b). Furthermore, *Foxp1*, *Foxp2* and *Foxp4* mRNA levels were significantly reduced in p10 islets (ESM Fig. 3c). As with conditional ablation of other islet TFs (e.g. *Pdx1*, *Nkx6-1*, *Mafa* [24–26]), we hypothesised that FOXP1, FOXP2 and/or FOXP4 was involved in maintaining blood glucose levels. Interestingly, only the triple FOXP gene knockout mutant, *Foxp1/2/4* cKO, displayed a glucose homeostasis phenotype (Fig. 1), presumably because of functional redundancy of the remaining FOXP factors in the double- and single-gene knockout models. Notably, the pancreas to body weight ratio and overall body weights were unchanged in 4W *Foxp1/2/4* cKO mice relative to controls (ESM Fig. 3d–e).

The physiological consequence of removing FOXP1/2/4 on glucose clearance was determined at 4 weeks by IPGTT. Strikingly, mutant mice were glucose tolerant (Fig. 1a, b) though they displayed a significant and non-sex-specific reduction in fasting blood glucose levels relative to controls (Fig. 1c), which persisted in 8-week-old (8W) adults
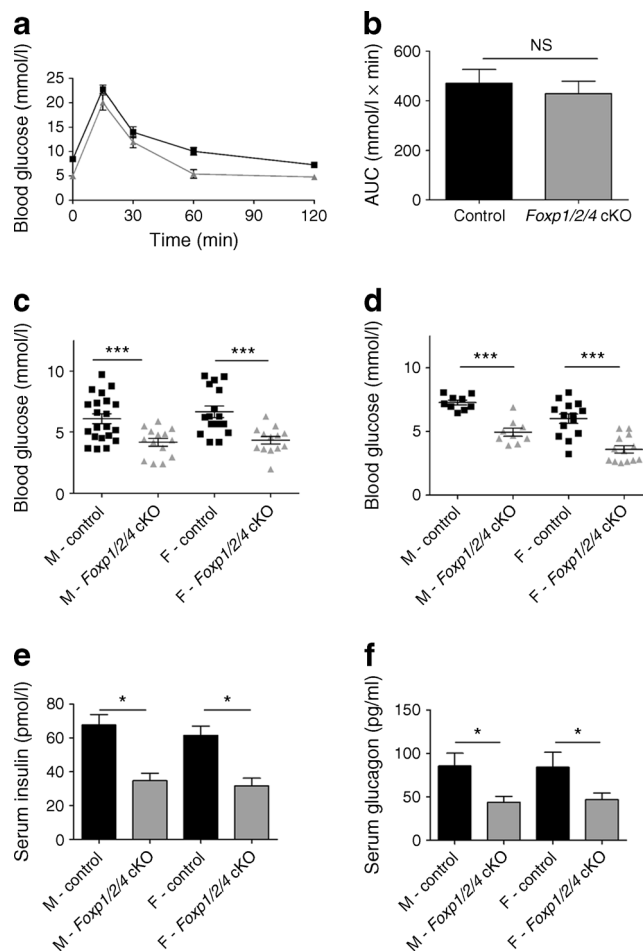

Fig. 1 *Foxp1/2/4* cKO mice are glucose tolerant, but have hypoglycaemia and hypoglucagonaemia. (**a**) 4W control and *Foxp1/2/4* cKO mice were subjected to IPGTT following 6 h fasting. Blood glucose measurements were taken at the indicated times. Results are expressed as mean±SEM (n=13 control [black lines]; n=8 *Foxp1/2/4* cKO [grey lines]). (**b**) AUC analysis of IPGTT from (**a**) (after normalising control and *Foxp1/2/4* cKO time point 0) shows no statistical difference in glucose clearance. (**c, d**) Blood glucose levels from 4W (**c**) and 8W (**d**) control and *Foxp1/2/4* cKO mice after a 6 h fast (n=21, male control; n=14, male *Foxp1/2/4* cKO; n=16, female control; n=13, female *Foxp1/2/4* cKO). (**e, f**) Serum insulin (**e**) and glucagon (**f**) levels from 4W control and *Foxp1/2/4* cKO mice following a 16 h fast (n=6, male control; n=7, male *Foxp1/2/4* cKO; n=10, female control; n=6, female *Foxp1/2/4* cKO). *p<0.05 and ***p<0.001 after two-tailed Student's t test. F, female; M, male

(Fig. 1d). We next determined whether the hypoglycaemic phenotype was a consequence of defects in insulin- and/or glucagon-secretion levels. Serum was collected from 4W control and *Foxp1/2/4* cKO mice following a 16 h fast (to exacerbate any potential problems). Serum insulin and glucagon levels were both diminished by nearly 50% (Fig. 1e, f). The decreased insulin is likely attributed to a normal physiological response to the fasting hypoglycaemia [27], as glucose tolerance in *Foxp1/2/4* cKO mice was normal (Fig. 1a, b). In contrast, the mechanism(s) governing glucagon secretion appeared dysregulated.

***Foxp1/2/4* cKO mice have a dramatic loss of islet alpha cells** Immunohistochemical analysis of 4W mouse pancreases showed a significant loss of glucagon^{+} alpha cells in *Foxp1/2/4* cKO pancreases, and no striking differences in beta or delta cells (Fig. 2a–f). Alpha, beta and delta cell mass analysis showed an ~85% decrease in alpha, an ~50% decrease in delta and no change in beta cells (Fig. 2g–i). The average number of insulin[+]/glucagon[+]/somatostatin^{+} cells per islet in *Foxp1/2/4* cKO pancreases was significantly decreased, suggesting the islets are smaller in size (ESM Fig. 4a). Furthermore, the reduction of the alpha cell population in 3- and 6-month-old *Foxp1/2/4* cKO pancreases shows that this change is permanent (ESM Fig. 4b, c). While it is unclear if serum somatostatin levels are reduced in parallel with delta cell numbers, this would likely not contribute to the hypoglycaemia and hypoglucagonaemia observed, as somatostatin suppresses insulin and glucagon release [28, 29]. Notably, alpha, beta and delta cell numbers were relatively unchanged in e18.5 *Foxp1/2/4* cKO pancreases, demonstrating that the change in islet cell production largely occurs postnatally (ESM Fig. 5a–c).

Islet alpha, beta and delta cell proliferation is decreased and cell cycle genes are dysregulated in *Foxp1/2/4* cKO islets Proliferative and apoptosis levels in *Foxp1/2/4* cKO pancreases were monitored to obtain insight into the mechanism(s) underlying the smaller islet size and severe loss of alpha cells. The level of the Ki67 proliferative marker was negatively affected in p10 *Foxp1/2/4* cKO alpha, beta and delta cells (Fig. 3a–e), but not in e18.5 pancreases (ESM Fig. 5d–f). There was no change in TUNEL apoptosis staining

in these samples, and hormone co-expression was not observed at e18.5, p10, 4W or 8W (data not shown). Overall, these data suggest that alpha cell deficiency reflects a postnatal proliferative defect, and not cell death or transdifferentiation.

Next, RNA was isolated from hand-picked p10 control and *Foxp1/2/4* cKO islets to examine for changes in cell cycle regulatory genes. Notably, the mRNA levels of *Ccna2*, *Ccnb1* and *Ccnd2* were significantly reduced, whereas the CDK inhibitor, *Cdkn1a*, was upregulated (Fig. 3f). FOXP1 was previously shown to reduce *Cdk1na* expression [30]. Collectively, these observations demonstrate that FOXP1/2/4 regulate the proliferative capacity of pancreatic endocrine cells postnatally.

Glucagon secretion, but not insulin, is abnormal in adult *Foxp1/2/4* cKO islets The hypoglycaemic *Foxp1/2/4* cKO phenotype was interesting as only 3% of the mouse islet alpha cell population is apparently required to maintain euglycaemia and normoglucagonaemia [31], yet the *Foxp1/2/4* cKO mutant retained roughly 15% of these cells (Fig. 2g). Islets from 4W control (25 islets per reaction) and *Foxp1/2/4* cKO (150 islets per reaction, to account for the significant decrease in alpha cell numbers) mice were challenged with 1 mmol/l glucose plus 10 mmol/l arginine (a potent glucagon secretagogue [32]) to directly determine if glucagon secretion was defective. These conditions were required because glucagon secretion was not observed in *Foxp1/2/4* cKO islets treated with 1 mmol/l glucose alone or equivalent number of islets to control (data not shown). The percentage of glucagon secreted from *Foxp1/2/4* cKO islets in the presence of arginine

Fig. 2 *Foxp1/2/4* cKO mice have dramatically reduced islet alpha cell mass. (**a**–**f**) Immunohistochemical analysis of glucagon (**a**, **d**), insulin (**b**, **e**) and somatostatin (**c**, **f**) of 4W control and *Foxp1/2/4* cKO pancreases. Scale bars, 40 μm. (**g**–**i**) Alpha (**g**), beta (**h**) and delta (**i**) cell mass was obtained from 4W control and *Foxp1/2/4* cKO pancreases (*n*=3 mice per genotype; six slides per hormone per mouse were analysed). *$p<0.05$ and ***$p<0.001$ after two-tailed Student's *t* test
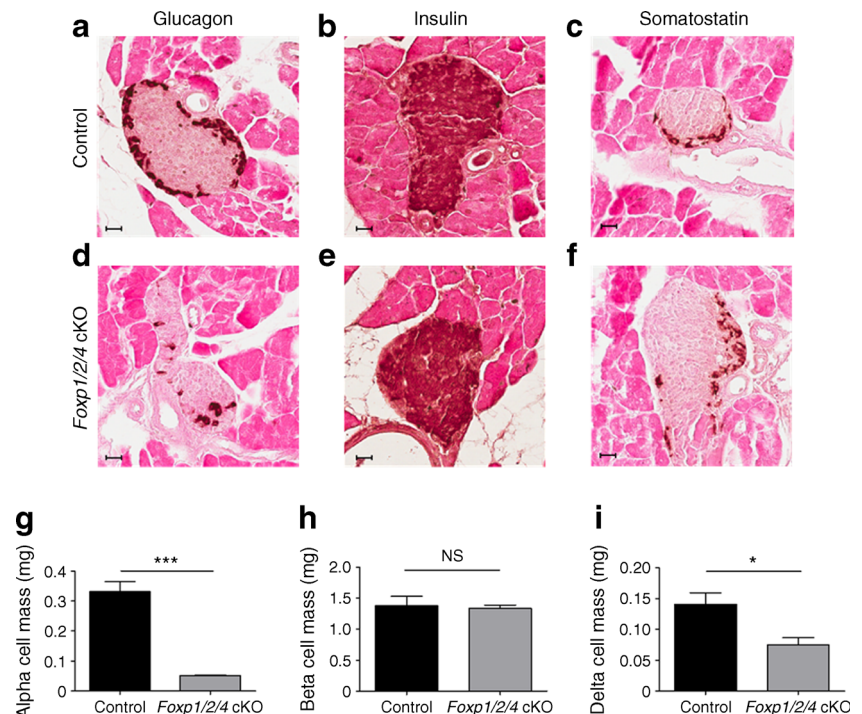
Fig. 3 Islet alpha, beta and delta cell proliferation is decreased and cell cycle gene mRNAs are altered in *Foxp1/2/4* cKO islets. (**a, b**) p10 control and *Foxp1/2/4* cKO pancreases were stained for insulin, Ki67 and glucagon (**a**) or insulin, Ki67 and somatostatin (**b**). In (**a**): white arrowheads, glucagon$^+$/Ki67$^+$; yellow arrowheads, Glucagon$^+$/Ki67$^-$. In (**b**): white arrowheads, somatostatin$^+$/Ki67$^+$; yellow arrowheads, somatostatin$^+$/Ki67$^-$. Scale bars, 20 μm. (**c–e**) Percentage of proliferating alpha (**c**), beta (**d**) and delta cells (**e**) from control and *Foxp1/2/4* cKO. (**f**) Quantitative PCR analysis of p10 control and *Foxp1/2/4* cKO islet RNA. mRNA levels in *Foxp1/2/4* cKO islets were normalised against *Gapdh* and compared directly with normalised control levels arbitrarily set at 1. Black bars, control; grey bars, *Foxp1/2/4* cKO; $n=4$. *$p<0.05$; **$p<0.01$ and ***$p<0.001$ after two-tailed Student's *t* test. GCG, glucagon; Ins, insulin; SST, somatostatin
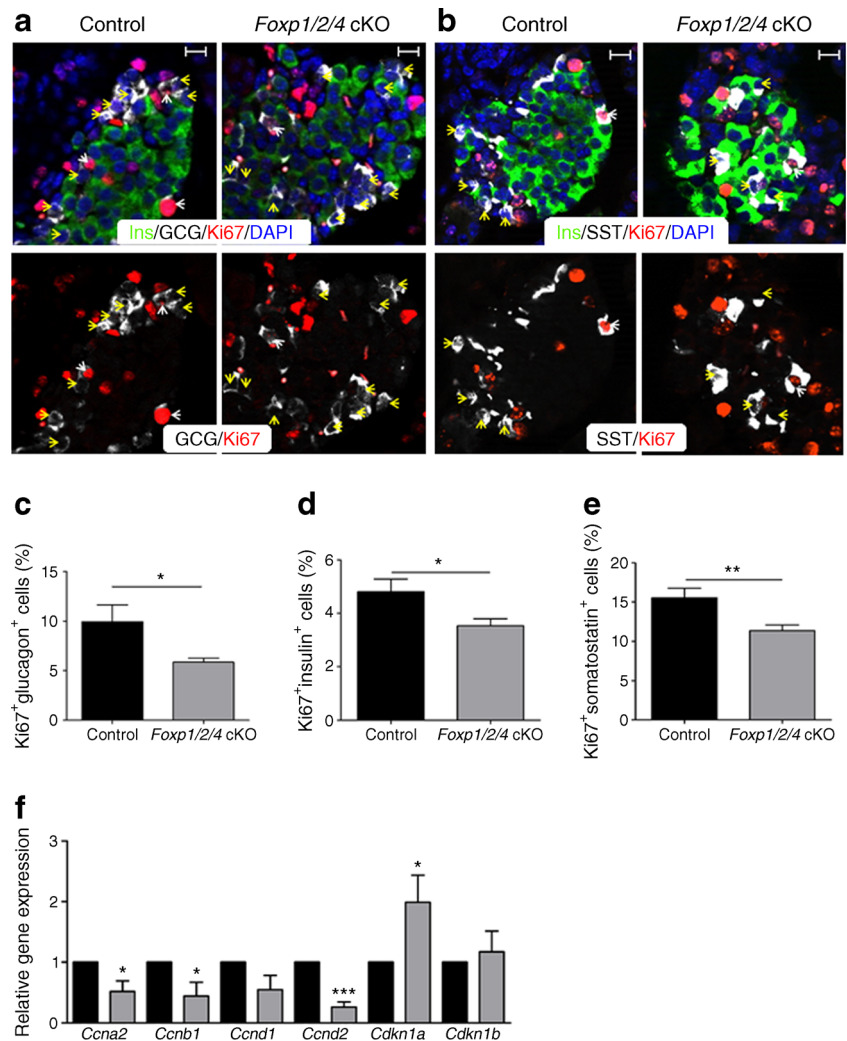
was significantly decreased relative to controls (Fig. 4b). Notably, the reduction of glucagon content per islet (Fig. 4d) was very similar to the reduction in glucagon$^+$ cell number (Fig. 2g) in *Foxp1/2/4* cKO mice. The alpha cell dysfunction in this mutant could also result from loss of MAFB, a potent driver of glucagon expression and secretion [33], or the likelihood that the alpha cells still express FOXP. However, immunofluorescence analysis demonstrated that glucagon$^+$ cells are FOXP1/P4$^-$ and MAFB$^+$ (ESM Fig. 6, Fig. 4a). Importantly, *Foxp1/2/4* cKO islets exposed to 2.8 mmol/l (low) and 16.7 mmol/l (high) glucose secreted similar amounts of insulin (Fig. 4c) and the insulin content per islet was unchanged (Fig. 4e), highlighting the specific nature of the glucagon secretion defects in *Foxp1/2/4* cKO islets.

Discussion

The glucagon-secreting alpha cell has recently moved to the forefront of efforts to combat type 1 and type 2 diabetes.

Importantly, there are only a few regulators known to specifically impact alpha cell mass or function (aristaless related homeobox [ARX], POU domain, class 3, transcription factor 4 [BRN4], MAFB, FOXA1 and FOXA2 [33–37]); however, the TFs directly impacting alpha cell proliferation have yet to be identified. For example, ARX is critical for maintaining alpha cell identity during development and in neonates, but does not influence alpha cell proliferation [38]. Here, we have shown that conditional removal of FOXP1/2/4 from developing islet endocrine cells significantly impacts glucose homeostasis through reduced postnatal alpha cell proliferation and a dramatic reduction of the alpha cell population.

Fasting hypoglucagonaemia and hypoglycaemia were found in *Foxp1/2/4* cKO mice, which contain ~15% of their alpha cell population. In contrast, Thorel et al concluded that approximately 3% of normal alpha cells are sufficient to maintain euglycaemia [31]. Comparatively, this suggested that most of the remaining alpha cells in *Foxp1/2/4* cKO islets were dysfunctional and unable to regulate glucagon secretion, which we found to be true (Fig. 4). It is widely accepted that
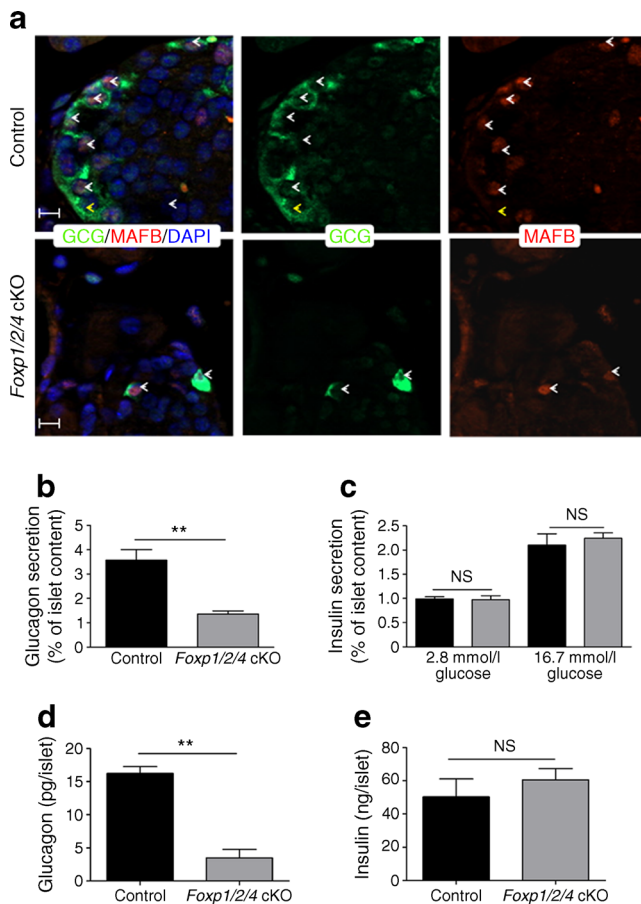
Fig. 4 *Foxp1/2/4* cKO islet alpha cells have defective glucagon secretion. (**a**) 4W control and *Foxp1/2/4* cKO pancreases were monitored by immunofluorescence for glucagon and MAFB. White arrowheads, glucagon⁺/MAFB⁺; yellow arrowheads, glucagon⁺/MAFB⁻. Scale bars, 20 μm. (**b, c**) Islets isolated from 4W control (25 islets/reaction) and *Foxp1/2/4* cKO (25 islets/reaction, insulin; 150 islets/reaction, glucagon) were incubated with either (**b**) 1 mmol/l glucose+10 mmol/l arginine (glucagon secretion) or (**c**) with 2.8 mmol/l or 16.7 mmol/l glucose (insulin secretion) for 45 min (black bars, control; grey bars, *Foxp1/2/4* cKO). $n=4$. Data are presented as the percentage of secreted hormone over total hormone content (% of islet content). (**d, e**) Total glucagon (**d**) and insulin (**e**) content per islet was calculated in 4W control and *Foxp1/2/4* cKO islets. **$p<0.01$ after two-tailed Student's t test. GCG, glucagon

hyperglycaemia in diabetes is a consequence of the loss and/or dysfunction of both the islet alpha and beta cell populations [39, 40]. Without the proper counter-regulatory role of insulin, glucagon action on the liver is unregulated and abnormal hepatic glucose output persists, thus unfavourably exacerbating hyperglycaemia. Mouse models of diabetes induced by streptozotocin (STZ), which is particularly toxic to rodent beta cells, illustrate the action of glucagon in the progression of diabetes. Lee et al have shown that inhibiting total glucagon action impedes the development of STZ-induced diabetes [41]. In contrast, the previously described acute alpha cell ablation model suggests that a few remaining alpha cells mediate normal glucagon signalling and cannot prevent STZ-

induced diabetes [31]. As the remaining 15% of *Foxp1/2/4* cKO alpha cells have dysregulated glucagon secretion, it will be interesting to examine if these mutant mice are resistant to STZ-induced diabetes. Importantly, *Foxp1/2/4* cKO hypoglycaemia is presumably not due to the modest decrease in delta cell mass because somatostatin suppresses both insulin and glucagon signalling [28, 29].

Animal models with glucagon action blocked by genetic inhibition of the glucagon receptor [42, 43] or reduced active glucagon synthesis [44] have alpha cell hyperplasia. In striking contrast, *Foxp1/2/4* cKO mice lack compensatory alpha cell hyperplasia, suggesting that levels of circulating glucagon are either sufficient to prevent alpha cell hyperplasia or, more likely, the remaining alpha cells lack the proliferative capacity. If the latter, it would be expected that alpha cell expansion would not occur upon blocking glucagon signalling in *Foxp1/2/4* cKO mice via treatment with monoclonal antibodies specific for glucagon receptor [45], or by crossing the *Foxp1/2/4* cKO line with the glucagon receptor knockout mice *(Gcgr⁻/⁻)* [42]. Based on earlier findings [30], it is likely that FOXP1, P2, and P4 recruitment of the nuclear receptor co-repressor 2 (NCOR2/SMRT) is required for inhibiting *Cdkn1a* promoter activity in islet cells.

The levels of a subset of islet-enriched TFs are reduced in human type 2 diabetic islets, specifically MAFA, MAFB, PDX1 and NK6 homeobox 1 (NKX6.1) [46]. This led us to consider that decreased alpha cell FOXP levels might also contribute to the altered glucagon secretion levels in *Foxp1/2/4* cKO islets. However, FOXP4 levels appear unchanged in both the alpha and beta cell population (ESM Fig. 7), suggesting that at least FOXP4 levels do not change in type 2 diabetic islets. FOX proteins are particularly important in the developing pancreas and islet function. In this regard, conditional inactivation of *Foxa* alleles from developing and mature beta cells negatively affects genes involved in metabolic processes, ion transport functions, as well as beta cell enriched enzymes [10, 47]. Furthermore, FOXA1 and FOXA2 were recently shown to control the expression of genes essential for glucagon production and secretion, including: glucagon, *MafB*, *Brn4* (also known as *Pou3f4*), *Pck2*, *Nkx2-2*, *Kir6.1* (also known as *Kcnj8*), *Sur1* (also known as *Abcc8*), and *Gipr* [47]. As the FOX family of TFs bind similar DNA response elements, we suspect there is overlap in FOXP1/2/4 and FOXA1/2 gene targets in alpha cells.

Contribution statement JMS, CSH and RS were responsible for the study design, data acquisition and analysis and wrote the manuscript. LB, MG, JF, MH, IS and BZS contributed to data acquisition and analysis. CAF, SEF and EEM contributed to the interpretation of data. All authors contributed to the critical revision of the manuscript and approved the final version to be published. RS is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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