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ARTICLE

Critical role of c-Kit in beta cell function: increased insulin secretion and protection against diabetes in a mouse model

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Abstract
Aims/hypothesis The receptor tyrosine kinase, c-Kit, and its ligand, stem cell factor, control a variety of cellular processes, including pancreatic beta cell survival and differentiation as revealed in *c-Kit*^{Wv} mice, which have a point mutation in the c-Kit allele leading to loss of kinase activity and develop diabetes. The present study further investigated the intrinsic role of c-Kit in beta cells, especially the underlying mechanisms that influence beta cell function.
Methods We generated a novel transgenic mouse model with *c-KIT* overexpression specifically in beta cells (*c-KitβTg*) to further examine the physiological and functional roles of c-Kit in beta cells. Isolated islets from these mice were used to investigate the underlying molecular pathway of c-Kit in beta cells. We also characterised the ability of c-Kit to protect animals from high-fat-diet-induced diabetes, as well as to rescue *c-Kit*^{Wv} mice from early onset of diabetes.
Results *c-KitβTg* mice exhibited improved beta cell function, with significantly improved insulin secretion, and increased beta cell mass and proliferation in response to high-fat-diet-induced diabetes. *c-KitβTg* islets exhibited upregulation of: (1) insulin receptor and IRSs; (2) Akt and glycogen synthase kinase 3β phosphorylation; and (3) transcription factors important for islet function. *c-KIT* overexpression in beta cells also rescued diabetes observed in *c-Kit*^{Wv} mice.
Conclusions/interpretation These findings demonstrate that c-Kit plays a direct protective role in beta cells, by regulating glucose metabolism and beta cell function. c-Kit may therefore represent a novel target for treating diabetes.

Keywords Beta cell function · Beta cell-specific *c-KIT* transgenic mice · *c-Kit Wv* mutation · High-fat-diet-induced diabetes · Insulin secretion

Abbreviations

c-KitβTg mice	Transgenic mouse model with *c-KIT* overexpression specifically in beta cells
eGFP	Enhanced green fluorescent protein
GSIS	Glucose-stimulated insulin secretion
GSK3β	Glycogen synthase kinase 3β
HFD	High-fat diet
IPGTT	Intraperitoneal glucose tolerance test
IPITT	Intraperitoneal insulin tolerance test
MAFA	v-Maf musculoaponeurotic fibrosarcoma oncogene family, protein A (avian)
PDX1	Pancreatic and duodenal homeobox 1

Z. C. Feng and J. Li contributed equally to this work.

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PI3K	Phosphoinositide-3-kinase
RIP	Rat insulin promoter
SCF	Stem cell factor

Introduction

Therapeutic strategies aimed at repopulating insulin-producing cells show great potential for restoring glycaemia in diabetes. Extensive studies have focused on ways to facilitate the differentiation of progenitor cells into beta cells [1–3], and to maintain their viability and function [4, 5]. It has been shown that the haematopoietic stem cell marker c-Kit is important in the development and function of islets of Langerhans, especially in support of beta cell proliferation, maturation and survival [6–12]. Upon binding to the stem cell factor (SCF), c-Kit undergoes dimerisation and autophosphorylation, followed by the recruitment of downstream signalling molecules to induce subsequent cell proliferation, differentiation, survival and migration [13, 14].

c-Kit is found in fetal and adult rodent pancreatic islets [6–8, 10]. We have demonstrated that human and rat fetal pancreatic ductal epithelial cells producing c-Kit display high proliferation and level of SCF [11, 12, 15]. After pancreatic duct ligation in the rat, c-Kit is activated in ductal cells during islet cell neogenesis, along with an increase of pancreatic and duodenal homeobox 1 (PDX1) levels [16]. Increased c-Kit and PDX1 abundance is also observed in islets from pancreases of streptozotocin-induced diabetic rats, suggesting that c-Kit is involved in beta cell regeneration [17].

Manipulation of rat islets in cell culture has further revealed that c-Kit-enriched cells can give rise to new beta cells that secrete insulin in a glucose-responsive fashion [18]. Fetal rat islets treated with SCF had a significant increase in insulin levels and DNA content [7]. Furthermore, INS-1 cells responded to SCF with increased cell proliferation [10]. Downregulation of *c-KIT* (also known as *KIT*) expression in human islet–epithelial clusters using small interfering RNA leads to significantly reduced mRNA and protein levels of PDX1 and insulin in conjunction with decreased cell proliferation, as well as increased cell death [12]. These studies reveal a remarkable correlation between the functions of c-Kit and enhanced beta cell development and function.

Homozygous *c-Kit*-null (*c-Kit*$^{W/W}$) mutant mice, display relatively normal islet morphology, but die shortly after birth and are not available for further functional studies [19]. We have previously characterised *c-Kit*^{Wv} mice, which have a point mutation in the c-Kit allele, disrupting receptor function. These mice exhibit a loss of beta cell mass and proliferation, resulting in early onset of diabetes [20]. In the present study, we describe a novel transgenic mouse model with *c-KIT* overexpression specifically in beta cells (*c-Kit*β*Tg*), which we used to investigate the underlying mechanism of c-Kit activity in beta cells, and to further delineate the physiological and functional role of c-Kit in normal, high-fat diet (HFD)-induced diabetic and *c-Kit*^{Wv} mice.

Methods

Generation and maintenance of c-Kitβ*Tg mice* Human *c-KIT* cDNA (2.9 kb pairs) followed by the IRES2-enhanced green fluorescence protein (eGFP) (Clontech, Palo Alto, CA, USA) sequence was inserted into the pKS/rat insulin promoter (RIP) plasmid [21] to generate the transgene. Transgenic mice (*c-Kit*β*Tg*) were generated using C57BL6/J embryos and identified by PCR [22] using the primers globin 5 and c-Kit, and further confirmed by a second set of primers, eGFP3 and globin 3 (Electronic supplementary material [ESM] Table 1). Five RIP–*c-KIT* transgenic founders were obtained and bred with C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME, USA) to establish independent mouse lines. Initial characterisation revealed that the mice displayed similar, if not identical, patterns of transgene expression and phenotype that eliminate any positional effect due to the location of transgene integration. We therefore used offspring from two independent transgenic lines for subsequent detailed analyses. All mice had free access to standard diet. The protocol used was approved by the University of Western Ontario Animal User Subcommittee in accordance with the guidelines of the Canadian Council of Animal Care.

Body weight, food intake and in vivo metabolic studies Body weight, blood glucose levels, intraperitoneal glucose tolerance tests (IPGTT) and intraperitoneal insulin tolerance tests (IPITT) were performed using *c-Kit*β*Tg* mice and their wild-type littermates from 4 to 40 weeks of age as described previously [20, 23]. Food intake was monitored at 6 weeks of age for a 2 week period. Blood glucose levels were examined under non-fasting, and after 4 h and overnight fasting (16 h) with free access to water. For the IPGTT and IPITT, an intraperitoneal injection of glucose (D-(+)-glucose; dextrose; Sigma, St Louis, MO, USA) at a dosage of 2 mg/g body weight or of human insulin (Humalin; Eli Lilly, Toronto, ON, Canada) at 1 U/kg body weight was administered and blood glucose levels were examined. The AUC was used to quantify responsiveness [20, 23].

Generation of HFD-induced diabetes and c-Kitβ*Tg:Wv mouse models* The HFD study was initiated at 6 weeks of age, with *c-Kit*β*Tg* and wild-type male mice receiving HFD

chow (D12492; Research Diets, New Brunswick, NJ, USA) for 4 weeks, followed by in vivo metabolic studies [24, 25]. Breeding *c-KitβTg* with *c-Kit*^{Wv} mice yielded four different mouse genotypes: (1) wild-type; (2) *c-KitβTg*; (3) *c-Kit*^{Wv} and (4) *c-KitβTg:Wv*. Presence of the *c-Kit Wv* allele was identified by the animal's characteristic fur pigmentation [20]; the *c-KitβTg* allele was identified by PCR. In vivo metabolic studies were performed on male mice groups at 8 weeks of age.

INS-1 cell culture INS-1 (832/13) cells were cultured in RPMI 1640 containing 10% FBS vol./vol., as described previously [26]. For the exogenous SCF study, INS-1 cells were cultured in RPMI 1640 plus 1% (wt/vol.) BSA, and treated for 24 h with SCF (50 ng/ml; ID Laboratory, London, ON, Canada), SCF plus wortmannin (100 nmol/l; Sigma) or non-SCF (control) [15]. Cells were collected for immunofluorescence staining and protein extraction. Cell proliferation was examined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and Ki67 staining [15]. Three different cell passages were used for each set of experiments, representing $n=3$.

In vivo and ex vivo glucose-stimulated insulin secretion assay and insulin ELISA For the in vivo glucose-stimulated insulin secretion **(**GSIS) assay, blood samples were collected following 4 h of fasting (0 min), and at 5 and 35 min after glucose loading [20, 23]. For the ex vivo GSIS assay, freshly isolated islets from mice were hand-picked and incubated for 1 h with RPMI 1640 plus 0.5% (wt/vol.) BSA containing 2.2 or 22 mmol/l glucose [20]. Insulin secretion was measured using an ultrasensitive (mouse) insulin ELISA (Alpco, Salem, NH, USA). A static GSIS index was calculated [20, 26]. The insulin content of isolated islets was measured and expressed as nanograms per microgram DNA.

Immunofluorescence and morphometric analyses Pancreases were fixed in 4% (wt/vol.) paraformaldehyde, and sections prepared from the entire length of the pancreas and stained with primary antibodies of dilutions as listed (ESM Table 2). Quantitative evaluations of alpha cell and beta cell mass were performed using Openlab image software (Improvision, Lexington, MA, USA) [20, 23]. Beta cell proliferation and levels of different transcription factors were determined by double immunofluorescence staining and quantification from at least 12 random islets per pancreatic section [20, 23].

RNA extraction and real-time RT-PCR RNA was extracted from isolated islets of *c-KitβTg* and wild-type mice with or without HFD using a kit (miRNeasy; Qiagen, Germantown, MD, USA) [23]. Sequences of PCR primers are listed in ESM Table 3. Real-time PCR analyses were performed using a kit (iQ SYBR Green Supermix; Bio-Rad Laboratories, Mississauga, ON, Canada). Relative levels of gene expression were calculated and normalised to the internal gene, 18S rRNA, with at least four repeats per age per experimental group [20, 23].

Protein extraction and Western blot analysis Islet proteins from *c-KitβTg* and wild-type pancreases were extracted in NP-40 lysis buffer [20, 26]. An equal amount of protein from each group was fractionated by 10% (wt/vol.) SDS-PAGE, transferred on to a nitrocellulose membrane (Bio-Rad) and incubated with primary antibodies as listed (ESM Table 2). Proteins were detected using western blot detection reagents (ECL-Plus; Perkin Elmer, Wellesley, MA, USA) and imaged using Versadoc Imaging System (Bio-Rad). Bands were densitometrically quantified by Image lab software (Bio-Rad).

Statistical analysis Data are expressed as means±SEM. Statistical significance was determined by unpaired Student's t test or ANOVA followed by Fisher's least significant differences post-hoc test. Differences were considered to be statistically significant at $p<0.05$.

Results

Generation of c-KitβTg mouse model We generated *c-KitβTg* mice on a C57BL/6J background using the RIP to direct overexpression of human *c-KIT* specifically in beta cells; human *c-KIT* was also linked with an IRES-eGFP to facilitate monitoring of transgene expression. Immunoblot analysis of eGFP in pancreas, liver, muscle, brain and isolated islet protein lysates showed positive signals in the pancreas and islets of *c-KitβTg* mice (Fig. 1a). The RIP did not lead to any aberrant transgene expression. Fluorescence microscopy showed that eGFP was present in islets freshly isolated from *c-KitβTg*, but not in those from wild-type littermates (Fig. 1b). This was further confirmed by immunofluorescence staining for eGFP and insulin (Fig. 1b). Human *c-KIT* mRNA was only detected in *c-KitβTg* mouse islets (Fig. 1c), a finding corroborated by elevated islet c-Kit levels (Fig. 1d) and c-Kit immunofluorescence staining (Fig. 1e). We also examined the abundance of SCF, a ligand of c-Kit, in *c-KitβTg* mouse islets (ESM Fig. 1). A significant increase of *Scf* (also known as *Kitl*) mRNA was noted in *c-KitβTg* islets compared with wild-type islets (ESM Fig. 1a); however, protein levels of SCF were not statistically different between *c-KitβTg* and wild-type islets (ESM Fig. 1b). Taken together, these results indicate that overexpression of human *c-KIT* under the transcriptional control of the RIP in *c-KitβTg* mice is specific to beta cells.

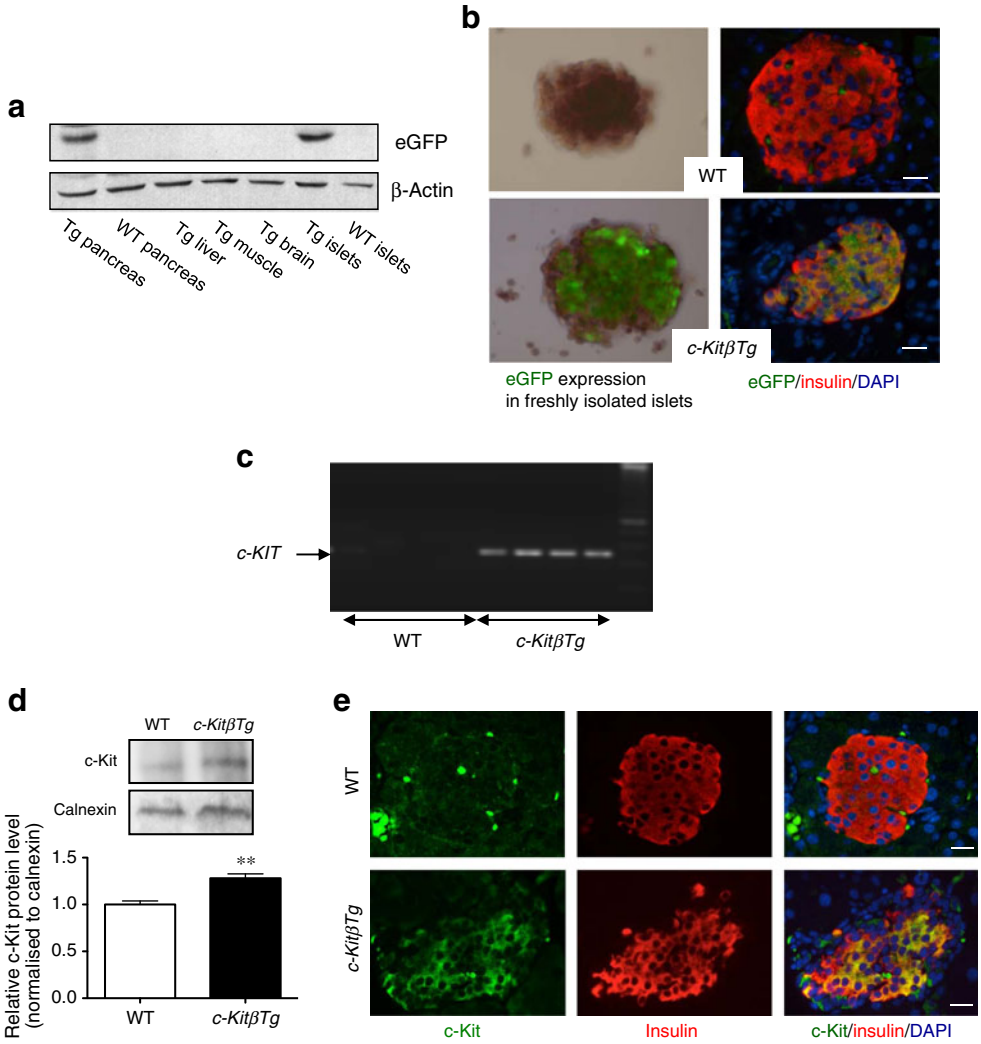

Fig. 1 Generation of C57BL/6J transgenic mice with *c-KIT* overexpression specifically in beta cells. **a** Western blot analysis of eGFP abundance in tissues as indicated and isolated islets (representative blots shown). **b** Abundance of eGFP on freshly isolated islets and pancreatic sections of 4-week-old male wild-type (WT) and *c-KitβTg* mice by double immunofluorescence staining for eGFP (green) with insulin (red). **c** RT-PCR analysis of human *c-KIT* mRNA in isolated islets of wild-type and *c-KitβTg* mice at 4 weeks of age. **d** Western blot analysis of c-Kit levels in isolated islets of wild-type and *c-KitβTg* mice at 8 weeks of age; values are mean±SEM; $n=3$; $^{**}p<0.01$ vs wild-type. **e** Immunofluorescence staining for c-Kit (green) on pancreatic sections from 4-week-old wild-type and *c-KitβTg* male mice. Nuclei were stained with DAPI (blue). Representative images are shown. Scale bars (**b**, **e**), 25 μm

Improved glucose tolerance and insulin secretion in c-KitβTg mice There were no significant differences in body weight during the 40 weeks of observation, with no changes in food intake, between *c-KitβTg* and wild-type mice (ESM Fig. 2). No significant differences in 4 h fasting plasma insulin and blood glucose levels (Fig. 2a, b, ESM Fig. 3a, b) were detected between *c-KitβTg* and wild-type mice at 8 weeks of age; however, the overnight fasting blood glucose levels were significantly lower in *c-KitβTg* mice than in wild-type littermates (Fig. 2b, ESM Fig. 3b). The IPGTT showed a relatively similar response capacity in *c-KitβTg* and wild-type male mice at 4 weeks (ESM Fig. 4a). However, significantly improved glucose tolerance was observed in male *c-KitβTg* mice at 8 and 20 weeks of age, along with significant decreases in the AUC during the IPGTT (Fig. 2c, ESM Fig. 4b); no significant change was observed in *c-KitβTg* female mice (ESM Fig. 3c, ESM Fig. 4b). No changes were observed in insulin tolerance between the experimental groups (Fig. 2d, ESM Fig. 3d). Furthermore, the effect of *c-KIT* overexpression on beta cell insulin secretion was significant, with isolated islets from male and female *c-KitβTg* mice releasing significantly more insulin than wild-type controls in response to 22 mmol/l glucose (Fig. 2e, ESM Fig. 3e). This was associated with higher insulin content in *c-KitβTg* islets (Fig. 2f). Since female *c-KitβTg* mice did not exhibit a significant alteration in the IPGTT, we focused on male *c-KitβTg* mice for detailed characterisation.

Increased islet transcription factors, beta cell mass and proliferation in c-KitβTg mice To further characterise the functional role of c-Kit in beta cells, we examined levels of transcription factors essential for islet growth, function and morphology in *c-KitβTg* and wild-type mice. At 8 weeks of age, we observed a slightly increased islet number (Fig. 3a), with a significant increase in the number of small (<500 μm^2) and large islets (>10,000 μm^2; Fig. 3b) in the pancreas of *c-KitβTg* mice. No significant alterations in alpha cell mass were detected between wild-type and *c-KitβTg* mice (Fig. 3c). However, beta cell mass in *c-KitβTg*

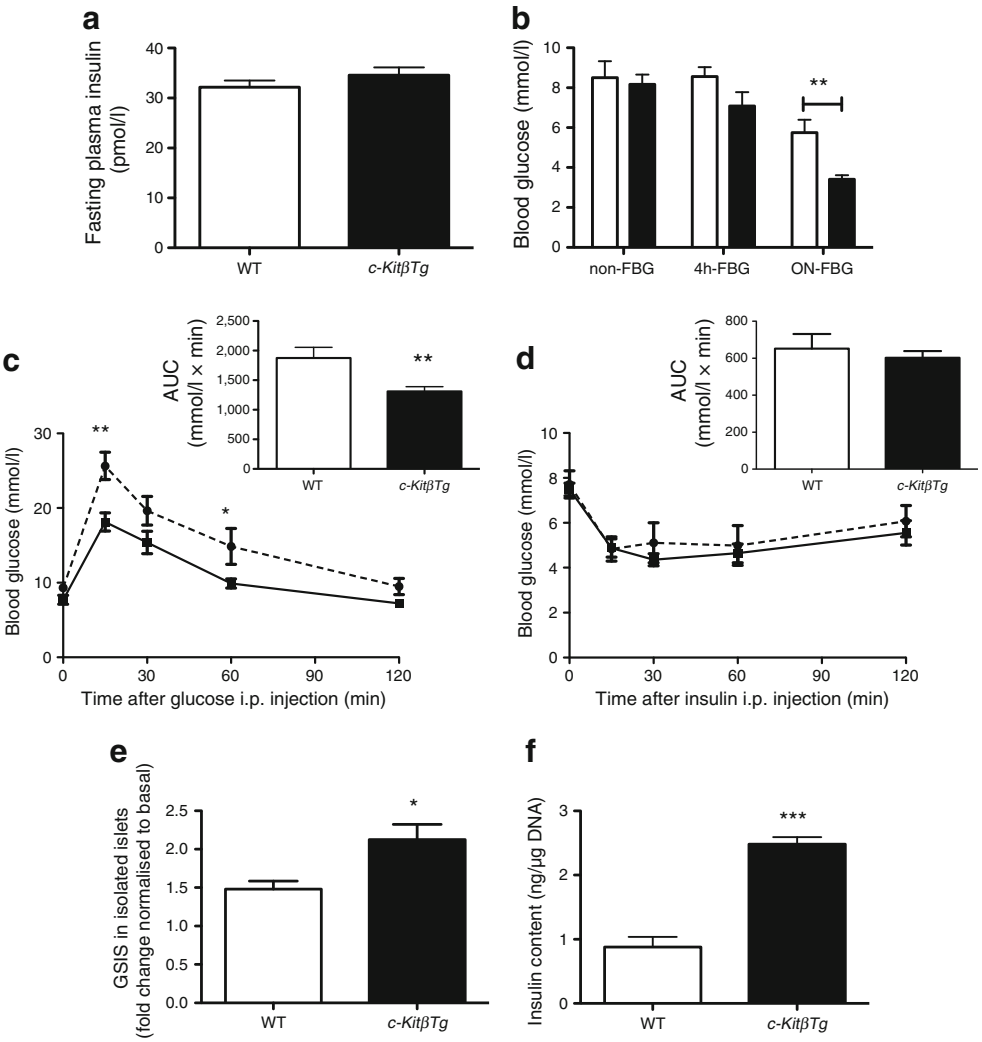

Fig. 2 Glucose tolerance and insulin secretion in 8-week-old *c-KitβTg* male mice. Fasting plasma insulin (**a**) and blood glucose **(b)** in wild-type (WT, white bars) and *c-KitβTg* (black bars) mice. **c** IPGTT and (**d**) IPITT in wild-type (black circles) and *c-KitβTg* (black squares) mice. The glucose responsiveness of the corresponding experimental groups is shown as the AUC of the IPGTT (**c**, insert) or IPITT (**d**, insert) graphs, with units of (mmol/l x min). Data (**a**–**d**) are expressed as mean±SEM; n=5–18; *p<0.05 and **p< 0.01 vs wild-type mice. **e** Insulin secretion of isolated islets from wild-type and *c-KitβTg* mice in response to a 22 mmol/l glucose challenge; data are expressed as fold change normalised to basal (2.2 mmol/l glucose) secretion; n=3–4. **f** Insulin content in isolated islets; data are normalised to DNA content; n=7; *p<0.05 and ***p<0.001 vs wild-type mice

mice was increased by 1.6-fold compared with wild-type (Fig. 3d). Increased beta cell mass was associated with an increase in beta cell proliferation in *c-KitβTg* mice (Fig. 3e, ESM Fig. 5). Quantitative real-time RT-PCR analysis of *Pdx1*, *Neurod1*, *Mafa*, *Pax6*, *Nkx2-2* and *Nkx6-1* showed significantly increased mRNA levels in *c-KitβTg* mice (Fig. 3f), with elevated intensity of the corresponding signals in the islets of *c-KitβTg* mice (ESM Fig. 6a). The expression of *Glut2* (also known as *Slc2a2*), *Ins1* and *Ins2*, *Gcg* and *Glp1r* mRNA in isolated *c-KitβTg* islets was also significantly increased (Fig. 3g) with relatively enhanced Glut2 and glucagon-like peptide 1 receptor (Glp1R) staining (ESM Fig. 6b).

Increased abundance of insulin receptor, phosphorylated IRS1/2 and their downstream signalling molecules in the islets of c-KitβTg mice Our previous study showed that the *c-Kit*^{Wv} mutation caused beta cell dysfunction [20] and that this mutation was associated with downregulation of the Akt–glycogen synthase kinase 3β (GSK3β)–cyclin D1 pathway [27]. We therefore examined whether signalling molecules up- and downstream of the phosphoinositide-3-kinase (PI3K)–Akt pathway or cell survival signals are altered in *c-KitβTg* islets. Interestingly, significant upregulation of *Insr* and *Irs1* mRNA was observed in *c-KitβTg* islets (Fig. 4a), with an increase of insulin receptor and phosphorylated IRS1/2 protein levels (Fig. 4b, c) compared with wild-type mice. Protein levels of phospho-Akt (Fig. 4d), phospho-GSK3β (Fig. 4e), cyclin D1 (Fig. 4f) and PDX1 (Fig. 4g) were also significantly increased in *c-KitβTg* islets compared with islets isolated from wild-type littermates. This observation was further characterised using the INS-1 cell line. When treated with exogenous SCF, INS-1 cells exhibited significantly increased cell proliferation (ESM Fig. 7a, b), with upregulation of the phospho-Akt–GSK3β pathway (ESM Fig. 7c), together with increased insulin receptor and phosphorylated IRS1/2 protein levels (ESM Fig. 7d). The increase in insulin receptor and phospho-IRS1/2 protein levels was regulated by the PI3K–Akt signalling pathway.

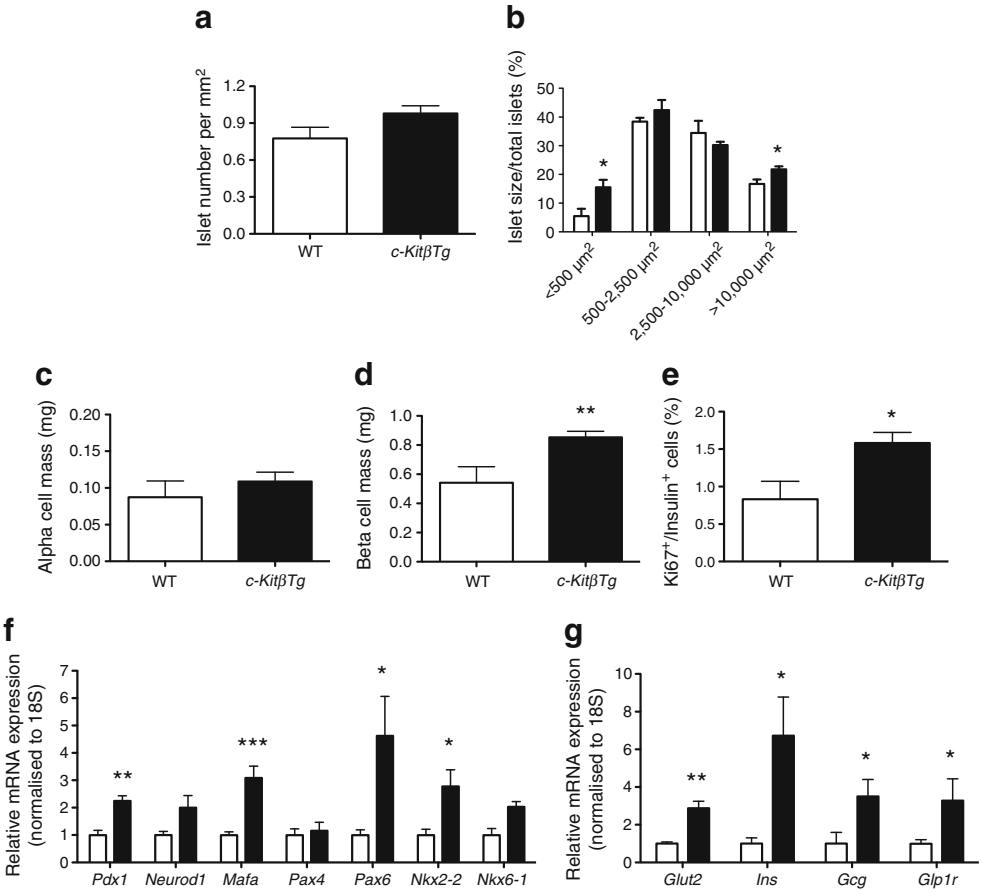

Fig. 3 Islet morphology in *c-KitβTg* male mice. Morphometric analysis of islet number (**a**), islet size (**b**), alpha cell mass (**c**), beta cell mass (**d**) and the percentage of $Ki67^+$ in beta cells (**e**) in wild-type (WT, white bars) and *c-KitβTg* (black bars) mice at 8 weeks of age. **f** Transcription factor and (**g**) islet gene expression in *c-KitβTg* and wild-type mice at 8 weeks of age, as analysed by quantitative RT-PCR. Data are expressed as means±SEM; n=4–8; *p<0.05, **p<0.01 and ***p<0.001 vs wild-type mice

c-KitβTg mice tolerated HFD-induced diabetes To investigate how beta cell-specific *c-KIT* overexpression would affect glucose homeostasis of mice under diabetic conditions, male *c-KitβTg* and wild-type littermates were subjected to a HFD. Similar food intake and body weight gains were observed in both experimental groups (Fig. 5a). Interestingly, after 4 weeks of HFD, the fat pad mass was significantly smaller (Fig. 5b) and the 4 h fasting glucose level was lower in *c-KitβTg* HFD mice than in wild-type HFD mice (Fig. 5c). Glucose intolerance was noted in wild-type HFD littermates, but was significantly improved in *c-KitβTg* HFD mice (Fig. 5d), with similar results for insulin response in wild-type HFD as determined by the IPITT (Fig. 5e). In vivo GSIS assays revealed slightly higher basal and 5 min plasma insulin levels in *c-KitβTg* HFD mice (Fig. 5f); moreover, at 35 min after glucose stimulation, the plasma insulin release was significantly reduced in wild-type HFD compared with *c-KitβTg* HFD mice (Fig. 5f). GSIS on isolated islets further demonstrated that *c-KitβTg* HFD islet insulin secretion was significantly increased in response to 22 mmol/l glucose compared with wild-type HFD islets (Fig. 5g). The insulin content was also significantly increased in *c-KitβTg* HFD islets (Fig. 5h).

Improvements in glucose tolerance in *c-KitβTg* HFD mice were associated with significantly increased *Pdx1*, *Mafa*, *Ins*, *Insr* and *Irs1* mRNA, as well as phospho-IRS1/2 levels in *c-KitβTg* HFD islets (Fig. 6a). The improved metabolic phenotype of *c-KitβTg* HFD mice was associated with a significant increase in the number of pancreatic islets and beta cell mass compared with wild-type HFD mice (Fig. 6b, d). A slightly increased alpha cell mass was observed in *c-KitβTg* HFD mice; however, the increase was not statistically significant (Fig. 6c). Nevertheless, a significant increase in beta cell mass was detected, with increased beta cell proliferation in *c-KitβTg* HFD mice compared with the wild-type HFD group (Fig. 6e).

$c\text{-}Kit^{Wv}$ mice with specific overexpression of c-Kit in beta cells displayed normal glucose metabolism $c\text{-}Kit^{Wv}$ mice were bred with *c-KitβTg* mice to determine whether *c-KIT* overexpression in beta cells could rescue animals with the *c-Kit Wv* mutation from early onset of diabetes. Wild-type, *c-KitβTg* and *c-KitβTg:Wv* mice exhibited similar fasting blood glucose levels, with a significant improvement in fasting blood glucose levels being noted in *c-KitβTg:Wv* compared with $c\text{-}Kit^{Wv}$ mice (Fig. 7a). *c-KitβTg:Wv* mice also had a similar glucose tolerance capacity to that of the wild-type and *c-KitβTg* groups, and also displayed significant decreases in the AUC (Fig. 7b). The IPITT revealed no differences between the experimental groups (Fig. 7c).

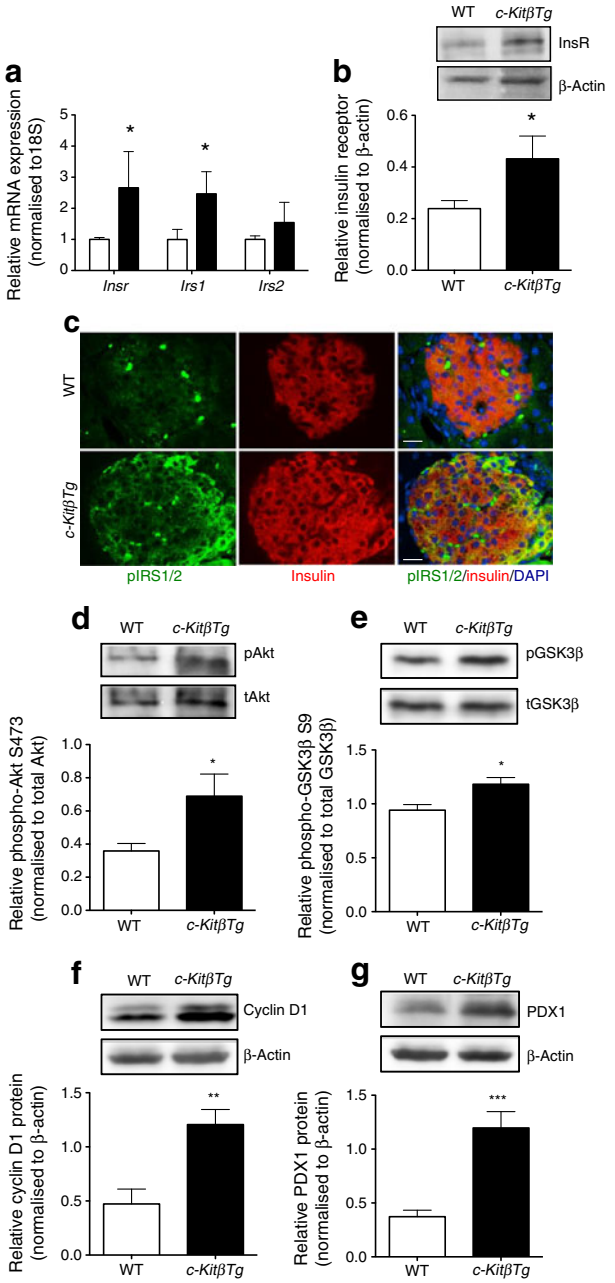

Fig. 4 Levels of phosphorylated Akt/GSK3β signalling and downstream signalling molecules in *c-KitβTg* male mice. **a** Quantitative RT-PCR analysis of *Insr*, *Irs1* and *Irs2* in isolated islets of wild-type (WT, white bars) and *c-KitβTg* (black bars) mice at 8 weeks of age. Data are expressed as mean±SEM; n=3–4. **b** Western blot analysis for insulin receptor (InsR) and (**c**) double immunofluorescence staining for phospho-IRS1/2 (green) and insulin (red) in pancreatic sections from 8-week-old wild-type and *c-KitβTg* mice. Nuclei were stained by DAPI (blue); representative images shown; scale bars 25 μm. **d** Western blot analysis of phosphorylated (p) and total (t) Akt, (**e**) pGSK3β and tGSK3β, (**f**) cyclin D1 and (**g**) PDX-1 abundance in isolated islets of wild-type and *c-KitβTg* mice at 8 weeks of age. Representative blots are shown. Data are normalised to total protein or loading control and expressed as means±SEM; n=4–5. *p<0.05, **p<0.01 and ***p<0.001 vs wild-type mice

Significantly improved GSIS was observed at 5 and 35 min in *c-KitβTg:Wv* mice compared with *c-Kit*^{Wv} mice (Fig. 7d). To further confirm islet function, an ex vivo GSIS study was conducted, in which insulin secretion in response to a 22 mmol/l glucose challenge in *c-KitβTg:Wv* islets was similar to that in wild-type and *c-KitβTg* islets (Fig. 7e), but significantly higher than that of *c-Kit*^{Wv} islets (Fig. 7e). Insulin content in isolated *c-KitβTg:Wv* islets was also higher than in *c-Kit*^{Wv} islets, but significantly lower than in controls (Fig. 7f). These results indicate that the *c-Kit* point mutation is directly responsible for defective beta cell function in *c-Kit*^{Wv} mice and that *c-KIT* overexpression was able to preserve beta cell function in *c-Kit*^{Wv} mice.

Discussion

Here, we demonstrated that *c-KIT* overexpression in beta cells confers improved glucose metabolism by enhancing insulin secretion, and increasing beta cell mass and proliferation, probably through activation of the PI3K–Akt signalling pathway. When *c-KitβTg* mice were subjected to a HFD, they displayed resistance to HFD-induced glucose intolerance and preserved beta cell function relative to wild-type littermates. Moreover, *c-KitβTg* mice were protected against early onset of diabetes. These results clearly indicate that c-Kit is intrinsic to beta cell function and proliferation. This effect is mediated by the regulation of key beta cell transcription factors (e.g. PDX1 and v-maf musculoaponeurotic fibrosarcoma oncogene family, protein A (avian) [MAFA]) and possibly through interaction with the insulin receptor to activate downstream PI3K–Akt signalling.

Compared with wild-type mice, *c-KitβTg* mice had significantly lower overnight fasting blood glucose levels, improved glucose tolerance and enhanced GSIS. The improvement in glucose metabolism of *c-KitβTg* mice was associated with an increase in beta cell mass and proliferation, as well as in *Glut2* and *Glp1r* expression. These results corroborate our previous finding of glucose intolerance in *c-Kit*^{Wv} mice [20]. We monitored the *c-KitβTg* mice up to 40 weeks of age and did not detect any abnormal cell growth or islet tumours, indicating that beta cell-specific *c-KIT* overexpression does not lead to malignancy. We detected a significant increase in *Scf* mRNA, but only a modest increase in the corresponding protein. This is likely to be due to our western blotting technique, which could only detect membrane-associated, not soluble, SCF. Nevertheless, the concentration of SCF may be in excess and is sufficient to interact with the increased number of c-Kit receptors (~25%) to transduce markedly enhanced intracellular signals in *c-KitβTg* mice. Our data indicate that c-Kit overabundance in beta cells enhances glucose tolerance and beta

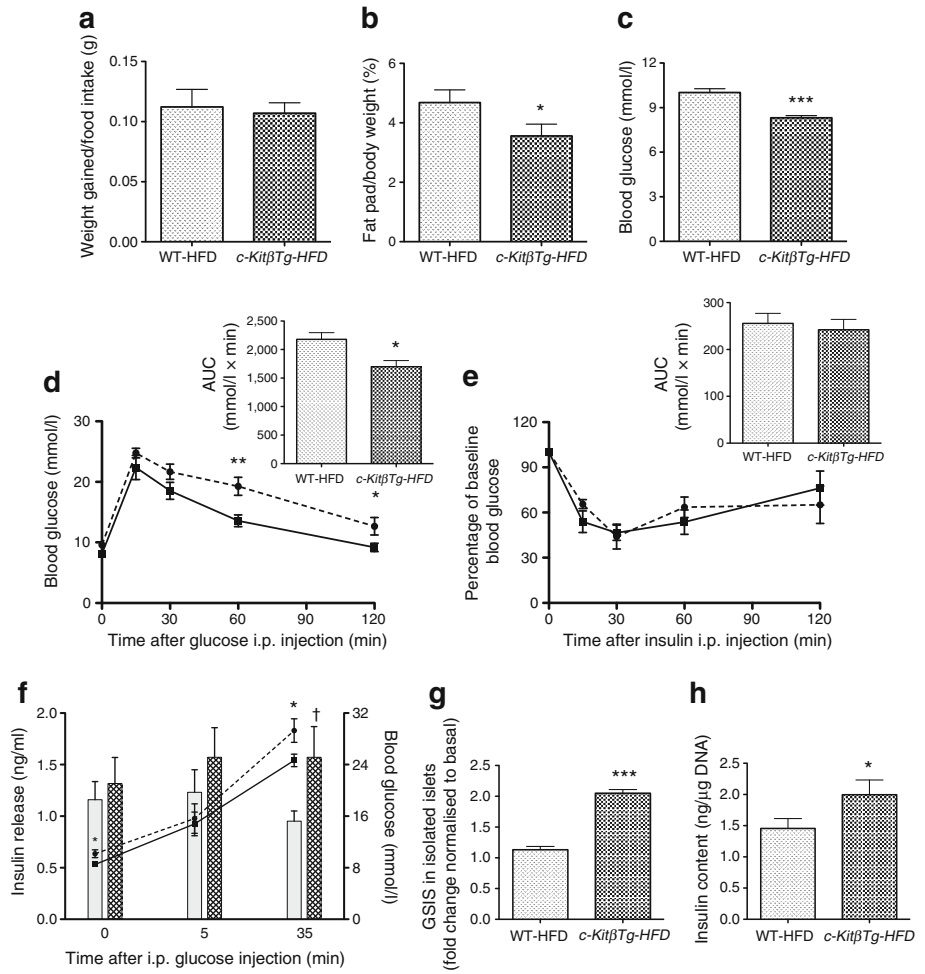

Fig. 5 Effect of a HFD on *c-KitβTg* mice. **a** Weight gained per food intake, (**b**) fat pad per body weight and (**c**) 4 h fasting blood glucose levels in *c-KitβTg* HFD and wild-type (WT) HFD mice. **d** IPGTTs and **(e)** IPITTs were performed on wild-type HFD (black circles) and *c-Kit*βTg HFD (black squares) mice. The glucose responsiveness of the corresponding experimental groups is shown as the AUC of the IPGTT (**d**, insert) or IPITT (**e**, insert) graphs, with units of (mmol/l x min). Data (**a**–**e**) are expressed as means±SEM; n=7–8. **f** In vivo GSIS of wild-type HFD and *c-KitβTg* HFD mice (n=6). Lighted bars, WT-HFD insulin; darked bars, *c-KitβTg* HFD insulin; black circles, WT-HFD glucose; black squares, *c-KitβTg* HFD glucose. **g** GSIS is improved in isolated islets from *c-KitβTg* HFD mice in response to a 22 mmol/l glucose challenge; data are expressed as fold change normalised to basal (2.2 mmol/l glucose) secretion (n=5). **h** Insulin content in isolated islets. Data are normalised to DNA content (n=10). *p<0.05, **p<0.01, ***p<0.001 and $^{\dagger}p$<0.05 vs wild-type HFD mice

cell function in males, while the effect is less significant in female mice. These sex-related differences in glucose metabolism are consistent with our previous results using *c-Kit*^{Wv} mice and conditional β1 integrin knockout mice [20, 23], and have also been described in other mouse models [28, 29]. We observed that isolated islets from female *c-KitβTg* mice had significantly enhanced insulin secretion in response to a high glucose challenge compared with wild-type females, indicating that the sex-related differences may involve other pathways, such as the contribution of oestrogen to glucose homeostasis in female rodents.

Significantly increased *Pdx1* mRNA and protein abundance was observed in *c-KitβTg* mice. We previously reported that SCF-stimulated c-Kit receptor activity leads to increased *PDX1* mRNA expression in human fetal islet–epithelial clusters [15], while *c-Kit*^{Wv} mice showed a significant reduction in *Pdx1* expression in islets [20]. It is well documented that PDX1 is integral to normal pancreas development and beta cell function [30]. In addition to beta cell proliferation [31], *Pdx1* expression is also required for modulation of insulin gene expression and glucose metabolism [32]. Our results showed that *c-KitβTg* islets exhibited high PDX1 levels and had increased beta cell proliferation and mass, confirming a correlation between PDX1 and islet beta cell replication. Indeed, the enhanced islet insulin secretion in response to a high glucose challenge and the improved glucose tolerance observed in *c-KitβTg* mice may also be due to increased islet PDX1 abundance. Interestingly, significant upregulation of

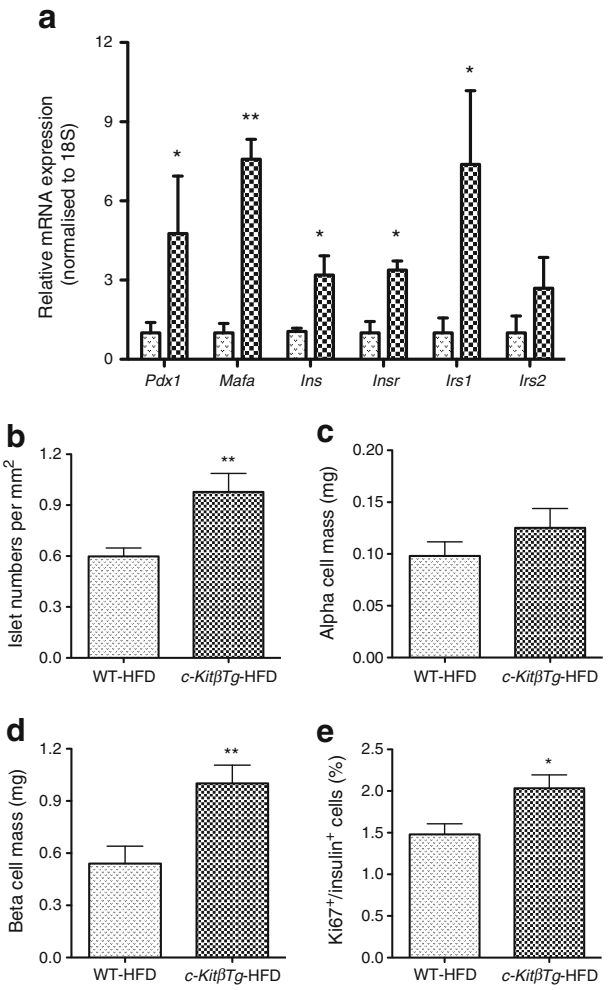

Fig. 6 Effect of a HFD on islet morphology of *c-KitβTg* mice. **a** Quantitative RT-PCR analysis of genes as indicated on isolated islets of wild-type HFD (light bars) and *c-KitβTg* HFD (dark bars) mice. Data are expressed as means±SEM; $n=3$. **b** Morphometric analysis of islet number, (**c**) alpha cell mass, (**d**) beta cell mass and (**e**) percentage of $Ki67^+$ in beta cells from wild-type HFD and *c-KitβTg* HFD mice. Data are expressed as means±SEM; $n=6$–7. **a**–**e** $*p<0.05$ and $**p<0.01$ vs wild-type HFD mice

Mafa mRNA was observed in both sexes of *c-KitβTg* mice. MAFA binds to the C1 element of the insulin gene to modulate insulin gene transcription and enhance beta cell maturation [33]. *Mafa*-null mice had a defect only in adult islet architecture and beta cell activity, while MAFA overproduction enhanced beta cell insulin biosynthesis and secretion through upregulation of important beta cell genes, including *Pdx1*, *Neurod1*, *Nkx6-1* and *Glp1r* [34]. Moreover, overexpression of *MAFA* in neonatal rat beta cells led to enhanced glucose-responsive insulin secretion and beta cell maturation [35]. Thus, improved glucose metabolism and insulin secretion in *c-KitβTg* mice may be due to increased c-Kit receptor stimulation of islet *Pdx1* and *Mafa* expression.

The molecular mechanisms associated with the phenotypic changes observed in *c-KitβTg* mice include significant upregulation of phospho-Akt and phospho-GSK3β in beta cells. Our previous in vitro study on human fetal islet–epithelial clusters demonstrated that increased c-Kit × SCF interactions resulted in upregulation of PI3K–Akt, but not of mitogen-activated protein kinase (MAPK)–extracellular signal-regulated kinase (ERK) pathway signalling [15]. In the current study, we also observed significant increases in insulin receptor and phospho-IRS1/2 levels, in addition to the increase in Akt and GSK3β phosphorylation together with upregulation of cyclin D1 in islets isolated from *c-KitβTg* mice. These results suggest the following possible mechanisms, whereby c-Kit might stimulate beta cell function and proliferation: (1) via direct activation of the Akt–GSK3β–cyclin D1 pathways [15, 36]; (2) via direct or indirect interaction between c-Kit and the insulin receptor and IRSs to establish cross-talk; and/or (3) by positive feedback of the insulin receptor via PI3K–Akt signalling in association with PDX1- and Mafa-induced insulin secretion. Islets of beta cell-specific *Gsk3β* ablated mice had increased beta cell mass with lower fasting blood glucose, along with improved glucose tolerance and GSIS [37]. Furthermore, beta cell-specific *Gsk3β* knockout mice had increased islet IRS-1 and -2 levels with significantly improved beta cell function [37], which is in agreement with our observations. It has been reported that insulin secreted by pancreatic beta cells positively regulates its own biosynthesis by enhancing insulin gene transcription in an autocrine manner via the beta cell insulin receptor and downstream signalling pathways [38, 39]. Specific knockout of *Insr* in pancreatic beta cells results in defective insulin secretion, similar to that observed in type 2 diabetes [40]. Taken together, these results suggest that Akt–GSK3β–cyclin D1 signalling downstream of c-Kit is essential for beta cell function.

HFD treatment is detrimental to beta cell function and insulin sensitivity in mice [24, 41], and leads to impaired glucose tolerance due to insulin resistance and insufficient beta cell insulin secretion [24, 41–43]. While *c-KitβTg* and wild-type mice maintained on a HFD showed similar food intake and weight gain, significantly less fat pad formation was observed in the former after 4 weeks on the HFD. Importantly, *c-KitβTg* HFD mice exhibited significantly improved glucose tolerance and GSIS, supporting the notion that c-Kit has a direct effect on beta cell function. Islets of *c-KitβTg* HFD mice also showed significantly increased levels of insulin receptor and insulin signals, suggesting a possible secondary mechanism, whereby c-Kit stimulates beta cell function by cross-talking to the insulin receptor via PI3K–Akt signalling. Therefore, overabundance of c-Kit in beta cells plays a primary role by increasing beta cell mass and proliferation, as well as a secondary role by increasing insulin secretion via upregulation of the insulin receptor through the PI3K–Akt signalling pathway, which enables *c-KitβTg* HFD mice to tolerate HFD-induced diabetes.

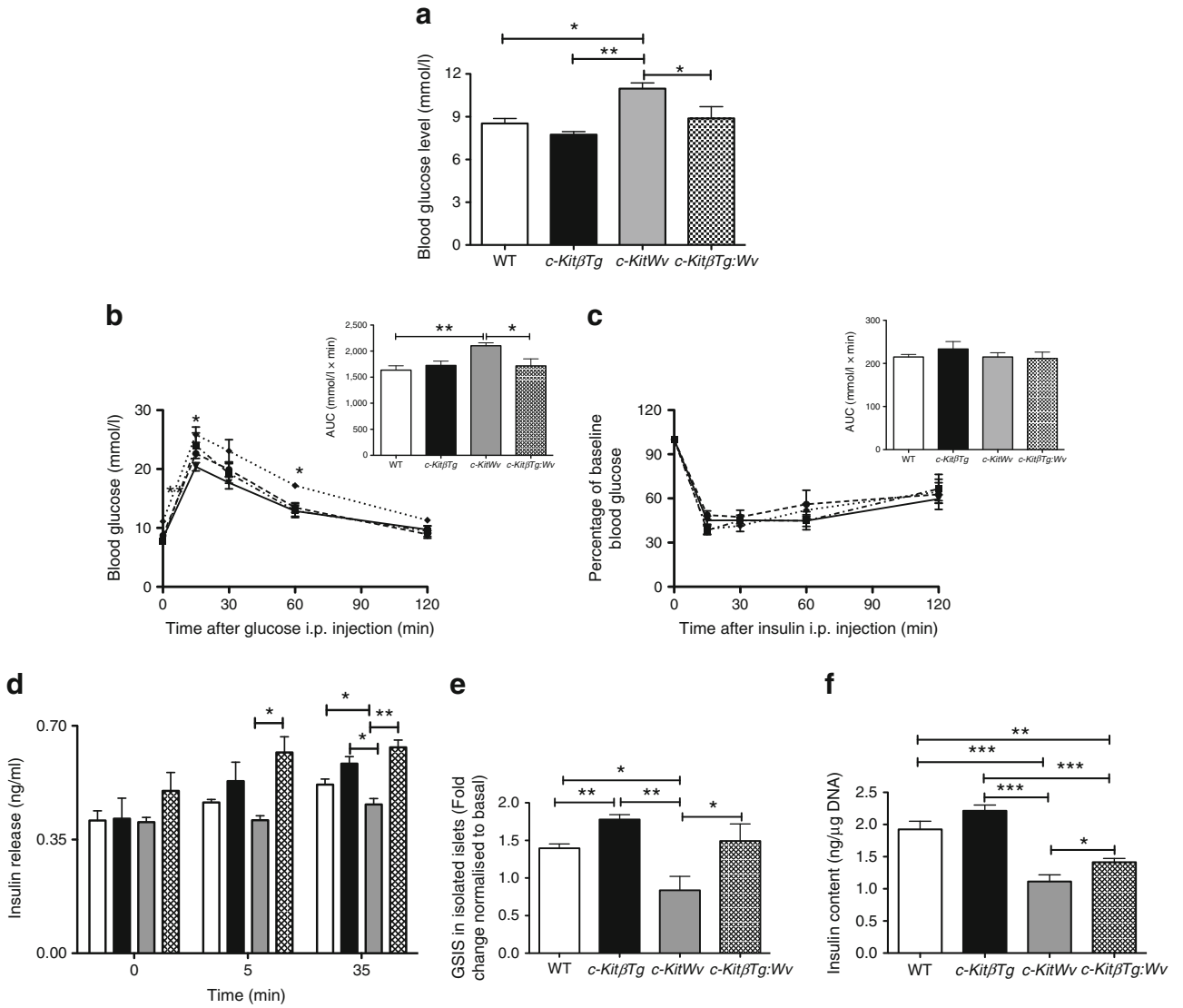

Fig. 7 Glucose tolerance and insulin secretion in *c-KitβTg:Wv* mice. **a** Fasting blood glucose levels, (**b**) IPGTT and (**c**) IPITT values in male wild-type (WT), *c-KitβTg*, *c-KitWv* and *c-KitβTg:Wv* mice at 8 weeks of age. The glucose responsiveness of the corresponding experimental groups is shown as the AUC of the (**b**, insert) IPGTT or (**c**, insert) IPITT graphs with units of (mmol/l x min). Data (**a**–**c**) are expressed as means±SEM; n=7–8. **d** In vivo GSIS of wild-type (white bars), *c-KitβTg* (black bars), *c-KitWv* (grey bars) and *c-KitβTg:Wv* (dotted bars) mice (n=3–4). **e** GSIS of isolated islets from *c-KitβTg:Wv* mice is improved in response to a 22 mmol/l glucose challenge; data are expressed as fold change normalised to basal (2.2 mmol/l glucose) secretion; n=3. **f** Insulin content in isolated islets; data are normalised to DNA content; n=6–8. *p<0.05, **p<0.01 and ***p<0.001

Finally, we bred *c-KitβTg* with *c-Kit*^{Wv} mice to determine whether *c-KIT* overexpression could prevent beta cell dysfunction in *c-Kit*^{Wv} mice. Our results showed that *c-KitβTg:Wv* mice displayed normal fasting glycaemia and glucose tolerance, as well as enhanced glucose-induced insulin secretion. This marked improvement in glucose metabolism in *c-KitβTg:Wv* mice provides direct evidence of a primary effect of the c-Kit receptor on beta cell function.

In summary, we showed that *c-KIT* overexpression in beta cells led to improved beta cell proliferation and function, and protected mice from HFD-induced diabetes. Furthermore, beta cell-specific overexpression of *c-KIT* was able to prevent beta cell defects in *c-Kit*^{Wv} mice. This study provides direct evidence to support the notion that c-Kit plays a primary physiological role in beta cells, and thus may help efforts to develop gene and cell therapeutic schemes for patients with diabetes.

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Contribution statement ZCF and JL contributed to the acquisition of data, data analysis and interpretation, manuscript drafting and final approval of the manuscript. BAT contributed to the data analysis and interpretation, manuscript drafting and final approval of the manuscript. MR contributed to analysis and interpretation of data, revising the article, and final approval of the manuscript. SPY contributed to the design of the transgenic model, analysis and interpretation of data, the provision of study materials, and the critical revision and final approval of the manuscript. RW contributed to the conception and design of the study, the collection and assembly of data, data analysis and interpretation, manuscript writing and final approval of the manuscript.

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