Disruption of CR6-interacting factor-1 (CRIF1) in mouse islet beta cells leads to mitochondrial diabetes with progressive beta cell failure

Yong Kyung Kim · Kyong Hye Joung · Min Jeong Ryu · Soung Jung Kim · Hyeonseok Kim · Hyo Kyun Chung · Min Hee Lee · Seong Eun Lee · Min Jeong Choi · Joon Young Chang · Hyun Jung Hong · Koon Soon Kim · Sang-Hee Lee · Gi Ryang Kweon · Hail Kim · Chul-Ho Lee · Hyun Jin Kim · Minh Shong

Received: 24 September 2014 / Accepted: 30 December 2014 / Published online: 8 February 2015
© Springer-Verlag Berlin Heidelberg 2015

Abstract

Aim/hypothesis Although mitochondrial oxidative phosphorylation (OxPhos) dysfunction is believed to be responsible for beta cell dysfunction in insulin resistance and mitochondrial diabetes, the mechanisms underlying progressive beta cell failure caused by defective mitochondrial OxPhos are largely unknown.

Methods We examined the in vivo phenotypes of beta cell dysfunction in beta cell-specific *Crif1* (also known as

Gadd45gip1)-deficient mice. CR6-interacting factor-1 (CRIF1) is a mitochondrial protein essential for the synthesis and formation of the OxPhos complex in the inner mitochondrial membrane.

Results *Crif1*^{beta-/-} mice exhibited impaired glucose tolerance with defective insulin secretion as early as 4 weeks of age without defects in islet structure. At 11 weeks of age, *Crif1*^{beta-/-} mice displayed characteristic ultrastructural mitochondrial abnormalities as well as severe glucose intolerance. Furthermore, islet area and insulin content was decreased by approximately 50% compared with wild-type mice. Treatment with the glucoregulatory drug exenatide, a glucagon-like peptide-1 (GLP-1) agonist, was not sufficient to preserve beta cell function in *Crif1*^{beta-/-} mice.

Conclusions/interpretation Our results indicate that mitochondrial OxPhos dysfunction triggers progressive beta cell failure that is not halted by treatment with a GLP-1 agonist. The *Crif1*^{beta-/-} mouse is a useful model for the study of beta cell failure caused by mitochondrial OxPhos dysfunction.

Electronic supplementary material The online version of this article (doi:10.1007/s00125-015-3506-y) contains peer-reviewed but unedited supplementary material, which is available to authorised users.

Y. K. Kim · K. H. Joung · M. J. Ryu · S. J. Kim · H. K. Chung · M. H. Lee · S. E. Lee · M. J. Choi · J. Y. Chang · H. J. Hong · K. S. Kim · M. Shong (✉)
Research Center for Endocrine and Metabolic Diseases, Chungnam National University School of Medicine, Daejeon 301-721, Korea
e-mail: minhos@cnu.ac.kr

H. Kim · S.-H. Lee · H. Kim
Graduate School of Medical Science and Engineering, Korea Advanced Institute of Science and Technology, Daejeon, Korea

G. R. Kweon
Department of Biochemistry, Chungnam National University School of Medicine, Daejeon, Korea

C.-H. Lee
Animal Model Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea

H. J. Kim (✉) · M. Shong
Department of Internal Medicine, Chungnam National University Hospital, Daejeon 301-721, Korea
e-mail: kimhj43@cnuh.co.kr

Keywords Beta cell failure · CRIF1 · Mitochondrial oxidative phosphorylation

Abbreviations

CRIF1	CR6-interacting-factor 1
CCCP	Carbonyl cyanide <i>m</i> -chlorophenyl hydrazone
GLP-1	Glucagon-like peptide 1
GTT	Glucose tolerance test
HSP-60	Heat shock protein 60
IPGTT	i.p. GTT
ITT	Insulin tolerance test
LC3	Microtubule-associated protein light chain 3

mtDNA	Mitochondrial DNA
OCR	Oxygen consumption rate
8-OHdG	8-Oxo-2'-deoxyguanosine
OxPhos	Oxidative phosphorylation
TEM	Transmission electron microscopy
TFB1M	Transcription factor B1 mitochondrial
VDAC1	Voltage-dependent anion-selective channel protein 1
WT	Wild-type

Introduction

Whole body glucose homeostasis depends on the dynamic functional capacity of beta cells in pancreatic islets [1]. Beta cell dysfunction, as defined by a decrease in individual beta cell function or whole beta cell mass, is a hallmark of diabetes [2–4]. However, the causes of beta cell dysfunction during the progression of insulin resistance and diabetes remain to be elucidated [5]. Dysfunction of cellular organelles, such as the endoplasmic reticulum and mitochondria, has long been studied as the underlying defect in beta cell dysfunction in diabetes [6, 7]. The mitochondria are implicated in several aspects of beta cell function and dysfunction in diabetes [8, 9]. Beta cell mitochondria not only generate metabolic signals for insulin exocytosis in response to glucose oxidation [10, 11] but also participate in the processes of apoptosis, necrosis and autophagy [12, 13]. In the context of mitochondrial dysfunction, these cellular processes are thought to be responsible for beta cell loss in the progression of diabetes [14].

To elucidate the roles of mitochondrial oxidative phosphorylation (OxPhos) dysfunction in the pathogenesis of diabetes, animal models harbouring mitochondrial OxPhos alterations in beta cells have been developed. Loss of mitochondrial transcription factor A (TFAM), an obligatory mitochondrial DNA (mtDNA) transcription factor in beta cells, results in spontaneous hyperglycaemia associated with reduced insulin secretion and beta cell loss [15]. Loss of the mitochondrial translation factor transcription factor B1 mitochondrial (TFB1M) specifically from beta cells leads to beta cell dysfunction and loss of beta cell mass [16]. Taken together, these findings support the hypothesis that impaired OxPhos function contributes to the pathogenesis of diabetes.

To analyse the role of mitochondrial OxPhos dysfunction in beta cells, we generated a mouse model of OxPhos dysfunction by specifically deleting the nuclear *Crif1* (also known as *Gadd45gip1*) gene in pancreatic beta cells. CR6-interacting-factor 1 (CRIF1) is a mitochondrial protein that specifically interacts with the protein components of the large subunit of the mitochondrial ribosome and plays an essential role in the synthesis of mtDNA-encoded OxPhos polypeptides and subsequent insertion of OxPhos subunits into the inner mitochondrial membrane [17]. Therefore, its disruption

results in marked failure of mitochondrial respiration caused by the loss of OxPhos complex formation in the mitochondrial inner membrane [18, 19]. Importantly, our animal model of CRIF1-deficiency in pancreatic beta cells exhibited accelerated and progressive beta cell failure phenotypes that will be useful for the study of beta cell failure associated with mitochondrial OxPhos dysfunction.

Methods

Ethics statement All animal experiments were reviewed and approved by the Committee on the Ethics of Animal Experiments of Chungnam National University Graduate School of Medicine and were performed according to the institutional guidelines for care and use of laboratory animals.

Mice *Ins2-Cre* transgenic mice (C57BL/6-Tg[*Ins2-cre*]25Mgn/J) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Floxed *Crif1* (*Crif1*^{flox/flox}) mice were generated as described previously [20]. *Crif1*^{flox/flox} mice were mated with *Ins2-Cre* transgenic mice to produce beta cell-specific *Crif1*-knockout (*Crif1*^{beta-/-}) mice. (see Electronic Supplementary Materials [ESM] [Methods](#) for further details). Exenatide (Byetta, Eli Lilly, Indianapolis, IN, USA) was administered to *Crif1*^{beta-/-} mice (10 nmol/kg) by daily subcutaneous injection for 4 weeks starting at 4 weeks of age.

Cell culture Mouse embryonic fibroblasts (MEFs) and MIN6 cells were maintained in DMEM containing 25 mmol/l glucose, 15% (wt/vol.) FBS, 1% (wt/vol.) penicillin, and a streptomycin cocktail (Roche Diagnostics, Mannheim, Germany).

Transmission electron microscopy Pancreatic tissues were sectioned using an EM UC6 ultramicrotome (LEICA) and stained with 4% (wt/vol.) uranyl acetate and citrate. Sections were observed using a Tecnai G2 Spirit Twin transmission electron microscope (FEI Company, OR, USA) and a JEM ARM 1300S high-voltage electron microscope (JEOL, Tokyo, Japan; see ESM [Methods](#) for further details).

Western blotting Approximately 100–150 isolated islets were cultured overnight [21], washed once with PBS, and lysed directly in SDS sample buffer supplemented with a protease inhibitor cocktail (Roche Diagnostics). Western blot analysis was performed according to standard methods with the indicated antibodies (see ESM [Methods](#) for further details).

Measurement of the oxygen consumption rate in islets The oxygen consumption rate (OCR) in isolated islets was measured using a Seahorse XF-24 Analyzer (Seahorse Bioscience, North Billerica, MA, USA) [22]. (See ESM [Methods](#) for further details).

Islet isolation, glucose-stimulated insulin secretion and pancreatic insulin content measurement Islets were isolated from 4-week-old and 11-week-old mice fed a normal chow diet as described previously [21]. Insulin concentrations were measured using an insulin ELISA kit (Alpco Diagnostics, Windham, NH, USA; see ESM [Methods](#) for further details).

Glucose tolerance test and insulin tolerance test For the glucose tolerance test (GTT), mice were fasted overnight (16 h) and glucose (2 g/kg) was administered by i.p. injection or gavage feeding. Blood glucose levels were measured with a glucometer (Accu-CHEK Active, Roche Diagnostics). For the ITT, mice were fasted for 6 h prior to receiving an i.p. injection of 0.75 U/kg insulin (Humalog, Eli Lilly, Indianapolis, IN, USA) and blood glucose levels were measured. Plasma insulin levels were measured using a mouse insulin ELISA kit (Alpco Diagnostics).

Morphometric analysis of islets The pancreas was excised, weighed, incubated in 10% (wt/vol.) neutral buffered formalin (NBF; Histoperfect fixation, BBC Biochemical, WA, USA) for 4 h, and embedded in paraffin. Sections (4 μ m thick) were cut at 60 μ m intervals, processed by haematoxylin and eosin staining, and observed by light microscopy (Olympus). The surface area of islets was analysed using Image Inside software [23] (see ESM [Methods](#) for further details).

Determination of the beta cell proliferation rate The proliferation rate was based on the percentage of cells positive for both Ki67 and insulin. A minimum of 4,000 insulin-positive cells were counted per pancreatic section.

Statistical analysis Statistical analyses were completed using SPSS version 21 (IBM, Chicago, IL, USA). We used the unpaired, two-tailed Student's *t* test for two group comparisons. For three group comparisons, we used one-way ANOVA followed by the Tukey honest significant difference (HSD) test for multiple comparisons. A level of $p < 0.05$ was considered to be statistically significant. All data were expressed as means $\pm$ SEM.

Results

Homozygous loss of *Crif1* causes mitochondrial dysfunction and structural abnormalities in MEFs and mouse pancreatic beta cells CRIF1 is a mitochondrial protein associated with the large subunit of the mitochondrial ribosome and plays an essential role in the synthesis of mtDNA-encoded OxPhos polypeptides (ESM Fig. 1a,b) [17]. Mitochondria isolated from homozygous *Crif1*-deficient MEFs ($-/\Delta$) showed a markedly reduced level of nascent translation products

Fig. 1 CRIF1 deficiency causes a loss of mitochondrial OxPhos function in vitro and in vivo. **(a)** Immunofluorescence analysis of endogenous CRIF1. MIN6 cells were immunostained for CRIF1 (red) and VDAC1 (green). CRIF1 localised to mitochondria. Magnification $\times 1,000$. **(b)** Representative western blot analysis of CRIF1 protein expression in islets of 11-week-old WT, *Crif1*^{beta+/-} and *Crif1*^{beta-/-} mice ($n=5$ per group). **(c)** Representative transmission electron micrographs of beta cells from 11-

week-old WT, *Crif1*^{beta+/-} and *Crif1*^{beta-/-} mice, as indicated. Arrows indicate mitochondria. Scale bar, 2 μ m. **(d)** Number of mitochondria per 100 μ m² of beta cells. Images were randomly acquired of 30 beta cells from 11-week-old WT, *Crif1*^{beta+/-} and *Crif1*^{beta-/-} mice. ** $p < 0.01$ as indicated. **(e)** Representative western blot analysis of HSP-60 and VDAC1 protein expression in islets isolated from 11-week-old WT, *Crif1*^{beta+/-} and *Crif1*^{beta-/-} mice ($n=5$ per group)

(ESM Fig. 1c). In immunofluorescence analysis, CRIF1 colocalised with voltage-dependent anion-selective channel protein 1 (VDAC1) in MIN6 cells (Fig. 1a), indicating that it is present in the mitochondria of beta cells. Thus, we induced beta cell-specific *Crifl* knockout using a *Cre-loxP* system (ESM Fig. 2a) to investigate the in vivo effect of mitochondrial OxPhos dysfunction [24, 25]. *Crifl*-floxed mice (*Crifl^{f/f}*) were crossed with *Ins2-Cre* mice to obtain *Crifl^{beta-/-}* (*Crifl^{flox/flox};Ins2-Cre^{+/-}*) mice (ESM Fig. 2b; see ESM Methods for genomic DNA PCR analysis). Lack of CRIF1 protein expression in the pancreatic islets of *Crifl^{beta-/-}* mice confirmed efficient *Cre*-mediated deletion of *Crifl* gene from beta cells (Fig. 1b).

Transmission electron microscopy (TEM) demonstrated that mitochondria of *Crifl*-deficient MEF and *Crifl^{beta-/-}* beta cells exhibited structural abnormalities characterised by marked intra-cristal swelling and reduced electron density in the mitochondrial matrix (Fig. 1c, ESM Fig. 3). An increased

number of mitochondria and higher expression of VDAC1 indicated that mitochondrial biogenesis was upregulated in *Crifl^{beta-/-}* beta cells. The concomitant quantitative increase in heat shock protein 60 (HSP-60), which acts as a marker of mitochondrial stress, suggested increased mitochondrial biogenesis in *Crifl^{beta-/-}* beta cells caused by an adaptive response to mitochondrial dysfunction due to *Crifl* loss (Fig. 1d, e).

Impaired mitochondrial function results in glucose intolerance and defective insulin secretion in *Crifl^{beta-/-}* mice Food intake and body weight did not significantly differ between wild-type (WT; *Crifl^{+/+};Ins2-Cre^{+/-}*), *Crifl^{beta+/-}* (*Crifl^{flox/+};Ins2-Cre^{+/-}*) and *Crifl^{beta-/-}* mice (Fig. 2a–c). To determine the effect of mitochondrial dysfunction caused by beta cell-specific *Crifl* knockout, *Crifl^{beta-/-}* mice were subjected to i.p. glucose tolerance tests GTT (IPGTT) at 4 and 11 weeks of age. *Crifl^{beta-/-}* mice developed glucose

Fig. 2 CRIF1 is necessary for normal insulin secretion in response to glucose. (a, b) Weights of 4-week-old and 11-week-old WT, *Crifl^{beta+/-}* and *Crifl^{beta-/-}* mice ($n=7$ per group). (c) Food intake was measured for 2 weeks starting at 4 weeks of age WT (white squares), *Crifl^{beta+/-}* (black squares) and *Crifl^{beta-/-}* (white circles) ($n=3$ per group). (d) IPGTTs were performed on WT (white squares), *Crifl^{beta+/-}* (black squares) and *Crifl^{beta-/-}* (white circles) mice at 4 weeks of age ($n=5$ per group). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs WT. (e) Plasma insulin levels were measured at the indicated time points before and after i.p. injection of glucose in WT (white bars), *Crifl^{beta+/-}* (grey bars) and *Crifl^{beta-/-}* (black bars) mice. ** $p<0.01$ as indicated. (f) IPGTTs were performed on 11-week-old WT (white squares),

Crifl^{beta+/-} (black squares) and *Crifl^{beta-/-}* (white circles) mice ($n=5$ per group). * $p<0.05$, ** $p<0.01$ vs WT. (g) Plasma insulin levels were measured at the indicated time points before and after i.p. injection of glucose in WT (white bars), *Crifl^{beta+/-}* (grey bars) and *Crifl^{beta-/-}* (black bars) mice. ** $p<0.01$ as indicated. (h) ITTs were performed on 11-week-old mice that had been fasted for 4 h ($n=4-5$ mice per group). Blood glucose levels were measured at the indicated time points before and after i.p. injection of human regular insulin (0.75 U/kg) and glucose disposal was calculated. Data are means ± SEM. NS; WT vs *Crifl^{beta+/-}* and WT vs *Crifl^{beta-/-}*. WT (white squares), *Crifl^{beta+/-}* (black squares) and *Crifl^{beta-/-}* (white circles)

intolerance at 4 weeks of age (Fig. 2d), which was characterised by a decrease in insulin secretion in response to glucose stimulation during IPGTT (Fig. 2e). At 11 weeks of age, glucose level at the same time point was higher than that of 4-week-old mice (Fig. 2f), although the deficiency in insulin secretion during IPGTT was similar to that of 4-week-old mice (Fig. 2g). Despite severe glucose intolerance, the insulin sensitivity of *Crifl*^{beta-/-} mice remained normal at 11 weeks of age (Fig. 2h). Thus, *Crifl*^{beta-/-} mice developed diabetes with insufficient insulin secretion in response to glucose, suggesting an essential role for *Crifl* in beta cell function.

To delineate the relationship between abnormal mitochondrial function and defective insulin secretion in *Crifl*^{beta-/-} mice, we measured the OCR in purified islets (Fig. 3a). Basal OCR was similar in WT and *Crifl*^{beta-/-} islets. However *Crifl*^{beta-/-} islets showed different OCRs after 20 mmol/l glucose stimulation. Glucose stimulation increased OCR by 2.1-fold in WT islets, while *Crifl*^{beta-/-} islets showed only a 1.3-fold increase. In *Crifl*^{beta-/-} islets, the response to 5 μ mol/l oligomycin (an ATP synthase inhibitor) was normal, but the response to carbonyl cyanide *m*-chlorophenyl

hydrazone (CCCP; a mitochondrial respiration uncoupler) was delayed, and the maximum capacity was decreased (a 2.4-fold increase in WT mice vs a 1.8-fold in *Crifl*^{beta-/-} mice; ESM Fig. 4). The decreased OCR in *Crifl*^{beta-/-} islets following glucose stimulation suggested that impaired OxPhos function in beta cells could lead to inappropriate insulin secretion in response to glucose because normal mitochondrial OxPhos function is required for glucose-stimulated insulin secretion.

To determine the effect of *Crifl* knockout on insulin secretion per se, we calculated fractionated insulin secretion from each islet and confirmed that insulin secretion from *Crifl*^{beta-/-} islets upon either glucose or KCl stimulation was reduced in both 4-week-old and 11-week-old mice (Fig. 3b, c). Since KCl can directly stimulate insulin secretion without increasing ATP production in mitochondria, these data suggest that beta cells of *Crifl*^{beta-/-} mice have more profound defects than simple mitochondrial OxPhos dysfunction.

Progressive loss of beta cell mass associated with autophagy in *Crifl*^{beta-/-} mice The worsening of glucose tolerance in *Crifl*^{beta-/-} mice with age and loss of KCl-dependent insulin secretion in *Crifl*^{beta-/-} islets suggested that mitochondrial dysfunction in beta cells could progressively deteriorate the functional integrity of the beta cells. Thus, we examined the pancreatic islets of *Crifl*^{beta-/-} mice in an age-specific manner. Immunofluorescence staining for insulin and glucagon revealed no difference in immunoreactivity among the different groups: WT, *Crifl*^{beta+/+} and *Crifl*^{beta-/-} mice at 4 weeks of age (Fig. 4a). However, *Crifl*^{beta-/-} mice showed extensive loss of insulin-positive beta cells at 11 weeks of age (Fig. 4a). Pancreatic insulin content was reduced in *Crifl*^{beta-/-} mice by 20% at 4 weeks of age and by 68% at 11 weeks of age (Fig. 4b, c). Islet area was comparable among all groups at 4 weeks of age but decreased by 45% in *Crifl*^{beta-/-} mice compared with WT and *Crifl*^{beta+/+} mice ($12.7 \times 10^5 \mu\text{m}^2$ vs $21.9 \times 10^5 \mu\text{m}^2$ vs $20.9 \times 10^5 \mu\text{m}^2$, respectively) at 11 weeks of age (Fig. 4d, e). These findings indicate that *Crifl*^{beta-/-} mice exhibit loss of beta cell mass.

To determine the underlying mechanism responsible for the progressive loss of beta cell mass, we measured beta cell proliferation using immunofluorescent staining for Ki67, a marker of cell proliferation (Fig. 5a). The proportions of Ki67-positive cells decreased significantly in *Crifl*^{beta-/-} mice at both 4 weeks and 11 weeks of age (Fig. 5b, c).

To examine the level of beta cell loss in *Crifl*^{beta-/-} mice, we first measured the level of apoptosis in the islets and found no significant differences in TUNEL staining (data not shown) or immunostaining for cleaved caspase 9 (ESM Fig. 5a,b). Interestingly, in TEM images of *Crifl*^{beta-/-} beta cells, we observed the characteristic features of autophagosomes, which were surrounded by double membranes and contained small mitochondria, suggesting the occurrence of mitophagy

Fig. 3 Impaired OCR results in increased beta cell dysfunction. **(a)** OCR of isolated islets from 11-week-old mice was measured using a Seahorse XF-24 Analyzer (50 islets per well). WT (grey circles), *Crifl*^{beta-/-} (black circles). Data are means $\pm$ SEM ($n=7$ per group). $*p<0.05$ vs WT. **(b, c)** The rate of insulin secretion is expressed as normalised insulin release per total islet insulin content in 4-week-old and 11-week-old mice, respectively ($n=3$, 10 islets per well). The x-axis shows concentration of treated glucose. WT (white bars), *Crifl*^{beta+/+} (grey bars) and *Crifl*^{beta-/-} (black bars). Data are means $\pm$ SEM of three experiments, each performed in triplicate. $*p<0.05$ as indicated and NS; WT vs *Crifl*^{beta+/+}

Fig. 4 Progressive loss of beta cell mass in *Crifl*^{β-/-} mice. **(a)** Pancreatic sections from 4-week-old and 11-week-old mice were co-stained with DAPI (blue), an anti-insulin antibody (red) and an anti-glucagon antibody (green). Scale bars, 50 μm. **(b, c)** Pancreatic insulin was extracted using acid and ethanol ($n=3$) and analysed using an ELISA kit. Insulin concentrations were normalised to the level of total cellular protein. Data are

means±SEM. * $p<0.05$, ** $p<0.01$ as indicated. **(d, e)** Histomorphometric quantification of the islet area (μm²) divided by the total pancreas weight (mg) was performed in five sections per mouse, each separated by 60 μm ($n=3$). Data are means±SEM. * $p<0.05$ as indicated. WT (white bars), *Crifl*^{β+/-} (grey bars) and *Crifl*^{β-/-} (black bars)

(Fig. 5d) [26]. In association with these findings, the endoplasmic reticulum was distended around the swollen mitochondria (ESM Fig. 5c) [27]. Western blot analysis showed increased microtubule-associated protein light chain 3 (LC3)-II immunoreactivity (Fig. 5e), and immunohistochemical staining for LC3B revealed the strong induction of LC3B expression in the pancreatic islets of *Crifl*^{β-/-} mice (Fig. 5f). Immunofluorescent staining for LC3 revealed an increase in LC3-puncta in the beta cells of 11-week-old *Crifl*^{β-/-} mice (ESM Fig. 6). These results indicate that autophagy is associated with the features of islets in *Crifl*^{β-/-} mice, which agrees with previous studies showing reduced beta cell mass without evidence of increased apoptosis in patients with mitochondrial diabetes [28, 29].

Treatment with the glucagon-like peptide 1 agonist, exenatide, did not increase beta cell mass in *Crifl*^{β-/-} mice The glucagon-like peptide-1 (GLP-1) agonist, exenatide, is known to improve insulin secretion [30, 31], stimulate pancreatic beta cell proliferation [32, 33] and prevent beta cell death [34, 35]. To determine whether exenatide might have a protective effect on the progressive deterioration of beta cell function in *Crifl*^{β-/-} mice, we treated WT and *Crifl*^{β-/-} mice with daily s.c. injections of exenatide (10 nmol/kg) or saline for

4 weeks, beginning at 4 weeks of age, when the beta cell mass is comparable between the two groups. In WT mice with normal beta cell morphology, exenatide treatment increased beta cell proliferation and mass (Fig. 6a, b and ESM Fig. 7). However, exenatide did not increase beta cell proliferation or mass in *Crifl*^{β-/-} mice and no beneficial effect on islet morphology was observed (Fig. 6a, b and ESM Fig. 7). In addition, neither glucose tolerance nor insulin secretion was improved in *Crifl*^{β-/-} mice treated with exenatide (Fig. 6c, d). These data suggest that GLP-1 agonists or dipeptidyl peptidase 4 (DPP-4) inhibitors might not have protective effects in patients presenting with deteriorated mitochondrial dysfunction.

Discussion

CRIF1 is a mitochondrial protein that associates with the large mitoribosomal subunits located close to the polypeptide exit tunnel and facilitates the co-translational insertion of mtDNA-encoded nascent OxPhos polypeptides into the inner membrane [17]. Therefore, homozygotic and heterozygotic loss of *Crifl* in specific organs results in markedly or marginally reduced OxPhos capacities, respectively [18, 36]. For

Fig. 5 Reduced beta cell proliferation in *Crifl*^{beta-/-} mice. **(a)** Representative images of beta cell replication in 4-week-old and 11-week-old mice using Ki67 as a replication marker (red). Sections were co-stained for insulin (green). Scale bars, 50 μ m. **(b, c)** Quantification of the rate of beta cell proliferation in WT (white bars), *Crifl*^{beta+/+} (grey bars) and *Crifl*^{beta-/-} (black bars) mice. Proliferation rate was based on the percentage of cells positive for both Ki67 and insulin. Data are means $\pm$ SEM. * p < 0.05 as indicated. NS; WT vs *Crifl*^{beta+/+}. **(d)**

Representative transmission electron micrographs of beta cells from 11-week-old *Crifl*^{beta-/-} mice. Red squares indicate autophagosomes and are magnified on the right: magnification $\times$ 10,000 (left) and $\times$ 25,000 (right). Scale bars, 1 μ m. **(e)** Representative western blot analysis of LC3-II protein expression in islets isolated from 11-week-old WT, *Crifl*^{beta+/+} and *Crifl*^{beta-/-} mice (n = 5 per group). **(f)** Representative immunohistochemical images of tissues stained for LC3B (experiments were repeated three times). Scale bar, 50 μ m

example, homozygotic and heterozygotic *Crifl*-deficiency in adipose tissue produces widely variable disease phenotypes in vivo [18]. These results demonstrate that genetic ablation of *Crifl* in a tissue-specific fashion is a useful model for the study of tissue responses to variable degrees of reduced OxPhos capacity.

An interesting finding of the present study was that diabetic phenotypes in *Crifl*^{beta-/-} mice developed as early as 4 weeks of age and persisted until 11 weeks of age. The beta cell mass was not different from that in WT mice, but islets isolated from 4-week-old *Crifl*^{beta-/-} mice showed reduced glucose-stimulated insulin secretion. Therefore, the main cause of glucose intolerance in 4-week-old *Crifl*^{beta-/-} mice can be attributed to secretory defects in insulin secretion in response to glucose. The decreased insulin content in 4-week-old *Crifl*^{beta-/-} mice, without a decrease in beta cell mass, may be owing to decreased synthesis or defective maturation or processing of pre-proinsulin in the presence of mitochondrial dysfunction. Heterozygous superoxide dismutase 2 (SOD2) deficiency in mice resulted in impaired glucose-stimulated insulin secretion, suggesting that oxidative stress might be

linked with diminished insulin secretion [37]. Based on higher expression of 8-oxo-2'-deoxyguanosine (8-OHDG) in the pancreatic islets of *Crifl*^{beta-/-} mice (data not shown), increased oxidative stress might contribute to the decrease of beta cell secretory capacity. Taken together, the early phase of CRIF1 loss in pancreatic beta cells resulted in defects in metabolic coupling sufficient to develop into systemic glucose intolerance.

It is clear that the pancreatic islets in *Crifl*^{beta-/-} mice were significantly reduced in terms of beta cell mass. These changes reflect progressive cellular dysfunction caused by *Crifl* loss in beta cells. Previous reports suggest that the rapid progressive loss of beta cells may be caused by accelerated cell death via apoptosis and necrosis [38, 39]. However, our failure to detect apoptosis markers in the islets of *Crifl*^{beta-/-} mice may indicate that active apoptosis is not the principal cause of beta cell failure. Increased mitophagy is prevalent in cells with dysfunctional mitochondria [40, 41]. Consistent with this finding, TEM revealed that beta cells in the islets of *Crifl*^{beta-/-} mice contained an increased number of autophagosomes. However, our experimental findings did not determine the role of

Fig. 6 Exenatide does not increase islet area or insulin secretion in *Crifl*^{β-/-} mice. **(a)** Histomorphometric quantification of the islet area (μm²) divided by the total pancreas weight (mg) was performed in five sections per mouse, each separated by 40 μm (*n*=3). **p*<0.05 as indicated. **(b)** Quantification of beta cell proliferation in WT and *Crifl*^{β-/-} mice. ***p*<0.01 as indicated. Proliferation rate was based on the percentage of cells positive for both Ki67 and insulin. **(c)** OGTTs were performed in exenatide-treated WT mice (10 nmol/kg; white squares), saline-treated *Crifl*^{β-/-} mice (white triangles) and exenatide-treated *Crifl*^{β-/-} mice (10 nmol/kg; black circles) (*n*=5 per group). **p*<0.05 WT vs exenatide-treated *Crifl*^{β-/-}. **(d)** Plasma insulin levels were measured at the indicated time points before and after glucose administration. Saline-treated *Crifl*^{β-/-} mice (white bars) and exenatide-treated *Crifl*^{β-/-} mice (10 nmol/kg; black bars). Data are means±SEM

increased beta cell autophagy, which may have contributed to beta cell loss in CRIF1-deficiency. Previous reports indicate that beta cell proliferation can compensate for the loss of beta cell mass or function to maintain normal glucose tolerance [42, 43]; however, homozygotic *Crifl*-deficient mice did not exhibit this proliferative response (Fig. 5b). Consequently, the diabetic phenotypes in 11-week-old *Crifl*^{β-/-} mice can be explained by both accelerated beta cell loss and failure of compensatory beta cell proliferation.

Restoration and maintenance of beta cell mass by preventing the progressive loss of, or enhancing the proliferative potential of, beta cells may be a viable therapeutic option for the treatment of diabetes [44, 45]. Exenatide (synthetic exendin-4) has the potential to prevent the progressive loss of beta cell function and beta cell mass associated with type 2 diabetes [46]. GLP-1 promotes beta cell proliferation and

inhibits beta cell apoptosis [34, 35, 47, 48]. Therefore, we treated *Crifl*^{β-/-} mice with exenatide and observed glucose tolerance and beta cell proliferation. The proliferative effects of exenatide were observed in WT mice, but this effect was absent in *Crifl*^{β-/-} mice. Based on these data, exenatide does not appear to be a candidate for the treatment of diabetes caused by mitochondrial dysfunction.

As mentioned previously, beta cell-specific deficiency of *Tfam*, an essential factor for the transcription of mtDNA-encoded OxPhos polypeptides, causes diabetes from approximately 5 weeks of age, characterised by impaired release of insulin from islets in response to glucose stimulation. Older beta cell-specific *Tfam*-knockout mice exhibit increased oxidative stress and reduced beta cell mass, without increased apoptosis in islets. The reduced beta cell mass of *Tfam*- and *Crifl*-knockout mice may not be owing to increased apoptosis (ESM Fig. 5). Insufficient compensation for islet beta cell loss in *Crifl*^{β-/-} mice may result in reduced islet mass.

A common variant (rs950994) of the *TFB1M* gene was recently reported to be associated with reduced insulin secretion, elevated postprandial glucose levels, and an increased risk of developing type 2 diabetes [49]. Accordingly, mice heterozygous for *Tfb1m* deficiency exhibit reduced levels of TFB1M in islets, impaired mitochondrial function and reduced insulin release in response to glucose in vivo and in vitro. Additionally, in mice in which *Tfbim* is specifically knocked out in beta cells, ATP is depleted and the level of reactive oxygen species (ROS) is increased, which led to loss of beta cell function and mass owing to increased apoptosis and necrosis [16].

Animal models of mitochondrial dysfunction in beta cells, including *Crifl*^{β-/-} mice, show sequential beta cell dysfunction and beta cell mass loss, reminiscent of the development and progression of type 2 diabetes in humans. Further studies are required to determine the clinical implication of *CRIF1* deficiency in human disease and to analyse the function and mass of beta cells in *Crifl*^{β-/-} mice.

In summary, these findings indicate that knocking out *Crifl* in beta cells, which results in impaired OxPhos function, may trigger progressive beta cell failure and negate the proliferative effects of exenatide. The homozygotic *Crifl*^{β-/-} mouse model showed accelerated progressive beta cell failure, which will be useful for the study of beta cell failure caused by mitochondrial dysfunction.

Funding MS acknowledges financial support from the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2012M3A9B2027958), MOE, Korea. This research was supported by the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (2012R1A2A1A03002833). The funders had no role in the study design, data collection and analysis, decision to publish or preparation of the manuscript.

Duality of interest statement The authors declare that there is no duality of interest associated with this manuscript.

Author contributions YKK, HJK, HaK, C-HL and MS made substantial contributions to the conception and design of the study. YKK, HJH, MJR, SJK, HyK, KSK and SHL were responsible for the acquisition of data and provided materials for animal experiments. HKC, MHL, SEL, MJC, KHJ and GRK helped with the analysis and interpretation of data. YKK, MJR, JYC, HaK, HJK and MS wrote the manuscript. All authors contributed to the discussion and revised the article and all approved the final version of the manuscript. MS is responsible for the integrity of the work as a whole.

References

- Langin D (2001) Diabetes, insulin secretion, and the pancreatic beta-cell mitochondrion. *N Engl J Med* 345:1772–1774
- Lingohr MK, Buettner R, Rhodes CJ (2002) Pancreatic beta-cell growth and survival—a role in obesity-linked type 2 diabetes? *Trends Mol Med* 8:375–384
- Mathis D, Vence L, Benoist C (2001) Beta-cell death during progression to diabetes. *Nature* 414:792–798
- Rhodes CJ (2005) Type 2 diabetes—a matter of beta-cell life and death? *Science* 307:380–384
- Muoio DM, Newgard CB (2008) Mechanisms of disease: molecular and metabolic mechanisms of insulin resistance and beta-cell failure in type 2 diabetes. *Nat Rev Mol Cell Biol* 9:193–205
- Back SH, Kaufman RJ (2012) Endoplasmic reticulum stress and type 2 diabetes. *Annu Rev Biochem* 81:767–793
- Maechler P, Wollheim CB (2001) Mitochondrial function in normal and diabetic beta-cells. *Nature* 414:807–812
- Supale S, Li N, Brun T, Maechler P (2012) Mitochondrial dysfunction in pancreatic beta cells. *Trends Endocrinol Metab* 23:477–487
- Lowell BB, Shulman GI (2005) Mitochondrial dysfunction and type 2 diabetes. *Science* 307:384–387
- Henquin JC (2000) Triggering and amplifying pathways of regulation of insulin secretion by glucose. *Diabetes* 49:1751–1760
- Wiederkehr A, Wollheim CB (2012) Mitochondrial signals drive insulin secretion in the pancreatic beta-cell. *Mol Cell Endocrinol* 353:128–137
- Donath MY, Ehses JA, Maedler K et al (2005) Mechanisms of beta-cell death in type 2 diabetes. *Diabetes* 54(Suppl 2):S108–S113
- Jung HS, Chung KW, Won Kim J et al (2008) Loss of autophagy diminishes pancreatic beta cell mass and function with resultant hyperglycemia. *Cell Metab* 8:318–324
- Larsson NG, Clayton DA (1995) Molecular genetic aspects of human mitochondrial disorders. *Annu Rev Genet* 29:151–178
- Silva JP, Kohler M, Graff C et al (2000) Impaired insulin secretion and beta-cell loss in tissue-specific knockout mice with mitochondrial diabetes. *Nat Genet* 26:336–340
- Sharoyko VV, Abels M, Sun J et al (2014) Loss of TFB1M results in mitochondrial dysfunction that leads to impaired insulin secretion and diabetes. *Hum Mol Genet* 23:5733–5749
- Kim SJ, Kwon MC, Ryu MJ et al (2012) CRIF1 is essential for the synthesis and insertion of oxidative phosphorylation polypeptides in the mammalian mitochondrial membrane. *Cell Metab* 16:274–283
- Ryu MJ, Kim SJ, Kim YK et al (2013) Crif1 deficiency reduces adipose OXPHOS capacity and triggers inflammation and insulin resistance in mice. *PLoS Genet* 9:e1003356
- Ryu MJ, Kim SJ, Choi MJ et al (2013) Mitochondrial oxidative phosphorylation reserve is required for hormone- and PPARgamma agonist-induced adipogenesis. *Mol Cells* 35:134–141
- Kwon MC, Koo BK, Moon JS et al (2008) Crif1 is a novel transcriptional coactivator of STAT3. *EMBO J* 27:642–653
- Li DS, Yuan YH, Tu HJ, Liang QL, Dai LJ (2009) A protocol for islet isolation from mouse pancreas. *Nat Protoc* 4:1649–1652
- Wikstrom JD, Sereda SB, Stiles L et al (2012) A novel high-throughput assay for islet respiration reveals uncoupling of rodent and human islets. *PLoS One* 7:e33023
- Claiborn KC, Sachdeva MM, Cannon CE, Groff DN, Singer JD, Stoffers DA (2010) Pcf1l modulates Pdx1 protein stability and pancreatic beta cell function and survival in mice. *J Clin Invest* 120:3713–3721
- Kulkarni RN, Bruning JC, Winnay JN, Postic C, Magnuson MA, Kahn CR (1999) Tissue-specific knockout of the insulin receptor in pancreatic beta cells creates an insulin secretory defect similar to that in type 2 diabetes. *Cell* 96:329–339
- Gannon M, Shiota C, Postic C, Wright CV, Magnuson M (2000) Analysis of the Cre-mediated recombination driven by rat insulin promoter in embryonic and adult mouse pancreas. *Genesis* 26:139–142
- Quan W, Jung HS, Lee MS (2013) Role of autophagy in the progression from obesity to diabetes and in the control of energy balance. *Arch Pharm Res* 36:223–229
- Bachar-Wikstrom E, Wikstrom JD, Kaiser N, Cerasi E, Leibowitz G (2013) Improvement of ER stress-induced diabetes by stimulating autophagy. *Autophagy* 9:626–628
- Kobayashi T, Nakanishi K, Nakase H et al (1997) In situ characterization of islets in diabetes with a mitochondrial DNA mutation at nucleotide position 3243. *Diabetes* 46:1567–1571
- Otobe S, Yasuda K, Mori Y et al (1999) Molecular and histological evaluation of pancreata from patients with a mitochondrial gene mutation associated with impaired insulin secretion. *Biochem Biophys Res Commun* 259:149–156
- Schmidt WE, Siegel EG, Creutzfeldt W (1985) Glucagon-like peptide-1 but not glucagon-like peptide-2 stimulates insulin release from isolated rat pancreatic islets. *Diabetologia* 28:704–707
- Fridolf T, Ahren B (1991) GLP-1(7–36) amide stimulates insulin secretion in rat islets: studies on the mode of action. *Diabetes Res* 16:185–191
- Stoffers DA, Kieffer TJ, Hussain MA et al (2000) Insulinotropic glucagon-like peptide 1 agonists stimulate expression of homeodomain protein IDX-1 and increase islet size in mouse pancreas. *Diabetes* 49:741–748
- Perfetti R, Zhou J, Doyle ME, Egan JM (2000) Glucagon-like peptide-1 induces cell proliferation and pancreatic-duodenum homeobox-1 expression and increases endocrine cell mass in the pancreas of old, glucose-intolerant rats. *Endocrinology* 141:4600–4605
- Farilla L, Hui H, Bertolotto C et al (2002) Glucagon-like peptide-1 promotes islet cell growth and inhibits apoptosis in Zucker diabetic rats. *Endocrinology* 143:4397–4408
- Wang Q, Li L, Xu E, Wong V, Rhodes C, Brubaker PL (2004) Glucagon-like peptide-1 regulates proliferation and apoptosis via activation of protein kinase B in pancreatic INS-1 beta cells. *Diabetologia* 47:478–487
- Shin J, Lee SH, Kwon MC et al (2013) Cardiomyocyte specific deletion of Crif1 causes mitochondrial cardiomyopathy in mice. *PLoS One* 8:e53577
- Kang L, Dai C, Lustig ME et al (2014) Heterozygous SOD2 deletion impairs glucose-stimulated insulin secretion, but not insulin action, in high-fat-fed mice. *Diabetes* 63:3699–3710
- Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA, Butler PC (2003) Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. *Diabetes* 52:102–110
- Tamura Y, Chiba Y, Tanioka T et al (2011) NO donor induces Nec-1-inhibitable, but RIP1-independent, necrotic cell death in pancreatic beta-cells. *FEBS Lett* 585:3058–3064

40. Rodriguez-Enriquez S, Kim I, Currin RT, Lemasters JJ (2006) Tracker dyes to probe mitochondrial autophagy (mitophagy) in rat hepatocytes. *Autophagy* 2:39–46
41. Kim I, Rodriguez-Enriquez S, Lemasters JJ (2007) Selective degradation of mitochondria by mitophagy. *Arch Biochem Biophys* 462: 245–253
42. Georgia S, Bhushan A (2004) Beta cell replication is the primary mechanism for maintaining postnatal beta cell mass. *J Clin Invest* 114:963–968
43. Nir T, Melton DA, Dor Y (2007) Recovery from diabetes in mice by beta cell regeneration. *J Clin Invest* 117:2553–2561
44. Bonner-Weir S (2000) Perspective: postnatal pancreatic beta cell growth. *Endocrinology* 141:1926–1929
45. Edlund H (2002) Pancreatic organogenesis—developmental mechanisms and implications for therapy. *Nat Rev Genet* 3:524–532
46. Furman BL (2012) The development of Byetta (exenatide) from the venom of the Gila monster as an anti-diabetic agent. *Toxicol Off J Int Soc Toxicology* 59:464–471
47. Brubaker PL, Drucker DJ (2004) Minireview: glucagon-like peptides regulate cell proliferation and apoptosis in the pancreas, gut, and central nervous system. *Endocrinology* 145:2653–2659
48. Baggio LL, Drucker DJ (2007) Biology of incretins: GLP-1 and GIP. *Gastroenterology* 132:2131–2157
49. Koeck T, Olsson AH, Nitert MD et al (2011) A common variant in TFB1M is associated with reduced insulin secretion and increased future risk of type 2 diabetes. *Cell Metab* 13:80–91