# DPP4 inhibitor vildagliptin preserves β-cell mass through amelioration of endoplasmic reticulum stress in C/EBPB transgenic mice

**Shinobu Shimizu[1], Tetsuya Hosooka[2], Tomokazu Matsuda[2], Shun-ichiro Asahara[2], Maki Koyanagi-Kimura[2], Ayumi Kanno[2], Alberto Bartolome[3], Hiroaki Etoh[1], Megumi Fuchita[1], Kyoko Teruyama[1], Hiroaki Takahashi[1], Hiroyuki Inoue[1], Yusuke Mieda[1], Naoko Hashimoto[2], Susumu Seino[2,4]** and **Yoshiaki Kido[1]**

[1]Division of Medical Chemistry, Department of Biophysics and [2]Division of Diabetes and Endocrinology, Department of Internal Medicine, Kobe University Graduate School of Health Sciences, Kobe 654-0142, Japan

[3]Departamento de Bioquímica y Biología Molecular, Facultad de Farmacia, Universidad Complutense, Madrid, Spain

[4]Division of Cellular and Molecular Medicine, Department of Physiology and Cell Biology, Kobe University Graduate School of Medicine, Kobe, Japan

(Correspondence should be addressed to Y Kido; Email: kido@med.kobe-u.ac.jp)

## Abstract

The development of type 2 diabetes is accompanied by a progressive decline in β-cell mass and function. Vildagliptin, a dipeptidyl peptidase 4 inhibitor, is representative of a new class of antidiabetic agents that act through increasing the expression of glucagon-like peptide-1. The protective effect of this agent on β cells was studied in diabetic mice. Diabetic pancreatic β cell-specific C/EBPB transgenic (TG) mice exhibit decreased β-cell mass associated with increased apoptosis, decreased proliferation, and aggravated endoplasmic reticulum (ER) stress. Vildagliptin was orally administered to the TG mice for a period of 24 weeks, and the protective effects of this agent on β cells were examined, along with the potential molecular mechanism of protection. Vildagliptin ameliorated hyperglycemia in TG mice by increasing the serum concentration of insulin and decreasing the serum concentration of glucagon. This agent also markedly increased β-cell mass, improved aggravated ER stress, and restored attenuated insulin/IGF1 signaling. A decrease in pancreatic and duodenal homeobox 1 expression was also observed in β cells isolated from our mouse model, but this was also restored by vildagliptin treatment. The expression of C/EBPB protein, but not mRNA, was unexpectedly downregulated in vildagliptin-treated TG mice and in exenatide-treated MIN6 cells. Activation of the GLP1 pathway induced proteasome-dependent C/EBPB degradation in β cells as the proteasome inhibitor MG132 restored the downregulation of C/EBPB protein by exenatide. Vildagliptin elicits protective effects on pancreatic β cells, possibly through C/EBPB degradation, and has potential for preventing the progression of type 2 diabetes.

*Journal of Molecular Endocrinology* (2012) **49,** 125–135

## Introduction

Pancreatic β cell failure is a fundamental pathogenic feature of type 2 diabetes, and the progressive decline in β cell function and mass over the course of type 2 diabetes development is well known (Weyer *et al.* 1999, Butler *et al.* 2003, Rhodes 2005, Wajchenberg 2007). This occurs regardless of intervention with insulin and/or antidiabetic agents (UK Prospective Diabetes Study (UKPDS) Group 1998*a*,*b*). Recent studies have revealed that both attenuated β cell function, and the loss of β-cell mass contribute to the pathogenesis of type 2 diabetes, which has drawn attention to the need for agents capable of protecting β cells.

Dipeptidyl peptidase 4 (DPP4) inhibitors form a new class of antihyperglycemic agents that can be used for the treatment of type 2 diabetes. These agents act by increasing the incretin hormone glucagon-like peptide-1 (GLP1), which stimulates insulin secretion (Holst & Deacon 2004). In addition to this action, GLP1 and its analogs have a variety of other biological effects, including the suppression of glucagon secretion and food intake, as well as the inhibition of gastric emptying (Drucker & Nauck 2006). Furthermore, GLP1 and its analogs have been shown to enhance β-cell mass by increasing the proliferation and decreasing the apoptosis of β cells in animal models (Xu *et al.* 1999, Wang & Brubaker 2002, Li *et al.* 2003, Stoffers 2004). By contrast, despite clear evidence of the effects of GLP1 and its analogs, only a small number of reports to date have examined whether DPP4 inhibitors also enhance β-cell mass (Mu *et al.* 2009, Cho *et al.* 2011, Duttaroy *et al.* 2011) or whether the increase in GLP1 levels elicited by DPP4 inhibition leads to the suppression of serum glucagon concentrations (Balas *et al.* 2007, Ahren *et al.* 2010).


0952–5041/12/049–125 
DOI: 10.1530/JME-12-0039
Online version via http://www.endocrinology-journals.org

Endoplasmic reticulum (ER) stress is thought to play an important role in the development of β cell failure in type 2 diabetes (Harding & Ron 2002). We have recently shown that the accumulation of transcription factor C/EBPB in pancreatic β cells contributes to β cell failure by enhancing susceptibility to ER stress (Matsuda *et al.* 2010). In our previous study, we established two lines of transgenic (TG) mice specifically overexpressing C/EBPB in pancreatic β cells, in which C/EBPB was expressed approximately ten- and three-fold more relative to C57BL/6J mice. These mice exhibited decreased β-cell mass as a result of increased apoptosis and reduced cellular proliferation, and aggravated ER stress was also observed in the β cells of these mice (Matsuda *et al.* 2010). As a result, both lines of TG mice exhibited hyperglycemia along with decreased plasma insulin concentration in a manner dependent on the level of C/EBPB expression (Matsuda *et al.* 2010). The first line with a tenfold increase in C/EBPB expression exhibited blood glucose levels of >400 mg/dl in the fed state, while the second line with a threefold increase showed ∼200 mg/dl. In this study, the second line of TG mice was administered the DPP4 inhibitor vildagliptin for up to 24 weeks to examine whether this agent could exert a protective effect on β cells by preserving β-cell mass. The molecular mechanism by which vildagliptin exerted beneficial effects in TG mice was also examined.

## Materials and methods

### Mice

Pancreatic β cell-specific C/EBPB TG mice of the C57BL/6J background were generated as described previously by inserting the C/EBPB gene under control of the rat insulin promoter (Matsuda *et al.* 2010). Animals were maintained in a 12 h light:12 h darkness cycle and fed normal chow from weaning (3-week old), also as previously described (Kido *et al.* 2000, Hashimoto *et al.* 2005). All experiments used male mice, and WT and C/EBPB-TG mice were grouped and housed with either regular water or water continuously supplemented with 0·6 mg/ml vildagliptin (a gift from Novartis Institutes for Biomedical Research, Cambridge, MA, USA) from 4 weeks to 24 weeks of age. Blood glucose and plasma insulin concentrations were determined as described elsewhere (Kido *et al.* 2000, Hashimoto *et al.* 2005), while plasma glucagon levels were measured using a RIA kit (Millipore, Inc., Billerica, MA, USA). Plasma GLP1 levels were measured using a GLP1 ELISA kit (Shibayagi, Gunma, Japan). This study was performed according to the guidelines of the Animal Ethics Committee of Kobe University Graduate School of Medicine.

### Oral glucose tolerance tests

Mice were deprived of food for 16 h before the oral administration of glucose (1·5 mg/g body weight). Blood was collected immediately before and at 15, 30, 45, 60, and 120 min following administration.

### Real-time RT-PCR analysis

Total cellular RNA was extracted from islets isolated from control and C/EBPB-TG mice using an RNeasy kit (Qiagen). Real-time RT-PCR analysis of the total RNA pooled from four to five animals of each genotype was performed as described previously (Matsuda *et al.* 2010). Values were normalized to cyclophilin mRNA levels and expressed as fold induction. The primers (sense and antisense) used for gene amplification were as follows: 5′-ACCGGGTTTCGGGACTTGA-3′ and 5′-GTTGCGTAGTCCCGTGTCCA-3′ for mouse C/EBPB and 5′-CA-GACGCCACTGTCGCTTT-3′ and 5′-TGTCTTTGGAACTTTGTCTGCAA-3′ for cyclophilin.

### Infection with a retrovirus encoding C/EBPB

Platinum-E (PLAT-E) ecotropic packaging cells were transfected with the retroviral vector pMX-C/EBPB (encoding mouse C/EBPB) with the use of Lipofectamine 2000 Reagent (Invitrogen). Recombinant retroviruses released into the culture medium were harvested 48 h after transfection and added to MIN6 cells cultured in DMEM supplemented with 15% FBS. After culturing for 8 h, the infected cells were subjected to selection in medium containing puromycin.

### Western blot analysis

Lysates of isolated islets and C/EBPB-overexpressed MIN6 cells were prepared as described previously (Kido *et al.* 2000, Hashimoto *et al.* 2005) and probed with antibodies for C/EBPB, C/EBP homologous protein (CHOP) (Santa Cruz Biotechnology, Inc.,), phosphorylated c-jun, phosphorylated eIF2α, phosphorylated N-jun N-terminal kinases, the phospho-$Thr^{308}$ form of Akt, the phospho-$Ser^{473}$ form of Akt, the cleaved form of caspase-3, phosphorylated protein kinase-like ER kinase (PERK), phosphorylated cAMP response element binding (CREB), proliferating cell nuclear antigen (PCNA; Cell Signaling, Danvers, MA, USA), insulin receptor substrate 2 (IRS2) (Upstate, Lake Placid, NY, USA), β-actin (Sigma–Aldrich), and pancreatic and duodenal homeobox 1 (Pdx1; provided by T Nakamura, Shiga University of Medical Science). Quantitative analysis of the blots was performed using Multi Gauge Version 3.0 software (Fujifilm, Tokyo, Japan) and normalized to β-actin levels.

### Incubation with exenatide and MG132 in MIN6 cells

C/EBPB-overexpressed MIN6 cells were incubated with 100 nM exenatide for the indicated time. C/EBPB-overexpressed MIN6 cells were pretreated with 20 μM MG132 for 30 min before exenatide incubation.

### Immunostaining and morphometric analysis

Following removal, the pancreas was immersed in Bouin's solution, embedded in paraffin, and sliced into 4 μm thick sections, which were stained with antibody to insulin, glucagon, and PCNA (Dako, Carpinteria, CA, USA). Immune complexes were detected with secondary antibodies conjugated with either Cy3 or FITC (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Quantification of β-cell mass was performed as described previously (Kido *et al.* 2000, Hashimoto *et al.* 2005).

### Assessment of food intake

The food intake of four mice in each group caged separately was measured for 5 days before and at 8 weeks after vildagliptin treatment.

### Islet isolation and insulin content measurement

Islets were isolated from the pancreas by collagenase digestion and handpicked under stereoscopy. For measurement of islet insulin content, ten size-matched islets from each group were solubilized in acid–ethanol solution (75% ethanol, 1·4% HCl) overnight at 4 °C, and insulin concentrations were measured. Data were normalized to total protein content.

### Statistical analysis

All results are expressed as mean ± S.E.M. Statistical significance was assessed by ANOVA and, when appropriate, the Student's *t*-test. A $P$ value of $<0{\cdot}05$ was considered statistically significant.

## Results

### Effect of vildagliptin on blood glucose levels and serum insulin concentrations in pancreatic β cell-specific C/EBPB TG mice

We first examined plasma GLP1 level after 12 weeks of vildagliptin treatment. GLP1 levels in untreated TG mice showed a decreased tendency compared with control mice (Fig. 1A). Vildagliptin increased the GLP1 level by 2·4-fold in control mice and by fourfold in TG

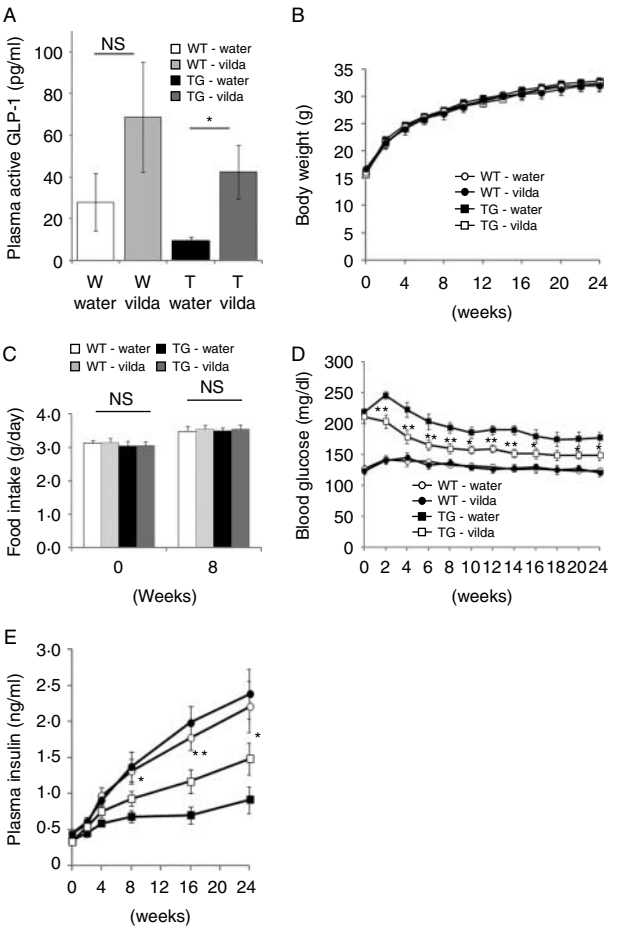


**Figure 1** Vildagliptin (vilda) decreases blood glucose and increased serum insulin concentrations in C/EBPB TG mice. (A) Plasma active GLP1 levels after 12 weeks of treatment ($n$=5–6). (B) Body weight during the 24-week treatment period ($n$=7–9 per group). (C) Food intake at the beginning and after 8 weeks of treatment ($n$=4 per group). (D) Blood glucose levels and (E) plasma insulin concentrations during the 24-week treatment period ($n$=9–12). *$P<0{\cdot}05$, **$P<0{\cdot}01$.

mice, although the increase in control mice did not reach statistical significance (Fig. 1A). Vildagliptin treatment did not alter food intake or body weight in either pancreatic β cell-specific C/EBPB TG mice or controls (Fig. 1B and C). As previously reported, blood glucose levels were found to be increased and serum insulin concentrations were decreased in TG mice compared with controls (Fig. 1D and E; Matsuda *et al.* 2010). Treatment with vildagliptin significantly improved blood glucose levels in TG mice as early as 2 weeks following the initiation of administration. Although the glucose levels of the TG mice did not reach those observed in the controls, this effect was maintained throughout the experimental period for up to 24 weeks (Fig. 1D). Vildagliptin treatment in TG mice was also found to result in a significantly increased

serum insulin concentration compared with untreated mice (Fig. 1E). Neither blood glucose level nor serum insulin concentration in controls was affected by vildagliptin treatment (Fig. 1D and E).

Similar results were observed when an oral glucose tolerance test (OGTT) was performed on the TG mice after 8 weeks of vildagliptin treatment. While the TG mice showed increased blood glucose levels associated with decreased insulin levels during the OGTT, vildagliptin treatment resulted in markedly decreased blood glucose levels and increased serum insulin concentrations (Fig. 2A and B). This treatment did not alter either parameter in controls (Fig. 2A and B). The OGTT was also performed after 24 weeks of treatment to examine whether the effects of vildagliptin were maintained for a longer period. After this longer period, improved glucose tolerance was again found to be associated with an increase in serum insulin concentrations in vildagliptin-treated TG mice, suggesting that the effect of vildagliptin on glucose metabolism in these mice is preserved for at least 24 weeks (Fig. 2C and D).

### Effect of vildagliptin on serum glucagon concentration in TG mice

GLP1 and its analogs are well known to suppress glucagon secretion of α cells (Drucker & Nauck 2006), although few reports have examined whether the increase in GLP1 level, elicited by DPP4 inhibition, has such an effect (Balas *et al.* 2007, Ahren *et al.* 2010). We therefore examined whether vildagliptin treatment had an impact on serum glucagon concentration in TG mice. OGTTs revealed that the basal serum glucagon concentration was significantly increased in vildagliptin-untreated TG mice and was suppressed to a lesser extent following the administration of glucose compared with controls. This suggests that α-cell dysfunction contributes to glucose intolerance in TG mice (Fig. 2E). The basal glucagon concentration and abnormal trend observed after glucose load were significantly improved by vildagliptin treatment in TG mice (Fig. 2E). These effects were also confirmed by the area under the curve during the OGTT (Fig. 2F).

### Effect of vildagliptin on β-cell mass in TG mice

We next studied whether vildagliptin treatment resulted in increased β-cell mass in TG mice. Consistent with our previous report (Matsuda *et al.* 2010), TG mice exhibited a 60% decrease in β-cell mass compared with controls (Fig. 3A and B). A marked increase in β-cell mass was observed in the TG mice after 8 weeks of treatment with vildagliptin (Fig. 3A). When the β-cell mass was quantified, the vildagliptin-treated TG mice showed an 80% increase compared with untreated TG mice (Fig. 3B). We also examined whether the protective effect of vildagliptin on β-cell mass was preserved for a longer period than the initial 8 weeks, and it was found to be maintained in TG mice over

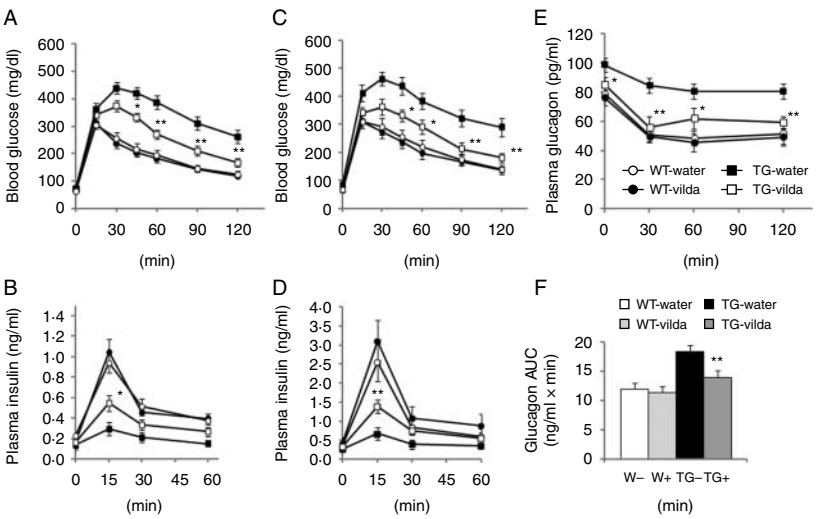


**Figure 2** Vildagliptin (vilda) improves glucose intolerance in TG mice. (A and C) Blood glucose levels, (B and D) plasma insulin levels, (E) plasma glucagon levels, and (F) area under the curve (AUC 0–120 min) of plasma glucagon levels of overnight (16 h) fasted mice after oral glucose load (1·5 mg/g body weight) following 8 weeks (A, B, E, and F) and 24 weeks (C and D) of vildagliptin treatment ($n$=7–9 per group). *$P$<0·05, **$P$<0·01.

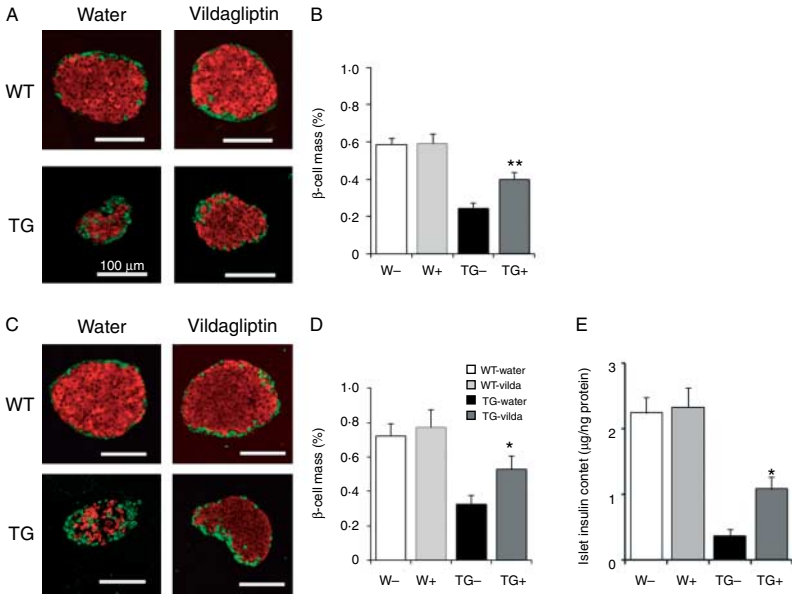


**Figure 3** Vildagliptin increases β-cell mass and insulin content in TG mice. (A) Immunofluorescence staining of pancreas sections for insulin (red) and glucagon (green) following 8 weeks of vildagliptin administration. (B) Quantification of β-cell mass in WT and TG mice. (C and D) Immunofluorescence staining of pancreas sections and quantification of β-cell mass in WT and TG mice following 24 weeks of vildagliptin administration. (E) Insulin content of pancreatic islet cells from WT and TG mice ($n=5$ per group). *$P<0{\cdot}05$, **$P<0{\cdot}01$.

the 24 weeks of treatment (Fig. 3C and D). The extent of protection was equivalent between 8 and 24 weeks of treatment (Fig. 3A, B, C, and D). While the insulin content of the islet cells was also found to be significantly decreased in TG mice compared with controls (Fig. 3E), vildagliptin treatment significantly increased the insulin content by ∼2·5-fold in the TG mice (Fig. 3E). We also examined the α cell mass because serum glucagon concentration was increased in TG mice and because vildagliptin treatment improved this alteration in these mice. α cell mass in TG mice showed an increased tendency compared with control mice, although it did not reach statistical significance (Fig. 3A and C, and data not shown). Furthermore, vildagliptin treatment affected α cell mass in neither TG mice nor control mice (Fig. 3A and C, and data not shown).

To further examine the mechanism involved in the increased β-cell mass observed in the TG mice following vildagliptin treatment, β cell proliferation and apoptosis were evaluated. The number of PCNA-positive cells in untreated TG mice was found to be decreased relative to controls (Fig. 4A and B), while vildagliptin partially, but significantly, increased the number of PCNA-positive cells in TG mice (Fig. 4A and B). Although the abundance of PCNA was also found to be decreased in the islets of TG mice, levels were again restored following vildagliptin treatment (Fig. 4C). When apoptosis was assessed, upregulation of CHOP protein and increased abundance of the cleaved form of caspase-3 were observed in the islet cells of TG mice, although these apoptosis markers were significantly inhibited by treatment with vildagliptin (Fig. 5A and C). These results suggest that both increased β cell proliferation and decreased β cell apoptosis contribute to the increased β-cell mass observed following vildagliptin treatment in TG mice.

## Effect of vildagliptin on gene expression and signaling pathways in the islets of TG mice

To clarify the molecular mechanism by which vildagliptin ameliorates β cell failure in TG mice, alterations in the expression or phosphorylation status of various genes were examined in islets isolated from the TG mice. First, we checked ER stress signaling, which was composed of the PERK, IRE1a, and ATF6a pathways. Consistent with our previous report (Matsuda *et al.* 2010), the phosphorylation levels of PERK and eukaryotic translation initiation factor 2A (eIF2α) were increased in untreated TG mice (Fig. 5A and C). IRE1a signaling was also increased in untreated TG mice as shown by the increase in the spliced form of XBP1, which is a downstream target of IRE1a

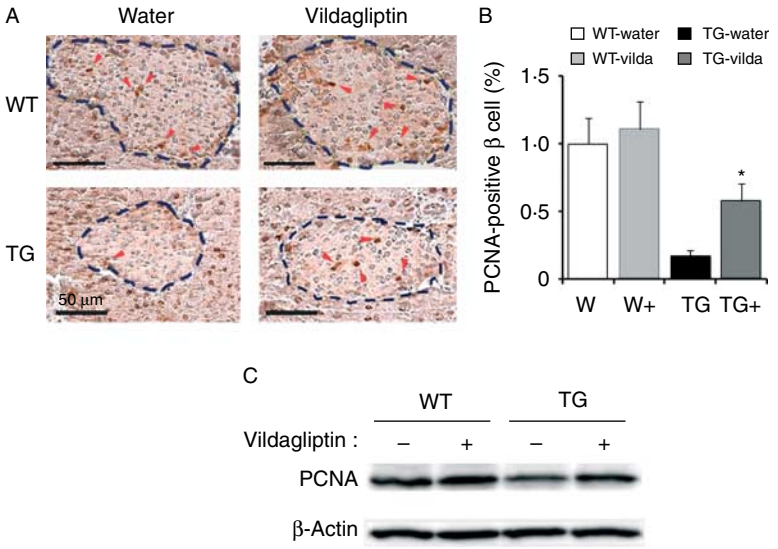


**Figure 4** Vildagliptin increases β cell proliferation in TG mice. Detection of the expression of cell proliferation-related protein after 8 weeks of vildagliptin treatment. (A) Pancreatic sections stained with antibodies to PCNA (arrowheads indicate PCNA-positive cells). (B) Quantification of PCNA-positive β cells to total number of β cells. (C) Western blot analysis of islets cells using antibodies to PCNA ($n=5$ per group). *$P<0.05$.

(Fig. 5E and F). Vildagliptin treatment suppressed activation of these pathways in TG mice (Fig. 5A and C). GRP78 expression, which is regulated mainly by the ATF6a pathway, was not altered in TG mice or by vildagliptin treatment (data not shown). Similarly, the increased expression of proteins related to apoptosis, such as CHOP and cleaved caspase-3, observed in TG mice was substantially normalized following vildagliptin treatment, in a manner consistent with the increase in β-cell mass in TG mice (Fig. 5A). In addition, while TG mice exhibited a reduction in the phosphorylation of CREB, a transcription factor known to be activated by GLP1 in a cAMP-dependent manner, vildagliptin treatment reversed this decreased phosphorylation (Fig. 5B and D). Given that CREB regulates IRS2 expression (Jhala *et al.* 2003), which is a key component of insulin signaling in β cells, and that insulin/IGF1 signaling in β cells plays a pivotal role in maintaining β-cell mass (Withers *et al.* 1998, Kulkarni *et al.* 1999, Kubota *et al.* 2000, Hashimoto *et al.* 2006, Ueki *et al.* 2006), we also examined the alteration of insulin/IGF1 signaling in the islets of TG mice. As expected, these mice showed reduced IRS2 expression and attenuated Akt phosphorylation, while vildagliptin treatment increased both IRS2 expression and Akt phosphorylation (Fig. 5B and D). Finally, the PDX1 transcription factor is also essential for regulating β cell development and function (McKinnon & Docherty 2001). While PDX1 expression was markedly reduced in TG mice, vildagliptin almost completely restored PDX1 expression (Fig. 5B and D). Given that insulin/IGF1 signaling also regulates PDX1 expression in β cells (Kitamura *et al.* 2002), the upregulation of PDX1 expression by vildagliptin is thought to be attributable to the activation of insulin/IGF1 signaling via the cAMP/CREB pathway. C/EBPB expression in TG mice was unexpectedly decreased by vildagliptin treatment, although the mRNA expression of C/EBPB was not altered. This indicates that vildagliptin can elicit posttranscriptional modifications of C/EBPB.

### Effect of the GLP1 analog exenatide on gene expression and signaling pathways in C/EBPB overexpressing β cells

We next sought to examine whether the protective effect of vildagliptin on β cell failure in TG mice occurs as a direct result of the activity of this drug on β cells. MIN6 cells were infected with a retrovirus to induce overexpression of C/EBPB before being treated with the GLP1 analog exenatide. The expression levels and activation of various proteins in these cells were then examined. The phosphorylation of proteins involved in ER stress, such as protein kinase RNA-like ER kinase, eIF2a, and c-jun, as well as the expression of apoptosis-related proteins, including CHOP and cleaved caspase-3, was found to be markedly upregulated by C/EBPB overexpression, although these alterations were reversed by incubation with

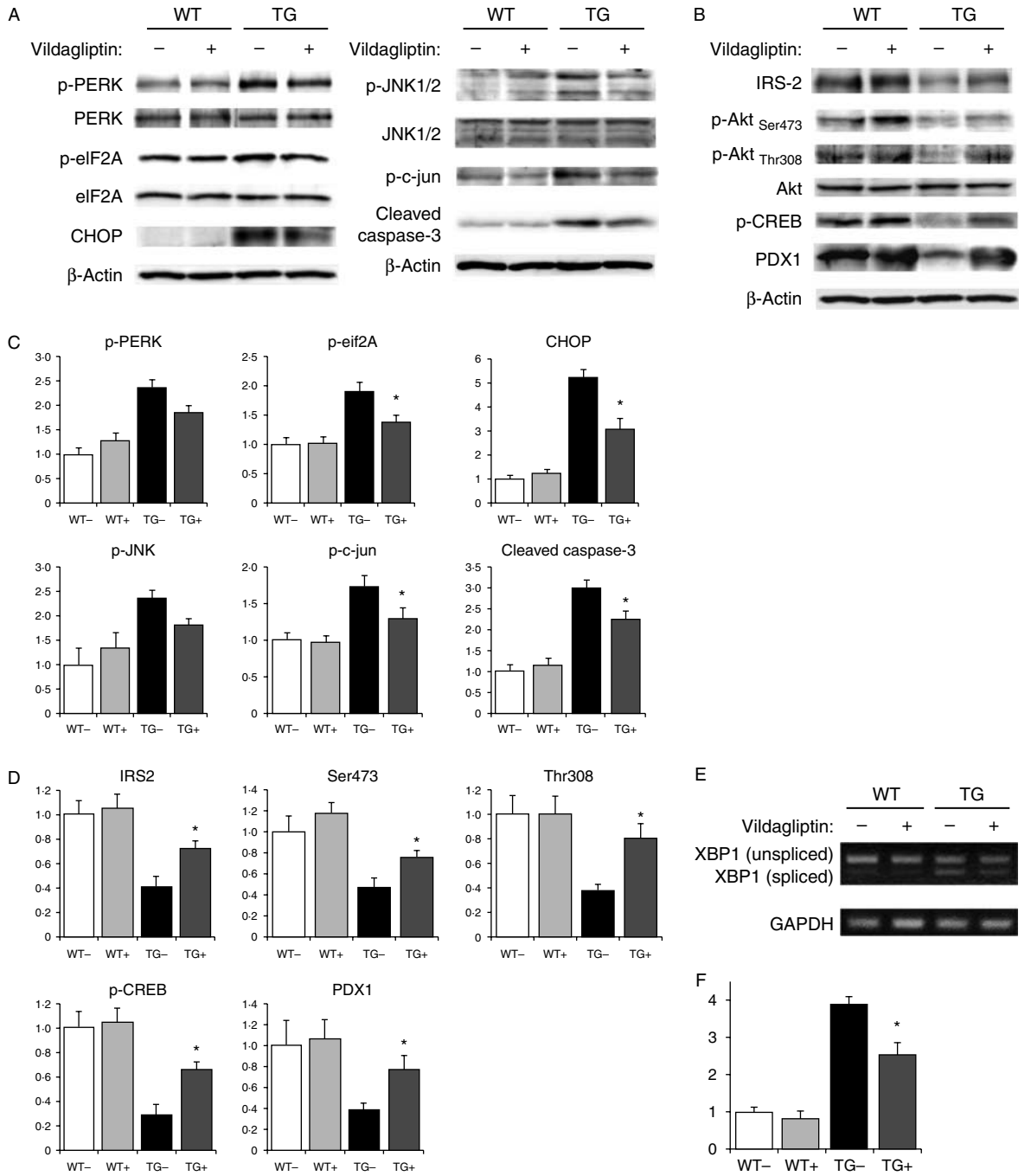


**Figure 5** Vildagliptin reduces ER stress and apoptosis and restores insulin signaling in TG mice. Western blot analysis of islets isolated from TG mice by collagenase digestion following 8 weeks of vildagliptin treatment to detect (A) ER stress-related proteins and apoptosis signaling proteins and (B) insulin signal-related proteins ($n=5$ per group). (C and D) Quantification of the western blot in A and B. (E) The amounts of unspliced form and spliced form of XBP1 in isolated islets and (F) quantification of the spliced form of XBP1 in E normalized to GAPDH. $^*P<0{\cdot}05$.

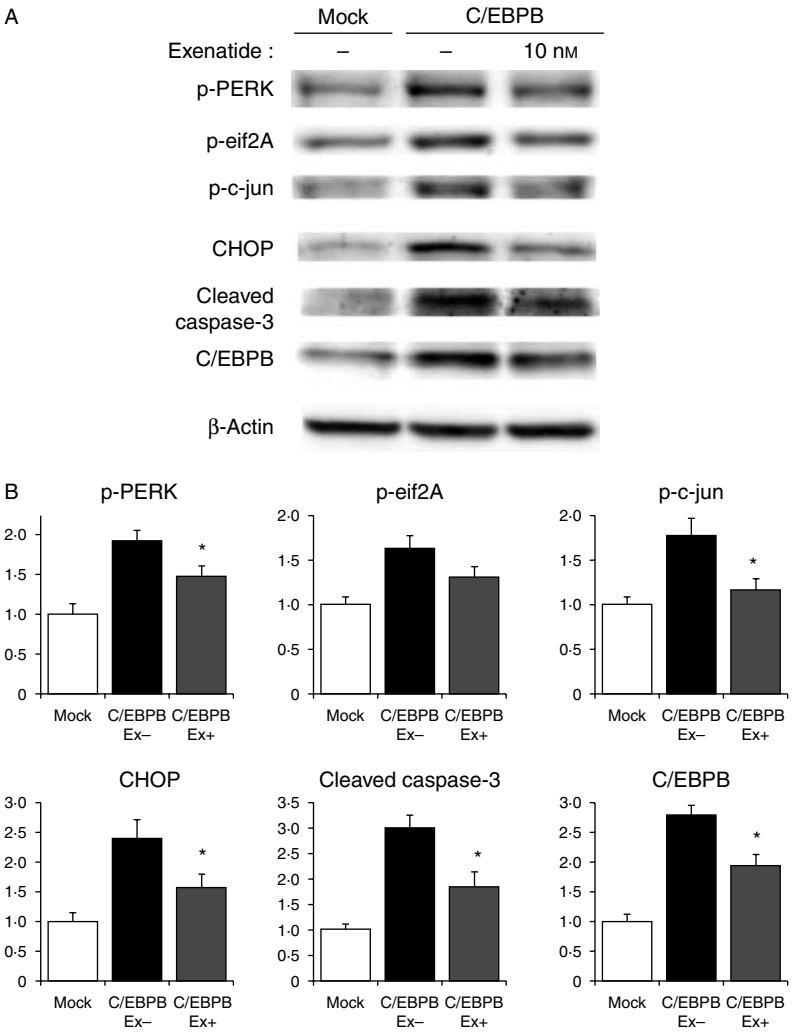


**Figure 6** Vildagliptin suppresses C/EBPB protein expression *in vitro*. (A) Western blot analysis of total cell protein extracted from MIN6 cells infected with a retrovirus encoding C/EBPB or the corresponding empty vector (Mock) following incubation in the presence or absence of 10 nM exenatide for 24 h. Data are representative of three independent experiments. (B) Quantification of western blot in A. *$P<0{\cdot}05$.

10 nM exenatide (Fig. 6A and B). These findings suggest that vildagliptin primarily exerts its protective effect on β cells in TG mice, independent of improved glucose metabolism.

### Activation of GLP1 pathway induces proteasome-dependent C/EBPB degradation

Exenatide treatment significantly attenuated C/EBPB expression in MIN6 cells (Fig. 6A and B), which was consistent with the results observed in islets isolated from vildagliptin-treated TG mice (Fig. 7A, B, and C). To study the mechanism by which activation of the GLP1 pathway attenuates the expression of C/EBPB protein, we tested the effect of a proteasome inhibitor, MG132, on exenatide-induced downregulation of C/EBPB. Exenatide again attenuated C/EBPB expression in these cells; however, MG132 restored expression of this protein, suggesting that activation of the GLP1 pathway induces proteasome-dependent degradation of C/EBPB protein in β cells (Fig. 7D).

## Discussion

We used pancreatic β cell-specific C/EBPB TG mice to examine whether vildagliptin exerts a protective effect on β cells. As TG mice manifest aggravated ER stress and decreased β-cell mass associated with increased apoptosis and reduced proliferation that are common

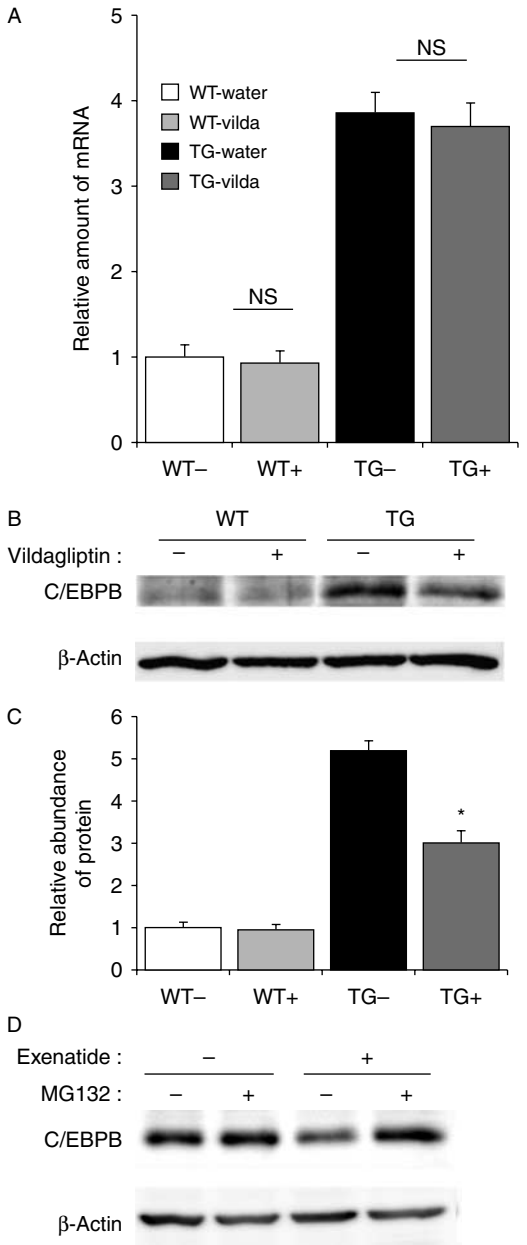


**Figure 7** Activation of the GLP1 pathway suppresses C/EBPB protein expression in TG mice. (A) The amount of C/EBPB mRNA normalized to cyclophilin in isolated islets from TG mice ($n=5$ per group). (B) Western blot analysis of C/EBPB in isolated islets from TG mice and (C) quantification of C/EBPB normalized to β-actin in B ($n=5$ per group). (D) C/EBPB-overexpressed MIN6 cells were pretreated with MG132 and incubated with 100 nM of exenatide for 6 h. C/EBPB expression was measured by western blot analysis. Data are representative of three independent experiments; quantitative data represent the mean±S.E.M. *$P<0{\cdot}05$.

pathogenic features observed in type 2 diabetes in humans (Harding & Ron 2002, Butler *et al.* 2003), we reasoned that TG mice would be a useful model for evaluating the beneficial effects of this agent. Although evidence has shown that incretin hormones such as GLP1 and its analogs can increase β-cell mass in animal models via blockading apoptosis or enhancing proliferation and neogenesis (Xu *et al.* 1999, Wang & Brubaker 2002, Li *et al.* 2003, Stoffers 2004), reports of whether DPP4 inhibitors increase β-cell mass have only recently started to emerge. We demonstrated that vildagliptin markedly improves insulin response to glucose and increases β-cell mass, leading to an alleviation of glucose metabolism in TG mice. We also showed that these effects are preserved over a relatively long term. Such observations indicate that vildagliptin could protect against the progressive decline in β cell function and mass over the course of type 2 diabetes in humans.

DPP4 inhibitors increase the circulating level of GLP1 and GIP through inhibiting degradation of these incretin hormones. As activation of the GLP1 pathway by exenatide improves aggravated ER stress and decreases insulin/IGF1 signaling by C/EBP overexpression in cultured β cells, the beneficial effect of vildagliptin observed in TG mice is thought to be attributed mainly to activation of the GLP1 pathway. However, the GIP pathway might also contribute to such an effect because GIP has been reported to protect pancreatic β cells (Widenmaier *et al.* 2010). The underlying molecular mechanism by which DPP4 inhibitors lead to an increase in β-cell mass is not fully understood. Insulin/IGF1 signaling in β cells has been shown to play a pivotal role in maintaining β-cell mass (Withers *et al.* 1998, Kulkarni *et al.* 1999, Kubota *et al.* 2000, Hashimoto *et al.* 2006, Ueki *et al.* 2006). In our study, the reduced β-cell mass observed in the TG mice was associated with attenuated insulin/IGF1 signaling, which could be restored by vildagliptin treatment and the resulting increase in the expression of IRS2. In addition to enhancing calcium-dependent insulin secretion (Holst & Gromada 2004), an increase in intracellular cAMP levels leads to the activation of the CREB transcription factor, which is responsible for the regulation of IRS2 expression in β cells (Jhala *et al.* 2003). In fact, activation of the GLP1 pathway by vildagliptin enhances CREB phosphorylation in β cells in TG mice. Therefore, the CREB-dependent upregulation of IRS2 expression is likely to be involved in the activation of insulin/IGF1 signaling by vildagliptin. This mechanism is supported by a recent study that demonstrated that IRS2 signaling is required for exenatide to have protective effects in β cells (Park *et al.* 2006). As PDX1 also plays an essential role in β cell development and the maintenance of mature β cell function (McKinnon & Docherty 2001), upregulation of PDX1 expression by vildagliptin also likely contributes to increased β-cell mass and improved insulin response to glucose elicited by this drug. PDX1 expression is known to be regulated by insulin/IGF1

signaling (Kitamura *et al.* 2002), and it is thus possible that vildagliptin directly upregulates PDX1 in β cells through activation of the insulin/IGF1 pathway.

ER stress is suggested to play an important role in the development of β cell failure in type 2 diabetes (Harding & Ron 2002). TG mice established in one of our previous studies were shown to manifest pancreatic β cell failure due to aggravated ER stress, such as increased eIF2a and c-jun phosphorylation (Matsuda *et al.* 2010). A previous study has additionally shown that exenatide directly modulates the ER stress response through induction of ATF4 expression and accelerates recovery from ER stress-mediated translational repression in cultured β cells in a cAMP-dependent manner (Yusta *et al.* 2006). Furthermore, exendin-4 and forskolin have been shown to protect β cells against free fatty acid- and salubrinal-induced ER stress and apoptosis by enhancing the expression of genes such as *BiP* (*Hspa5*), *Bcl2*, and *JunB* involved in cellular defenses (Cunha *et al.* 2009). However, the mechanism by which ER stress is regulated by GLP1 signaling remains unclear. In this study, we showed that activation of GLP1 signaling by either vildagliptin or exenatide improves aggravated ER stress in TG mice as well as in C/EBPB overexpressing β cells. As C/EBPB itself plays important roles in ER stress in β cells, we checked C/EBPB expression in vildagliptin-treated TG mice and in exenatide-treated C/EBPB overexpressing β cells. Unexpectedly, we observed that the expression of C/EBPB protein, but not mRNA, was downregulated in these experiments. We found that activation of the GLP1 pathway induced the proteasome-dependent C/EBPB degradation in β cells as MG132 restored the downregulation of C/EBPB protein by exenatide. Although we could not specify the molecular link between the GLP1 pathway and C/EBPB degradation in this study, GLP1 activation might alleviate ER stress in β cells possibly through inducing C/EBPB degradation. Future studies will be needed to uncover the pathway of GLP1-dependent C/EBPB degradation.

In conclusion, vildagliptin was shown to ameliorate hyperglycemia in TG mice by enhancing β-cell mass. It is likely that both the alleviation of ER stress and the enhancement of insulin signaling in β cells contribute to the beneficial effects of this agent. Collectively, our findings support the potential utility of vildagliptin in the protection of pancreatic β cells in patients with type 2 diabetes.

## Declaration of interest

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

## Funding

This work was supported by a grant for a Grant-in-Aid for Scientific Research from MEXT to Y K (22590981) and a Grant-in-Aid for Young Scientists from MEXT to T M (10015786), a Grant-in-Aid for Young Scientists from MEXT to N H (22790863), and a Novartis research grant to Y K.

## Author contribution statement

S Shimizu, M K-K, S-i A, T M, T H, S Seino, and Y K conceived and designed the experiments. S Shimizu, A K, M F, H E, H T, K T, H I, and Y M performed the experiments. S Shimizu, A B, M K-K, S-i A, T M, T H, N H, S Seino, and Y K analyzed the data. S Shimizu, A K, A B, M F, and H E contributed reagents/materials/analysis tools. T H, S Shimizu, and Y K wrote the manuscript.

## Acknowledgements

The authors thank D Kawamori (Osaka University) for excellent technical advice and M Nagano for technical assistance.

## References

Ahren B, Foley JE, Ferrannini E, Matthews DR, Zinman B, Dejager S & Fonseca VA 2010 Changes in prandial glucagon levels after a 2-year treatment with vildagliptin or glimepiride in patients with type 2 diabetes inadequately controlled with metformin monotherapy. *Diabetes Care* **33** 730–732. (doi:10.2337/dc09-1867)

Balas B, Baig MR, Watson C, Dunning BE, Ligueros-Saylan M, Wang Y, He YL, Darland C, Holst JJ, Deacon CF *et al.* 2007 The dipeptidyl peptidase IV inhibitor vildagliptin suppresses endogenous glucose production and enhances islet function after single-dose administration in type 2 diabetic patients. *Journal of Clinical Endocrinology and Metabolism* **92** 1249–1255. (doi:10.1210/jc.2006-1882)

Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA & Butler PC 2003 β-Cell deficit and increased β cell apoptosis in humans with type 2 diabetes. *Diabetes* **52** 102–110. (doi:10.2337/diabetes.52.1.102)

Cho JM, Jang HW, Cheon H, Jeong YT, Kim DH, Lim YM, Choi SH, Yang EK, Shin CY, Son MH *et al.* 2011 A novel dipeptidyl peptidase IV inhibitor DA-1229 ameliorates streptozotocin-induced diabetes by increasing β cell replication and neogenesis. *Diabetes Research and Clinical Practice* **91** 72–79. (doi:10.1016/j.diabres.2010.10.012)

Cunha DA, Ladriere L, Ortis F, Igoillo-Esteve M, Gurzov EN, Lupi R, Marchetti P, Eizirik DL & Cnop M 2009 Glucagon-like peptide-1 agonists protect pancreatic β cells from lipotoxic endoplasmic reticulum stress through upregulation of BiP and JunB. *Diabetes* **58** 2851–2862. (doi:10.2337/db09-0685)

Drucker DJ & Nauck MA 2006 The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. *Lancet* **368** 1696–1705. (doi:10.1016/S0140-6736(06)69705-5)

Duttaroy A, Voelker F, Merriam K, Zhang X, Ren X, Subramanian K, Hughes TE & Burkey BF 2011 The DPP-4 inhibitor vildagliptin increases pancreatic β-cell mass in neonatal rats. *European Journal of Pharmacology* **650** 703–707. (doi:10.1016/j.ejphar.2010.10.062)

Harding HP & Ron D 2002 Endoplasmic reticulum stress and the development of diabetes: a review. *Diabetes* **51** (Suppl 3) S455–S461. (doi:10.2337/diabetes.51.2007.S455)

Hashimoto N, Kido Y, Uchida T, Matsuda T, Suzuki K, Inoue H, Matsumoto M, Ogawa W, Maeda S, Fujihara H *et al.* 2005

PKClambda regulates glucose-induced insulin secretion through modulation of gene expression in pancreatic β cells. *Journal of Clinical Investigation* **115** 138–145. (doi:10.1172/JCI200522232)

Hashimoto N, Kido Y, Uchida T, Asahara S, Shigeyama Y, Matsuda T, Takeda A, Tsuchihashi D, Nishizawa A, Ogawa W *et al.* 2006 Ablation of PDK1 in pancreatic β cells induces diabetes as a result of loss of β-cell mass. *Nature Genetics* **38** 589–593. (doi:10.1038/ng1774)

Holst JJ & Deacon CF 2004 Glucagon-like peptide 1 and inhibitors of dipeptidyl peptidase IV in the treatment of type 2 diabetes mellitus. *Current Opinion in Pharmacology* **4** 589–596. (doi:10.1016/j.coph.2004.08.005)

Holst JJ & Gromada J 2004 Role of incretin hormones in the regulation of insulin secretion in diabetic and nondiabetic humans. *American Journal of Physiology. Endocrinology and Metabolism* **287** E199–E206. (doi:10.1152/ajpendo.00545.2003)

Jhala US, Canettieri G, Screaton RA, Kulkarni RN, Krajewski S, Reed J, Walker J, Lin X, White M & Montminy M 2003 cAMP promotes pancreatic β cell survival via CREB-mediated induction of IRS2. *Genes and Development* **17** 1575–1580. (doi:10.1101/gad.1097103)

Kido Y, Burks DJ, Withers D, Bruning JC, Kahn CR, White MF & Accili D 2000 Tissue-specific insulin resistance in mice with mutations in the insulin receptor, IRS-1, and IRS-2. *Journal of Clinical Investigation* **105** 199–205. (doi:10.1172/JCI7917)

Kitamura T, Nakae J, Kitamura Y, Kido Y, Biggs WH, Wright CV, White MF, Arden KC & Accili D 2002 The forkhead transcription factor Foxo1 links insulin signaling to Pdx1 regulation of pancreatic β cell growth. *Journal of Clinical Investigation* **110** 1839–1847.

Kubota N, Tobe K, Terauchi Y, Eto K, Yamauchi T, Suzuki R, Tsubamoto Y, Komeda K, Nakano R, Miki H *et al.* 2000 Disruption of insulin receptor substrate 2 causes type 2 diabetes because of liver insulin resistance and lack of compensatory β cell hyperplasia. *Diabetes* **49** 1880–1889. (doi:10.2337/diabetes.49.11.1880)

Kulkarni RN, Bruning JC, Winnay JN, Postic C, Magnuson MA & Kahn CR 1999 Tissue-specific knockout of the insulin receptor in pancreatic β cells creates an insulin secretory defect similar to that in type 2 diabetes. *Cell* **96** 329–339. (doi:10.1016/S0092-8674(00)80546-2)

Li Y, Hansotia T, Yusta B, Ris F, Halban PA & Drucker DJ 2003 Glucagon-like peptide-1 receptor signaling modulates β cell apoptosis. *Journal of Biological Chemistry* **278** 471–478. (doi:10.1074/jbc.M209423200)

Matsuda T, Kido Y, Asahara S, Kaisho T, Tanaka T, Hashimoto N, Shigeyama Y, Takeda A, Inoue T, Shibutani Y *et al.* 2010 Ablation of C/EBPβ alleviates ER stress and pancreatic β cell failure through the GRP78 chaperone in mice. *Journal of Clinical Investigation* **120** 115–126. (doi:10.1172/JCI39721)

McKinnon CM & Docherty K 2001 Pancreatic duodenal homeobox-1, PDX-1, a major regulator of β cell identity and function. *Diabetologia* **44** 1203–1214. (doi:10.1007/s001250100628)

Mu J, Petrov A, Eiermann GJ, Woods J, Zhou YP, Li Z, Zycband E, Feng Y, Zhu L, Roy RS *et al.* 2009 Inhibition of DPP-4 with sitagliptin improves glycemic control and restores islet cell mass and function in a rodent model of type 2 diabetes. *European Journal of Pharmacology* **623** 148–154. (doi:10.1016/j.ejphar.2009.09.027)

Park S, Dong X, Fisher TL, Dunn S, Omer AK, Weir G & White MF 2006 Exendin-4 uses Irs2 signaling to mediate pancreatic β cell growth and function. *Journal of Biological Chemistry* **281** 1159–1168. (doi:10.1074/jbc.M508307200)

Rhodes CJ 2005 Type 2 diabetes – a matter of β cell life and death? *Science* **307** 380–384. (doi:10.1126/science.1104345)

Stoffers DA 2004 The development of β-cell mass: recent progress and potential role of GLP-1. *Hormone and Metabolic Research* **36** 811–821. (doi:10.1055/s-2004-826168)

Ueki K, Okada T, Hu J, Liew CW, Assmann A, Dahlgren GM, Peters JL, Shackman JG, Zhang M, Artner I *et al.* 2006 Total insulin and IGF-I resistance in pancreatic β cells causes overt diabetes. *Nature Genetics* **38** 583–588. (doi:10.1038/ng1787)

UK Prospective Diabetes Study (UKPDS) Group 1998*a* Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34). UK Prospective Diabetes Study (UKPDS) Group. *Lancet* **352** 854–865. (doi:10.1016/S0140-6736(98)07037-8)

UK Prospective Diabetes Study (UKPDS) Group 1998*b* Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). UK Prospective Diabetes Study (UKPDS) Group. *Lancet* **352** 837–853. (doi:10.1016/S0140-6736(98)07019-6)

Wajchenberg BL 2007 β-Cell failure in diabetes and preservation by clinical treatment. *Endocrine Reviews* **28** 187–218. (doi:10.1210/10.1210/er.2006-0038)

Wang Q & Brubaker PL 2002 Glucagon-like peptide-1 treatment delays the onset of diabetes in 8 week-old db/db mice. *Diabetologia* **45** 1263–1273. (doi:10.1007/s00125-002-0828-3)

Weyer C, Bogardus C, Mott DM & Pratley RE 1999 The natural history of insulin secretory dysfunction and insulin resistance in the pathogenesis of type 2 diabetes mellitus. *Journal of Clinical Investigation* **104** 787–794. (doi:10.1172/JCI7231)

Widenmaier SB, Kim SJ, Yang GK, De Los Reyes T, Nian C, Asadi A, Seino Y, Kieffer TJ, Kwok YN & McIntosh CHS 2010 A GIP receptor agonist exhibits B-cell anti-apoptotic actions in rat models of diabetes resulting in improved B-cell function and glycemic control. *PLoS ONE* **5** e9590. (doi:10.1371/journal.pone.0009590)

Withers DJ, Gutierrez JS, Towery H, Burks DJ, Ren JM, Previs S, Zhang Y, Bernal D, Pons S, Shulman GI *et al.* 1998 Disruption of IRS-2 causes type 2 diabetes in mice. *Nature* **391** 900–904. (doi:10.1038/36116)

Xu G, Stoffers DA, Habener JF & Bonner-Weir S 1999 Exendin-4 stimulates both β cell replication and neogenesis, resulting in increased β-cell mass and improved glucose tolerance in diabetic rats. *Diabetes* **48** 2270–2276. (doi:10.2337/diabetes.48.12.2270)

Yusta B, Baggio LL, Estall JL, Koehler JA, Holland DP, Li H, Pipeleers D, Ling Z & Drucker DJ 2006 GLP-1 receptor activation improves β cell function and survival following induction of endoplasmic reticulum stress. *Cell Metabolism* **4** 391–406. (doi:10.1016/j.cmet.2006.10.001)

Received in final form 21 June 2012
Accepted 19 July 2012
Made available online as an Accepted Preprint 19 July 2012