WFS1-deficiency increases endoplasmic reticulum stress, impairs cell cycle progression and triggers the apoptotic pathway specifically in pancreatic β-cells

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SUMMARY

Wolfram syndrome, an autosomal recessive disorder associated with diabetes mellitus and optic atrophy, is caused by mutations in the *WFS1* gene encoding an endoplasmic reticulum (ER) membrane protein. Herein, we report that pancreatic islets of *wfs1*-deficient mice exhibit increases in phosphorylation of RNA-dependent protein kinase-like ER kinase, chaperone gene expressions and active XBP1 protein levels, indicating an enhanced ER stress response. We established *wfs1*-deficient MIN6 clonal β-cells by crossing *wfs1*-deficient mice with mice expressing simian virus 40 large T antigen in β-cells. These cells show essentially the same alterations in ER stress responses as *wfs1*-deficient islets, which were reversed by re-expression of WFS1 protein or overexpression of GRP78, a master regulator of the ER stress response. In contrast, these changes are not observed in heart, skeletal muscle, or brown adipose tissues with WFS1-deficiency. The increased ER stress response was accompanied by reduced BrdU incorporation and increased caspase-3 cleavage, indicating impaired cell cycle progression and accelerated apoptotic processes in the mutant islets. These changes are associated with increased expression of the cell cycle regulator $p21^{CIP1}$ in *wfs1*-deficient islets and clonal β-cells. Treatment of islets with thapsigargin, an ER stress inducer, caused upregulation of $p21^{CIP1}$. In addition, forced expression of $p21^{CIP1}$ resulted in reduced MIN6 β-cell numbers, suggesting the ER stress-induced increase in $p21^{CIP1}$ expression to be involved in β-cell loss in the mutant islets. These data indicate that WFS1-deficiency activates the ER stress response specifically in β-cells, causing β-cell loss through impaired cell cycle progression and increased apoptosis.

INTRODUCTION

Type 2 diabetes is caused by complex interactions between insulin resistance in peripheral tissues and impaired insulin secretion from pancreatic β-cells. There is a general consensus that the latter results from both impaired β-cell function and decreased β-cell mass (1-3). Adult β-cell mass is maintained by a balance between generation and death of β-cells. In patients with type 2 diabetes, new islet formation and β-cell replication are reportedly normal, and an increased rate of apoptosis has been suggested to underlie the loss of β-cell mass (4).

Recent studies using novel mutant mice have led to new insights into endoplasmic reticulum (ER) stress and maintenance of β-cell mass (5,6). The ER stress response, also known as the unfolded protein response (UPR), involves translational attenuation, transcriptional induction of chaperones and folding enzymes, as well as degradation of misfolded proteins, a process called ER-associated degradation (ERAD). When ER stress is strong and cellular survival mechanisms fail to correct the protein-folding defects, an ER stress-mediated apoptotic process is initiated (5-7). Mice with a homozygous null mutation of RNA-dependent protein kinase-like ER kinase (PERK) lose their ability to phosphorylate eukaryotic initiation factor 2α (eIF2α) and fail to attenuate translation in response to ER stress. These mice develop diabetes due to reduced β-cell mass (8). Importantly, mutations of the *EIF2AK3* gene encoding PERK in humans have been recognized as causing Wolcott-Rallison syndrome with diabetes mellitus in early infancy (9). A mouse model in which a Ser51Ala mutation of eIF2α prevents the protein from being phosphorylated by PERK and other eIF2α kinases, also displays a β-cell defect and impaired gluconeogenesis leading to lethal hypoglycemia

(10). Mice with a deletion mutation of $P58^{IPK}$, a cytosolic chaperone, were recently reported to exhibit β-cell failure and diabetes (11). These examples suggest that β-cells, producing large quantities of insulin and thus a greater load on the ER, are especially sensitive to ER stress.

Wolfram syndrome is a rare autosomal recessive disorder characterized by juvenile-onset diabetes mellitus, optic atrophy, diabetes insipidus and sensorineural deafness (12). This syndrome is caused by mutations in the *WFS1* gene (13,14), which encodes an ER resident membrane protein (15). Postmortem studies of the pancreas from subjects with Wolfram syndrome have shown β-cell loss (16). We recently established a line of mutant mice with a disrupted *wfs1* gene and found that these mice also exhibited impaired glucose homeostasis accompanied by a progressive reduction of β-cell mass (17). Thus, the *wfs1*-deficient mouse is a model for studying mechanisms of β-cell loss during the development of diabetes in Wolfram syndrome. We and others have also shown expression of WFS1 protein to be up-regulated by ER stress-inducing agents (18-20). A recent study employing IRE1α knockout and PERK knockout cells suggested that WFS1 is a component of the IRE1 and PERK signaling pathways (20). In addition, *wfs1*-deficient islets have been shown to exhibit increased DNA fragmentation in response to ER stress inducers (17), suggesting β-cell loss in Wolfram syndrome to be attributable to an inability to handle ER stress. A very recent study of islets conditionally lacking the *wfs1* gene in β-cells, demonstrated an increased GRP78 mRNA to GLUT2 mRNA ratio. This observation was interpreted as evidence of an enhanced ER stress response, on the assumption that GLUT2 mRNA levels represented the β-cell number in islets (21).

To further investigate the mechanisms underlying β-cell loss in Wolfram

syndrome, we conducted a systematic study of the UPR in *wfs1*-deficient islets as well as other tissues. We also created β-cell lines with WFS1-deficiency and studied UPR. We found all three UPR subpathways to be activated in *wfs1*-deficient islets and β-cell lines. Furthermore, we demonstrated increased cleavage of caspase-3, a hallmark of apoptosis, and impaired proliferation associated with enhanced expression of the cell cycle regulator $p21^{CIP1}$.

RESULTS

UPR activation in *wfs1*-deficient islets

A systematic study of the UPR was conducted using islets isolated from 6-week-old male *wfs1*-deficient mice with the B6 background. At 6 weeks of age, the β-cell mass of these mice begins to decrease (17). Accumulation of unfolded proteins in the ER is well known to induce dissociation of GRP78 from PERK, resulting in oligomerization and subsequent auto-phosphorylation of PERK. Activated PERK then phosphorylates eIF2α and suppresses general protein translation to reduce the ER load (5-7). In freshly isolated *wfs1*-deficient islets, PERK phosphorylation was increased (Fig. 1A). In addition, eIF2α phosphorylation was slightly but significantly enhanced with no alteration in total eIF2α levels in mutant islets (Fig. 1A). Thus, the ratio of phosphorylated eIF2α over total eIF2α levels analyzed by densitometry was increased by 27 ± 7% ($n = 4$ experiments, $p < 0.05$). These data indicate that one of three subpathways of the UPR arising from PERK phosphorylation is initiated in response to WFS1-deficiency in islets.

ER stress is also sensed by other ER resident proteins, IRE1 and ATF6, in addition to PERK (5-7). Activation of ATF6 via GRP78 dissociation and subsequent cleavage is known to induce the expressions of various chaperone genes, constituting another subpathway of the UPR (5-7). In *wfs1*-deficient islets, GRP94 mRNA levels were increased and those of GRP78 and $P58^{IPK}$ also tended to rise (Fig. 1B). Correspondingly, although the differences failed to reach statistical significance, levels of these chaperone proteins tended to be increased (Fig. 1C), suggesting that the ATF6 subpathway of the UPR is activated in response to WFS1-deficiency.

As shown in Fig. 1D, a shorter form of XBP1 mRNA was increased. This form is produced by 26-nucleotide splicing from primary XBP1 mRNA by the ribonuclease activity of IRE1, increasing active XBP1 protein levels in mutant islets (Fig. 1E). HRD1, a ubiquitin ligase involved in ERAD, is one of the XBP1 target genes (20). In *wfs1*-deficient islets, levels of HRD1 protein were markedly increased (Fig. 1E). In addition, mRNA levels of EDEM (ER-associated degradation-enhancing α-mannosidase-like protein) (23), another target of XBP1, were significantly increased in mutant islets (100 ± 5 arbitrary units (wild type) vs. 136 ± 18 (mutant), $n = 6$, $p < 0.05$). These data indicate that the IRE1-initiated subpathway of the UPR is also activated in *wfs1*-deficient islets.

Establishment of MIN6 β-cell lines deficient in WFS1

To examine the influence of WFS1-deficiency specifically in a homogenous β-cell population, β-cell lines were established by crossing $wfs1^{+/-}$ and $wfs1^{-/-}$ mice (17) with IT6 mice expressing simian virus 40 (SV40) large T antigen under the insulin promoter (24) and were designated MIN6wfs1$^{+/-}$ and MIN6wfs1$^{-/-}$, respectively (see Materials and Methods). IT6 mice were previously reported to develop insulinoma, from which the MIN6 cell line (24), one of the most highly differentiated β-cell lines, was generated. We established two cell lines each for the $wfs1^{+/-}$ and $wfs1^{-/-}$ genotypes. As shown in Fig. 2A, the two cell lines with the $wfs1^{-/-}$ genotype (MIN6wfs1$^{-/-}$-1 and 2) show similar UPR characteristics. Similarly, characteristics of two cell lines with the $wfs1^{+/-}$ genotype (MIN6wfs1$^{+/-}$) were indistinguishable (data not shown). Therefore, only one line of each genotype was used for subsequent analyses. We compared MIN6wfs1$^{-/-}$ with MIN6wfs1$^{+/-}$ at the same passage numbers (passages 5 to 8), but not with the original

MIN6 cells. This is because we were concerned that a difference in passage number between the original MIN6 and MIN6wfs1$^{-/-}$ cells, irrespective of WFS1-deficiency, might affect the protein expression profile, rendering the former an inappropriate control for the latter. After completion of a series of experiments, MIN6wfs1$^{+/-}$ cells reached passages 15 - 20, the same passage of original MIN6 cells we have. The function and survival of MIN6wfs1$^{+/-}$ cells are similar to those of wild-type MIN6 cells at similar passage numbers (data not shown).

Effects of WFS1-deficiency on UPR in β-cell lines

As shown in Fig. 2A, altered expressions of UPR-related proteins observed in *wfs1*-deficient islets were reproduced in MIN6wfs1$^{-/-}$ cells; PERK phosphorylation, as well as expressions of active XBP1 and HRD1, were increased in *wfs1*-deficient MIN6 cells. ATF4 levels were also shown to be increased in these cells. Furthermore, although GRP78 and GRP94 protein levels were similar (Fig. 2B, upper panel), the activity of the GRP78 promoter containing three ER stress response elements was greater in MIN6wfs1$^{-/-}$ cells than in MIN6wfs1$^{+/-}$ cells (Fig. 2B), strongly suggesting activation of the ATF6 subpathway of the UPR in MIN6wfs1$^{-/-}$ cells. To confirm that alterations in UPR-related proteins are due to WFS1-deficiency, wild-type human WFS1 protein was expressed in MIN6wfs1$^{-/-}$ cells. We took advantage of the tetracycline-inducible expression system. MIN6wfs1$^{-/-}$ cells were infected with the Tet-repressor expressing virus (AdCAG-TR) together with a recombinant adenovirus bearing wild-type human WFS1 cDNA under the CMV promoter containing the Tet-operator (AdCTO-WFS1). The cells were then treated with doxycycline (2 μg/ml). As shown in Fig. 2C, when WFS1 expression was restored to levels comparable to those of the original MIN6 cells,

the increase in PERK phosphorylation was prevented. In addition, overexpression of GRP78, a master regulator of the ER stress response, also resulted in normalization of PERK phosphorylation levels (Fig. 2D), clearly indicating the observed alteration in UPR-related proteins to be due to exacerbation of ER stress caused by WFS1-deficiency.

No UPR induction in heart, skeletal muscle or brown adipose tissues from *wfs1*-deficient mice

WFS1 protein is expressed in a variety of non-pancreatic tissues, though less abundantly than in islets (Fig. 3A). Therefore, we also examined expressions of UPR genes in tissues other than pancreatic islets. Cardiac function is reportedly not impaired in subjects with Wolfram syndrome (12) or in *wfs1*-deficient mice (17). Skeletal muscle and brown adipose tissue also appear essentially normal in mutant mice (data not shown). In contrast to islets, no UPR alterations were observed in these tissues from *wfs1*-deficient mice (Fig. 3B-D). Thus, UPR activation is tissue-specific in WFS1-deficiency.

Increased β-cell apoptotic response in *wfs1*-deficient islets

ER stress induces apoptosis through activation of various signaling molecules including JNK and pro-apoptotic proteins, such as CHOP (5-7). CHOP expression was increased at both the mRNA (Fig. 4A) and the protein level (Fig. 4B), in mutant as compared to wild-type islets. In contrast, JNK expression levels and phosphorylation states were not altered in *wfs1*-deficient islets (Fig. 4B). We also found increased levels of cleaved caspase-3, a hallmark of apoptosis, in mutant islets (Fig. 4B). CHOP expression and

cleaved caspase-3 levels were also increased in *wfs1*-deficient MIN6 cells (Fig. 4C), while no such changes were observed in heart, skeletal muscle or adipose tissue (data not shown).

We also measured apoptosis in MIN6wfs1$^{-/-}$ and MIN6wfs1$^{+/-}$ cells by counting adherent cells positive for annexin V staining under fluorescent microscope. We found 1 - 2% cells to be annexin V positive for both the *wfs1*$^{-/-}$ and the *wfs1*$^{+/-}$ genotype cultured under standard conditions, i.e. no differences between MIN6wfs1$^{-/-}$ and MIN6wfs1$^{+/-}$ cells. An increase in the number of apoptotic cells was observed when MIN6wfs1$^{-/-}$ cells were challenged with 0.5 μM thapsigargin for 24 hours, as compared to MIN6wfs1$^{+/-}$ cells under the same conditions (2.7 ± 1.0% (MIN6wfs1$^{+/-}$) vs. 6.2 ± 1.1% (MIN6wfs1$^{-/-}$), $n = 3$, $p < 0.05$). Therefore, MIN6wfs1$^{-/-}$ cells exhibited increased apoptosis susceptibility. These data, together, indicate that an ER stress mediated -apoptotic process is activated in *wfs1*-deficient β-cells.

Impaired β-cell proliferation in *wfs1*-deficient islets

In addition to increased apoptosis, decreased proliferation may contribute to loss of β-cell mass in *wfs1*-deficient mice. When β-cell proliferation activity was assayed by 5-bromodeoxyuridine (BrdU) incorporation in pancreases from wild-type and mutant mice, BrdU incorporation was found to be significantly reduced in *wfs1*-deficient β-cells (Fig. 5A and B). This observation suggested impaired proliferation, along with increased apoptosis, to contribute to β-cell loss in *wfs1*-deficient islets.

We next explored possible causes of the decreased β-cell proliferation in *wfs1*-deficient islets. The link between the UPR and cell cycle arrest was previously reported to be mediated by down-regulation of cyclin D1 due to general translational

suppression via eIF2α phosphorylation (25). However, neither expression of cyclin D1 nor that of cyclin D2, major isoforms of the D type cyclins in β-cells (26,27), was changed in mutant islets (data not shown). CHOP has also been recognized as causing cell cycle arrest and apoptosis (28,29). Since GADD34 is reportedly a target of CHOP (30) and is involved in cell growth and survival (31), GADD34 expression was examined. GADD34 transcript levels were found to be increased in *wfs1*-deficient islets (100 ± 11 vs. 151 ± 14, $p < 0.05$). Recent studies have demonstrated that cell cycle regulation is critical for maintenance of β-cell mass (25,26). Since GADD34 reportedly induces p53 phosphorylation and enhances expression of the cell cycle inhibitor $p21^{CIP1}$ (32), p53 and $p21^{CIP1}$ expressions were assessed. We found phosphorylation of p53 to be increased, though total p53 was not elevated (Fig. 5C). In addition, increased expressions of $p21^{CIP1}$ mRNA (100 ± 11 vs. 413 ± 32, $p < 0.01$) and $p21^{CIP1}$ protein (Fig. 5C) were observed in *wfs1*-deficient islets. We also examined the expression of another cell cycle inhibitor, $p27^{KIP1}$, and found no difference between wild-type and mutant islets (Fig. 5C). Increased expression of $p21^{CIP1}$ protein was also observed in *wfs1*-deficient MIN6 cells, SV40 large T antigen-transformed cells in which p53 activity was considered to be suppressed (Fig. 5D). Expression of $p21^{CIP1}$ protein was not increased in heart, skeletal muscle or brown adipose tissues from *wfs1*-deficient mice (data not shown).

In order to determine whether increased expression of $p21^{CIP1}$ is attributable to ER stress, wild-type islets were treated with thapsigargin (0.5 μM) for 12 hours. As shown in Fig. 5E, expression of $p21^{CIP1}$ was significantly increased. In addition, expression of $p21^{CIP1}$ was markedly increased in MIN6 cells treated with thapsigargin (Fig. 5F) or tunicamycin (data not shown). These data suggest 21^{CIP1} expression to be

induced by ER stress in β-cells.

Finally to assess the effects of $p21^{CIP1}$ expression on β-cell proliferation, $p21^{CIP1}$ was expressed in wild-type MIN6 cells in a tetracycline-inducible manner (Fig. 6A). Overexpression of $p21^{CIP1}$ suppressed a MIN6 cell number increase (Fig. 6B), suggesting that increased $p21^{CIP1}$ expression contributes to the reduced β-cell mass in *wfs1*-deficient islets.

DISCUSSION

We systematically investigated UPR in *wfs1*-deficient islets and MIN6 β-cells as well as heart, skeletal muscle and brown adipose tissues from the mutant mice in this study. Enhanced UPR was specifically observed in β-cells but not in other tissues examined. These findings indicate that diabetes in Wolfram syndrome is caused by increased ER stress in β-cells and establish Wolfram syndrome as an ER stress-based disease, as is the case in Wolcott-Rallison syndrome with PERK-deficiency (9). Furthermore, we found enhanced UPR to be associated with not only activation of the apoptotic pathway but also impaired cell cycle progression in β-cells. These observations provide evidence of novel mechanisms underlying ER stress-mediated β-cell loss.

We demonstrated activation of the PERK and IRE1 subpathways of the UPR. Increased activation of the GRP78 promoter indicates the ATF6 subpathway to be induced as well. GRP78 expression was also reportedly increased by knockdown of WFS1 expression in INS1 insulinoma β-cells (20). Collectively, these data indicate that all three UPR subpathways are activated by WFS1-deficiency in β-cells. The UPR is activated when ER homeostasis is perturbed by defective ER calcium homeostasis, mutations in ER resident proteins and/or abnormalities of the ERAD system. Disturbed ER homeostasis is also induced by defect(s) in components of the UPR system, as is the case in Wolcott-Rallison syndrome with PERK-deficiency. The present data suggest that impaired ER homeostasis does not result from defect(s) in a specific pathway(s) of the UPR. Our previous study demonstrated an abnormal cytosolic Ca^{2+} response in *wfs1*-deficient β-cells (17), suggesting that impaired ER Ca^{2+} homeostasis is a possible cause of ER stress associated with WFS1-deficiency.

We found that WFS1 protein is highly expressed in heart, skeletal muscle and brown adipose tissues. However, there is no UPR activation in these tissues from mutant mice. Thus, the UPR is tissue-specific in *wfs1*-deficient mice. One possible explanation of this tissue specificity is that a protein(s), compensating for loss of WFS1 protein function, is present in these tissues but not in β-cells. This interesting possibility merits further investigation and elucidation of WFS1 function is necessary to resolve the tissue-specific effects of WFS1-deficiency.

Our results demonstrate, in addition to the augmented apoptotic process evidenced by increased caspase-3 cleavage, that β-cell proliferation is decreased in *wfs1*-deficient mice. Impaired proliferation was also reported in BRIN-BD11 cells expressing the human WFS1 antisense transcript (33). Our observation is in contrast to that by Riggs et al., who detected no changes in the numbers of BrdU-positive cells in islets from β-cell specific *wfs1* knockout mice (21). The reason for this discrepancy is currently unclear, but may reflect differences in the ages of the mice studied: 6-week-old mice were used in the present versus 12- or 24-week-old animals in their study (21). Cell cycle dysregulation in *wfs1*-deficient islets was associated with increased expression of $p21^{CIP1}$, a cell cycle regulator. $p21^{CIP1}$ can serve, depending on which tissues or cells it is activated in, as both an inhibitor and an agonist of cell cycle progression (34). Our observation that forced expression of $p21^{CIP1}$ suppressed MIN6 β-cell proliferation suggests that $p21^{CIP1}$ operates as a cell cycle inhibitor in β-cells, although our results must be interpreted cautiously since forced overexpression of $p21^{CIP1}$ may produce effects different from those occurring in mutant β-cells with increased $p21^{CIP1}$ levels. A very recent study, demonstrating that $p21^{CIP1}$ acts as a molecular brake on mitogenic stimuli in β-cells (35), supports the notion of $p21^{CIP1}$

functioning as a cell cycle inhibitor in β-cells. ER stress inducers were recently reported to cause $p21^{CIP1}$ expression and cell cycle arrest in chondrocytes (36) and prostatic cancer cells (37), suggesting that cell cycle arrest associated with increased $p21^{CIP1}$ expression is a common feature in cells under ER stress. Furthermore, reduced proliferation associated with increased expression of $p21^{CIP1}$, in *wfs1*-deficient β-cells (the present study) and β-cells transgenic for hepatocyte growth factor and/or placental lactogen (35), highlights an important role for $p21^{CIP1}$ in regulation of β-cell mass in addition to the roles of $p27^{KIP1}$ recently reported (38).

CHOP induces GADD34 expression (32), which then activates p53 phosphorylation and $p21^{CIP1}$ transcription (36). Therefore, the CHOP --> GADD34 --> p53 pathway is a candidate for ER stress-mediated $p21^{CIP1}$ expression. Indeed, an increase in $p21^{CIP1}$ expression was associated with increased GADD34 expression and p53 phosphorylation in *wfs1*-deficient β-cells. However, induction of $p21^{CIP1}$ expression by thapsigargin was observed in MIN6 cells transformed with SV40 large T antigen, a well-known suppressor of p53. In addition, an ER stress-induced increase in $p21^{CIP1}$ expression was observed in p53-deficient prostatic cancer cells (37). Thus, ER stress appears to induce $p21^{CIP1}$ expression through both p53-dependent and -independent mechanisms.

Since β-cells are apparently much more sensitive to ER stress than other types of cells and tissues (39), ER stress might be a more common cause of β-cell failure than previously thought, especially in terms of the increased insulin demands of modern lifestyles. Our data indicate that both increased apoptosis and impaired proliferation, in β-cells, are mechanisms leading to β-cell loss in *wfs1*-deficient islets, a model of ER-stress mediated β-cell failure. Further studies designed to elucidate the molecular

mechanisms of β-cell loss under chronic ER stress are anticipated to contribute to future treatments for type 2 diabetes.

MATERIALS AND METHODS

Antibodies

The monoclonal antibody against $P58^{IPK}$ was a generous gift from Prof. M.G. Katze (University of Washington). Other antibodies were purchased from the indicated sources: anti-GRP94, anti-KDEL (Stressgen Biotechnologies), anti-GRP78, anti-XBP1, anti-$p21^{CIP1}$, anti-CHOP, anti-p53, anti-phosphorylated p53, and anti-ATF4 (Santa Cruz Biotechnology), anti-HRD1 (Abgent), anti-phosphorylated PERK, anti-JNK, anti-phosphorylated JNK, anti-eIF2α, anti-phosphorylated eIF2α, and anti-cleaved caspase-3 (Cell Signaling), and anti-$p27^{KIP1}$ (BD Transduction Laboratories).

Mouse islet isolation, real-time RT-PCR and Western blot

The *wfs1*-deficient mice used had a C57Bl/6 background and were described previously (17). All animal experiments were approved by the Tohoku University Institutional Animal Care and Use Committee (#15-45). Islets were isolated by collagenase infusion through the common bile duct and harvested by hand. Total RNA was prepared immediately after islet isolation using an RNAeasy kit (Qiagen). For real-time RT-PCR analysis, cDNA was synthesized by reverse transcription using the oligo $d(T)_{16}$ primer and subjected to PCR amplification with gene-specific primers (Table 1) using a SYBR Green 1 kit (Roche). Data are presented as relative values to actin mRNA. For detection of the spliced form of XBP1 mRNA, the primers were: 5'-TGAGAACCAGGAGTTAAGAAACGC-3' and 5'-TTCTGGGTAGACCTCTGGG AGTTCC-3'. For immunoblotting, islets from 3 to 4 mice were pooled, dissolved immediately after isolation in a lysis buffer (approximately 100 islets/15 μl) and

subjected to SDS-polyacrylamide gel electrophoresis. In several experiments, isolated islets were cultured overnight and treated with 0.5 μM thapsigargin for 12 h. All Western blot experiments were repeated at least three times, with different sets of samples, throughout this study. Immunoblot band intensities were analyzed using Scion image software (Scion Corporation) and normalized with those of actin.

Establishment of MIN6wfs1$^{-/-}$ and MIN6wfs1$^{+/-}$ cell lines

The *wfs1*$^{-/-}$ mice (17) were bred with IT6 mice expressing SV40 large T antigen under the human insulin promoter (24) and the resulting *wfs1*$^{+/-}$:SV40Tag/+ mice were further bred with *wfs1*$^{-/-}$ mice. Tumors from pancreases of 10- to 12-week-old *wfs1*$^{+/-}$:SV40Tag/+ and *wfs1*$^{-/-}$:SV40Tag/+ mice were carefully excised and placed in Dulbecco's Modified Eagle's Medium containing penicillin and streptomycin. Cells were expanded and frozen at passages 3 and 4. We used these cells at 5 to 8 passages in this study. For study of apoptosis, MIN6 cells were infected with AdRIPeGFP expressing enhanced green fluorescent protein under the insulin promoter to facilitate detection of cells under fluorescent microscope. Apoptosis was examined by staining with annexin V using the Annexin V-Cy3 apoptosis detection kit (Medical and Biological Laboratories). At least 1,000 cells per sample were counted for annexin V positive cells.

GRP78 promoter assay

The pGL3-promoter, pTK-RLuc and pGL3-basic plasmids were purchased from Promega. The mouse GRP78 promoter fragment spanning -172 to -21 (positions relative to the transcription start site) was amplified by PCR using oligonucleotides

5'-GACTCGAGGCCGCTTCGAATCGGCAG-3' and 5'-TCAAGCTTGGCCAGTATC GAGCGCGC-3'. This fragment contains three ER stress response elements (40) and the corresponding regions of human (40) and rat (41) GRP78 genes were shown to respond to ATF6 activation. A GRP78 promoter-driving luciferase reporter plasmid (designated pmGRP78pro(-172)-Luc) was constructed by subcloning this fragment into the *Xho*I and *Hind*III sites of the pGL3-basic vector. MIN6wfs1$^{+/-}$ or MIN6wfs1$^{-/-}$ cells were co-transfected with pGL3-promoter or pGRP78pro(-172)-Luc together with pTK-RLuc using the LipofectAMINE reagent (Invitrogen). Luciferase activities were assayed with Dual-Luciferase reporter system (Promega) using a Lumat LB9507 luminometer (Berthold).

BrdU incorporation assay

BrdU (100 mg/kg) was injected into the mice intraperitoneally. Six hours later, the mice were sacrificed and their pancreases were fixed with 4% paraformaldehyde. Immunohistochemical analyses were performed with a Cell Proliferation Assay kit (BD Pharmingen). Sections were also stained with anti-insulin. BrdU-positive β-cells were counted in at least 50 sections per mouse.

Recombinant adenovirus experiments

Human *GRP78* cDNA was purchased from Open Biosystems. Human *WFS1* cDNA was a generous gift from Prof. Y. Tanizawa (Yamaguchi University). The CMV promoter containing two Tet-operator sequences (designated CTO) was excised from pcDNA5/TO (Invitrogen) and ligated to these cDNAs. The Tet-repressor cDNA was excised from pcDNA6/TR (Invitrogen) and ligated to the CAG promoter unit (39).

These expression units were used to generate recombinant adenoviruses by a previously described method (40). The resulting viruses were designated AdCAG-TR for the Tet-repressor expressing virus and AdCTO-GRP78 for the GRP78 expressing virus under the CTO promoter, and so on. MIN6 and its derivative cells were infected with AdCAG-TR at a multiplicity of infection (m.o.i.) of 30 together with viruses with the CTO promoter at an m.o.i. of 100. One day after infection, cells were reseeded and divided into two groups. Two days thereafter, the cells were fed media with or without doxycycline (2 μg/ml). We have observed no adverse effects of infection of a control recombinant adenovirus expressing green fluorescence protein at an m.o.i. of less than 250 on MIN6 cell function in terms of cell proliferation and glucose-stimulated insulin secretion (data not shown). For the cell number assessment, MIN6 cells infected with AdCAG-TR and AdCTO-$p21^{CIP1}$ were seeded in 6-well plates at 2 x 10^5 per well, cultured in media with or without doxycycline (2 μg/ml) and harvested after the indicated intervals. Cells were then stained with trypan blue and counted.

Statistical analysis

Data are presented as means ± S.E. Differences between groups were assessed by Student's *t-test*.