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*.

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FIGURE LEGENDS

Figure 1. Activation of three subpathways of the UPR in *wfs1*-deficient islets.

A) Activation of the PERK/eIF2α pathway. Islets isolated from wild-type and *wfs1*-deficient mice were subjected to SDS-PAGE and probed with the indicated antibodies: P-PERK, phosphorylated-PERK; P-eIF2α, phosphorylated-eIF2α; T-eIF2α, total eIF2α.

B) Real-time RT-PCR analysis of GRP94, GRP78 and $P58^{IPK}$ gene expressions in wild-type (open columns) and *wfs1*-deficient (closed columns) mice. Relative mRNA levels were obtained after normalization to actin mRNA. $*p < 0.05$. $n = 6$.

C) Expressions of chaperone proteins in *wfs1*-deficient islets. Lysates of isolated islets were probed with the indicated antibodies.

D) Increased XBP1 mRNA splicing in *wfs1*-deficient islets. Amplification of XBP1 mRNA from islet total RNA with specific primers yields spliced (303 bp) and non-spliced (329 bp) XBP1 transcripts.

E) Activation of the IRE1/XBP1 pathway. Lysates of isolated islets were probed with the indicated antibodies.

Western blot data shown are representative of at least three experiments with different sets of samples.

Figure 2. Increased UPR and its reversal by expression of WFS1 or GRP78 in an SV40 transformed *wfs*1-deficient β-cell line (MIN6wfs1$^{-/-}$).

A) Expression of UPR-related proteins in various MIN6 cell lines. MIN6, MIN6wfs1$^{+/-}$-1, MIN6wfs1$^{-/-}$-1 and MIN6wfs1$^{-/-}$-2 cells were lysed and probed with the

indicated antibodies. Data shown are representative of at least three experiments with different sets of samples.

B) Expressions of chaperone proteins in MIN6wfs1$^{-/-}$ cells. (Upper panel) Cellular lysates were probed with anti-GRP78, anti-KDEL and anti-actin (loading control) antibodies. (Lower panel) MIN6wfs1$^{+/-}$ (open columns) and MIN6wfs1$^{-/-}$ (closed columns) cells were transiently transfected with the pGL3-promoter plasmid containing the SV40 promoter-luciferase (SV40pro: 0.5 μg) or pGRP78pro(-172)-Luc (GRP78pro: 0.5 μg) together with the reference plasmid pTK-RL (0.05 μg) encoding *Renilla* luciferase. Twenty-four hours after transfecion, cellular lysates were subjected to luciferase assay. The luciferase activity of the pGL3-promoter in MIN6wfs1$^{+/-}$ was defined as 1. The averages of three independent experiments, each performed in duplicate, are presented. *$p < 0.05$, $n = 3$.

C) Suppression of PERK phosphorylation by WFS1 re-expression in MIN6wfs1$^{-/-}$ cells. Cells were infected with AdCAG-TR expressing Tet-repressor (TR) and AdCTO-WFS1 harboring *WFS1* cDNA. WFS1 expression was induced by 48 hour doxycycline (DOX, 2 μg/ml) treatment. The experiment was repeated three times and similar results were obtained.

C) Suppression of PERK phosphorylation by GRP78 overexpression in MIN6wfs1$^{-/-}$ cells. Human GRP78 expression was induced by 48 hour DOX treatment. The experiment was repeated four times and similar results were obtained.

Figure 3. No UPR changes in heart, skeletal muscle or brown adipose tissue from *wfs1*-deficient mice.

A) WFS1 protein distribution in mice. Approximately 100 μg of protein from wild-type

mouse tissues were analyzed for the presence of WFS1 protein. BAT, brown adipose tissue; WAT, white adipose tissue.

B - D) UPR activation was not observed in heart (B), skeletal muscle (C) or BAT from *wfs1*-deficient mice. The Western blot data shown are representative of two experiments, each performed using three mice of each genotype.

Figure 4. Activation of apoptosis signaling in *wfs1*-deficient islets and MIN6 cells.

A) Real-time RT-PCR analysis of CHOP mRNA in wild-type (open column) and *wfs1*-deficient (closed column) islets. Relative mRNA levels were obtained after normalization to actin mRNA. *$p < 0.05$, $n = 6$.

B) Western blot analysis of apoptosis signaling proteins in *wfs1*-deficient islets. Lysates of islets were probed with the indicated antibodies: P-JNK, phospho-JNK; T-JNK, total-JNK. Data shown are representative of three experiments with different sets of samples.

C) Increased expression of CHOP and cleaved caspase-3 in *wfs1*-deficient MIN6 cells. Lysates of MIN6 cell derivatives were probed with the indicated antibodies. Data shown are representative of three experiments.

Figure 5. Impaired cell cycle progression and increased $p21^{CIP1}$ expression in *wfs1*-deficient islets.

A, B) Impaired cell cycle progression in *wfs1*-deficient β-cells. Incorporated BrdU and insulin were probed with specific antibodies (A) and BrdU positive β-cells were counted (B). Bars, 10 μm. *$p < 0.05$, $n = 4$ mice per group.

C, D) Increased $p21^{CIP1}$ expression in *wfs1*-deficient islets and MIN6 cells. Lysates of

wild-type and *wfs1*-deficient islets (C) or MIN6 cells (D) were probed with the indicated antibodies: T-p53, total-p53; P-p53, phospho-p53. Data shown are representative of three experiments with different sets of samples.

E, F) Induction of $p21^{CIP1}$ expression by thapsigargin (TG) in islets (E) and MIN6 cells (F). Wild-type islets were challenged with 0.5 μM TG for 12 h. MIN6 cells were also treated with 0.5 μM TG for the indicated durations. Lysates of islets or MIN6 cells were probed with the indicated antibodies. The experiment was repeated three times and similar results were obtained.

Figure 6. Decrease in MIN6 cell numbers in response to forced $p21^{CIP1}$ expression.

A) Forced expression of $p21^{CIP1}$ in MIN6 cells. Cells were either uninfected or infected with AdCAG-TR (m.o.i. of 30) and AdCTO-$p21^{CIP1}$ (m.o.i. of 100) harboring $p21^{CIP1}$ cDNA. Expression of $p21^{CIP1}$ was induced by 48 hour DOX (2 μg/ml) treatment. MIN6 cell lysates were subjected to immunoblot analysis using anti-$p21^{CIP1}$ and actin antibodies.

B) Numbers of MIN6 cells overexpressing $p21^{CIP1}$. One day after adenovirus transduction, cells were reseeded (2×10^5 per well) and divided into two groups, and, after two more days, treatment with (closed circles) or without (open circles) DOX (2 μg/ml) was commenced (day 0). Uninfected MIN6 cells (open squares) were also seeded two days before. Cells were then harvested on days 0, 2, 4 and 6, stained with trypan blue, and counted. Data are means ± S.E. for triplicate wells. $^{**}p < 0.01$ against both controls. The experiment was repeated three times and similar results were obtained.