Beta cell dysfunction due to increased ER stress in a stem cell model of Wolfram syndrome

Linshan Shang,1 Haiqing Hua,1,2 Kylie Foo,1 Hector Martinez,1 Kazuhisa Watanabe,2 Matthew Zimmer,1 David J Kahler,1 Matthew Freeby,2 Wendy Chung,2 Charles LeDuc,2 Robin Goland,2 Rudolph L. Leibel,2 and Dieter Egli1,*

1 The New York Stem Cell Foundation Laboratory, New York, NY 10032, USA
2 Division of Molecular Genetics, Department of Pediatrics and Naomi Berrie Diabetes Center, Columbia University, New York, NY 10032, USA

*Correspondence: d.egli@nyscf.org; Fax: 212-851-5423

ABSTRACT

Wolfram syndrome is an autosomal recessive disorder caused by mutations in WFSI and characterized by insulin-dependent diabetes mellitus, optic atrophy and deafness. To investigate the cause of beta cell failure, we used induced pluripotent stem (iPS) cells to create insulin-producing cells from individuals with Wolfram syndrome. WFS1-deficient beta cells showed increased levels of endoplasmic reticulum (ER) stress molecules, and decreased insulin content. Upon exposure to experimental ER stress, Wolfram beta cells showed impaired insulin processing and failed to increase insulin secretion in response to glucose and other secretagogues. Importantly, 4-phenyl butyric acid, a chemical protein folding and trafficking chaperone, restored normal insulin synthesis and the ability to upregulate insulin secretion. These studies show that ER stress plays a central role in beta cell failure
in Wolfram syndrome and indicate that chemical chaperones might have therapeutic relevance under conditions of ER stress in Wolfram syndrome and other forms of diabetes.

INTRODUCTION

All forms of diabetes are ultimately the result of an inability of pancreatic beta cells to provide sufficient insulin in response to ambient blood glucose concentrations. Stem cell-based models of diabetes should enable analysis of specific pathways leading to human beta cell failure and the testing of strategies to preserve or restore beta cell function.

Childhood-onset insulin-dependent diabetes can be caused by mutations in WFS1 gene (Wolframin), which is highly expressed in human islets as well as in the heart, brain, placenta and lung (1). Wolfram syndrome subjects are also affected by optic atrophy, deafness, ataxia, dementia and psychiatric illnesses (2). The disease is fatal and no treatments for the diabetes other than provision of exogenous insulin are available. Postmortem analyses of pancreata of Wolfram patients show a selective loss of pancreatic beta cells (3). In the mouse, loss of the WFS1 gene results in impaired glucose-stimulated insulin secretion and a reduction of beta cells in pancreatic islets (4; 5). But unlike human subjects, these mice develop only mild or no diabetes (4). Several molecular mechanisms by which WFS1 deficiency might affect beta cell function have been described. WFS1 deficiency reduces insulin processing and acidification in insulin granules of mouse beta cells, where low pH is necessary for optimal insulin processing and granule exocytosis (6). In human fibroblasts, WFS1 localizes to the endoplasmic reticulum (ER) (7), where it
increases free Ca$^{2+}$ (8) and interacts with calmodulin in a Ca$^{2+}$-dependent manner (9). In mouse islets, following stimulation with glucose, WFS1 is found on the plasma membrane, where it appears to stimulate cyclic adenosine monophosphate (cAMP) synthesis through an interaction with adenylyl cyclase, thereby promoting insulin secretion (10). In addition, WFS1 deficiency is accompanied by activation of components of the unfolded protein response (UPR), such as GRP78 (78 kDa glucose-regulated protein) / Bip (immunoglobulin-binding protein) and XBP-1 (X-box binding protein-1) and reduced ubiquitination of ATF6α (activating transcription factor-6α) (11; 12). Because the relevance of these molecular mechanisms to beta cell dysfunction is unclear, and because of phenotypic differences between mice and human subjects, there is a need for a biological model of the consequences of WFS1 deficiency in the human beta cells.

We generated insulin-producing cells from skin fibroblasts of patients with Wolfram syndrome and found that these WFS1-mutant cells display insulin processing and secretion in response to various secretagogues comparable to healthy controls, but have a lower insulin content and increased activity of UPR pathways. The chemical chaperone, 4-phenyl butyric acid (4PBA), reduced the activity of UPR pathways, and restored insulin content to levels comparable to controls. Experimental ER stress induced by exposure to low concentrations of thapsigargin (TG), impaired insulin processing and abolished insulin secretion in response to various secretagogues, while beta-cell function in control cells was unaffected. Importantly, genetic rescue of WFS1 restored insulin content and preserved the ability to secrete insulin under conditions of ER stress. These results demonstrate that ER stress plays a central role in beta cell dysfunction in Wolfram syndrome, and identify a potential approach to clinical intervention.
RESEARCH DESIGN AND METHODS

Research subjects and generation of induced pluripotent stem cells

Skin biopsies were obtained from subjects WS-1 (Biopsy # 1-088) and WS-2 (Biopsy # 1-071) at the Naomi Berrie Diabetes Center, using a 3mm AcuPunch biopsy kit (Acuderm Inc). Skin fibroblasts were derived and grown as previously described. Fibroblast cells from WS-3, WS-4 and WFS1 mutation carrier were obtained from Coriell Research Institute. Induced pluripotent stem cells were generated using the CytoTune™-iPS Sendai Reprogramming Kit (Invitrogen) (13) or using retroviral vectors (14). To genetically rescue the WFS1 locus, Wolfram iPS cell lines were transfected with lentivirus containing wild-type WFS1 cDNA sequence (from Addgene plasmid #13011) under murine stem cell virus (MSCV) promoter. Cell lines with stable integration were selected for and maintained by puromycin treatment.

Beta cell differentiation and analysis

Human embryonic stem (ES) or iPS cells were dissociated by Dispase (3-5 mins) and Accutase (5 mins, Sigma). Cells were suspended in human ES medium containing 10 µM Y27632, a ROCK inhibitor, and filtered through a 70 µm cell strainer. Then cells were seeded at a density of 800,000 cells/well in 12-well plates. After 1 or 2 days, when cells reached 80-90% confluence, differentiation was performed as previously described (15). To quantify the number of insulin positive cells within beta cell cultures, and to simultaneously quantify insulin and proinsulin content within beta cells, cells were dissociated to single cells and divided into three fractions: 20% of cells for quantification of positively stained cells using a Celigo Cytometer system (Cyntellect), 40% for RNA
analysis and 40% for ELISA assay to determine insulin content. For insulin content analysis, cells were lysed by M-PER protein extraction reagent (Thermo Scientific). Proinsulin and insulin content was measured with human proinsulin and insulin ELISA kits (Mercodia). To determine insulin and glucagon secretion in live cultures, beta cells were washed for 1 hour in CMRL medium, then incubated in CMRL medium containing 5.6 mM glucose for 1 hour and the medium was collected. After that, CMRL medium containing 16.9 mM glucose, or 15mM arginine, or 30 mM potassium, or 1µM Bay K8644, or 1 mM dibutyl cAMP (DBcAMP)+16.9mM glucose was added to the wells for 1 hour and the medium was collected. Human C-peptide concentration was measured by ultra-sensitive human C-peptide ELISA kit (Mercodia), which specifically detects C-peptide, but not insulin or proinsulin. Glucagon levels in medium were measured with a Glucagon ELISA kit (ALPCO Diagnostics).

**Unfolded protein response (UPR) analysis**

iPSCs, fibroblasts or beta cells were incubated for the indicated time and concentration of thapsigargin (TG) or tunicamycin (TM) (Sigma), Sodium 4-phenylbutyrate (4PBA) or tauroursodeoxycholate (TUDCA) (Both from EMD Chemicals Inc.) was added one hour prior to and during TG or TM treatment. For long-term 4PBA treatment, cells were incubated with 1 mM 4PBA from day 9 to day 15 of differentiation. Primers for PCR analysis of XBP-1 gel-imaging (16), sXBP-1 for QPCR (17), ATF4 (18), GRP78 and insulin are listed in Supplementary Table 1.

**Gene and Protein expression analysis**
RNA was isolated using RNAsesy plus kit (Qiagen). cDNA was generated by using RT kit (Promega). Cells were lysed by using M-PER Mammalian Protein Extraction Reagent and NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific). Protein levels were determined by western blot using the antibodies as listed: Rabbit-anti-ATF6α (ab37149, Abcam), Rabbit-anti-phospho-eIF2α (#3597, Cell signaling), mouse-anti-eIF2α (#2103, Cell signaling), mouse-anti GRP78 antibody (sc-166490, Santa Cruz).

**Electron Microscopic Analysis**

For electron microscopic analysis, differentiated beta cells were fixed in 2.5% glutaraldehyde in 0.1 M Sorenson’s buffer (pH 7.2) for one hour. Samples were processed and imaged by the Diagnostic Service, Department of Pathology and Cell Biology, Columbia University. Secretory granule structure and endoplasmic reticulum (ER) morphology were visually recognized. The number of granules per cell was determined using ImageJ software.

**Transplantation and glucose stimulated insulin secretion assay**

At 12 days of differentiation, 2-3 million cells were detached using TrypLE (Invitrogen), pelleted and mixed with 10-15 µl matrigel (BD Biosciences), before transplanted into kidney capsule of a NOD.Cg-Prkdc<sup>scid</sup> I2rg<sup>tm1Wjl</sup>/SzJ (NSG) mouse (Stock No. 005557, The Jackson Laboratory), using methods as previously described for the transplantation of islets (19). Glucose stimulation assay was performed 3 months after transplantation. Mice were deprived of food overnight (12-14 hours); water was available ad libitum. Each mouse was injected intraperitoneally with a glucose solution (1mg/g body weight).
Before and half an hour after glucose injection, capillary blood glucose was measured and venous blood samples were collected from the tail and submandibular vein. Human C-peptide concentration in the mouse serum was measured by using ultra-sensitive human C-peptide ELISA kit (Mercodia). Nephrectomy was performed on some mice after human C-peptide was detected in the mouse serum. For ER stress studies, thapsigargin was administrated by one dose injection of 1mg/kg of thapsigargin intraperitoneally.

**Statistical analysis**

One-way ANOVA was applied for multi-comparison among groups. Student’s t-test (2-tailed) was used to compare pairs of experiments. $P_{\alpha} < 0.05$ was considered statistically significant.

**Study Approval**

Protocols to obtain skin biopsies were approved by the Columbia Institutional Review Board and Embryonic Stem Cell Research Oversight committees. Human subjects gave signed informed consent. Animal protocols were approved by the Columbia Institutional Animal Care and Use Committee.

**RESULTS**

**Wolfram iPS cells differentiate into beta cells**

We obtained skin biopsies and established skin cell lines from two subjects with Wolfram syndrome designated WS-1 and WS-2. Sequencing of the *WFS1* locus revealed that WS-
2 is homozygous for a frameshift mutation 1230-1233delCTCT (V412fsX440), and that WS-1 is compound heterozygous for V412fsX440, and a missense mutation P724L, both mutations that had previously been reported in Wolfram subjects (1; 20). Three additional skin cell lines were obtained from Coriell Research Institute from two siblings with Wolfram syndrome: WS-3 and WS-4, and an unaffected parent (Carrier). Both WS-3 and WS-4 are compound heterozygous for the mutations W648X and G695V in the WFS1 protein (1) (Fig. 1A). All Wolfram subjects had childhood-onset diabetes, with the earliest onset at age 2 years for WS-2, and between ages 11-13 years for WS-1, WS-3 and WS-4. The relationship of these specific mutations to age of onset of diabetes is unknown. All subjects required treatment with exogenous insulin, and had optic atrophy. The patient WS-1 has no history of hearing loss or diabetes insipidus, but optic atrophy was discovered at age 14. For WS-2 patient, diabetes insipidus and optic atrophy developed at age 13. Progressive visual loss and cognitive disabilities have developed in both WS-1 and WS-2 patients. An embryonic stem cell line HUES42 (21) (Control-1) and an induced pluripotent stem cell (iPSC) line (Control-2) generated from a healthy individual were used as normal controls. DNA sequencing confirmed that these two control cell lines do not carry any coding mutations in \textit{WFS1}. To control for effects of genetic background, we included cells of the non-diabetic parent heterozygous for the mutation G695V in our analyses (Supplementary Table 2), and genetically rescued the \textit{WFS1} locus by expressing a cDNA transgene under the MSCV promoter.

We generated iPS lines from the four Wolfram and the control subjects using non-integrating Sendai virus vectors encoding the transcription factors Oct4, Sox2, Klf4 and
c-Myc (13). All iPS cell lines were karyotypically normal, expressed markers of pluripotency and differentiated into cell types and tissues of all three germ layers in vitro and after injection into immune-compromised mice (Supplementary Fig. 1). iPS cell lines from Wolfram and control subjects were differentiated into insulin-producing cells using stepwise differentiation into definitive endoderm (SOX17+ cells), pancreatic endoderm (PDX1+ cells), and beta cells (C-peptide+) cells (Fig. 1B). Differentiation efficiency of Wolfram cells was similar to controls: after 8 days of differentiation, about 80% of all cells expressed PDX1, a marker of pancreatic endocrine progenitors, and after 13 days of differentiation, about 20% of all cells expressed C-peptide (Fig. 1C, Supplementary Fig. 2). Immunostaining revealed that WFS1 was specifically expressed in insulin-producing cells, but not in glucagon-positive cells (Fig. 1D), which is consistent with the expression pattern reported for mouse islets (5).

Activated UPR reduces insulin synthesis in Wolfram beta cells

To investigate the effects of WFS1 mutations on beta-cell function, we quantified insulin mRNA and protein content in Wolfram and control stem cell-derived beta cells. WFS1 deficiency was associated with a 40%-50% reduction in insulin mRNA levels compared to controls (Fig. 2A), a 30%-40% decrease of insulin protein content (Fig. 2B), and a 30%-40% decrease in the number of secretory granules (Fig. 2C and D). Insulin production was restored to levels of control cells by a transgene encoding wild type WFS1, indicating that WFS-1 deficiency, and not other genetic differences, resulted in reduced insulin content (Fig. 2A to D).
To determine the cause of the decreased insulin content, we analyzed the ratio of proinsulin/insulin in beta cells. We found that the proinsulin/insulin ratio in Wolfram beta cells was between 0.4 and 0.55, similar to control cells (0.4-0.47) (Fig. 3A, Supplementary Figure 3). We next investigated the expression of components of the unfolded protein response (UPR) in Wolfram and control cells. Three branches of the UPR, inositol-requiring protein 1α (IRE1α), protein kinase RNA-like ER kinase (PERK) and activating transcription factor 6 (ATF6), sense increases in unfolded protein, and respond with modulation of cellular, transcriptional and translational activities (22). It has previously been reported that chronic hyper-activation of IRE1 leads to decreased insulin mRNA and protein level in INS-1 cells (23). Using quantitative PCR and Western blotting, we found that in fibroblasts, iPS cells, and beta cells, components of the three major UPR pathways were increased in Wolfram subject samples in comparison to controls, including sXBP1 (spliced XBP1) mRNA, which is downstream of IRE1; ATF4 (activating transcription factor-4) mRNA; and nuclear ATF6α, p-eIF2α (eukaryotic translation initiation factor-2α) and GRP78 protein (Fig. 3B-E, Supplementary Fig. 4). These differences were amplified by the imposition of experimental ER stress. Thapsigargin (TG), which impairs protein folding by interfering with calcium homeostasis in the ER (24), caused a greater increase of GRP78 mRNA levels in Wolfram (4-fold) than in control stem cells (2-fold) (Supplementary Fig. 4A). A stronger UPR response in Wolfram cells than in control cells is also observed with the application of another ER stress inducer, tunicamycin (TM), an inhibitor of N-linked glycosylation (25) (Supplementary Fig. 4B and C). Experimentally-induced ER stress also affected ER morphology: the ER was greatly dilated in Wolfram beta cells in the presence of low
dose of TG, while control cells were unaffected (Fig. 3G, Supplementary Fig. 5). Insulin processing was similarly affected; treatment with low doses of thapsigargin resulted in the accumulation of proinsulin in Wolfram but not in control cells (Fig. 3A). The upregulation of UPR pathways, including increased spliced XBP-1 mRNA, a downstream target of IRE-1 alpha suggests that the UPR response is likely responsible for the reduced insulin content and insulin processing in beta cells.

To determine if preventing the activation of the unfolded protein response could restore normal insulin content in Wolfram cells, we generated beta cells in the presence of the chemical chaperone sodium 4-phenylbutyrate (4PBA) during days 9 to 15 of differentiation. 4PBA can assist protein folding and thereby reduce the activity of UPR pathways (26). 4PBA (27; 28) and tauroursodeoxycholate (TUDCA) (29) reduced GRP78 mRNA levels in Wolfram cells treated with TG (Fig. 3F). Nuclear ATF6α, p-eIF2α protein levels, and sXBP-1 as well as ATF4 mRNA levels were reduced by 30-50% in comparison to no 4PBA vehicle controls (Fig. 3B-E). Strikingly, insulin mRNA levels in Wolfram cells were increased by 1.9-fold, and insulin content by 1.5-1.9 fold, to levels comparable to those in control cells without 4PBA (Fig. 2A and B). The number of secretory granules in Wolfram beta cells was also substantially increased with the treatment of long-term 4PBA, or with the expression of exogenous wild-type WFS1 protein (Fig. 2C and D). When control cells were exposed to the same 7d treatment with 4PBA during beta-cell differentiation, no significant increase of insulin production was observed (Fig. 2A and B). Also insulin processing was normalized by the treatment of 4PBA after exposure to thapsigargin (Fig. 3A). These results demonstrate that activation
of the UPR mediates the reduced insulin content in \textit{WFSI} mutant beta cells. The activation of all three UPR pathways suggests that WFS1 acts upstream of the unfolded protein response, likely to maintain ER function under protein folding stress.

\textbf{ER stress affects stimulated insulin secretion in \textit{WFSI} mutant beta cells}

To determine whether a specific insulin secretion pathway was affected in Wolfram cells, we exposed them to various insulin secretagogues, including glucose, arginine, potassium and the cAMP analog, dibutyryl cAMP (DBcAMP). Others have previously reported that glucose stimulates insulin secretion by ATP generation, resulting in the closing of ATP-sensitive potassium channels and reduction of potassium efflux, which depolarizes the cell, stimulating Ca\(^{2+}\) influx and exocytosis of insulin granules (30). Arginine induces insulin secretion by triggering Ca\(^{2+}\) influx, without reducing potassium efflux (31). cAMP influences insulin secretion through activation of PKA (protein kinase A) and/or Epac (exchange proteins activated by cAMP) (32); and finally, extracellular potassium directly depolarizes the plasma membrane, resulting in the release of insulin granules (33). To assess insulin secretion in response to glucose, we incubated cells in 5.6 mM glucose for 1 hour, followed by incubation in 16.9 mM glucose for 1 hour. Control and the \textit{WFSI} mutation carrier beta cells showed a 1.6 to 1.7-fold increase in C-peptide release in 16.9 mM glucose (versus 5.6 mM glucose). A similar 1.5 to 1.9-fold increase was seen in the four \textit{WFSI} mutant cell lines (Fig. 4A and B). Similarly, arginine, potassium, and DBcAMP, increased C-peptide secretion (2 to 4-fold) equally in control and \textit{WFSI} mutant cells (Fig. 4A). Therefore, \textit{WFSI} mutant beta cells displayed functional
responses to secretagogues acting at different points in pathways of metabolic sensing and insulin release comparable to control cells.

We next determined whether WFS1 deficiency affected stimulated insulin secretion under imposed ER stress. When TG-treated cells were exposed to high ambient glucose (16.9 mM), Wolfram cells failed to increase C-peptide secretion, while control beta cells increased C-peptide output by 1.6 fold (versus 5.6 mM glucose). Incubation with 4PBA prevented the effect of TG on Wolfram beta cells (Figure 4A, Supplementary Fig. 6). The reduction in stimulated C-peptide secretion by TG was seen with all secretagogues tested. Independent of the secretagogues applied, the chemical chaperone 4PBA prevented the decrease in C-peptide secretion upon application of ER stressor (Fig. 4A). A reduction in stimulated C-peptide secretion was observed for beta cells generated from all four Wolfram subjects, but not for the WFS1 mutation carrier or the non-mutant control iPSC line (Fig. 4B and C, Supplementary Fig. 7). The reduced beta cell function was not specific to TG: a comparable reduction in insulin secretion was also observed in tunicamycin (TM)-treated Wolfram beta cells upon potassium or glucose stimulation (Fig. 4B and C). Importantly, a wild-type WFS1 transgene reduced sensitivity to the ER stress agent thapsigargin, showing a response to both arginine and DBcAMP stimulation comparable to controls (Supplementary Figure 8).

To determine if the reduction in insulin secretion was due to beta cell death, we quantified the number of insulin positive cells over 72 hours, with or without TG treatment. Insulin cell number remained constant after 72 hours incubation with 10nM
TG (Supplementary Fig. 9A). We further tested the ER stress-related cell death marker gene expression, such as CHOP (C/EBP-homologous protein, also known as DNA damage-inducible transcript 3 encoded by the \textit{DDIT3} gene) (34), and TXNIP (thioredoxin-interacting protein) (35), both proteins involved in ER-stress dependent apoptosis. A higher increase of CHOP mRNA expression was observed in Wolfram cells compared to control cells after TG treatment for 12 hours, but expression level of TXNIP mRNA showed no significant changes in control and Wolfram cells (Supplementary Fig. 9B-C). Therefore, ER stress did not reduce insulin secretion by inducing cell death during the time frame studied, but rather by affecting the ability of beta cells to secrete insulin.

Because of the specific expression of \textit{WFS1} in beta cells (Fig. 1D), but not in glucagon-expressing cells, we anticipated that \textit{WFS1} mutations differentially affect beta and alpha cell function. We differentiated Wolfram cells into clusters containing both glucagon- and insulin-expressing cells (Fig. 1D), and stimulated these cells with arginine. As arginine stimulates both endocrine cell types, we were able to assess stimulated hormone secretion in the same cell cluster, in the presence and absence of TG. TG treatment reduced arginine-stimulated glucagon secretion in control and \textit{WFS1} mutant cells by 28% and 24%, respectively. In contrast, a reduction of stimulated insulin secretion occurred only in \textit{WFS1} mutant cells (3% increase in control cells, versus 43% decrease in \textit{WFS1} cells) (Fig. 4D). These results show that the insulin secretion phenotypes in \textit{WFS1} mutant cells are not due to changes in glucagon release in adjacent cells (36), and demonstrate that \textit{WFS1} (which is not expressed in islet alpha cells) specifically protects beta cells from the detrimental effects of ER stress.
**Impaired response to glucose of Wolfram beta cells in vivo**

*In vitro* studies may not fully recapitulate all relevant molecular-cellular circumstances in a systemic physiological environment. The transplantation of beta cells allows testing functionality of beta cells *in vivo* over a time period of several months. After 12 days of *in vitro* differentiation, cell culture containing 70-80% PDX1+ cells, were transplanted under the kidney capsule of immune-deficient mice. Human C-peptide was detected 13 weeks post transplantation in the serum of 13/16 mice transplanted with control cells and 14/20 mice transplanted with Wolfram cells. Basal C-peptide serum levels were comparable for both Wolfram and control HUES42-derived cells (Supplementary Fig. 10A). Human C-peptide was graft-derived, as human C-peptide became undetectable in blood 2 days after the removal of the kidney containing the transplanted cells (Supplementary Fig. 10B). Cells contained in the graft co-expressed C-peptide and urocortin 3, which labels functional mature beta cells (37) (Fig. 5A). Upon systemic glucose challenge, Wolfram-derived cells were able to respond to glucose administered intraperitoneally by increasing human C-peptide secretion. However, they displayed significantly reduced responsiveness compared to human islets and beta cells derived from control stem cells (Fig. 5B). We then performed repeated measurement of human C-peptide over a one-month period, starting from 3 months post transplantation surgery, when the mice were found to show glucose-stimulated human insulin secretion. During this one-month period, human islets and control grafts remained unchanged in both fasting and glucose-stimulated C-peptide secretion. In contrast, both fasting C-peptide level and glucose responsiveness of Wolfram beta cells further decreased (Fig. 5C and
To determine the level of ER stress in the transplanted cells, we isolated the grafts and performed immunohistochemistry for ATF6α and CHOP. Both ATF6α and CHOP staining were more intense in Wolfram beta cells, while control beta cells, human islets, as well as the surrounding grafted cells and mouse tissue showed lower intensity of staining (Fig. 5E and F). To test whether ER stress can mediate decreased C-peptide secretion from Wolfram beta cells in vivo, we challenged transplanted mice with thapsigargin. Basal C-peptide secretion from Wolfram cells was significantly decreased, while secretion of C-peptide from human islets remained constant (Supplementary Fig. 10C-D).

**DISCUSSION**

Here we report the first human cell model of an ER related disorder, a stem-cell based model of Wolfram syndrome. This model allowed us to study the consequences of ER stress on beta cell function. Beta cells of Wolfram subjects produce less insulin and have increased activity of three major UPR pathways, including PERK, IRE1 and ATF6. Addition of the chemical chaperone 4PBA reduced levels of UPR signaling pathway molecules and increased insulin content. These results demonstrate that WFS1 protects beta cells from protein folding stress and ER dysfunction, acting upstream of UPR signaling and not by regulating the activity of a particular component of the UPR pathway. Interestingly, low levels of UPR signaling affected insulin content, but did not affect insulin processing or stimulated insulin secretion in vitro. However, following imposition of chemical ER stress, insulin processing, ER morphology and stimulated insulin secretion were greatly affected. These phenotypes observed in vitro were
recapitulated *in vivo* after cell transplantation: Wolfram-derived cells displayed significantly reduced responsiveness to increased circulating glucose and the levels of immuno-histochemical staining of ER stress-associated protein ATF6α and CHOP were increased in Wolfram implants in comparison to controls, consistent with previous observations in a rodent model (12). Graft function of Wolfram, but not control cells decreased over the time frame of one month, suggesting that Wolfram cells have a reduced ability to cope with a challenging metabolic environment *in vivo*, characterized by fluxes of glucose, free fatty acids and other metabolites. Disease progression in human subjects may follow a similar course, from elevated UPR signaling resulting in reduced insulin content, to chronic ER stress and beta cell failure. Our data suggests that unresolved ER stress will ultimately lead to ER dysfunction, reduced processing of insulin, and blunted insulin secretion.

In type 1 diabetes, a decreasing number of beta cells endeavor to meet metabolic demand for insulin, and in most instances of type 2 diabetes, the demand for insulin is increased because of peripheral insulin resistance. Beta cells of type 2 diabetes and type 1 diabetes subjects may possess greater intrinsic ability to increase insulin synthesis in response to metabolic demand than Wolfram beta cells, but eventually encounter a similar situation, where metabolic demand requires insulin production at levels inducing a UPR response. If the disparity between demand and production remains unresolved, beta cell demise ensues. Increased expression of ER stress mediators has been observed in the islets of type 1 diabetic mice (38) and humans (39). Activation of ER stress-associated genes (i.e. PERK and GRP78) has also been observed in the livers of mouse models of type 2
diabetes (40); and ER stress also appears to contribute to beta cell apoptosis in type 2 diabetes patients (41). Reducing the demand for insulin by intensive insulin therapy improves endogenous beta cell function in type 1 diabetes (42), and improving insulin sensitivity by PPARγ (Peroxisome proliferator-activated receptor γ) inhibitors or by weight loss meliorates type 2 diabetes, in part because beta cell function is improved (43; 44). In the aggregate, these earlier studies and those reported here support a role for ER stress in mediating aspects of the susceptibility and response of beta cells to failure in the context of diabetes.

Common alleles of WFS1 are associated with increased diabetes risk (45), suggesting that our model of beta cell failure in Wolfram syndrome is also relevant to other forms of diabetes. Significantly, our model provides a platform for drug discovery and testing. We found that the chemical chaperone 4PBA is effective at reverting ER stress-associated phenotypes in beta cells. In addition to insulin therapy, this molecule - or compounds with similar activity - may be useful in preventing or delaying beta-cell dysfunction in Wolfram syndrome, and possibly other forms of diabetes.

ACKNOWLEDGMENTS

We are grateful to the research subjects who enabled this research. We thank Ellen Greenberg for assistance with subject recruitment and study coordination, Dr. Kristy Brown (Department of Pathology and Cell Biology, Columbia University) for electron microscope study assistance, and Dr. Fumihiko Urano (Washington University School of Medicine) for providing the WFS1 antibody and for helpful discussions. This work was
supported by The New York Stem Cell Foundation, The Leona M. and Harry B. Helmsley Charitable Trust, The Berrie Foundation Program in Cellular Therapies of Diabetes, NIH DK52431, P30DK063608, and The Hunter Eastman Scholar Award in Translational Diabetes Research. No potential conflicts of interest relevant to this article were reported.

Author Contributions: L.S., H.H., R.L., D.E. designed research. L.S. wrote the manuscript and researched data. H.H., K.F., H.M., K.W., M.Z., D.K., M.F., W.C., C.L. researched data and contributed to discussion. R.G., R.L., D.E. reviewed/edited manuscript. Dr. Dieter Egli is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
REFERENCES


8. Takei D, Ishihara H, Yamaguchi S, Yamada T, Tamura A, Katagiri H, Maruyama Y, Oka Y: WFS1 protein modulates the free Ca(2+) concentration in the endoplasmic reticulum. FEBS letters 2006;580:5635-5640


transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome. Proc Jpn Acad Ser B Phys Biol Sci 2009;85:348-362
27. Yam GH, Gaplovksa-Kysela K, Zuber C, Roth J: Sodium 4-phenylbutyrate acts as a chemical chaperone on misfolded myocilin to rescue cells from endoplasmic reticulum stress and apoptosis. Invest Ophthamelmol Vis Sci 2007;48:1683-1690
28. de Almeida SF, Picarote G, Fleming JV, Carmo-Fonseca M, Azevedo JE, de Sousa M: Chemical chaperones reduce endoplasmic reticulum stress and prevent mutant HFE
29. Berger E, Haller D: Structure-function analysis of the tertiary bile acid TUDCA for
the resolution of endoplasmic reticulum stress in intestinal epithelial cells. Biochem
Biophys Res Commun 2011;409:610-615
30. Ashcroft SJ: Glucoreceptor mechanisms and the control of insulin release and
biosynthesis. Diabetologia 1980;18:5-15
31. Herchuelz A, Lebrun P, Boscher AC, Malaisse WJ: Mechanism of arginine-
stimulated Ca2+ influx into pancreatic B cell. The American journal of physiology
1984;246:E38-43
32. Furman B, Ong WK, Pyne NJ: Cyclic AMP signaling in pancreatic islets. Adv Exp
Med Biol 2010;654:281-304
33. Matthews EK, Shotton PA: Efflux of 86Rb from rat and mouse pancreatic islets: the
34. Oyadomari S, Mori M: Roles of CHOP/GADD153 in endoplasmic reticulum stress.
Cell death and differentiatation 2004;11:381-389
35. Oslowski CM, Hara T, O'Sullivan-Murphy B, Kanekura K, Lu S, Hara M, Ishigaki S,
Zhu LJ, Hayashi E, Hui ST, Greiner D, Kaufman RJ, Bortell R, Urano F: Thioredoxin-
interacting protein mediates ER stress-induced beta cell death through initiation of the
glucagon release of human islets in vitro: effects of chronic exposure to glucagon. The
Journal of endocrinology 1997;152:239-243
37. Blum B, Hrvatin SS, Schuetz C, Bonal C, Rezania A, Melton DA: Functional beta-
cell maturation is marked by an increased glucose threshold and by expression of
38. Tersey SA, Nishiki Y, Templin AT, Cabrera SM, Stull ND, Colvin SC, Evans-Molina
C, Rickus JL, Maier B, Mirmira RG: Islet beta-cell endoplasmic reticulum stress
precedes the onset of type 1 diabetes in the nonobese diabetic mouse model. Diabetes
2012;61:818-827
NG, Eizirik DL: Expression of endoplasmic reticulum stress markers in the islets of
patients with type 1 diabetes. Diabetologia 2012;55:2417-2420
40. Ozcan U, Cao Q, Yilmaz E, Lee AH, Iwakoshi NN, Ozdelen E, Tuncman G, Gorgun
C, Glimcher LH, Hotamisligil GS: Endoplasmic reticulum stress links obesity, insulin
41. Laybutt DR, Preston AM, Akerfeldt MC, Kench JG, Busch AK, Biankin AV, Biden
TJ: Endoplasmic reticulum stress contributes to beta cell apoptosis in type 2 diabetes.
Diabetologia 2007;50:752-763
42. Shah SC, Malone JI, Simpson NE: A randomized trial of intensive insulin therapy in
newly diagnosed insulin-dependent diabetes mellitus. The New England journal of
medicine 1989;320:550-554
43. Gastaldelli A, Ferrannini E, Miyazaki Y, Matsuda M, Mari A, DeFronzo RA:
Thiazolidinediones improve beta-cell function in type 2 diabetic patients. American
journal of physiology Endocrinology and metabolism 2007;292:E871-883
44. Dixon JB, Dixon AF, O'Brien PE: Improvements in insulin sensitivity and beta-cell
function (HOMA) with weight loss in the severely obese. Homeostatic model assessment.
Diabetic medicine : a journal of the British Diabetic Association 2003;20:127-134
FIGURE LEGENDS

Figure 1. iPS cells from Wolfram subjects differentiated into insulin-producing cells.
A. Diagram of WFS1 structure showing the mutation sites and Sanger sequencing profiles in the four Wolfram subjects described here. Arrows indicate the four deleted nucleotides (CTCT). B. Immunostaining of Wolfram cultures differentiated into endoderm (SOX17), pancreatic endoderm (PDX1) and C-peptide positive cells. Scale bar: 50 µm. C. Differentiation efficiency in controls and WFS1 mutant cells determined by imaging (N=10 for each of 3 independent experiments). D. Immunostaining of WFS1, glucagon and C-peptide in iPS-derived pancreatic cell cultures at d15 of differentiation. Scale bar: 20 µm.

Figure 2. UPR dependent reduction of insulin content in Wolfram beta cells.
A. Insulin mRNA levels in control and Wolfram beta cells normalized to TBP (TATA-binding protein) mRNA levels and to the number of C-peptide positive cells used for analysis. B. Insulin protein content in the beta cells normalized to the number of C-peptide positive cells. Error bars represents 3 independent experiments with three replicates in each experiment. *: P<0.05 for Vehicle of WS vs. Control cells. C. Transmission electron microscope (TEM) images of beta cells (Scale bar: 2 µm), and D. Quantification of granule numbers per cell. Three independent differentiation experiments with n=20 sections for each subject of each experiment. *: P<0.05 for WS-1 vs. Control or WS-1 cells treated with 4PBA for 7 days or WS-1 cells carrying Wild-type WFS1 expression vector.
Figure 3. increased UPR pathway activity, and increased sensitivity of the ER to ER stress in Wolfram beta cells.

A. Proinsulin to insulin ratio determined by ELISA. N=6 for each of two independent experiments. B-F. Quantification of components of the unfolded protein response displayed as fold change compared to vehicle treated control cells (set to 1). B, C. Western blot analysis and quantification for nuclear ATF6α (N=3) (B) and phosphorylated eIF2α (C) (N=3) in beta cells. D-F. Quantitative PCR for mRNA levels of spliced XBP-1, ATF4 in beta cells and GRP78 in iPSCs. (N=3 for each experiment) * P<0.05. G. TEM showing endoplasmic reticulum morphology in control and Wolfram cells after 12 hours treatment with 10 nM TG. Arrows point to the ER. Scale bar: 500 nm. TG: thapsigargin; beta cells were treated with TG 10 nM for 12 hours. 4PBA: Sodium 4-phenylbutyrate; TUDCA: tauroursodeoxycholate. 1 mM 4PBA or 1mM TUDCA was added one hour prior to and during TG treatment. 4PBA 7d refers to treatment from day 9 to day 15 of beta cell differentiation.

Figure 4. Differential effect of ER stress on insulin secretion in Wolfram and control cells.

A. Fold change of human C-peptide secretion in response to indicated secretagogues. Cells were incubated in 5.6 mM glucose for 1 hour followed by 16.9 mM glucose, 15mM arginine, 30 mM potassium, or 1 mM DBcAMP+16.9mM glucose for an additional hour. Fold change of human C-peptide secretion was calculated as the ratio of C-peptide concentration occurring in secretagogue-stimulated media over the concentration in 5.6
mM glucose. Results represent three independent experiments with n=3 for each experiment. * P<0.05 of TG vs. Vehicle; # P<0.05 of TG+4PBA vs. TG. B. Fold change of human C-peptide secretion to 16.9 mM glucose stimulation. N=3 for each of two independent experiments. C. Fold change of human C-peptide secretion in response to 30mM potassium stimulation upon TM treatment. Results represent three independent experiments with n=3 for each experiment. D. Fold change of human C-peptide and glucagon in control and Wolfram cells under indicated conditions. N=3 for each of 3 independent experiments. TG: thapsigargin; TM: tunicamycin; 4PBA: Sodium 4-phenylbutyrate.

Figure 5. ER stress and reduced glucose-stimulated insulin secretion of Wolfram cells in vivo.

A. Immunohistochemistry for insulin and Urocortin 3 in transplants. Scale bar: 20 µm.
B. Fold change of human C-peptide in the sera of mice transplanted with human islets, control and Wolfram cells after intraperitoneal glucose injection. Bars show the median.
C. Human C-peptide levels in the sera of transplanted mice over one month time period. Error bars show standard deviation. D. Fold change of human C-peptide in the sera of transplanted mice over a one month time period. Error bars show standard deviation. E-F. Immunohistochemistry for insulin and ATF6α (E) and CHOP (F) in transplants. Scale bar: 20 µm.
Figure 1

A

B

C

D

563x191mm (300 x 300 DPI)
Figure 2

A

Relative insulin mRNA level

B

Insulin content (fmol/cell)

C

Control-1  WS-1  WS-1

WR-1+4PRA 7d  WR-1 (carry WFS1 vector)

D

Granule number per cell

Control-1  Ctrl vector  WFS1 vector +4PRA 7d  WS-1

Diabetes
Figure 3

A

B

C

D

E

F

G

299x474mm (300 x 300 DPI)
Figure 4

A

B

C

D

169x106mm (300 x 300 DPI)
Figure 5

A

<table>
<thead>
<tr>
<th></th>
<th>Insulin</th>
<th>Urocortin 3</th>
<th>Merge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human islets</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
</tr>
<tr>
<td>Control-1</td>
<td><img src="image4" alt="Image" /></td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
</tr>
<tr>
<td>WS-1</td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
<td><img src="image9" alt="Image" /></td>
</tr>
</tbody>
</table>

B

Fold change of Human C-PEP (Glucose injection/ fasting) for Human islets, Control-1, and WS-1. N=10, N=10, N=11.

C

<table>
<thead>
<tr>
<th></th>
<th>Human islets</th>
<th>Control-1</th>
<th>WS-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human C-PEP level (pM)</td>
<td><img src="image10" alt="Graph" /></td>
<td><img src="image11" alt="Graph" /></td>
<td><img src="image12" alt="Graph" /></td>
</tr>
<tr>
<td>N=6</td>
<td>N=4</td>
<td>N=6</td>
<td></td>
</tr>
</tbody>
</table>

D

Change of human C-PEP level (glucose injection/ fasting) for Human islets, Control-1, and WS-1. N=6, N=4, N=6.

E

<table>
<thead>
<tr>
<th></th>
<th>Insulin</th>
<th>ATF6a</th>
<th>Merge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human islets</td>
<td><img src="image13" alt="Image" /></td>
<td><img src="image14" alt="Image" /></td>
<td><img src="image15" alt="Image" /></td>
</tr>
<tr>
<td>Control-1</td>
<td><img src="image16" alt="Image" /></td>
<td><img src="image17" alt="Image" /></td>
<td><img src="image18" alt="Image" /></td>
</tr>
<tr>
<td>WS-1</td>
<td><img src="image19" alt="Image" /></td>
<td><img src="image20" alt="Image" /></td>
<td><img src="image21" alt="Image" /></td>
</tr>
</tbody>
</table>

F

<table>
<thead>
<tr>
<th></th>
<th>Insulin</th>
<th>CHOP</th>
<th>Merge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human islets</td>
<td><img src="image22" alt="Image" /></td>
<td><img src="image23" alt="Image" /></td>
<td><img src="image24" alt="Image" /></td>
</tr>
<tr>
<td>Control-1</td>
<td><img src="image25" alt="Image" /></td>
<td><img src="image26" alt="Image" /></td>
<td><img src="image27" alt="Image" /></td>
</tr>
<tr>
<td>WS-1</td>
<td><img src="image28" alt="Image" /></td>
<td><img src="image29" alt="Image" /></td>
<td><img src="image30" alt="Image" /></td>
</tr>
</tbody>
</table>
Supplementary Table and Figures:

Supplementary Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>XBP-1 (for gel imaging)</td>
<td>forward 5’ GAAGCCAAGGGGAATGAAGT 3’</td>
</tr>
<tr>
<td></td>
<td>reverse 5’ GGAAGGGGCATTTGAAGAAC 3’</td>
</tr>
<tr>
<td>Spliced-XBP-1 (for QPCR)</td>
<td>forward 5’ CTGAGTCGCAAGCAGGTG 3’</td>
</tr>
<tr>
<td></td>
<td>reverse 5’ TGCCCAACAGGATATCAGACT 3’</td>
</tr>
<tr>
<td>ATF4</td>
<td>forward 5’ GCCAAATGAGCTCTCTGA 3’</td>
</tr>
<tr>
<td></td>
<td>reverse 5’ ACCCATGAGTTGAAGTGC 3’</td>
</tr>
<tr>
<td>GRP78</td>
<td>forward 5’ CACAGTGCCCTGACCAAGA 3’</td>
</tr>
<tr>
<td></td>
<td>reverse 5’ TGGATCTCCGATGTACGGA 3’</td>
</tr>
<tr>
<td>Insulin</td>
<td>forward 5’ TCTCACACACCCAGACCCG 3’</td>
</tr>
<tr>
<td></td>
<td>reverse 5’ CAATGCCACGCTCTCGG 3’</td>
</tr>
<tr>
<td>WFS1 mutation detection</td>
<td>Forward: 5’ TCACGAGCCGGGGAAGA -3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’ AGAACGGCCAGGTTACT -3’</td>
</tr>
<tr>
<td></td>
<td>Forward: 5’ GCACCGTGGGTCAGTCTC -3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’ CAGGCCGCCGACGGAATGG -3’</td>
</tr>
</tbody>
</table>

Supplementary Table 1. Primer sequences used in this study.
## Supplementary Table 2

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Source</th>
<th>Sex</th>
<th>Age of onset/diagnosis</th>
<th>Mutations in WFS1 gene</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>WS-1</td>
<td>Naomi Berrie Diabetes Center</td>
<td>Male</td>
<td>12</td>
<td>1230-1233delCTCT (V412fsX440), P724L</td>
<td>Diabetes; Optic atrophy; Insulin dependent</td>
</tr>
<tr>
<td>WS-2</td>
<td>Naomi Berrie Diabetes Center</td>
<td>Female</td>
<td>2</td>
<td>1230-1233delCTCT (V412fsX440)</td>
<td>Diabetes; Optic atrophy; Insulin dependent</td>
</tr>
<tr>
<td>WS-3</td>
<td>Corriell Research Institute (GM01610)</td>
<td>Female</td>
<td>11</td>
<td>W648X, G695V</td>
<td>Diabetes; Optic atrophy; Insulin dependent</td>
</tr>
<tr>
<td>WS-4</td>
<td>Corriell Research Institute (GM01611)</td>
<td>Female</td>
<td>13</td>
<td>W648X, G695V</td>
<td>Diabetes; Optic atrophy; Insulin dependent</td>
</tr>
<tr>
<td>Carrier</td>
<td>Corriell Research Institute (GM01701)</td>
<td>Male</td>
<td>Not affected</td>
<td>G695V</td>
<td>Non-diabetic; Father of WS-3 and WS-4</td>
</tr>
<tr>
<td>Control-1 (HUES42)</td>
<td>Harvard University</td>
<td>Male</td>
<td>Not affected</td>
<td>None</td>
<td>Non-diabetic</td>
</tr>
<tr>
<td>Control-2 (iPSCs)</td>
<td>Naomi Berrie Diabetes Center</td>
<td>Male</td>
<td>Not affected</td>
<td>None</td>
<td>Non-diabetic</td>
</tr>
</tbody>
</table>

Supplementary Table 2. Information on genotypes and phenotypes of the research subjects.
Supplementary Figure 1. Induced pluripotent stem (iPS) cells generated from Wolfram fibroblasts using Sendai virus vectors. A. Wolfram subject fibroblasts and Wolfram subject iPS cells. B. Karyotypes of the iPS cells of four Wolfram research subjects. C. The Wolfram iPS cells expressed pluripotent marker genes, shown are SSEA4, SOX2, TRA-1-60, NANOG, TRA-1-81, OCT4, by immunocytochemistry. D. Immunohistochemistry of embryonic body cultures and histological analysis of teratomas derived from iPS cells.
Supplementary Figure 2

Supplementary Figure 2. FACS analysis for quantification of differentiated beta cells. At day 14 of differentiation, cells were stained with human C-peptide antibody and subjected to standard FACS preparation. Gating was set up based on fluorescent intensity of negative control cells.
Supplementary Figure 3

Supplementary Figure 3. Proinsulin to insulin ratio of Control and Wolfram beta cells determined by ELISA. N=3 for each of two independent experiments.
Supplementary Figure 4. Enhanced unfolded protein response in Wolfram cells. A. Fold change of GRP78 mRNA level in control and Wolfram iPS cells after 6 hours of thapsigargin (TG) treatment. * P< 0.05. B. Gel images showing spliced XBP-1 mRNA level in control and Wolfram iPS cells under indicated conditions and quantification represents the results from studies of 4 Wolfram subject lines of three independent experiments. Band intensities for vehicle and TM+4PBA conditions in control cells were below detection threshold. C. Western blot analysis showing fold change of GRP78 expression level in control and Wolfram fibroblasts under indicated conditions. Quantification represents the results from studies of 2 Wolfram subjects (WS-1 and WS-2) of three independent experiments. TM: tunicamycin; 4PBA: Sodium 4-phenylbutyrate. Cells were treated with TM or TG for 6 hours. 1 mM 4PBA was added one hour prior to and during TM or TG treatment.
Supplementary Figure 5

Supplementary Figure 5. Transmission Electron Microscope showing endoplasmic reticulum morphology in Wolfram cells with or without treatment of 10 nM TG. Arrows point to the ER. Obvious ER dilation was observed in Wolfram cells after treatment with TG. Note the Scale bar: 500 nm.
Supplementary Figure 6

Supplementary Figure 6. Wolfram disease phenotypes can also be observed using iPS cells made by retroviral vectors, instead of Sendai virus. A. Expression from the retroviral transgenes in different cell lines as indicated. This shows that the viral vectors expression was silenced in the iPS cells. B. Expression of endogenous pluripotent genes in different cell lines as indicated. C. Fold change of human C-peptide secretion when the concentration of glucose in the media was changed from 5.6 mM to 16.9 mM glucose in control and Wolfram beta cells. N=3 for each experiment of three independent experiments.
Supplementary Figure 7. Fold change of human C-peptide secretion to 15mM arginine (Arg) or 1μM Bay K8644 stimulation in Control-2 and WS-3 beta cells upon thapsigargin (TG) treatment. N=3 for each experiment of three independent experiments. Bay: Bay K8644, a calcium channel agonist. 4PBA: Sodium 4-phenylbutyrate. * P<0.05 of TG vs. Vehicle; # P<0.05 of TG+4PBA vs. TG.
Supplementary Figure 8. WFS1 gene expression and insulin secretion of WS-1 cells carrying either control Lenti-virus vector without a cDNA, or with a wild-type WFS1 cDNA driven by MSCV promoter. A. Quantitative PCR for WFS1 mRNA level (N=3). B. Fold change of human C-peptide secretion to 15 mM arginine or 1 mM DBcAMP+16.9mM glucose stimulation in WS-1 cells (N=3).
Supplementary Figure 9. A. Control and Wolfram insulin positive cell number over 72 hours with or without treatment of 10nM thapsigargin (TG). B and C. CHOP (C/EBP-homologous protein, also known as DNA damage-inducible transcript 3 encoded by the DDIT3 gene) and TXNIP (thioredoxin-interacting protein) mRNA level in control and Wolfram cells with or without 10nM thapsigargin (TG) treatment for 12 hours.
Supplementary Figure 10

A. Human C-peptide levels in the sera of mice transplanted with control or Wolfram cells. B. Human C-peptide levels in the sera of transplanted mice before and after nephrectomy. Negative control represents mice with sham surgery procedure. C. Fasting human C-peptide levels in mice transplanted with human islets and Wolfram cells before and after one dosage of 1mg/kg thapsigargin (TG) injection. D. Fold change of human C-peptide levels in transplanted mice before and after one dosage of 1mg/kg thapsigargin (TG) injection.
**Supplementary Information:**

**Cell culture**

Biopsy plating medium contained DMEM, FBS, GlutaMAX, Anti-Anti, NEAA, 2-Mercaptoethanol and nucleosides and culture medium was composed of DMEM, FBS, GlutaMAX and Pen-Strep (all from Invitrogen). For generation of induced pluripotent stem cells using Sendai viruses, 50,000 fibroblast cells were seeded in a well of six-well dish at passage three in fibroblast medium. Next day, Sendai viruses expressing human transcription factors Oct4, Sox2, Klf4 and C-Myc were mixed in fibroblast medium to infect fibroblast cells according to the manufacturer’s instructions. 2 days later, the medium was exchanged to human ES medium supplemented by the MEK inhibitor PD0325901 (0.5 µM; Stemgent), ALK5 inhibitor SB431542 (2 µM; Stemgent), and thiazovivin (0.5 µM; Stemgent). Each iPS cell line was expanded from a single colony and cultured on MEF feeder cells with human ES medium. Human ES medium contained the following: KO-DMEM, KSR, GlutaMAX, NEAA, 2-Mercaptoethanol, PenStrep and bFGF (all from Invitrogen). Karyotyping of the cells was performed by Cell Line Genetics Inc. (Wisconsin).

**Spontaneous differentiation of iPS cells**

To generate embryoid bodies, 1-2 million iPS cells of each line were detached by TrypLE (Invitrogen) treatment and cultured in a low-attachment 6-well culture dish for 3 weeks. Cells assumed a spherical shape and were collected for immunostaining. For teratoma analysis, 1-2 million cells of each iPS cell line were collected and injected subcutaneously into dorsal flanks of a NOD.Cg-Prkdc<sup>scid</sup> Il2rg<sup>tm1Wjl</sup>/SzJ (NSG) mouse (Stock No. 005557, The Jackson Laboratory). 8-12 weeks after injection, teratomas were collected, fixed overnight with 4% paraformaldehyde and processed for paraffin
embedding according to standard procedures. Then the samples were sectioned and HE (hematoxylin and eosin) stained.

**Immunohistochemistry and microscopy**

Cells and tissues were fixed by 4% paraformaldehyde. Tissues were embedded in OCT or paraffin and sectioned at 10 µm. Cells or sections were blocked in 5% normal donkey serum and stained with primary antibodies: mouse-anti-SSEA4 (MAB1435; R&D systems), rabbit-anti-SOX2 (09-0024; stemgent), mouse-anti-TRA1-60 (MAB4360; Millipore), goat-anti-NANOG (AF1997; R&D systems), mouse-anti-TRA1-81 (MAB4381; Millipore), mouse-anti-OCT4 (sc-5279; Santa Cruz Biotechnology), rabbit-anti-AFP (A000829; DAKO), mouse-anti-SMA (A7607; Sigma), rabbit-anti-TUJ1 (T3952; Sigma), goat-anti-SOX17 (AF1924; R&D systems), goat-anti-PDX1 (AF2419; R&D systems), mouse-anti-C-peptide (05-1109; Millipore), rabbit-anti-glucagon (A056501; DAKO), mouse-anti-ATF6α (sc-166659, Santa Cruz), rabbit-anti-Urocortin 3 (HPA038281, Sigma), Ddit3 (ab11419, Abcam). Anti-WFS1 antibody was generously provided by Dr. Urano, Fumihiko (Washington University School of Medicine). Secondary antibodies were obtained from Molecular Probes (Invitrogen). For fluorescence-activated cell sorting (FACS) analysis, cells were collected and fixed in 4% PFA for 15 min at room temperature and analyzed using a FACSARiaII. Images were acquired with an Olympus IX71 fluorescence microscope and confocal microscope (Zeiss LSM5 PASCAL).