Silencing of the WFS1 gene in HEK cells induces pathways related to neurodegeneration and mitochondrial damage

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WOLFRAM SYNDROME (WS, MIM222300) is an autosomal recessive disorder most frequently characterized by diabetes insipidus, diabetes mellitus, optic atrophy, and deafness (DIDMOAD), first described by Wolfram and Wagenner as a juvenile diabetes mellitus with optic atrophy (40, 46). Only insulin-dependent diabetes mellitus and progressive optic atrophy are necessary to confirm WS, and both of these syndromes may be present in childhood or adolescence (3). WS can be characterized as polyendocrinopathy with significant pituitary deficiency and atrophy (13, 31). In addition to these diagnostic syndromes, most WS patients have highly variable clinical symptoms including several neurological abnormalities such as nystagmus, mental retardation, and seizures (3). Moreover, several studies have shown diffuse and widespread atrophy in the brain (32, 36). Central respiratory failure due to brain-stem atrophy has been described as a common cause of death, indicating the significance of neurodegeneration in WS (3, 36). In addition to the neurological manifestations, psychiatric illnesses have often been found in WS patients. The most prominent psychiatric manifestations in WS homozygous individuals are depression, violent or assultive behavior, and organic brain syndromes (41).

WS is caused by mutations in the WFS1 gene, but the molecular function of WFS1 protein is not fully known. It is a transmembrane protein with 9–11 segments and is located in the endoplasmic reticulum (ER) (16). There is evidence that this protein plays a role in the regulation of ER calcium levels (30, 42). WFS1 is involved in the unfolded protein response (UPR), which is an adaptive response that counteracts ER stress (9). ER stress is defined as an imbalance between the actual folding capacity of the ER and the demand (24). Induction of ER stress with thapsigargin and tunicamycin causes significant upregulation of WFS1 expression (9). WFS1 seems to act as a survival factor; it is upregulated when ER stress is present, and its deficiency leads to more pronounced apoptosis (19).

WFS1 seems to be involved in the activation and secretion of bioactive peptides, including insulin. There is evidence that WFS1 is related to the processing of vasopressin in the hypothalamus (12). Increased insulin demand has been shown to promote apoptosis in WFS1-deficient mice (1). Our previous study indicated that WFS1 knockout (KO) mice exhibit impaired glucose tolerance and are significantly smaller than their wild-type littermates despite elevated growth hormone (GH) (42) and IGF-1 levels (22, 29). These mice have lower plasma insulin and higher proinsulin levels, indicating potential insulin-processing problems. A recent study confirmed localization of WFS1 protein to the secretory granule and its role in the prohormone processing (15). Therefore, there is link between WFS1 gene function, peptide processing, and apoptosis. However, the available information is still fragmented and based on the diseased or gene targeted tissue samples, where compensatory changes may hide pathogenetic causal pathways. To add more causative information, time course-related effects of WFS1 silencing should be analyzed.

The aim of this study was to describe altered functional genetic networks in human embryonic kidney (HEK) cells after silencing the WFS1 gene and to follow the changes in the gene expression profile over time. This approach could give additional information on potential causative relations.
Materials and Methods

Materials. BSA, TRIzol, Dulbecco’s modified Eagle’s medium (DMEM) (PAA Laboratories, Pasching, Austria), penicillin-streptomycin, L-glutamine, fetal calf serum, and Effectene transfection kit were purchased from Qiagen (Hilden, Germany). TRIzol reagent and SuperScript III First-Strand Synthesis System were from Invitrogen (Carlsbad, CA). Anti-wfs1 antibody was made in collaboration with the University of Copenhagen (25).

Mice. Wfs1 KO mice, as described in our previous publications (22), were housed under standard laboratory conditions on a 12-h light-dark cycle (lights on at 07:00 AM) with free access to food and water. All animal experiments in this study were performed in accordance with the European Communities Directive (86/609/EEC) and permit (no. 86, August 28, 2007) from the Estonian National Board of Animal Experiments, which reviewed and approved all procedures on animals.

HEK cell line culture. HEK cells were grown in DMEM supplemented with 10% fetal calf serum, and 100 U/ml penicillin-streptomycin. The day before transfection, 2–8 × 10⁴ cells were seeded per 60 mm dish in 5 ml of DMEM growth medium containing serum and antibiotics. The cells were grown at 37°C and 5% CO₂. The dishes were 80% confluent on the day of transfection.

siRNA transfection. WFS1 gene was silenced with three different short interfering RNAs (siRNA) using Effectene transfection reagent from Qiagen (cat. #301425) according to the manufacturer’s protocol. siRNAs were predesigned and inventoried Silencer Select siRNAs from Qiagen (cat. #301425) according to the manufacturer’s protocol. Short interfering RNAs (siRNA) using Effectene transfection reagent procedures on animals.

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Gene expression proﬁling. Total RNA was isolated from transfected cells at different time intervals (24, 48, 72, and 96 h) using TRIzol reagent (Invitrogen, 15596-018) following the manufacturer’s protocol. Yield and purity of RNA was determined by absorbance using a Nanodrop spectrophotometer (Thermo Scientific). Subsequently, 1 µg of total RNA from each sample was subjected to cDNA synthesis using SuperScript III First-Strand Synthesis System (Invitrogen, 18080-051) following the manufacturer’s protocol.

The gene expression level of WFS1 was detected applying TaqMan Assay-On-Demand (Hs00903610_m1, FAM/MGB probe; Life Technologies, Foster City, CA) and TaqMan Universal PCR Master Mix (Life Technologies) in the ABI Prism 7900 HT Sequence Detection System (Life Technologies). In addition, we performed conﬁrmatory RT-PCR analysis on RNA samples extracted from the kidneys of Wfs1 KO mice. The expression of Tomm20, Epsap1, Ptpmt1, and Adam19 genes was analyzed. Reactions were carried out in 10 µl reaction volumes in four replicates. Data are presented as 2-ΔCT calculated in relation to the Hprt1. TaqMan Endogenous Control assay for Hprt1 (HGPRT, VIC/MGB probe, part 4326321E, primer limited) was used as reference in all QRT-PCR reactions.

Protein preparation and Western blotting. Total cell protein was isolated from transfected cells at the same time point and the same sample from which total RNA was isolated using TRIzol reagent following the manufacturer’s protocol. The protein pellet was dissolved in 1% SDS (200 µl) by incubating the sample at 50°C for 1 h and followed by centrifugation at 10,000 g for 10 min at 4°C to sediment any insoluble material. Protein concentration was measured by DC Protein Assay (Bio-Rad, 500-0111). For Western blot analysis equivalent amounts of total protein were separated by SDS-PAGE on 10% polyacrylamide gels and then transferred to Hybond-P PVDF Transfer Membranes (Amersham Biosciences, UK) in 0.1 M Tris base, 0.192 M glycine and 10% (wt/wt) methanol using an electrophoretic transfer system. The membranes were blocked with 5% (wt/vol) nonfat dried milk in Tris-buffered saline containing 0.1% (vol/vol) Tween 20 at room temperature for 1 h. After being blocked, the membranes were incubated overnight with primary antibodies (mouse anti-actin 1:4,000 and rabbit anti-WFS1 1:1,000) and then washed and incubated with appropriate horseradish-peroxidase (HRP)-conjugated secondary antibody (1:4,000, Pierce) for 1 h at room temperature. Immunoactive bands were detected by enhanced chemiluminescence (ECL, Amersham Biosciences, UK) using medical X-ray film blue (Agfa). The blots probed for proteins of interest

Scale chr4: 1 6,270,000 6,275,000 6,280,000 6,285,000 6,290,000 6,295,000 6,300,000 6,305,000 6,310,000

WF51
WF51
WF51

UCSC Genes (RefSeq, UniProt, CCDS, Rfam, tRNAs & Comparative Genomics)

Fig. 1. Alignment map of the siRNAs we used for silencing of WFS1 gene illustrates the areas of the WFS1 gene targeted by siRNA interference. We used 3 siRNAs (red lines and red labels) to interfere with WFS1 expression. IDs of these siRNAs are (from left to right): s14858 (siRNA3), s14857 (siRNA2) and s14856 (siRNA1).
were analyzed with a QuantityOne 710 System densitometer (Bio-Rad).

Data analysis. The normalized, background subtracted, and modeled expression [robust microarray analysis (RMA)] data were further analyzed with the limma package implemented in the statistical software R (http://www.r-project.org/) without further transformation (18, 38). General data structure and factors are listed in the Table 1. We had three factors: siRNA (siRNA1, siRNA2, siRNA3, nc, cell), “time” (24, 48, 72, and 96 h), and “treatment” (siRNA, nc, cell). Level “cell” means the HEK cells without any treatment and was used as an additional control for the effect of siRNA treatment. The dataset was divided into two parts: one included all samples (also “cell”) and consisted of 68 chips. Another sample consisted of 54 chips where the cell-treated group was removed (siRNA1, siRNA2, siRNA3, and nc). Most of the analyses were performed on the sample of 54 chips. For differential expression analysis linear modeling was used with two main factors in the analysis design (time and treatment). False discovery rate (FDR) adjustment and q-values were used for multiple testing correction (39).

Time-course analysis was performed by linear modeling separately for each different siRNA groups [basic formula: ANOVA irresistible one + time)]. The microarray data have been reannotated with the latest Entrez13 CDF. Data for the four culture time points (24, 48, 72, and 96 h) were calculated by subtracting [data in log(2) scale] the mean of the two control replicates from the mean of all six siRNA cultures for each time point. The value for each gene at time 0 was defined as 0.

Figures were produced with R, GraphPad 5.0, Cytoscape, and Ingenuity Pathway Analysis (IPA).

Functional annotation and network analysis. To define the functional networks of the differentially expressed genes, data were run through IPA (Ingenuity Systems, http://www.ingenuity.com). A data set containing Affymetrix probe-set identifiers and corresponding fold change values was uploaded to IPA. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Knowledge Base and was considered for the analysis. Canonical pathways in the Ingenuity Knowledge Base were considered for the analysis. The significance of the association between the data set and the canonical pathway was measured in two ways: 1) a ratio of the number of molecules from the data set that map to the pathway divided by the total number of molecules that map to the canonical pathway was displayed. 2) Fisher’s exact test was used to calculate a P value determining the probability that the association between the genes in the dataset and the canonical pathway was explained by chance alone.

Functional analysis was performed on the entire dataset and filtered to get only FDR corrected significant genes (14 genes). Statistical significance filtering was used to increase the focus and specificity of analysis.

RESULTS

Silencing of the WFS1 gene in HEK cells. We used three siRNA constructs targeting WFS1 to silence transcription in a cultured HEK cell line. In addition, nonsense siRNA-treated and untreated cells were cultured in parallel as controls. To verify silencing of WFS1, we performed quantitative PCR in the Ingenuity Knowledge Base were considered for the analysis. The significance of the association between the data set and the canonical pathway was measured in two ways: 1) a ratio of the number of molecules from the data set that map to the pathway divided by the total number of molecules that map to the canonical pathway was displayed. 2) Fisher’s exact test was used to calculate a P value determining the probability that the association between the genes in the dataset and the canonical pathway was explained by chance alone.

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Table 2. Genes regulated by siRNA knock-down of WFS1

<table>
<thead>
<tr>
<th>Symbol</th>
<th>GeneID</th>
<th>Regulation</th>
<th>FDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>WFS1</td>
<td>7466</td>
<td>down</td>
<td>1.76×10^{-4}</td>
</tr>
<tr>
<td>H11004</td>
<td>51015</td>
<td>up</td>
<td>2.52×10^{-4}</td>
</tr>
<tr>
<td>H11003</td>
<td>9804</td>
<td>up</td>
<td>2.52×10^{-4}</td>
</tr>
<tr>
<td>H11002</td>
<td>114971</td>
<td>up</td>
<td>9.62×10^{-4}</td>
</tr>
<tr>
<td>H11006</td>
<td>55312</td>
<td>up</td>
<td>2.43×10^{-3}</td>
</tr>
<tr>
<td>H11002</td>
<td>1831</td>
<td>up</td>
<td>7.37×10^{-3}</td>
</tr>
<tr>
<td>H11002</td>
<td>8728</td>
<td>down</td>
<td>8.37×10^{-3}</td>
</tr>
<tr>
<td>H11003</td>
<td>3516</td>
<td>up</td>
<td>9.57×10^{-3}</td>
</tr>
<tr>
<td>H11002</td>
<td>23673</td>
<td>up</td>
<td>1.97×10^{-2}</td>
</tr>
<tr>
<td>H11002</td>
<td>2034</td>
<td>down</td>
<td>2.16×10^{-2}</td>
</tr>
<tr>
<td>H11002</td>
<td>6622</td>
<td>up</td>
<td>2.39×10^{-2}</td>
</tr>
<tr>
<td>H11002</td>
<td>813</td>
<td>up</td>
<td>2.39×10^{-2}</td>
</tr>
<tr>
<td>H11002</td>
<td>8886</td>
<td>up</td>
<td>2.39×10^{-2}</td>
</tr>
<tr>
<td>H11002</td>
<td>892</td>
<td>up</td>
<td>2.74×10^{-2}</td>
</tr>
</tbody>
</table>

FDR, false discovery rate.

(QRT-PCR) from three replicates. Downregulation of WFS1 RNA expression was clearly evident (Fig. 2). Twenty-four hours after incubation, none of the siRNAs induced statistically significant downregulation of WFS1. WFS1 RNA was significantly downregulated after 48, 72, and 96 h of siRNA incubation. After 72 h of incubation only siRNA2-induced suppression was not statistically significant. Western blot analysis further confirmed downregulation of the expression of WFS1 protein by the siRNA treatment (Fig. 3).

Microarray expression analysis. RNA from all cultures was hybridized to Human Gene 1.0 ST expression microarrays. Quality control analysis indicated consistent and similar intensities between different arrays. Array data were preprocessed with the RMA algorithm using updated probe annotations (see MATERIALS AND METHODS).

We found no significant difference in WFS1 expression between cell-only and negative siRNA controls over the time course in a two-way ANOVA (main effect of treatment \( F_{1,25} = 3.12, P = 0.09 \)), indicating that the negative control siRNA sequence had no unexpected silencing effect on the target gene.

After normalizing expression data for the siRNA-treated arrays to the mean of the negative control arrays at each time point, we showed that there was no significant effect of the different siRNA sequences (\( F_{2,36} = 2.03, P = 0.15 \)). Therefore, we considered samples from all three siRNA sequence types as replicates.

We then calculated multiple two-way ANOVA for time and treatment for all genes. There were 14 genes with a significant main effect of treatment after correction for multiple testing (\( P < 0.05 \), corrected by the FDR method of Benjamini and Hochberg [3a]). These results are summarized in Table 2.

Interestingly, 11 of these regulated genes were upregulated in response to WFS1 silencing and only two (besides WFS1 itself) were downregulated.

Time-course analysis. To investigate the cascade of events downstream of WFS1 silencing, we looked for genes exhibiting time-delayed patterns of regulation over the time series studied. The normalized data were filtered using multiple two-way ANOVA (main effect of time \( P < 0.05 \) after FDR correction), and the resulting 37 profiles were matched to predefined patterns. The best match of each profile to eight patterns (up-or downregulated at 1, 2, 3, or 4 days) was used to link genes, such that, for example, a gene upregulated on day 1 was considered potentially causal for the upregulation of a gene on day 2, and so on. Figure 4 shows the resulting network. Each of the links between genes suggests a causal hypothesis that can be later experimentally tested. As an example of such a hypothesis, the transcription factor Krüppel-like transcription factor 4 (KLF4) that is downregulated on the first day following WFS1 knock-down. Known to regulate cell proliferation via its role in telomere maintenance, KLF4 might be affecting transcription of WRAP53, a prosurvival protein required for telomere elongation (26, 45, 47). Another potential pathway...
Fig. 5. This figure illustrates that genes with highest expressional changes (filter set to $P < 0.05$ after FDR correction) after WFS1 silencing belong to the "protein trafficking, cell morphology, cellular function and maintenance" functional network. Gene expression profiles were uploaded to the Ingenuity Analysis Software and based on the differential expression of these genes, the most relevant biochemical network with functional links is found. Red symbols are upregulated genes, green symbols are downregulated genes, and the numbers reflect the $t$-value of the statistical comparison with Bayesian moderated $t$-test. Figure generated through the use of Ingenuity Pathway Analysis (IPA, Ingenuity Systems, http://www.ingenuity.com).
involves the transporter SLC7A5 (downregulated on day 1), which may modulate expression of SLC7A11 (downregulated on day 2). Both SLC7A5 (also known as LAT1) and SLC7A11 (also known as xCT) are known to have important roles in neuronal function (20, 37).

Functional analysis. Ingenuity functional pathway analysis software was used for more general functional annotation of the differential gene sets. These are the genes with highest differential expression after the WFS1 silencing. Network analysis of the 14 genes with lowest P values (filter set to P < 0.05 after FDR correction) revealed significant enrichment of protein trafficking, cell morphology, cellular function, and maintenance network (score 37, Fig. 5). All 14 genes are part of this functional network, and therefore we concluded that silencing of WFS1 gene significantly influences the protein trafficking network.

Canonical pathways are idealized or generalized pathways, which represent common properties of a particular signaling module or pathway (http://stke.sciencemag.org/about/help/cm.dtl). Analysis of differential gene expression profiles for the canonical pathways indicated the enrichment of Parkinson’s Signaling (Fig. 6). Another canonical pathway that was significantly enriched was riboflavin metabolism pathway. Taken together, based on canonical analysis, WFS1 silencing seems to disturb basic metabolic pathways important for cellular survival.

Real-time PCR confirmation in Wfs1 KO mice. To determine whether similar changes in gene expression could be observed in kidney cells obtained from conventional WFS1 silencing (e.g., in the Wfs1 KO mice), we performed real-time PCR analysis from the kidneys of Wfs1 KO mice. As HEK cells are also kidney derived, we choose to use the same tissue from mutant mice.

We analyzed expression of Tomm20, Epas1, Ptpmt1, and Adam19 genes and the values of wild-type (WT) and Wfs1 mutant (KO) animals were compared with t-test. Statistically significant (***P < 0.001) differences were found in case of Tomm20 and Epas1 (Fig. 7). The change in Tomm20 expression was in the same direction, but the change in Epas1 expression was in the opposite direction compared with the microarray study.

DISCUSSION

WFS1 gene deletion models WS, a rare familial disorder characterized by multiple endocrine system failure (8, 46). As in humans, the mice have a very severe phenotype after deletion of Wfs1, starting to progress from the age of 20 wk (22, 29). We have described the growth failure and alterations in the GH pathway in these mice (22). Wfs1 mutant mice have impaired glucose homeostasis and diabetes (19, 34). In addition, we have described reduced fertility in male Wfs1 mutant mice (28). Description of gene expression profiles in mutant mice is a straightforward approach, but the time-dependent temporal resolution of molecular changes is lost, as are the causal relationships between the markers. Therefore, combination of data from gene targeting and siRNA experiments, could provide invaluable information.

WFS1 is involved in the UPR and ER stress response (9, 10). Deletion of WFS1 leads to a permanent activation of the ER stress pathway what involves upregulation of cell survival genes (e.g., heat shock proteins) and downregulation of the normally active transcriptional network (34, 48). Therefore, changes in WFS1 expression can lead to the changes in the transcriptional network. Inactivation of WFS1 leads to a permanent suppression of the physiological processes in cells and permanent upregulation of genes enhancing cell survival. These changes have been described in Wfs1 KO mice in earlier studies (22, 34, 48). In the present study, we analyzed the effect of acute reduction of WFS1 expression on the transcriptional network of HEK cells. We expected to see changes that were not influenced by compensatory changes induced by permanent deletion of this gene. As WFS1 reduction is related to the induction of ER stress, the genes we discovered by this approach should reflect the early effects of ER stress on transcription (9).

The main finding of this study was the activation (positive log FC values) of genes related to mitochondrial dysfunction.
The complement system is an early response system that is an event related to the UPR, ER stress, and degeneration. This supports the previously held view that WS was a mitochondrial disease (6, 7). Moreover, WS is a syndrome characterized by degeneration in multiple tissues: pancreas, inner ear, eyes, brain, etc. (2). Therefore, changes in mitochondria, a fundamental element of the cell, could result in such a widespread system breakdown.

Interestingly, in our previous study using the gene expression profiling in the temporal lobe, we found significant down-regulation of the transthyretin (Ttr) in WFS1 mutant mice (22). Ttr is a gene that has a very strong connection with amyloid-related degeneration: amyloid polyneuropathy, cardiac amyloidopathy, and amyloidosis in general (11, 14, 33). As WFS1 mutation is related to the degeneration of several organs (islets, brain, heart, etc.), our previous and present findings indicate a number of interesting molecular networks that may mediate the brain degeneration seen in the WS. Functional annotation of gene expression profiles detected upregulation of the molecular network named “protein trafficking, cell morphology, cellular function and maintenance network” (Fig. 5). Protein trafficking is an event related to the UPR, ER stress, and degeneration. Therefore, functional annotation also indicated activation of degenerative molecular pathways.

Moreover, another analysis found statistically significant upregulation of the C4b (complement component 4B) gene in the hypothalamus of Wfs1-deficient mice (23). This gene is particularly interesting, as it is mostly known for its role in the immune response as a factor for the classical activation pathway. However, several studies suggest it also has a role in the brain pathologies, especially in the degenerative disorders (27). Thus, C4b has been implicated in Alzheimer’s disease, autism, prion disease, and multiple sclerosis (4, 5, 21, 43, 44). Neurodegeneration is one of the most important features of WS (3, 35). The complement system is an early response system that is the part of innate immune response, and its role in neurobiology has been recognized (17).

The real-time PCR experiment did not confirm our findings entirely. While there were clear differences in Tomm20 and Epa1, we didn’t see statistically significant differences in the other genes identified in the microarray experiment. This discrepancy can be caused by the different experimental set-up. Suppression of gene expression with siRNA should reflect acute or temporary changes in the transcriptome, while gene targeting induces chronic/permanent changes where adaptation (secondary) changes are mixed with primary effects. However, our findings suggest that Tomm20 may be a key gene influenced by Wfs1 expression.

In conclusion, WFS1 invalidation induces upregulation of genes within a pathway related to the mitochondrial dysfunction, the activation of which can lead to cell destruction and degeneration. WFS1 silencing initiates mitochondrial dysfunctioning and unfolded protein response followed by apoptotic events in the cells. This can be the potential cellular mechanism leading to the degeneration in case of WFS1 deficiency, but more detailed studies are needed.

**GRANTS**

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

Author contributions: S.K. conception and design of research; S.K., M.I., U.S., M.G., and C.F. performed experiments; S.K. and R.W.O. analyzed data; S.K. and L.C.S. interpreted results of experiments; R.W.O. prepared figures; R.W.O., E.V., and L.C.S. edited and revised manuscript; L.C.S. approved final version of manuscript.
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Wfs1 SILENCING IN HEK CELLS

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