Ubiquitin-fold modifier 1 inhibits apoptosis by suppressing the endoplasmic reticulum stress response in Raw264.7 cells

XIAOLEI HU, QI PANG, QIONGNA SHEN, HUIFANG LIU, JIANGPING HE, JING WANG, JIE XIONG, HUIJIE ZHANG and FENGLING CHEN

Department of Endocrinology, Shanghai 3rd People's Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 201999, P.R. China

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Abstract. Ubiquitin-fold modifier 1 (Ufm1) is a new member of the ubiquitin-like protein family, and its biological function remains largely unknown, particularly in macrophages. In this study, we demonstrate that Ufm1 expression is increased in diabetic mouse resident peritoneal macrophages (RPMs) and in the mouse macrophage cell line, Raw264.7, subjected to endoplasmic reticulum (ER) stress. Following treatment of the cells with the ER stress inducers, thapsigargin (TG) or tunicamycin (TM), the lentiviral short hairpin RNA (shRNA)-mediated knockdown of Ufm1 increased the apoptosis of Raw264.7 cells. Furthermore, these cells had higher expression levels of immunoglobulin heavy chain-binding protein (BiP) and C/EBP homologous protein (CHOP), which are markers of the ER stress response. The overexpression of Ufm1 induced by lentiviral infection in the Raw264.7 cells treated with the ER stress inducers, TG or TM, resulted in the opposite effect. Taken together, our results suggest that Ufm1 is upregulated in diabetic mouse RPMs and in Raw264.7 cells in response to ER stress and that Ufm1 protects macrophages against apoptosis. Thus, Ufm1 may be a novel gene that protects against ER stress-induced apoptosis in macrophages.

Introduction

The endoplasmic reticulum (ER) is an organelle in eukaryotic cells that is involved in the synthesis, maturation, quality control and trafficking of a wide range of proteins (1, 2). Disruptions in ER function resulting from increased protein synthesis, misfolded protein overload, changes in calcium concentration or hypoxia induce ER stress and activate the ER stress response [also known as the unfolded protein response (UPR)] to restore ER homeostasis and protect the cell. Mechanisms activated during the ER stress response include translational attenuation, activation of ER chaperones, such as immunoglobulin heavy chain-binding protein (BiP, also known as GRP78) and the degradation of unfolded proteins (1,3). Chronic or unresolved ER stress, however, can lead to apoptosis. Apoptosis resulting from ER stress is mediated by factors including C/EBP homologous protein (CHOP), IRE1α and caspase-12 (4-6). Accumulating evidence indicates that ER stress-induced apoptosis contributes to the pathogenesis of various diseases, such as type 2 diabetes mellitus (T2D), atherosclerosis, liver disease, neurodegenerative disorders and cancer (2,7-12).

It has also been shown that many cellular regulatory processes depend on the post-translational functions of ubiquitin and ubiquitin-like proteins (Ubls), including transcription, DNA repair, signal transduction, autophagy, cell proliferation, differentiation, apoptosis, ER regulation, inflammation, antigen processing and stress responses (13-15). Ubiquitin-fold modifier 1 (Ufm1) has recently been identified as a novel Ubl with a molecular mass of 9.1 kDa, and it appears to have a similar tertiary structure to ubiquitin, despite having little amino acid sequence identity (16). Similar to the process of protein ubiquitination, Ufm1 is first synthesized in a pro-form and cleaved at the C terminus by the specific cysteine proteases, UfSP1 and UfSP2, to expose the conserved glycine residue that is essential for its subsequent conjugating reactions (17). The mature form of Ufm1 is successively activated by a specific E1-activating enzyme (Uba5), an E2-conjugating enzyme (Ufc1) and an E3-ligating enzyme (Ufl1), and then it is covalently attached to a cellular target protein, such as C20orf116 (also known as UFBP1), CDK5RAP3 and mitochondrial trifunctional protein (MTP) (18-20). Finally, Ufm1 is cleaved by UfSPs from the conjugation (16,18).

Previous studies have suggested that there is an association between Ufm1 expression and the ER stress response in mice suffering from ischemic heart injury and in the pancreatic islets of an animal model of T2D (21,22). Recently, it has been shown that Ufm1 expression is induced by ER stress and protects against ER stress-induced apoptosis in the mouse pancreatic β-cell line, INS-1E (19). Moreover, ER stress upregulates the Ufm1 system in multiple cancer cell lines, and the knockdown of the Ufm1 system results in the activation of the UPR (23). These results indicate that Ufm1 is linked to ER stress.

Correspondence to: Professor Fengling Chen, Department of Endocrinology, Shanghai 3rd People's Hospital, School of medicine, Shanghai Jiao Tong University, 280 Mohe Road, Shanghai 201999, P.R. China
E-mail: cfl1993@126.com

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Macrophages are found in all tissues and have been shown to play important roles in development, metabolic homeostasis, tissue repair and immunity (24). These findings also indicated that macrophages play important roles in T2D and atherosclerosis, and a number of studies have focused on the role of macrophage ER stress-induced apoptosis in diabetic macrovascular diseases, such as atherosclerosis (25-28). The expression pattern and potential biological function of Ufm1 in macrophages under ER stress, however, remains unclear. Therefore, in the present study, we evaluated Ufm1 expression in diabetic mouse resident peritoneal macrophages (RPMs), as well as the effects of Ufm1 on the ER stress response in the cultured mouse macrophage cell line, Raw264.7. Furthermore, the lentiviral short hairpin RNA (shRNA)-mediated knockdown of Ufm1 increased the apoptosis of Raw264.7 cells exposed to ER stress inducers and these cells had higher expression levels of BiP and CHOP, which are markers of the ER stress response. The overexpression of Ufm1 by the lentiviral infection of Raw264.7 cells treated with ER stress inducers resulted in the opposite effect. Thus, our results demonstrate that Ufm1 suppresses macrophage apoptosis by inhibiting the ER stress response *in vitro*.

Materials and methods

Materials. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, phosphate-buffered saline (PBS) and puromycin were purchased from Gibco BRL (Grant Island, NY, USA). Lipofectamine 2000, Opti-MEM and TRIzol reagent were obtained from Invitrogen (Carlsbad, CA, USA). The GoScript™ Reverse Transcription kit and the GoTaq® qPCR Master mix kit were purchased from Promega Corp. (Madison, WI, USA). Ethylenediaminetetraacetic acid (EDTA), trypsin, thapsigargin (TG) and tunicamycin (TM) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Animals. For our experiments, 8- to 12-week-old male db/db (C57BL/6J-Lepr^{db}/Lepr^{db}) mice and littermate control db/m (C57BL/6J background) mice were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China) and fed standard chow (LabDiet 5001; LabDiet, St. Louis, MO, USA). All the mice were kept in a specific pathogen-free facility and allowed free access to food and water. The experiments were performed in strict accordance with the Institutional Guidelines for the Care and Use of Laboratory Animals of Shanghai Jiao Tong University (Shanghai, China).

Isolation and culture of mouse RPMs. RPMs from db/db mice and db/m mice were isolated 3 days following an intraperitoneal injection of 4% thioglycollate solution, as previously described (29). Briefly, approximately 5 ml of cold serum-free DMEM were injected intraperitoneally after the mice under were euthanized ether anesthesia. Following a gentle massage, the peritoneal fluid containing the cells was harvested and centrifuged at 700 x g for 5 min at 4˚C. The pellet was resuspended in 5 ml of DMEM containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin and then cultured at 37˚C in a 60-mm dish (Corning Life Sciences, Oneonta, NY, USA) for 1 h. Only the attached macrophages were used for the following experiments.

Cell culture. The human embryonic kidney 293T (HEK293T) cell line and mouse macrophage cell line, Raw264.7, were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). The cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37˚C. When the cells reached approximately 70-80% confluency, they were dissociated with trypsinization and subcultured.

RNA isolation and quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The cells were collected and washed with ice-cold PBS. Total RNA was isolated from the cells using TRIzol reagent. cDNA was synthesized using the GoScript™ Reverse Transcription System according to the manufacturer's instructions and then used as the template for qPCR. The GoTaq® qPCR Master mix kit was used for the qPCR assays. The thermal cycling conditions consisted of 2 min at 95˚C, followed by 40 cycles of 15 sec at 95˚C and 1 min at 60˚C. Each sample was run in duplicate on an ABI ViiA 7 Real-Time PCR system and analyzed by the relevant software. The $2^{-\Delta\Delta CT}$ method was used to analyze the relative changes in gene expression. Either GAPDH or β-actin was used as an internal standard. The following primers for each target gene were used: Ufm1 forward, 5'-TTCCTG CAGCTACAAGTGCG-3' and reverse, 5'-TCCAACTCGGT CTCTAGGAATGAT-3'; β-actin forward, 5'-TGTGACGTT GACATCCGTAAAGAC-3' and reverse, 5'-TCCACACAGAG TACTTGCGCTC-3'; BiP forward, 5'-GAGTTCTTCAA TGGCAAGGAGC-3' and reverse, 5'-GGACAAACATCAA GCAGTACCAGAT-3'; CHOP forward, 5'-CTCATCCCCA GGAAACGAAGAG-3' and reverse, 5'-TTGGGATGTGCG TGTGACC-3'; and GAPDH forward, 5'-TGGTGAAGGT CGGTGTGAAC-3' and reverse, 5'-GCTCCTGGAAGAT GGTGATGG-3';

Western blot analysis. The cells were harvested in RIPA lysis buffer supplemented with phenylmethanesulfonyl fluoride (PMSF) and protease inhibitor cocktail. Proteins were separated by 10-15% SDS-PAGE, transferred onto PVDF membranes, and stained with the following antibodies: anti-Ufm1 (1:10,000; Abcam, Cambridge, MA, USA), anti-BiP (1:1,000), anti-CHOP (1:1,000), anti-cleaved caspase-3 (1:1,000) (all from Cell Signaling Technology, Inc., Danvers, MA, USA), anti-GAPDH (1:5,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and anti-β-actin (1:10,000; Cell Signaling Technology). Secondary antibodies conjugated to horseradish peroxidase and ECL western blot analysis reagents (Millipore Corp., Billerica, MA, USA) were used for detection. Immunoreactive bands were quantified using Gel-Pro 32 software (Media Cybernetics, Inc., Rockville, MD, USA).

Construction of recombinant lentivirus and cell infection. Three candidate small interfering RNA (siRNA) sequences for mouse Ufm1 were designed following the procedure from the Dharmacon siDESIGN Center: siRNA-1 (5'-CGGCTCAGAA CTGAGAATCAT-3'), siRNA-2 (5'-CGCCGTTCACAGCAGT GCTAA-3'), and siRNA-3 (5'-AGTTGGAAGCTGCTAAT ATAT-3'). Scrambled siRNA (5'-TTCTCCGAACGTGTC ACGT-3') was used as the negative control. shRNAs that corresponded to the siRNA sequences for Ufm1 and the

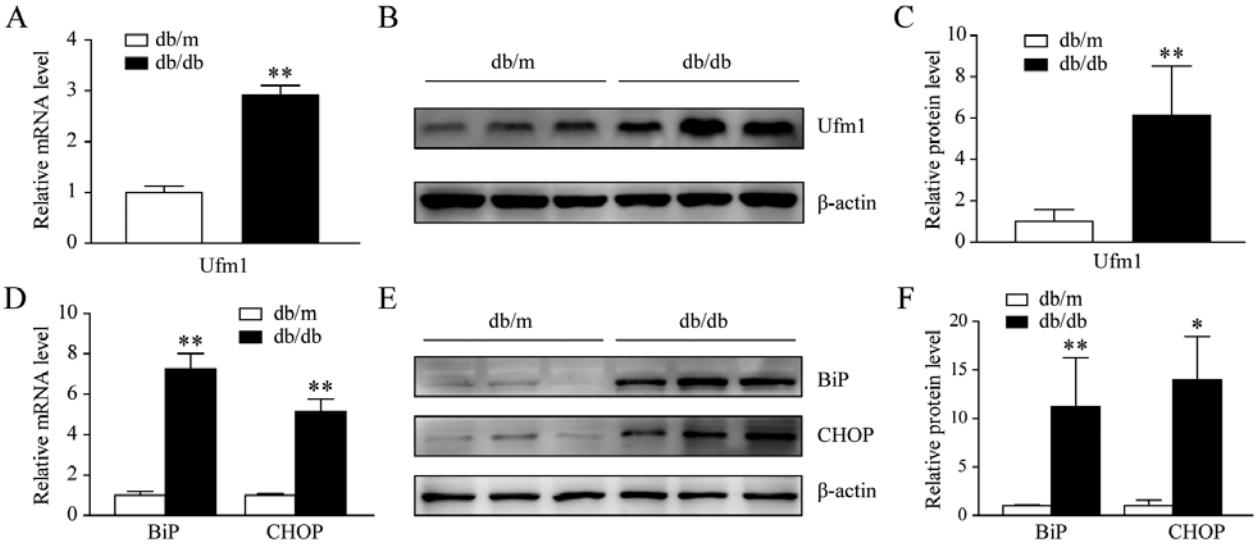

Figure 1. Increase in ubiquitin-fold modifier 1 (Ufm1) expression in the resident peritoneal macrophages (RPMs) of db/db mice. (A-C) mRNA and protein expression levels of Ufm1, (D-F) immunoglobulin heavy chain-binding protein (BiP) and C/EBP homologous protein (CHOP) in RPMs from db/m and db/db mice. β-actin was used as an internal control. Data are presented as the means ± standard deviation (SD), n=3-6; $^{*}p<0.05$, $^{**}p<0.01$ vs. db/m.

negative control were then generated. These shRNAs were inserted into the lentiviral vector, pFU-GW-RNAi (Genechem Co., Ltd., Shanghai, China). The vectors containing the Ufm1-shRNA sequences were designated as pFU-GW-RNAi-Ufm1 and the vector containing the negative control was designated as pFU-GW-RNAi-NC. Lentiviruses were generated by the triple transfection of 80% confluent HEK293T cells with the modified pFU-GW-RNAi plasmid and pHelper 1.0 and pHelper 2.0 helper plasmids (Genechem Co.) using Lipofectamine 2000 according to the manufacturer's instructions. Lentiviruses were harvested 72 h after transfection. The lentiviruses were purified using ultracentrifugation and the titers of the lentiviruses were determined. The Raw264.7 cells were infected with either the Ufm1-shRNA lentivirus (Lv-shUfm1) or the negative control lentivirus (Lv-shNC).

The sequence of the mouse Ufm1 gene was obtained from GenBank (NM_026435) and oligonucleotides were designed and synthesized based on this sequence. A restriction enzyme site (*Not*I and *Nsi*I) was added to each end of the oligonucleotides. PCR was performed to connect the synthesized oligonucleotides into a complete sequence. Following a *Not*I/*Nsi*I (Fermentas, Glen Burnie, MD, USA) digestion, the produced fragment was ligated into the pLV5 vector (GenePharma Co.), producing the pLV5-UFM1 construct. The pLV5 vector was used as a negative control. The lentiviral vector (pLV5-UFM1 or pLV5) was added to Opti-MEM, and then packing plasmids (pGag/Pol, pRev, pVSV-G; GenePharma Co.) and Lipofectamine 2000 were added sequentially. The resulting mixture was co-transfected into the HEK293T cells, which were incubated for 72 h. The supernatant was collected, centrifuged and filtered, and the titers of the lentiviruses were determined. The Raw264.7 cells were then infected with either the Ufm1 lentivirus (Lv-Ufm1) or the negative control lentivirus (Lv-NC).

Detection of apoptosis by flow cytometry. Apoptosis was analyzed by double staining with Annexin V-PE and 7-AAD (BD Biosciences, Franklin Lakes, NJ, USA), which was detected by a FACSCalibur flow cytometer (BD Biosciences). Briefly, the cells were stained with 100 μl binding buffer containing 5 μl Annexin V-PE and 5 μl 7-AAD at room temperature in the dark for 10-15 min. The data were analyzed using CellQuest software (BD Biosciences).

Statistical analysis. All data are expressed as the means ± standard deviation (SD) of 2 or 3 independent experiments. The Student's t-test was used to evaluate the differences in each group using SPSS 17.0 software. A value of $p<0.05$ was considered to indicate a statistically significant difference.

Results

Increase in Ufm1 expression in the RPMs of db/db mice. To investigate the functional role of Ufm1 in T2D macrophages, we determined the Ufm1 mRNA and protein expression levels in the RPMs from the db/db mice and the littermate controls, db/m mice. We first identified the RPMs using the previously reported surface antigen F4/80^{+} detection technique (29). Total RNA and protein were extracted from the RPMs of the db/db and db/m mice. The results from qRT-PCR and western blot analysis showed that there was a significant increase in the Ufm1 mRNA and protein expression levels (Fig. 1A-C). We also determined the expression levels of BiP and CHOP, which are markers of the ER stress response. As shown in Fig. 1D-F, their expression levels in the RPMs from the db/db mice were higher than those from the db/m mice. These results indicated that the Ufm1 mRNA and protein levels were higher in the diabetic mice and that the ER stress response was activated in the RPMs from the db/db mice.

Ufm1 expression is upregulated by ER stress inducers in Raw264.7 cells. It has been reported that the Ufm1 expression level is higher in animal models and cultured cells under ER stress (19,21-23); however, the effects of ER stress on Ufm1

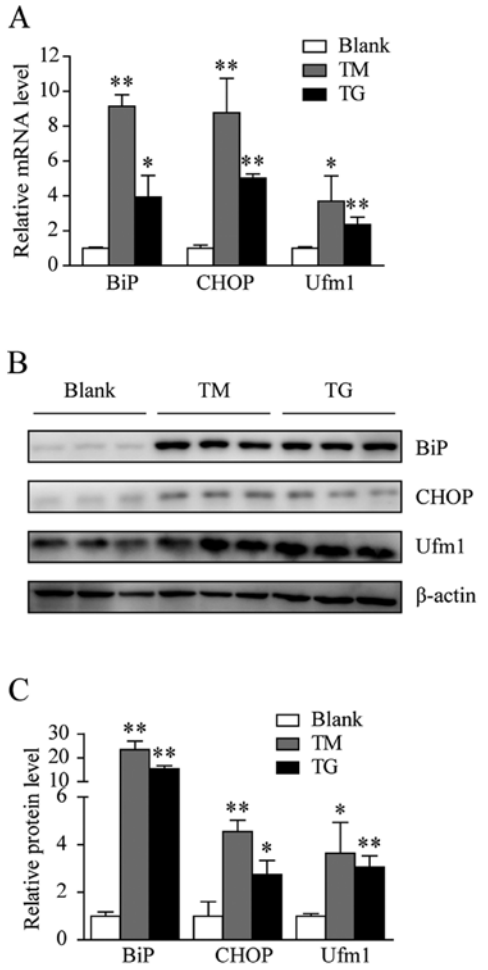

Figure 2. Ubiquitin-fold modifier 1 (Ufm1) expression is upregulated by endoplasmic reticulum (ER) stress inducers in Raw264.7 cells. Ufm1, binding immunoglobulin protein (BiP) and C/EBP homologous protein (CHOP) mRNA and protein expression levels were detected by (A) qRT-PCR and (B and C) western blot analysis, after the Raw264.7 cells were treated with 0.5 μM thapsigargin (TG) or 8 μg/ml tunicamycin (TM) for 12 h. β-actin was used as an internal control. Data are presented as the means ± standard deviation (SD), n=6; *p<0.05, **p<0.01 vs. blank. Blank, untreated cells.

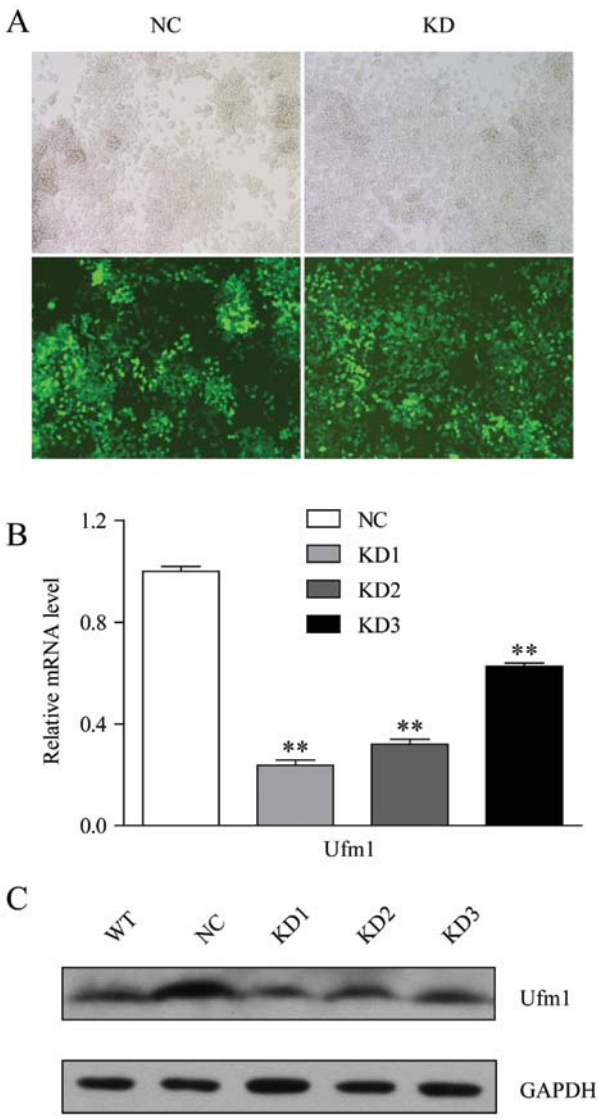

Figure 3. Verification of ubiquitin-fold modifier 1 (Ufm1) knockdown efficiency following the lentiviral infection of Raw264.7 cells. (A) Raw264.7 cells were transfected with lentivirus containing short hairpin RNA (shRNA) targeting the Ufm1 gene (Lv-shRNA-Ufm1) or negative lentivirus (Lv-shRNA-NC). After 5 days of transfection, the infection efficiency of the lentivirus was estimated to be approximately 90% as determined by the percentage of cells expressing eGFP protein by fluorescence microscopy (magnification, x100). (B and C) Knockdown efficiency of Ufm1 was analyzed by (B) qRT-PCR and (C) western blot analysis. GAPDH was used as an internal control. Data are presented as the means± standard deviation (SD), n=3; **p<0.01 vs. NC. WT, wild-type normal cells; NC, negative control cells; KD, cells in which Ufm1 was knocked down with the 3 shRNAs.

expression in macrophages remain unknown. In this study, to investigate the association between Ufm1 expression and macrophage ER stress, we examined whether Ufm1 expression is affected by ER stress in Raw264.7 cells. We used TG, an inhibitor of ER Ca^{2+} ATPase, and TM, an inhibitor of glycosylation, to induce the ER stress response in Raw264.7 cells. As shown in Fig. 2A, Ufm1 mRNA expression increased by >2-fold in the Raw264.7 cells treated with 0.5 μM TG for 12 h and by approximately 4-fold in those treated with 8 μg/ml TM for 12 h. In addition, the Ufm1 protein level increased (Fig. 2B and C). To confirm that the Raw264.7 cells treated with either TG or TM were under ER stress, we also examined the BiP and CHOP expression levels. As expected, their expression levels had markedly increased (Fig. 2A-C), and this result is consistent with the results previously discussed in this study. Taken together, these data suggest that an upregulation in Ufm1 expression is associated with the ER stress response.

ER stress-induced apoptosis increases following the knockdown of Ufm1 expression in Raw264.7 cells. Apoptosis is triggered when the ER stress response fails to restore ER homeostasis. To further analyze the contributions of Ufm1 to the regulation of macrophage ER stress, as well as the effects of a reduced Ufm1 expression level on macrophage function, we examined the ER stress response and ER stress-induced apoptosis in Raw264.7 cells in which Ufm1 expression had been knocked down by lentivirus-mediated shRNA. Following viral infection, >90% of the cells were eGFP-positive, which indicated a high efficiency of infection (Fig. 3A). Among the 3 shRNAs examined, shUfm1-1 was the most effective in reducing the Ufm1 expression level (Fig. 3B and C). Therefore, this shRNA sequence was selected for further study. According to the results from

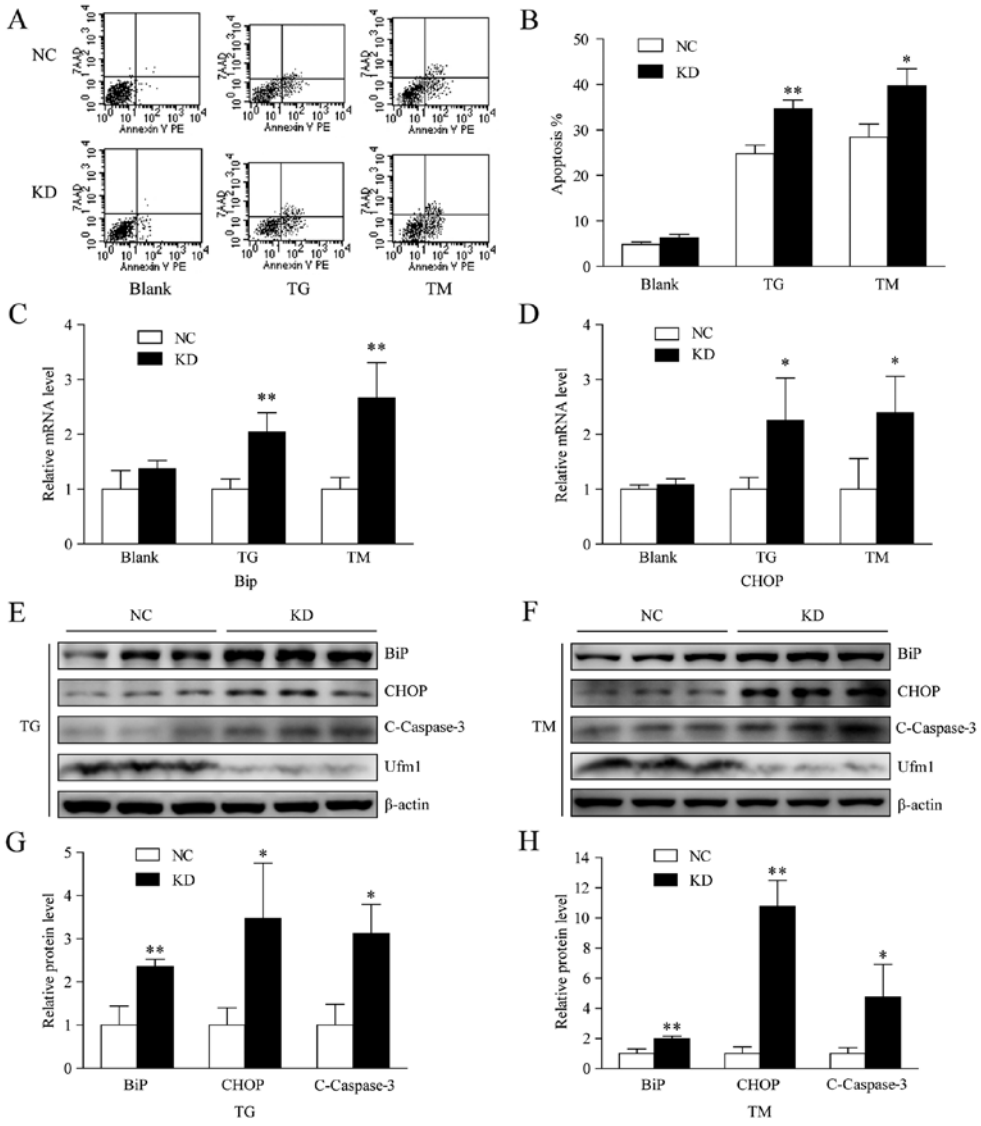

Figure 4. Endoplasmic reticulum (ER) stress-induced apoptosis increases after the knockdown of ubiquitin-fold modifier 1 (Ufm1) expression in Raw264.7 cells. The most effective shRNA to knockdown Ufm1 was selected and the cells and negative control cells were treated with 0.5 μM thapsigargin (TG) or 8 μg/ml tunicamycin (TM) for 12 h. (A) Representative images of flow cytometry for the analysis of apoptosis by staining with Annexin V-PE and 7-AAD. (B) The percentage of apoptotic cells was determined according to the number of cells expressing Annexin-V. (C and D) Immunoglobulin heavy chain-binding protein (BiP) and C/EBP homologous protein (CHOP) mRNA expression levels were detected by qRT-PCR. (E-H) BiP, CHOP and cleaved-caspase-3 protein levels were detected by western blot analysis. β-actin was used as an internal control. Data are presented as the means± standard deviation (SD), n=3-6; *p<0.05, **p<0.01 vs. NC. Blank, untreated cells; NC, negative control cells; KD, cells in which Ufm1 was knocked down; C-Caspase-3, cleaved caspase-3.

flow cytometry, decreasing the Ufm1 expression level markedly enhanced TG- and TM-induced apoptosis by 10 and 12%, respectively, but it had no significant effect on the basal apoptosis when compared with the negative control (Fig. 4A and B). The sensitization of macrophages to ER stress-induced apoptosis was also confirmed by the level of cleaved caspase-3 detected by western blot analysis (Fig. 4E-H). Furthermore, when the Ufm1 expression was knocked down, there was no effect on the expression levels of BiP and CHOP under no treatment conditions (Fig. 4C and D), but after the cells were treated with TG or TM, there was a higher expression level of BiP and CHOP compared with the negative control (Fig. 4C-H). These results indicate that Ufm1 knockdown sensitizes macrophages to ER stress. Taken together, these data demonstrate that Ufm1 is at least partially involved in regulating ER stress-induced apoptosis.

Overexpression of Ufm1 prevents ER stress-induced apoptosis in Raw264.7 cells. To further substantiate the effects of Ufm1 on ER stress-induced macrophage apoptosis, we constructed a lentiviral vector system that expresses Ufm1 cDNA and GFP and is puromycin resistant. The Raw264.7 cells were transfected with the Lv-Ufm1 or Lv-NC vectors, and after 4 days of transfection, >80% of the cells expressed GFP (Fig. 5A), which indicated that the lentiviral infections were effective. After another 5 days of 4 μg/ml puromycin selection, the Ufm1 mRNA and protein expression levels in the Lv-Ufm1-infected cells were markedly increased, when compared with those in the Lv-NC-infected cells (Fig. 5B and C). To determine whether the overexpression of Ufm1 affects ER stress-induced macrophage apoptosis, the Lv-Ufm1 and Lv-NC cells were exposed to 0.5 μM TG or 8 μg/ml TM for 12 h, and the expression levels of BiP, CHOP and cleaved caspase-3 were determined. As predicted, in comparison

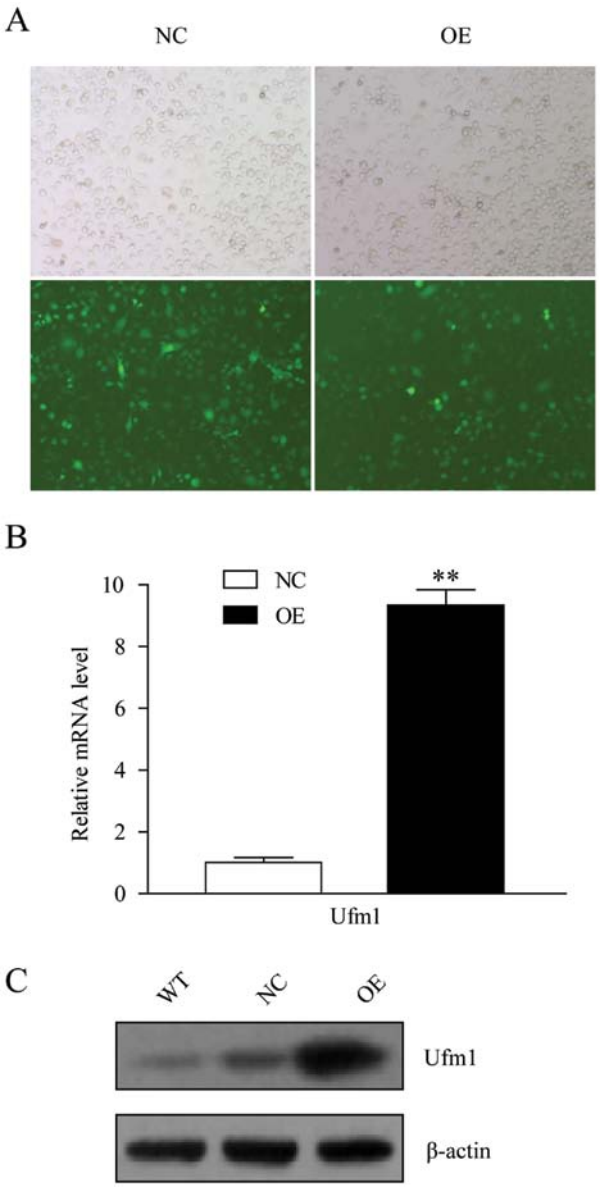

Figure 5. Verification of ubiquitin-fold modifier 1 (Ufm1) overexpression efficiency following lentiviral infection of Raw264.7 cells. Raw264.7 cells were transfected with lentivirus containing the Ufm1 gene (Lv-Ufm1) or negative lentivirus (Lv-NC). (A) After 4 days of transfection, the infection efficiency of the lentivirus was estimated to be approximately 80% as determined by the percentage of cells expressing GFP protein using fluorescence microscopy (magnification, x100). (B and C) Another 5 days of 4 μg/ml puromycin selection, the Ufm1 expression levels were analyzed by (B) qRT-PCR and (C) western blot analysis. β-actin was used as an internal control. Data are presented as the means ± stanadard deviation (SD), n=3; **p<0.01 vs. NC. WT, wild-type normal cells; NC, negative control cells; OE, Ufm1 overexpressing cells.

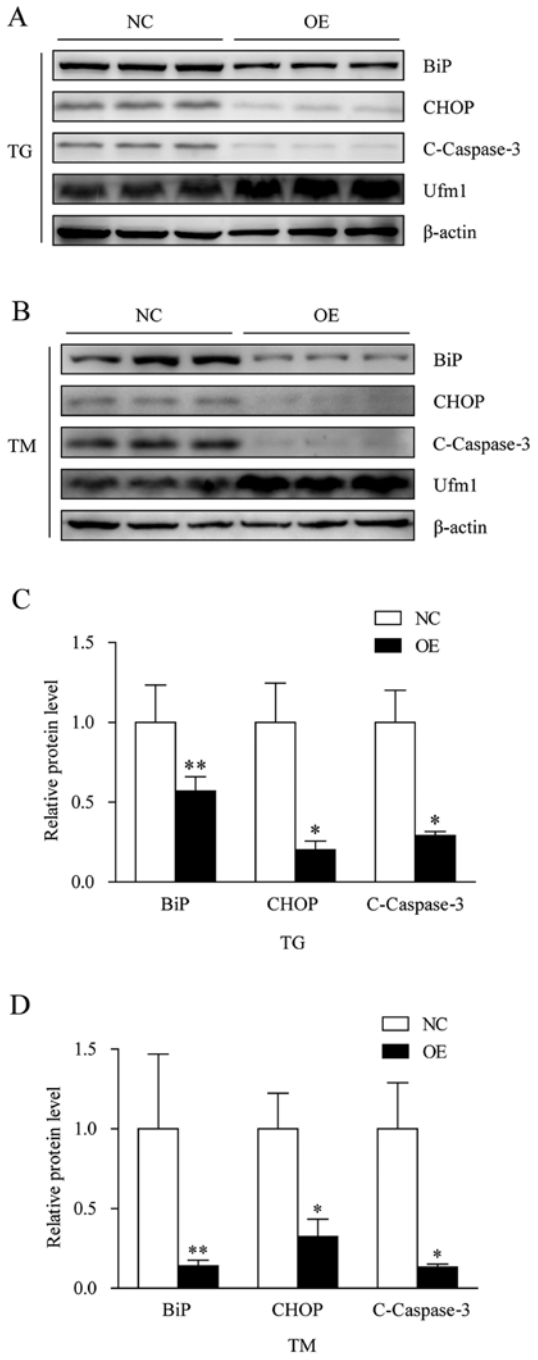

Figure 6. Overexpression of ubiquitin-fold modifier 1 (Ufm1) prevents endoplasmic reticulum (ER) stress-induced apoptosis in Raw264.7 cells. (A-D) Ufm1 overexpressing and negative control cells were exposed to 0.5 μM thapsigargin (TG) or 8 μg/ml tunicamycin (TM) for 12 h. Immunoglobulin heavy chain-binding protein (BiP), C/EBP homologous protein (CHOP) and cleaved-caspase-3 protein levels were analyzed by western blot analysis. β-actin was used as an internal control. Data are presented as the means ± standard deviation (SD), n=6; *p<0.05, **p<0.01 vs. NC. NC, negative control cells; OE, Ufm1 overexpressing cells. C-Caspase-3, cleaved caspase-3.

with the negative control, the overexpression of Ufm1 attenuated macrophage ER stress and prevented the apoptosis induced by exposure to TG or TM (Fig. 6A-D).

Discussion

In the present study, we investigated the expression pattern and potential biological function of Ufm1 in macrophages. We found that Ufm1 expression was markedly upregulated in diabetic RPMs, and its expression increased in response to ER stress inducers in cultured macrophages. Further investigation revealed that ER stress-induced macrophage apoptosis increased after the knockdown of Ufm1 and that the overexpression of Ufm1 attenuated ER stress-induced macrophage apoptosis.

Ufm1 is a new member of the Ubl family, whose biological functions are poorly understood, particularly in macrophages.

It has been reported that Ufm1 is expressed in many tissues and cell lines (16,19) and that an upregulation in Ufm1 expression is linked with the activation of the ER stress response in ischemic heart injury and T2D (21,22). Consistent with these previous studies, our results demonstrate that Ufm1 is expressed in RPMs from normal mice and that its expression is upregulated in RPMs from diabetic mice. Additionally, the expression levels of BiP and CHOP were upregulated in the RPMs from diabetic mice compared with those from the control mice. Elevated BiP and CHOP expression levels may potentially result from insulin resistance or glucose and lipid metabolic abnormalities, which are known ER stress inducers and may contribute to impeding macrophage ER function. These findings are also consistent with those of our previous studies using cDNA microarray expression profiling in RPMs from diabetic mice (unpublished data). Chemicals such as TG and TM are commonly used to induce the ER stress response in cultured cells and animals for experimental purposes (30). In our study, we used TG and TM to induce the ER stress response in the Raw264.7 macrophage cell line. Following exposure to TG or TM, the Ufm1 mRNA and protein expression levels increased; these results are consistent with those of previous studies (19,23) and the results presented above. The BiP and CHOP expression levels were also higher in the TG- or TM-treated groups, which indicated that the ER stress response was activated in these cells. Taken together, these findings suggest that Ufm1 is associated with macrophage ER stress.

It has been reported that prolonged ER stress can trigger apoptosis. CHOP is a member of the C/EBP family of bZIP transcription factors (5). Mounting evidence suggests that CHOP plays an essential role in regulating macrophage ER stress-induced apoptosis, including the release of ER calcium, the mitochondrial release of apoptogens and the activation of the death receptor, Fas (31-35). Caspase-3 is a well-known apoptosis effector, and it has been reported to be involved in ER stress-induced apoptosis (36,37). Based on our findings that Ufm1 expression was upregulated by ER stress inducers and that the Ufm1 expression pattern was similar to that of several UPR genes and the known functions of activated CHOP, we initially hypothesized that Ufm1 may be a negative regulator in macrophage ER stress-induced apoptosis and that the knockdown of Ufm1 may reduce the amount of ER stress and apoptosis observed following treatment with TG or TM. To our surprise, however, our flow cytometry and western blot analysis results revealed that the knockdown of Ufm1 expression exacerbated ER stress-induced apoptosis in the cultured macrophages. To confirm this finding, we established an Ufm1 overexpression model. The overexpressing of Ufm1 significantly reduced the BiP and CHOP expression levels, as well as the cleaved-caspase-3 expression level under ER stress conditions and reduced ER stress-induced apoptosis. These results are slightly different from those of a previous study in which there was no change in the UPR when the Ufm1 expression was knocked down in INS-1E cells, even though Ufm1 knockdown sensitized β-cells to apoptosis (19). Our results, however, are similar to the findings of a recent study by Zhang *et al* (23). The authors found that knocking down the expression of Ufm1 components in U2OS cells triggered the activation of the UPR and amplified the ER network (23). This discrepancy between studies may be attributed to differences in the protocols used in each study, such as the cell lines, chemical inducers, RNA interference and target gene knockdown efficiency. Additionally, Ufm1 is expressed in many tissues and cells under physiological conditions (16,19), and its Uba5 plays a critical role in cell differentiation (38), which provides indirect evidence that Ufm1 may play a key role in normal cellular function and that Ufm1 knockdown may affect macrophage function. Taken together, these findings establish a link between Ufm1 and ER stress and suggest that Ufm1 is a novel protective gene whose expression is upregulated in response to ER stress-induced macrophage apoptosis. Further studies are required to elucidate the precise mechanisms involved.

In conlcusion, our study demonstrates that Ufm1 expression is involved in macrophage ER stress and that Ufm1 inhibits apoptosis by suppressing the ER stress response in macrophages. These results demonstrate the significance of Ufm1 in regulating macrophage function during ER stress, indicating that an increase in Ufm1 expression protects a compensatory response in diabetic mice and suggests that Ufm1 be considered as a potential therapeutic target against diabetes.

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