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Knockdown of ERp44 leads to apoptosis via activation of ER stress in HeLa cells

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ABSTRACT

ERp44, an endoplasmic reticulum (ER) resident protein, regulates intracellular Ca^{2+} release and involves in the maturation of many proteins in mammalian cells. In this study, we investigated the effects and mechanism of ERp44 on cell apoptosis by using ERp44 knockdown stable HeLa cell lines. We found that ERp44 knockdown resulted in increases in cell apoptosis rate more than one fold higher than that of control; using serum starvation, caspase-3 protein level was significantly up-regulated in ERp44 knockdown cells compared to the control cells. Furthermore, we demonstrated that in response to serum starvation, the protein levels of CHOP and GRP78 were also largely raised in ERp44 knockdown cells. Moreover, caspase-12 was activated, which suggested cell apoptosis was induced by ER stress. Taken together, our results indicate that knockdown of ERp44 leads to cell apoptosis through the activation of ER stress.

1. Introduction

Endoplasmic Reticulum (ER) is a specialized organelle that plays a critical role in several biological processes such as protein maturation, lipid biosynthesis and calcium homeostasis [1]. There are hundreds of molecular chaperones and folding enzymes in ER lumen that participate in protein folding, glycosylation, movement to Golgi apparatus and extracellular matrix [2].

ERp44, an ER resident protein, was firstly identified as ER chaperon of thioredoxin family by co-immunoprecipitation with Ero1-Lα in HeLa cells [3]. It has been reported that ERp44 exhibits its chaperon activity by retention of formylglycine-generating Enzyme (FGE) in ER [4], and involves in the maturation of several proteins, such as serotonin Transporter (SERT) [5], IgM [6] and adiponectin [7]. Mikoshiba and his colleagues reported that ERp44 directly binds to the L3V domain of IP_3R1, a vital Ca^{2+} channel on ER membrane, and regulates intracellular Ca^{2+} concentrations [8]. Our previous work showed that IP_3-induced Ca^{2+} release (IICR) is decreased in ERp44 overexpressed HeLa cells and C160/212 of L3V domain influences the binding capacity between IP_3R1 and ERp44 [9].

ER stress is kind of condition that ER homeostasis is disrupted due to excessive unfolded proteins accumulation and alterations in the redox or calcium balance [10]. To cope with ER stress, unfolded protein response (UPR) is initiated including the increase of ER chaperon expression, the lower translation level of general proteins and the degradation of misfolded proteins [11]. There are three main branches of UPR which named by three typeltransmembrane proteins: protein kinase RNA-like ER kinase (PERK), inositol requiring protein-1 (IRE1) and activating transcription factor-6 (ATF6) [12]. When ER stress is prolonged, ER stress-mediated apoptosis is initiated [13]. There are three well-known apoptotic pathways named extrinsic, intrinsic [14] and perforin/granzyme pathway [15]. The intrinsic pathway has crosstalk with PERK pathway that activated CHOP negatively regulates B cell lymphoma-2 (Bcl-2) [16]. Besides, ASK1 and JNK pathway is triggered by IRE1 [17]. Finally, released cytochrome C initiates the formation of apoptosome and results in the activation of caspase-9 and the downstream caspase cascade [18].

In the present study, we investigated the role and mechanism of ERp44 in cell apoptosis in HeLa cells. We found that knockdown of ERp44 causes remarkable cell apoptosis which due to the activation of ER stress.

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2. Materials and methods

2.1. Construction of vector and generation of virus

pSUPER vector was used to suppress the endogenous ERp44 gene according to a protocol described previously [19]. The target sequences were SiControl-3U (5′-AACAGCACCAUCGACCAACGU-3′), SiERp44-3U (5′-AACAGCAGCAUCAACCUACGU-3′). These oligonucleotides were annealed and ligated downstream of the H1 promoter. The packaging cell line Phoenix-293 was cultured in high glucose Dulbecco's modified Eagle medium (H-DMEM) supplemented with 10% fetal calf serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin in standard conditions (37 °C in 5% humidified CO_2 incubator). After placing to 10 cm dishes, cells were transfected by 60 μL lipofectamine 2000((Invitrogen, USA) with 12 μg pSUPER-siControl or 12 μg pSUPER-siERp44. Forty-eight hours after transfection, cell supernatant was collected and stored at −80 °C.

2.2. Infection of the cells

HeLa cells were grown in H-DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin in standard conditions. The day before infection, 3×10^5 cells were planted per well of 6-well plates. The next day, virus supernatant was added with polybrene (5 μg/ml final concentration) and selected with 2.5 μg/ml puromycin for 10 days. Resistant clones were picked up and expanded for an additional one month. ERp44 protein levels were then analyzed by western blot.

2.3. Measurement of intracellular Ca^{2+}

Ca^{2+} imaging was performed according to the previous report [20]. Stable cells were planted on sheet plate for more than 24 h. Then the cells were loaded with 10 μM Fluo4-AM (Molecular Probes, Eugene, OR, USA) diluted in extracellular solution (140 mM NaCl, 5.5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Hepes, 3 mM Glucose) for 10 min in CO_2 incubator. After washing three times with extracellular solution, cells were stimulated by 3 μM ATP. Fluo-4 fluorescence was recorded using a laser scanning confocal head attached to an inverted microscope (Leica SP-5).

2.4. Detection of cell apoptosis

Apoptosis was assessed by using Annexin V, a protein that binds to phosphatidylserine (PS) residuces which are exposed on the cell surface of apoptotic cell. Cells were cultured with L-DMEM without serum for 4 or 6 days. After treatment, cells were re-suspended at 10^6 cells/mL in 195 μL/tube 1 × Annexin-V binding buffer and 5 μL of Annexin-V FITC and incubated at room temperature for 10 min in the dark, 1000 g centrifuged 5 min, discarded supernatant, added 190 μL 1 × Annexin-V Binding Buffer and 10 μL of Propidium Iodide (PI) buffer to each tube and analyzed the cells by flow cytometry within 1 h of staining.

2.5. Western blot analysis

To determine the expression of associated proteins in stable cells, cell lysate was subjected to the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis. Cells were collected and cell lysates were prepared using the RIPA lysis buffer containing 50 mM Tris (pH 7.4), 150 mM sodium chloride, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS. After centrifugation to remove cell debris, the lysates were prepared using protein loading buffer, and then loaded onto a 12% PAGE in running buffer. The proteins were transferred to a PVDF membrane (Millipore, USA), and then were probed with specific antibodies. β-actin and GAPDH proteins were detected as control to ensure equal protein loading in experiments. Protein bands were detected by Immobilon™ Western chemiluminescence HRP substrate reagent (Millipore, USA). Primary antibodies were rabbit GRP78 (#3183), rabbit cleaved caspase-3 (#9661), rabbit caspase12 (#2202), rabbit GAPDH (#2118), rabbit β-actin (#4967) from cell signaling technology.

2.6. Statistical analysis

All experiments were repeated at least three times. Statistical significance was determined by a one-way Welch's t-test. A difference was considered statistically significant when $p < 0.05$.

3. Results

3.1. Knockdown of ERp44 induces cell apoptosis

To investigate the role of ERp44, adenovirus was prepared in Phoenix-293 cell line with shRNA of ERp44 or non-specific shRNA (as control). In this study, we established HeLa cell stable lines infected by adenovirus and kept in cell culture with antibiotic added. Several positive cell clones were screened out with different knockdown efficiency of ERp44 (Fig. 1A). By the proof of western blot, the relative intensity of ERp44 protein expression blot to

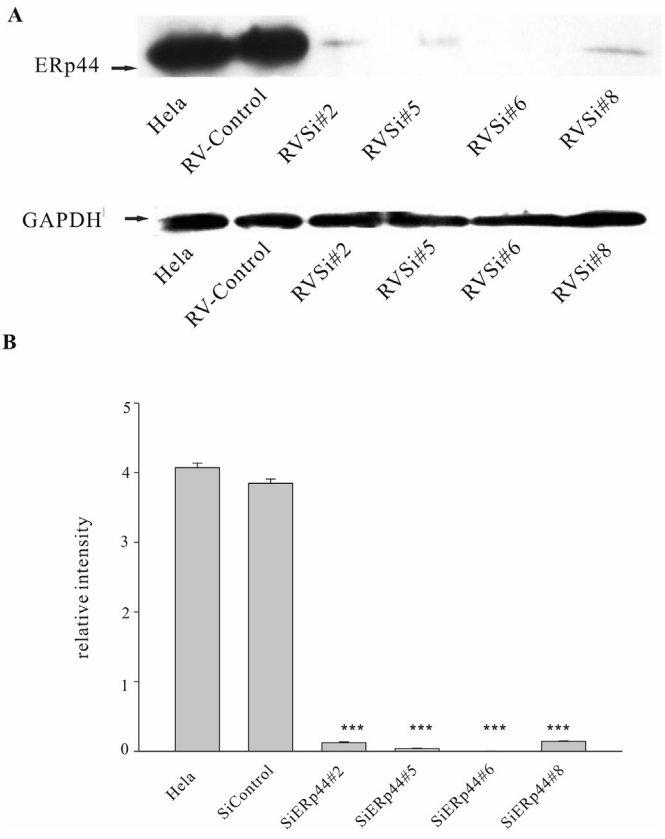

Fig. 1. Establishment of ERp44 knockdown stable cell lines. Adenovirus were packed as tools to loss of function of ERp44 in HeLa cells. (A) Western blot results showed ERp44 protein expression level of HeLa cells without treatment, HeLa cells transfected by adenovirus without targeting siRNA inserted (RV-Control) and HeLa cells transfected by adenovirus with human ERp44 siRNA construct (RVSi). Housekeeping gene GAPDH was used as protein loading control. (B) Statistics of ERp44 protein blot intensity to GAPDH were showed. Data were presented as the mean ± SEM, n = 3, ***P < 0.001.

GAPDH in HeLa cells without transfection, HeLa cells with control adenovirus transfection and HeLa cells with ERp44 knockdown adenovirus transfection #2, #5, #6, #8 were 4.1, 3.97, 0.32, 0.09, 0.003 and 0.37, respectively (Fig. 1B). The efficiency of ERp44 silencing in stable line #2, #5, #6 and #8 was 92%, 98%, 99% and 91% separately. We observed that knockdown of ERp44 caused cell shrinking, rounding and losing adhersion to culture dish compared to the control (Fig. 2A); there were more chromatins which underwent condensation, polynucleosomal DNA fragmentation and nuclear shrinking observed in Hoechest staining slice (Fig. 2B). Flow cytometry assay showed that the apoptosis rate in HeLa cells, shRNA control, HeLa cell stable lines #5 and #6 was 5.71%, 5.68%, 12.38% and 11.17%, respectively (Fig. 2C). Compared to the control, the number of apoptotic cells was increased to 2.5 and 2.2 folds in ERp44 knockdown stable line #5 and #6, respectively.

Next, we examined the intracellular apoptotic signaling pathway. The results indicated that cleaved caspase-3 which is an active form was significantly activated in ERp44 knockdown cells in response to serum starvation (Fig. 3A), suggesting that cell apoptosis induced by knockdown of ERp44 was mediated by caspase-3.

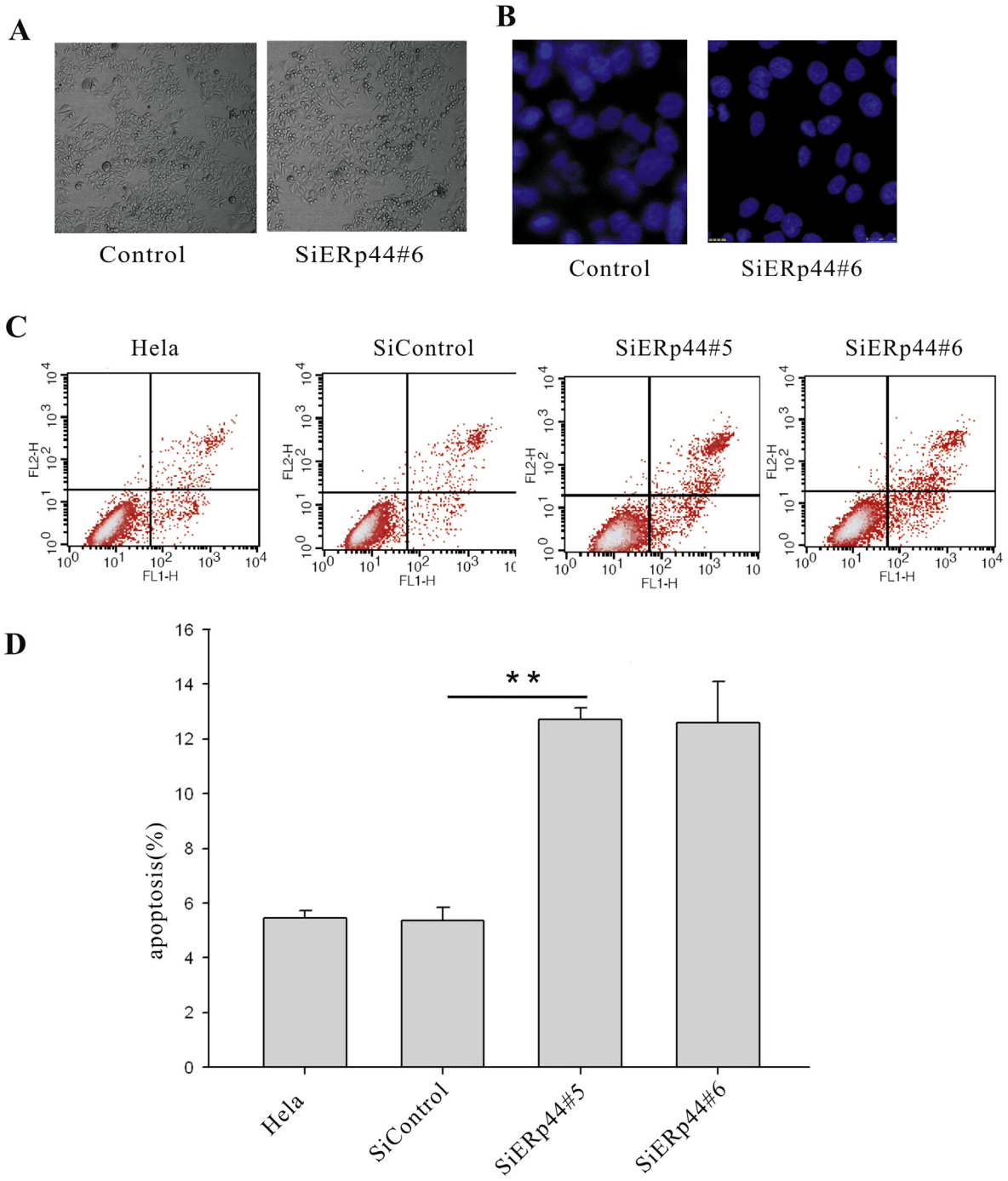

Fig. 2. ERp44 knockdown induces cell apoptosis in HeLa cells. (A) Cultured stable HeLa cells were imaged under light field. (B) Hoechest staining (C) Si-Control cells and ERp44 RNAi cells were treated with serum starvation for 6 days and subjected to Annexin V/PI staining following by flow cytometry analysis to assess apoptosis. (D) Percentage of apoptosis cells in HeLa, Si-Control, Si-ERp44#5, si-ERp44#6 was counted separately. The mean ± SEM of three independent determinations were showed. $^{**}p < 0.01$.

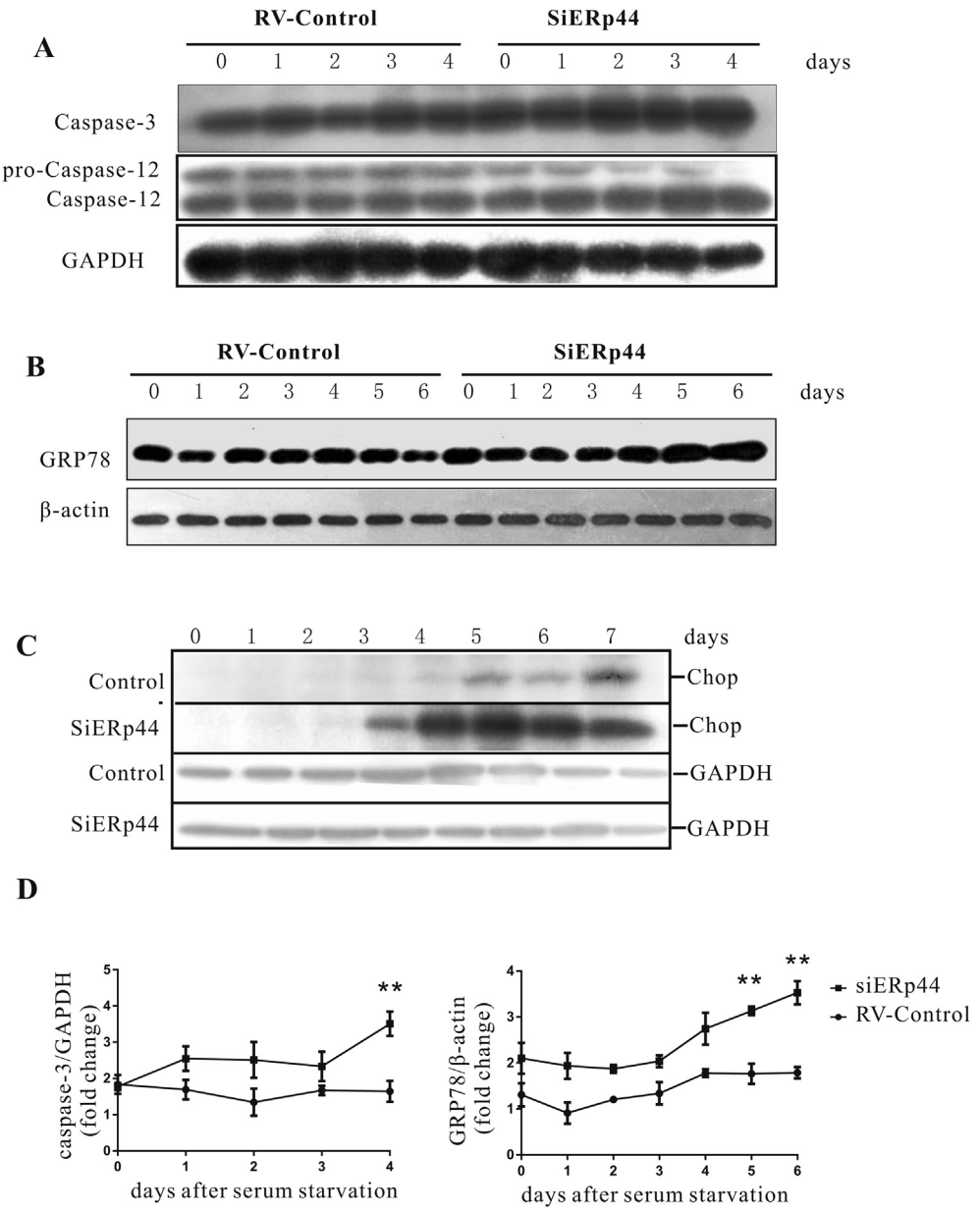

Fig. 3. ER stress mediates ERp44 knockdown induced cell apoptosis. (A) Stable HeLa cells with ERp44 knockdown or control were treated with serum starvation in L-DMEM culture for four days. Whole cellular protein were extracted as western blot sample with antibody against cleaved caspase-3 and caspase-12. GAPDH was used to ensure equal whole protein loading. (B) The time of starvation was elongated to six days and the whole cellular protein samples were evaluated for GRP78, a marker of ER stress, with β-actin as loading control. (C) CHOP protein level (D) Statistics of caspase-3 and GRP78 protein blot intensity to loading control and the mean ± SEM of three independent determinations were showed. $^{**}P < 0.01$.

3.2. ERp44 knockdown induced apoptosis is mediated by activation of ER stress

Among the three pathways, the intrinsic one possibly involved in HeLa cell apoptosis induced by ERp44 knockdown. To test this, we conducted experiments of ER stress. As showed in Fig. 3B, GRP78, an ER marker, was increased to 2 folds in HeLa cells treated with serum starvation in day 6, indicating that HeLa cells were more sensible to ER stress induced by ERp44 knockdown. CHOP, a downstream of ER stress, is a regulator of Bcl-2 family located on mitochondria membrane and its activation serves as a break to the pro-apoptotic and anti-apoptotic balance with the consequence of cell apoptosis. Our results demonstrated that the protein level of CHOP was greatly up-regulated in ERp44 knockdown cells (Fig. 3C). The caspase-12, a member of caspase family, is confined to ER stress induced apoptosis pathway [21]. Here, we found that the pro-caspase-12 (60 kD) protein expression level was attenuated but the cleaved form (35 kD) was raised (Fig. 3A) in response to serum starvation. These results illuminated that cell apoptosis caused by ERp44 knockdown were mediated by ER stress.

3.3. Knockdown of ERp44 enhances ATP induced Ca^{2+} oscillation in HeLa cells

It has been previously reported that ERp44 binds to IP_3R1 and affects intracellular Ca^{2+} release [8]. In this study, we examined the effect of ERp44 knockdown on Ca^{2+} release in HeLa cells. Confocal microscope Ca^{2+} imaging results showed that Ca^{2+} oscillation was

strikingly activated by adding 3 μM extracellular ATP in both ERp44 knockdown and control cells within the duration of 85 s (Fig. 4A). However, the properties of Ca^{2+} release were significantly different between ERp44 knockdown and control cells. The peak amplitude of Ca^{2+} oscillation in cell line siERp44#5 was increased to 1.25 folds compared to that of the control cells (Fig. 4B).

4. Discussion

In this study, we determined the role of ERp44 in cell apoptosis process. We observed cell apoptosis phenotype and cleaved caspase-3 protein level up-regulated in ERp44 knockdown HeLa stable cell lines; the protein level of GRP78, CHOP and cleaved caspase-12 in ERp44 knockdown cells were increased in response to serum starvation. We also found that Ca^{2+} oscillation induced by extracellular ATP was enhanced in ERp44 knockdown cells. Cell apoptosis caused by ERp44 knockdown was induced by ER stress.

ERp44 is expressed especially in ER lumen, part of Golgi apparatus and MAM due to its RDEL (Arg–Asp–Glu–Leu) sequence on C terminal. It has been previously reported that ERp44 inhibits IP_3R1 activation by physical interaction with L3V domain of IP_3R1 in mammalian cells [8]. IP_3R1, a vital Ca^{2+} channel located on ER membrane, has effect on Ca^{2+} release in response to IP_3. Our results indicated that knockdown of ERp44 enhanced ATP induced Ca^{2+} release. Since the activities of ER located enzymes and chaperones are Ca^{2+} dependent, decrease of ER luminal Ca^{2+} by IP_3R inhibition affects ER function and ER associated cellular processes. It is well known that loss of ER luminal Ca^{2+} causes ER stress [22] and activates unfolded protein response (UPR). Our data of ER stress indicated that ERp44 knockdown cells were more sensitive to ER stress stimulation. This phenotype might be due to ER luminal Ca^{2+} decrease caused by IP_3R activation in ERp44 knockdown cells. Therefore, ERp44 influences ER stress through blocking IP_3R1 activity.

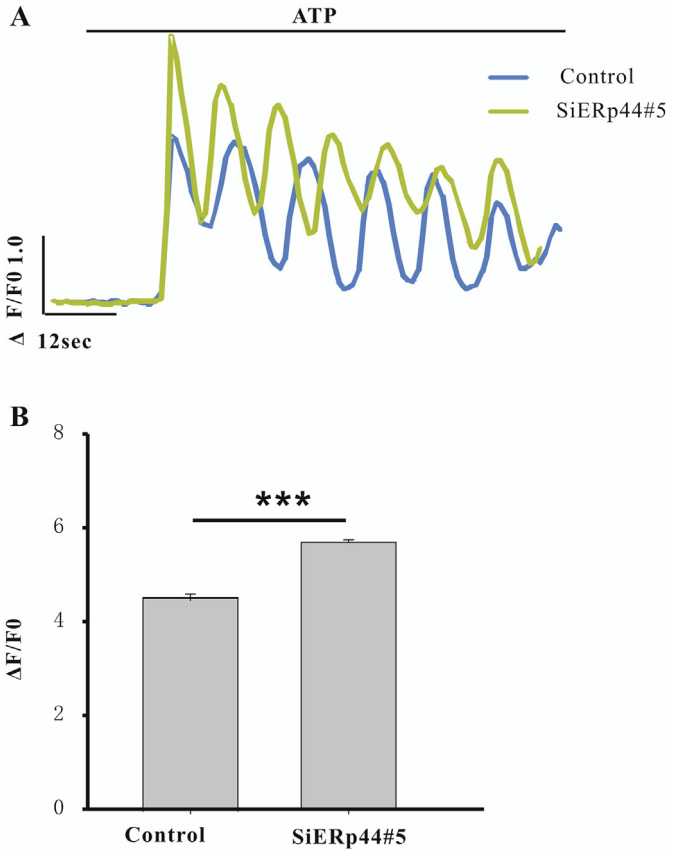

Fig. 4. ERp44 knockdown enhances extracellular ATP induced Ca^{2+} release. (A) 3 μM ATP was used to stimulate Ca^{2+} oscillations after Fluo4 loading in stable ERp44 knockdown cell line and control. Fluorescence signal of siERp44#5 stable line and RV-control were measured. (B) Statistical results of Ca^{2+} oscillation proportion during ATP stimuli in HeLa cells (n = 40 from 3 independent experiments). Data were presented as the mean ± SEM, ***$p < 0.001$.

How ER stress induces cell apoptosis? One mechanism is that the adaptor molecule TNF-receptor-associated factor 2 (TRAF2) recruited by activated IRE1 activates apoptosis-signal-regulating kinase1 (ASK-1) resulting in active form of caspase-12 release [23]. It had been also reported that activated JNK phosphorylates Bcl-2 and Bim followed by enhanced cell apoptosis. CHOP (growth-arrest- and DNA-damage-inducible gene 153, GADD153) is induced by three branches of UPR but PERK-eIF2-ATF4 is essential. Some target genes of CHOP such as Bcl-2, GADD34, ERO1 and TRB3 have been identified. Bax and Bak, the pro-apoptotic member of Bcl-2 family are activated by CHOP or JNK, leading to caspases activation [24]. In this study we found that the active form of caspase-3 was significantly elevated by CHOP implicating that cell apoptosis occurred due to knockdown of ERp44. This is confirmed by our further findings that knockdown of ERp44 increased in the GRP78 levels and initiated ER stress. Moreover, cleavage of caspase-12 relies on its interaction with TRAF2 in response to ER stress [23]. In our data, pro-caspase-12 protein expression diminished and cleaved caspase-12 increased in ERp44 knockdown cells at the late period of serum starvation, which suggests that the relationship between the cell apoptosis and ER stress induced by knockdown of ERp44. Collectively, our study indicates that human ERp44 playes an important role in ER stress induced cell apoptosis.

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