Silencing of both ATF4 and PERK inhibits cell cycle progression and promotes the apoptosis of differentiating chondrocytes

ZHMENG WU*, MEILING LI*, WEI ZHENG*, QIN HU, ZHI CHENG and FENGJIN GUO

Department of Cell Biology and Genetics, Core Facility of Development Biology, Chongqing Medical University, Chongqing 400016, P.R. China

Received March 16, 2016; Accepted April 25, 2017

DOI: 10.3892/ijmm.2017.2985

Abstract. In the current study, we demonstrate that the silencing of protein kinase R (PKR)-like endoplasmic reticulum (ER) kinase (PERK) and activating transcription factor 6 (ATF4) (using small interfering RNA expression constructs) inhibits the chondrocyte cell cycle and proliferation in vitro and ex vivo. The silencing of PERK alone using siRNA against PERK (siPERK) led to arrest in the G1 phase, it decreased the number of cells in the S phase, and delayed progression to the G2-M phase. Co-transfection with siRNA against ATF (siATF4) led to a more profound inhibitory effect on cell cycle progression. Moreover, transfection with siPERK was associated with enhanced endoplasmic reticulum (ER) stress-induced apoptosis during bone morphogenetic protein 2 (BMP2)-induced chondrogenesis, and transfection with siATF4 exacerbated ER stress-related cell death. Data from flow cytometry (FCM), immunohistochemistry and TUNEL assays supported these findings in vitro and ex vivo. As shown by our results, the combined effect of the silencing of ATF4 and PERK led to the activation of an ER stress-specific caspase cascade in the cartilage tissue. On the whole, these findings reveal a new crucial combined effect of the silencing of PERK and ATF4 in modulating ER stress-mediated apoptosis during chondrocyte differentiation and proliferation.

Introduction

The normal function of the endoplasmic reticulum (ER) is essential for numerous cellular processes and, ultimately, cell survival. Conditions that inhibit the protein folding capacity of the endoplasmic reticulum (ER) lead to the accumulation of misfolded or unfolded proteins in the ER lumen, generating a potentially toxic state referred to as ER stress. ER stress is attenuated through the activation of a complex adaptive cellular response, known as the unfolded protein response (UPR). Three transmembrane proteins, inositol-requiring enzyme 1 (IRE1), protein kinase R (PKR)-like ER kinase (PERK) and activating transcription factor (ATF)6, are responsible for detecting ER stress and the initiation of the UPR. Prolonged stress or failure to adapt to ER stress ultimately culminates in ER stress-induced apoptosis, and ER stress has been associated with various neurodegenerative, cardiovascular and orthopedic diseases (1-4). Additionally, ER stress has been demonstrated to play an important role in cellular differentiation during developmental processes. Accordingly, characterizing molecular mediators of the signaling switch between the protective and apoptotic responses to ER stress, and how these molecules interact to influence cell differentiation and proliferation, is an important endeavor which may reveal key aspects of developmental regulation and cellular pathologies.

Several studies have clearly demonstrated that physiological stressors influence cell differentiation and survival during musculoskeletal developmental and reparative processes, including chondrocyte differentiation, chondrogenesis and endochondral ossification (5-7). Chondrocyte sensitivity to ER stress has been documented and a clear association between ER stress and several diseases affecting connective tissue is readily observable through murine genetic knockout studies and the analysis of diseased human tissues (8,9); however, the mechanisms through which ER stress specifically affects differentiation programs in chondrocytes remain poorly understood.

Of note, bone morphogenetic protein 2 (BMP2), a pre-eminent cytokine, plays critical roles in embryogenesis, cell growth, differentiation, bone development and the repair of bone fractures. It also activates UPR transducers, such as PERK, old astrocyte specifically-induced substance (OASIS) and ATF6 (10-12). Notably, PERK is a major transducer of the ER stress response and directly phosphorylates eukaryotic initiation factor 2α (eIF2α), which specifically promotes...
the translation of ATF4. PERK and ATF4 have been shown to play important roles in osteoblast differentiation and bone formation. Specifically, Saito et al, as well as others revealed that ER stress occurred during BMP2-induced osteoblast differentiation and activated the PERK-eIF2α-ATF4 signaling pathway, followed by the promotion of gene expression essential for osteogenesis (13-15). In an effort to disentangle the dual association of PERK/ATF4 signaling with both pro-survival and pro-apoptotic responses during ER stress, Walter et al, and others, investigated the association between cell fate and the temporal activation of PERK/ATF4 in live cells and found that the shift from cell survival to apoptosis was determined by the timing of PERK/ATF4 signaling relative to that of IRE1/XBP1, another UPR signaling pathway (16,17).

However, whether PERK/ATF4 signaling participates in ER stress-mediated apoptosis during the course of chondrocyte differentiation, and the potential underlying mechanism(s), remain unknown. Thus, the current study aimed to better define the molecular mediators of cell survival during cartilage development with special regard to molecules associated with chondrocyte differentiation and ER stress-induced apoptosis. Specifically, the data presented herein elucidate the involvement of PERK and ATF4 in cell cycle progression and ER stress-mediated apoptosis during the course of chondrogenesis. Furthermore, the combined effect(s) of PERK and ATF4 upon the regulation of the cell cycle and apoptosis were investigated.

**Materials and methods**

**Ethics statement.** All animal experiments were designed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Science Foundation of China and conducted with the prior approval of the Chongqing Medical University Institutional Animal Care and Use Committee (permit nos. SYXK 2007-0001 and SCXK 2007-0002) and the Committee on the Ethics of Animal Experiments of Chongqing Medical University. Mice were housed under controlled temperatures in a 12 h light/dark cycle with easy access to food and water.

**Adenoviruses.** To generate PERK and ATF4 small interfering RNA (siRNA; siPERK and siATF4, respectively) expression constructs, siRNA corresponding to the coding sequence of the PERK and ATF4 genes (siPERK forward, 5'-ACCTCCAA GACCAACCATTTCCT-3' and reverse, 5'-AAAGTGGTT GGTCTTGGAGGTTTT-3'; and siATF4 forward, 5'-AGGA GCAAACACAGACTTTCCT-3' and reverse, 5'-ATGC TGTCCTTGTGTTGCTCTTTT-3') were cloned into the pSes-HUS vector (an adenoviral shuttle vector for siRNA expression, a gift from Professor Tangni, Chongqing Medical University) according to the manufacturer's instructions (18,28). All constructs were verified by nucleic acid sequencing; subsequent analysis was performed using BLAST software (National Institutes of Health, available at http://www.ncbi.nlm.nih.gov/blast/).

**Cell culture.** To examine the effect of knocking down PERK and ATF4 on chondrogenesis, ATDC5 chondrogenic cells (ATCC®; PCS-500-051™) and C3H10T1/2 embryonic fibroblasts (a gift from Dr Chuanju Liu, New York University School of Medicine, New York, NY, USA) were infected with adenoviral vector containing siPERK or siATF4 or siPERK + siATF4 or a control RFP adenovirus before micromass culture. To examine the effect of the silencing of ATF4 and PERK on chondrogenesis, ATDC5 cells and C3H10T1/2 cells were infected with siATF4 (MOI=60) or siPERK (MOI=80) adenovirus or control RFP adenovirus prior to micromass culture. Uninfected cells were used as the negative controls (NC). Micromass culture was performed as described previously (19,20). The ATDC5 and C3H10T1/2 cells were briefly trypsinized and resuspended in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% FBS at a concentration of 10^6 cells/ml, and 6 drops (approximately 120 µl) of suspended cells were placed in a 60-mm tissue culture dish (Becton-Dickinson, San Diego, CA, USA). After 2 h of incubation at 37°C, 1 ml of DMEM containing 10% FBS and BMP2 (300 ng/ml) was added to the culture medium. The medium was changed every 2-3 days.

**RNA extraction and reverse transcription (RT)-PCR.** Total RNA was extracted from the cultured cells using the RNeasy Mini kit (Qiagen, Hilden, Germany) and reverse transcribed using the SuperScript pre-amplification system (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The following sequence-specific primers were synthesized: 5'-AGCACTCAGATGGAGAGAGTGCA-3' and 5'-GCT ATGCGGAGTTCTGACTGT-3' for PERK; 5'-TGGCGTT CCTGCGGCTCC-3' and 5'-CTGCTCCTCTCCCTCTTGG AGC-3' for ATF4. GAPDH was employed as an internal control using the following oligonucleotides: 5'-ACC ACAGTCATGACCATCAG-3' and 5'-TCCACCACTCTTG TTGCTGTA-3'. The identity of each targeted PCR amplification product was confirmed by DNA sequence analysis of gel-purified bands (Qiagen).

**Western blot analysis.** Proteins in total cell extract from micromass cultures of BMP2-treated (300 ng/ml) ATDC5 or C3H10T1/2 cells were resolved on a 10% SDS-polyacrylamide gel and electroblotted onto nitrocellulose membranes. After blocking in 10% non-fat dry milk in Tris-buffered saline TWEEN-20 [10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% TWEEN-20], the blots were incubated with either mouse monoclonal anti-PERK antibody (sc-377400; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and anti-C/EBP homologous protein (CHOP) antibody (ab14119; diluted 1:1,000; Abcam, Cambridge, UK) or rabbit anti-caspase-3 antibody (ab32351; Abcam) and anti-p-c-Jun N-terminal kinase (JNK) antibody (sc-293138; Santa Cruz Biotechnology, Inc.,) for 1 h. After washing, the respective secondary antibody [horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulin or HRP-conjugated anti-rabbit immunoglobulin; Sigma. St. Louis, MO, USA; A9044] was added, and the bound antibody was visualized using an enhanced chemiluminescence system (Amersham Biosciences, Uppsala, Sweden).

**Culture of fetal mouse bone explants and immunohistochemistry.** Metatarsals were isolated from 8 newborn C57BL/6j mice and cultured in DMEM (Gibco) containing 1% heat-inactivated fetal calf serum (Invitrogen) and 100 U/ml penicillin-strep-
tomycin in the presence of BMP2 (300 ng/ml) and siPERK, siATF4, or siPERK + siATF4 for 5 days, followed by histological examination. Affinity-purified anti-caspase-3 (ab32351; Abcam), caspase-12 (ab62484; Abcam), anti-CHOP (ab1419; Abcam) and anti-p-JNK (sc-293138; Santa Cruz Biotechnology, Inc.) were used for visualization, and the sections were counterstained with Mayer's hematoxylin (H9627; Sigma).

**TUNEL assay.** Metatarsals from 5 newborn C57BL/6J mice, as well as micromass cultures of C3H10T1/2 and ATDC5 cells, were cultured for 5 days in the presence of conditioned medium containing BMP2 (300 ng/ml) and siPERK, siATF4, or siATF4 + siPERK followed by the detection of apoptosis in accordance with the manufacturer's recommended protocol using the DeadEnd™ Fluorometric TUNEL System (Promega Corp., Madison, WI, USA). Localized green fluorescence of apoptotic FITC-labeled TUNEL-positive cells was imaged using a fluorescence microscope (S/N:2109; Kramer Scientific Corp., Amesbury, MA, USA).

**Apoptosis analysis by flow cytometry (FCM).** At 48 h following infection with siPERK, siATF4, or siATF4 + siATF4, ATDC5 and C3H10T1/2 cells in micromass culture were then cultivated in BMP2 (300 ng/ml) for 1, 3, and 5 days. Each culture media of BMP2-treated ATDC5 and C3H10T1/2 cells were then collected for FCM analysis. Briefly, following incubation with RNAse (1 mg/ml; Qiagen) in the dark at 37°C for 1 h, the cells were stained with propidium iodide (30 mg/ml; Sigma) and analyzed using a flow cytometer (FACSCalibur; Becton-Dickinson) to determine cell cycle distribution and detect cellular apoptotic rate. The experiments were performed in triplicate.

**Statistical analysis.** Statistical analysis was performed using SPSS 10.0.1 software for Windows. Data are expressed as the means ± SD from at least 3 independent experiments. The Student's t-test was used to determine whether two sets of data were significantly different from each other. Data for multiple variable comparisons were analyzed by one-way analysis of variance (ANOVA). A value of P<0.05 was deemed to indicate a statistically significant difference.

**Results**

**Measurement of ATF4 and PERK expression following transfection with specific siRNA constructs.** siPERK and siATF4 adenoviral vectors were constructed and identified using endonuclease digestion and DNA sequencing (data not shown). The ATDC5 and C3H10T1/2 cells infected with siPERK, siATF4, or siPERK + siATF4 were examined by RT-PCR and western blot analysis. The mRNA level of PERK markedly decreased in the ATDC5 and C3H10T1/2 cells transfected with siPERK, as compared with the untransfected controls (Fig. 1A). The protein level was also significantly decreased in the siPERK-infected cells, as compared with the control cells (Fig. 1B). Furthermore, as shown in Fig. 1C and D, the mRNA expression of ATF4 decreased compared to the controls, in the siATF4-infected ATDC5 and C3H10T1/2 cells. The ATF4 protein level was also significantly decreased in the siATF4-infected ATDC5 and C3H10T1/2 cells compared with the respective control cells. These results confirm the successful construction of the adenoviral vectors and the silencing of PERK and ATF4.

Silencing of ATF4 by transfection with siATF4 decreases the expression of PERK in chondrogenesis. To further investigate the function of PERK in chondrogenesis, we examined the expression of PERK in a BMP2-induced micromass culture of ATDC5 cells by western blot analysis. As shown in Fig. 2A, the silencing of PERK by transfection with siPERK markedly decreased the expression of PERK in the BMP2-stimulated ATDC5 cells after 3 days of culture. Furthermore, concurrent silencing of the expression of PERK and ATF4 in the BMP2-stimulated ATDC5 cells further decreased PERK expression compared to the cells in which only PERK was silenced. These results remained consistent after 5 days of the BMP2-induced micromass culture of ATDC5 cells as assessed by western blot analysis. As shown in Fig. 2B, in the cells treated with BMP2 + siATF4 + siPERK PERK expression was markedly decreased compared with the BMP2 + siPERK and BMP2 + siATF4 groups. These data clearly indicate that the silencing of ATF4 by transfection with siATF4 regulates endogenous PERK protein expression, and further reduces the expression of PERK inhibited by siPERK. Micromass cultures of these cells were incubated in the presence of 300 ng/ml BMP2 for the induction of chondrogenesis.

**Combined effect of the silencing of ATF4 and PERK on cell growth in chondrocyte differentiation.** To examine whether the knockdown of PERK and/or ATF4 can influence the cell cycle profile during chondrogenesis, FCM analysis was used to determine the cell cycle distributions of ATDC5 and C3H10T1/2 cells during BMP2-induced chondrogenesis in the presence of siPERK, siATF4 or siATF4 + siPERK.

At the time points of 1,3, and 5 days, the proportion of ATDC5 cells in the S phase following treatment with BMP2 + siPERK was decreased compared to that of the ATDC5 cells cultured under the influence of BMP2 alone (Fig. 3A and B). In the ATDC5 cells treated with BMP2 + siPERK, the cell number in the S phase was 36.03, 34.21 and 28.17% on days 1, 3, and 5, respectively; for the ATDC5 cells treated with BMP2 + siATF4, 32.17, 30.78, and 29.25% of the cell population was in the S phase on days 1, 3, and 5, respectively. The decreased percentage of cells in the S phase was even more significant in the ATDC5 cells treated with BMP2 + siATF4 + siPERK, with S phase cells accounting for only 29.78, 26.52 and 17.82% of the cell population at 1, 3, and 5 days, respectively.

Similarly, in the C3H10T1/2 cells treated with BMP2 + siPERK, the percentage of cells in the S phase was 42.35, 44.07, 31.21%; and 33.05, 29.01, 27.65% in C3H10T1/2 cells compared with the respective control cells. These results confirm the successful construction of the adenoviral vectors and the silencing of PERK and ATF4.

The difference between the S phase cell distribution data for each treatment group and that of the corresponding control groups reached statistical significance (P<0.05). These data
indicate that the knockdown of PERK inhibits cell cycle distribution during chondrogenesis, and that the silencing of ATF4 enhances the inhibitory effect of the silencing of PERK on cell growth during chondrocyte differentiation.
The FCM data also revealed that in the ATDC5 cells treated with BMP2 + siATF4 + siPERK, the percentage of cells in the G2 phase was 18.82±0.91, 9.31±1.02, 6.07±0.85% on culture days 1, 3, and 5 days, respectively. In the C3H10T1/2 cells treated with BMP2 + siATF4 + siPERK, 17.75±0.92, 8.51±0.92, and 5.87±0.94% of cells were in the G2 phase at 1, 3, and 5 days of culture, respectively (Fig. 3, P<0.05). Collectively, these results indicate that the silencing of PERK affects cell cycle distribution by reducing the number of cells in the S phase cells during chondrogenesis. Specifically, the knockdown of PERK inhibited cell proliferation during chondrocyte development with arrest in the G1 phase, a decrease in the number of cells in the S phase and the delay of the progression to the G2-M phase. Furthermore, the silencing of ATF4 enhanced the inhibitory effect of the silencing of PERK on cell cycle progression during chondrocyte differentiation.

Combined effect of the silencing of ATF4 and PERK on ER stress-mediated apoptosis. We then sought to determine whether the silencing of PERK (using siPERK), ATF4 (using siATF4) or both (using siATF4 + siPERK) affects cell apoptosis. As shown in Fig. 4, the cell apoptotic rate was markedly increased after micromass culture of the ATDC5 cells for 1, 3 and 5 days following treatment with BMP2 + siATF4 + siPERK, as compared with the BMP2 + siPERK, BMP2 + siATF4 and BMP2 treatment groups. The cell apoptotic rate was 3.72, 5.35 and 21.85% in the BMP2 + siPERK-treated ATDC5 cells, and 4.01, 4.26 and 16.73% in the BMP2 + siATF4-treated ATDC5 cells on 1, 3 and 5 days, which clearly reflects a larger population of apoptotic cells as compared with the cells treated with BMP2 alone. In the BMP2 + siATF4 + siPERK-treated ATDC5 cells, the cell apoptotic rate was markedly increased to 5.52, 10.73 and 38.25% on days 1, 3 and 5, respectively. The differences between each treatment group and the BMP2 control group reached statistical significance (P<0.05 and P<0.01, Fig. 4A and B).

Additionally, in the micromass culture of C3H10T1/2 cells, the cell apoptotic rate was also increased in the BMP2 + siPERK group (3.19, 5.45 and 19.78%, days 1, 3 and 5) and BMP2 + siATF4 group (4.75, 5.32 and 18.12%, days 1, 3 and 5) compared to the BMP2 group (2.7, 1.97 and 2.95%, days 1, 3 and 5). In the C3H10T1/2 cells transfected with both siPERK and siATF4, the apoptotic rate was 6.51, 23.63 and 47.73% on 1, 3 and 5 days, respectively. The differences between each treatment group and the BMP2 control group reached statistical significance (P<0.05 and P<0.01, Fig. 4C and D).

Figure 3. Analysis of cellular proliferation by FCM. (A and C) FCM images with propidium iodide staining and analysis on cell cycle distribution. Micromass culture of (A) ATDC5 and (C) C3H10T1/2 cells after treatment with NC/BMP2 (300 ng/ml)/BMP2 + siPERK/BMP2 + siATF4/BMP2 + siPERK + siATF4. FCM analysis showed that the percentage of the BMP2 + siPERK + siATF4 ATDC5 cells in the S phase was decreased significantly compared to that of the cells in the BMP2 + siPERK, BMP2 + siATF4 and BMP2 control groups during BMP2-induced chondrogenesis at 1, 3, and 5 days. The results for the C3H10T1/2 cells were similar. Experiments were repeated 3 times, and samples were analyzed by the Student's t-test and statistical significance with P<0.05. Representative images are shown. (B and D) FCM analysis showed that the percentages of ATDC5 and C3H10T1/2 cells in the BMP2 + siPERK + siATF4 groups in the S phase were decreased significantly compared with those of the cells in the BMP2 + siPERK, BMP2 + siATF4 and BMP2 control groups. *P<0.05 compared with the control. FCM, flow cytometry; BMP2, bone morphogenetic protein 2.
Taken together, these data demonstrate that the knockdown of PERK using siPERK or ATF4 using siATF4 enhances ER stress-mediated apoptosis in BMP2-induced chondrocyte differentiation. Further, the combined application of siPERK and siATF4 further promoted ER stress-mediated apoptosis in chondrocyte differentiation induced by BMP2, generating an additive ‘push’ toward apoptosis.

To confirm the influence of ER stress-mediated apoptosis by transfection with siPERK, siATF4 and siATF4 + siPERK in BMP2-stimulated ATDC5 cells, the expression of ER stress-mediated apoptotic molecules, including CHOP, caspase-3, caspase-12 and p-JNK, was detected by western blot analysis in the ATDC5 cells stimulated with BMP2 for 3 and 5 days. The expression of CHOP, caspase-3, caspase-12 and p-JNK was markedly increased in the ATDC5 cells following infection with siPERK, siATF4 and siATF4 + siPERK, and BMP2 treatment over 3 and 5 days. As shown in Fig. 5, both siPERK and siATF4 induced a marked increase in the expression levels of p-JNK, active (cleaved) caspase-3, caspase-12 and CHOP in the ATDC5 cells transfected with siPERK. Furthermore, the expression levels of p-JNK, cleaved caspase-3, caspase-12 and CHOP were markedly increased following 3 and 5 days of BMP2 induction in the siATF4 + siPERK-infected ATDC5 cells. These results indicated that transfection with siPERK, siATF4 and siATF4 + siPERF4 increased the expression of ER stress-mediated apoptosis signaling pathway molecules during chondrocyte differentiation. The individual effects of the silencing of PERK and ATF4 exerted a more robust, additive combined effect as observed with the implementation of the siPERK + siATF4 treatment condition.

The silencing of both ATF4 and PERK (siATF4 + siPERK) increases ER stress-mediated apoptosis in vitro. The high-density culture system was then incubated in the absence (CTR) or presence of 300 ng/ml BMP2, BMP2 + siPERK, BMP2 + siATF4 and BMP2 + siATF4 + siPERK for 3 days, at which point, a TUNEL assay was performed to examine the effects of siPERK and siATF4 on apoptosis during chondrogenesis. As shown in Fig. 6, during BMP2-induced chondrocyte differentiation, the number of TUNEL-positive cells increased significantly in the ATDC5 cells transfected with BMP2 + siPERK, BMP2 + siATF4 and BMP2 + siATF4 + siPERK for 3 days, compared with the ATDC5 cells treated with BMP2 only (15.53%), and the number of TUNEL-positive cells was further enhanced in the ATDC5 cells treated with BMP2 + siATF4 + siPERK (69.71%).
Figure 5. Effects of transfection with siPERK + siATF4, siPERK and siATF4 on the expression of ER stress-mediated apoptosis in BMP2-stimulated ATDC5 cells for 3 and 5 days. (A) Expression of p-JNK in the course of chondrogenesis in a micromass culture of BMP2-stimulated ATDC5 cells for 3 and 5 days. Whole cell lysates were prepared from ATDC5 cells and treated with 300 ng/ml BMP2 for 3 and 5 days. The lysates were resolved by SDS-PAGE and then immunoblotted with antibodies against p-JNK and total JNK. (B) Expression of cleaved caspase-3 in the course of chondrogenesis in a micromass culture of BMP2-stimulated ATDC5 cells for 3 and 5 days. Whole cell lysates were prepared from ATDC5 cells and treated with 300 ng/ml BMP2 for 3 and 5 days. The lysates were resolved by SDS-PAGE and then immunoblotted with antibodies against cleaved caspase-3 and caspase-3. (C) Expression of CHOP in the course of chondrogenesis in a micromass culture of BMP2-stimulated ATDC5 cells for 3 and 5 days. Whole cell lysates were prepared from ATDC5 cells and treated with 300 ng/ml BMP2 for 3 and 5 days. The lysates were resolved by SDS-PAGE and then immunoblotted with antibodies against CHOP and β-actin. (D) Expression of caspase-12 in the course of chondrogenesis in a micromass culture of BMP2-stimulated ATDC5 cells for 3 and 5 days. Whole cell lysates were prepared from ATDC5 cells and treated with 300 ng/ml BMP2 for 3 and 5 days. The lysates were resolved by SDS-PAGE and then immunoblotted with antibodies against caspase-12 and β-actin. BMP2, bone morphogenetic protein 2; JNK, c-Jun N-terminal kinase; CHOP, C/EBP homologous protein.

Figure 6. Transfection with siATF4 + siPERK increases ER stress-mediated apoptosis in micromass culture of ATDC5 and C3H10T1/2 cells during BMP2-induced chondrogenesis. (A) Following treatment with 300 ng/ml BMP2, BMP2 + siPERK, BMP2 + siATF4 or BMP2 + siPERK + siATF4 in micromass culture of (a-e) ATDC5 and (f-j) C3H10T1/2 cells, the cells were then analyzed for apoptosis by TUNEL staining. Representative photographs of TUNEL staining in cells. The FITC-labeled TUNEL-positive cells were imaged under a fluorescence microscope (x200 magnification). The cells with green fluorescence were recognized as apoptotic cells, and the scale bars represent 200 µm. Representative images from TUNEL analysis are shown. (B) Analysis of results of cell apoptosis. Data are the means ± SD for relative apoptosis normalized to control cells for 3 independent experiments. Columns are the means of 3 separate experiments; bars represent SD. *P<0.05 as determined by Student’s t-test, vs. BMP2 and BMP2 + siPERK group; BMP2 and BMP2 + siATF4 group; BMP2 + siATF4/BMP2 + siPERK and BMP2 + siPERK + siATF4 group. Representative images from TUNEL analysis are shown. BMP2, bone morphogenetic protein 2.
In addition, in the C3H10T1/2 cells treated with BMP2 + siPERK, and those treated with BMP2 + siATF4, the number of TUNEL-positive cells increased (32.83 and 39.36%) compared with that of the C3H10T1/2 BMP2-treated cells (17.68%). The number of TUNEL-positive C3H10T1/2 cells treated with BMP2 + siATF4 + siPERK (66.52%) was also significantly greater than that recorded from either the C3H10T1/2 BMP2 + siPERK-treated cells or BMP2 + siATF4-treated cells. TUNEL assay was repeated in triplicate. The differences between the BMP2 + siATF4 + siPERK, BMP2 + siPERK, BMP2 + siATF4 and BMP2 groups reached statistical significance (P<0.05, Fig. 6). It should be noted that the silencing of PERK in BMP2 + siATF4 activated ER stress-mediated apoptosis during chondrocyte differentiation induced by BMP2; the silencing of both ATF4 and PERK (siATF4 + siPERK) enhanced ER stress-mediated apoptosis to a level exceeding that induced by the silencing of ATF4 or PERK alone.

In order to verify whether the silencing of PERK (using siPERK), ATF4 (using siATF4) or both (siATF4 + siPERK) affects growth plate chondrocytes in developing tissue, TUNEL assay was undertaken to determine the effect of siPERK, siATF4 and siATF4 + siPERK on apoptosis in cartilage tissue (Fig. 7). The result show that the TUNEL-positive cells in the BMP2 + siATF4 + siPERK group were significantly increased compared with the BMP2 + siPERK, BMP2 + siATF4 and the BMP2 group. These results further demonstrate that a cumulative effect of siATF4 and siPERK in pushing chondrocytes toward an apoptotic cell fate.

Silencing of ATF4 and PERK induces ER stress-mediated caspase activation in chondrocyte tissue. To further understand the molecular events of ER stress-mediated apoptosis induced by the silencing of ATF4 and PERK (siATF4 + siPERK) in chondrogenesis, the effect of transfection with siATF4 + siPERK on endochondral bone formation was examined by implementing cultures of metatarsals isolated from newborn mice as an ex vivo model of bone formation. Firstly, the metatarsals were cultured for 5 days in the presence of conditioned medium containing 300 ng/ml BMP2 (control), BMP2 + siPERK, BMP2 + siATF4 or BMP2 + siATF4 + siPERK adenovirus. Western blot analysis was then used to examine the expression of ATF4 and PERK in the metatarsal culture extracts. The protein level of ATF4 was markedly decreased in the siATF4 and siPERK + siATF4-infected culture extracts, as compared with the protein level of ATF4 in the BMP2 and BMP2 + siPERK-treated culture extracts. Likewise, the protein level of PERK was markedly decreased in the siPERK and siPERK + siATF4-infected culture extracts, as compared to the BMP2 and BMP2 + siATF4 treatment group (Fig. 8A).

We then detected the expression of ER stress-specific caspasess. At the time of explantation, these explants consisted of undifferentiated cartilage. Over a 5-day culture period, these explants underwent all sequential stages of endochondral bone formation. As shown in Fig. 8B, treatment with siATF4 + siPERK increased the expression of apoptosis-related proteins, such as cleaved caspase-3, CHOP, p-JNK and caspase-12.

These results demonstrated the activation of caspase-3, p-JNK, CHOP and caspase-12 by ER stress during chondrogenesis and that the silencing of ATF4 and PERK increased the expression of ER stress-mediated apoptosis signaling pathway molecules. Taken together, these data demonstrated that the combined silencing of ATF4 and PERK enhanced ER stress-mediated apoptosis in BMP2-induced chondrogenesis.

**Discussion**

In eukaryotic cells, signaling pathways relay information between the ER, cytosol and nuclei to restrict the accumulation of unfolded proteins in the ER. A number of studies have shown that factors influencing cell fate and/or differentiation are activated during ER stress. In mammalian cells, the UPR plays a fundamental role in maintaining cellular homeostasis and is therefore at the center of many normal physiological responses and pathologies (21-24).

Cells respond to ER stress via ER stress sensors, leading to the UPR. PERK is a major transducer of the ER stress response and directly phosphorylates eIF2α, resulting in translational attenuation (16,25,26). Whether and how PERK/ATF4...
Our current data indicate that PERK/ATF4 influences cell cycle distribution in chondrogenesis. Firstly, the application of adeno viral vectors carrying siPERK and siATF4, and infected the ATDC5 and C3H10T1/2 cells. Protein analysis of whole cell extracts validated our approach, as the expression of PERK and ATF4 was markedly decreased in each of the cells expressing the relevant adenoviral vectors (Fig. 1). Furthermore, we demonstrated that the silencing of ATF4 was able to regulate endogenous PERK and ATF4 gene expression, evidenced by the further reduction in PERK expression in the cells co-transfected with siATF4 and siPERK, as compared to the cells transfected with siPERK alone (Fig. 2).

We previously reported that BMP2 mediates mild ER stress during chondrogenesis and activates the IRE1α-XBP1 pathway; X-box binding protein 1 spliced (XBP1s) in turn enhances chondrocyte hypertrophy by functioning as a co-factor of RUNX2. We also previously found that BMP2 activates UPR-signaling molecules in chondrogenesis, such as XBP1s, BiP and IRE1α (27,28). Herein, we expanded upon our previous findings by defining the role of PERK/ATF4 in ER stress-mediated apoptosis during chondrocyte differentiation. Our current data indicate that PERK/ATF4 influences cell cycle distribution in chondrogenesis. Firstly, the application of siPERK and siATF4 inhibited cell proliferation in chondrocyte development with G1 phase arrest, a reduction in the number of cells in the S phase and the delay of G2-M phase progression. The joint application of siATF4 and siPERK resulted in the enhanced disruption of cell cycle distribution (Fig. 3).

Beta-actin served as the internal control. We demonstrated that the joint application of siATF4 and siPERK resulted in the inhibition of cells in the S phase and the delay of G2-M phase progression, as evidenced by the reduced expression of PCNA and cyclin D1 and the increased expression of p21 (Fig. 3). The joint application of siATF4 and siPERK also inhibited cell proliferation in chondrocyte differentiation. Additionally, the expression of ER stress-mediated apoptosis participates in ER stress-mediated apoptosis in the process of chondrocyte differentiation, and the mechanisms of how ER stress-mediated apoptosis is regulated in chondrogenesis remain unknown.

ER stress-induced cell death is a new, exciting apoptotic pathway, the full impact of which, particularly in development and the pathology of disease, remains undetermined (29,30). It is known that caspases, a family of cysteine proteases including caspase-3, -9, and -12, act as a common death effect or molecules in various forms of apoptosis. Caspase-12 is an ER-associated proximal effector in the caspase activation cascade, and cells lacking this enzyme are partially resistant to inducers of ER stress (31-33). Three pathways have been identified as being involved in ER stress-mediated apoptosis: the caspase-12/caspase-4 pathway, the CHOP pathway and the IRE1-JNK pathway. Caspase-12 and -4 have been proposed as caspases that initiate ER stress-induced cell death with caspase-12 reported to directly cleave pro-caspase-9 and induce apoptosis (34-37). CHOP induces ER stress-induced cell death, at least in part, by suppressing the expression of Bcl-2 and inducing Bim expression. It has been reported that IRE1α also participates in ER stress-induced cell death by activating JNK. These findings support the notion that ER stress leads to several redundant pathways for caspase activation (38-40).

In order to gauge the activation of ER-stress-mediated apoptotic pathways, we detected the expression of phosphorylated JNK. C3H10T1/2 cells were co-transfected with siPERK or siATF4 or BMP2 + siPERK + siATF4 for 5 days and subjected to Western blot analysis. The expression levels of phosphorylated JNK, cleaved caspase-3, C3H10T1/2 cells expressing BMP2 + siPERK + siATF4 were increased in the cells treated with BMP2 + siPERK + siATF4 as compared with those treated with BMP2 + siPERK or BMP2 + siATF4 or BMP2 alone (Fig. 4). Additionally, the expression of ER stress-mediated apoptosis participates in ER stress-mediated apoptosis in the process of chondrocyte differentiation, and the mechanisms of how ER stress-mediated apoptosis is regulated in chondrogenesis remain unknown.

ER stress-induced cell death is a new, exciting apoptotic pathway, the full impact of which, particularly in development and the pathology of disease, remains undetermined (29,30). It is known that caspases, a family of cysteine proteases including caspase-3, -9, and -12, act as a common death effect or molecules in various forms of apoptosis. Caspase-12 is an ER-associated proximal effector in the caspase activation cascade, and cells lacking this enzyme are partially resistant to inducers of ER stress (31-33). Three pathways have been identified as being involved in ER stress-mediated apoptosis: the caspase-12/caspase-4 pathway, the CHOP pathway and the IRE1-JNK pathway. Caspase-12 and -4 have been proposed as caspases that initiate ER stress-induced cell death with caspase-12 reported to directly cleave pro-caspase-9 and induce apoptosis (34-37). CHOP induces ER stress-induced cell death, at least in part, by suppressing the expression of Bcl-2 and inducing Bim expression. It has been reported that IRE1α also participates in ER stress-induced cell death by activating JNK. These findings support the notion that ER stress leads to several redundant pathways for caspase activation (38-40).

In order to gauge the activation of ER-stress-mediated apoptotic pathways, we detected the expression of phosphorylated JNK, cleaved caspase-3, C3H10T1/2 cells expressing BMP2 + siPERK + siATF4 were increased in the cells treated with BMP2 + siPERK + siATF4 as compared with those treated with BMP2 + siPERK or BMP2 + siATF4 or BMP2 alone (Fig. 4). Additionally, the expression of ER stress-mediated apoptosis participates in ER stress-mediated apoptosis in the process of chondrocyte differentiation, and the mechanisms of how ER stress-mediated apoptosis is regulated in chondrogenesis remain unknown.
signaling pathway-associated molecules was also increased in the BMP2 + siPERK group and BMP2 + siATF4 group as compared to the BMP2 treatment control group. Accordingly, we demonstrated that transfection with siPERK and siATF4 increased the expression of ER stress-mediated apoptotic signaling pathway molecules during chondrogenesis and that co-transfection with siATF4 enhanced the upregulated expression of apoptotic molecules induced upon treatment with siPERK.

Furthermore, the results of TUNEL assay and immuno-histochemistry revealed that the BMP2 + siATF4 + siPERK group featured many more apoptotic cells as compared with the BMP2 + siPERK group, BMP2 + siATF4 group and the BMP2 group, demonstrating that the silencing of PERK and ATF4 increased the expression of ER stress-mediated apoptosis signaling pathway molecules during chondrogenesis (Figs. 6-8).

In conclusion, our data indicate that the silencing of PERK and ATF4 enhance ER stress-mediated apoptosis during chondrogenesis and that the joint silencing of ATF4 and PERK leads to a more profound promotion of apoptotic signaling that is observed following the silencing of either PERK or ATF4 alone.

Acknowledgements

The authors would like to thank Aubryanna Hettingham (Department of Orthopaedic Surgery and Cell Biology, New York University School of Medicine) for critically reading the manuscript. This study was supported by the National Natural Science Foundation of China (81371928 and 81171697); New Century Excellent Talent Support Project of Education Ministry of China (NCET-12-1090).

References

1. Pluquet O, Portier A and Abbadie C: The unfolded protein response and cellular senescence. A review in the theme: Cellular mechanisms of endoplasmic reticulum stress signaling in health and disease. Am J Physiol Cell Physiol 308: C415-C425, 2015.
2. Grootjans J, Kaser A, Kaufman RJ and Blumberg RS: The unfolded protein response in immunity and inflammation. Nat Rev Immunol 16: 469-484, 2016.
3. Karali E, Bellou S, Stellas D, Klinakis A, Murphy C and Fotis S: VEGF signals through ATF6 and PERK to promote endothelial cell survival and angiogenesis in the absence of ER stress. Mol Cell Biol 25: 559-572, 2005.
4. Murakami T, Saito A, Hino S, Kondo S, Kanemoto S, Chihara K, Sekiya H, Tsumagari K, Ochiai K, Yoshinaga K, et al: Signalling mediated by the endoplasmic reticulum stress transducer OASIS is involved in bone formation. Nat Cell Biol 11: 1205-1211, 2009.
5. Horiuchi K, Tohmatsu T and Morikawa H: The unfolded protein response in skeletal development and homeostasis. Cell Mol Life Sci 73: 2851-2869, 2016.
6. Zuscik MJ, Hilton MJ, Zhang X, Chen D and O’Keefe RJ: Regulation of chondrogenesis and chondrocyte differentiation by stress. J Clin Invest 118: 429-438, 2008.
7. Saito A and Imazum K: Endoplasmic reticulum stress response in osteogenesis. Clin Calcium 23: 1569-1575, 2013 (In Japanese).
8. Saito A, Hino S, Murakami T, Kanemoto S, Kondo S, Saito M, Nishimura R, Yoneda T, Furuiuchi T, Ikegawa S, et al: Regulation of endoplasmic reticulum stress response by a BBF2H7-mediated Sec23a pathway is essential for chondrogenesis. Nat Cell Biol 11: 1197-1204, 2009.
9. Rajpar MH, McDermott B, Kung L, Eardley R, Knowles L, Heenan M, Thornton DJ, Wilson R, Bateman JF, Poulsom R, et al: Targeted induction of endoplasmic reticulum stress induces cartilage pathology. PLoS Genet 5: e1000691, 2009.
10. Rosen V. BMP2 is essential in bone development and repair. Cytokine Growth Factor Rev 20: 475-480, 2009.
11. Canalis E, Economides AN and Gazzero E: Bone morphogenetic proteins, their antagonists, and the skeleton. Endocrin Rev 24: 218-235, 2003.
12. Saito A, Ochiai K, Kondo S, Tsumagari K, Murakami T, Cavener DR and Imazum K: Endoplasmic reticulum stress-response mediated by the PERK-eIF2α-ATF4 pathway is involved in osteoblast differentiation induced by BMP2. J Biol Chem 287: 905-915, 2012.
13. Saito A, Ochiai K, Kondo S, Tsumagari K, Murakami T, Cavener DR and Imazum K: Endoplasmic reticulum stress-response mediated by the PERK-eIF2α-ATF4 pathway is involved in osteoblast differentiation induced by BMP2. J Biol Chem 286: 4809-4818, 2011.
14. Liu CY, Schröder M and Kaufman RJ: Ligand-independent dimerization activates the stress response kinases IRE1 and PERK in the lumen of the endoplasmic reticulum. J Biol Chem 275: 24881-24885, 2000.
15. Tsai SF, Tao M, Ho LI, Chiou TW, Lin SZ, Su HL and Harn HJ: Isochauhaulactone-induced DDIT3 causes ER stress-PERK independent apoptosis in glioblastoma multiforme cells. Oncotarget, 2016.
16. Walter F, Schmid J, Düssmann H, Concannon CG and Prehn JH: Imaging of single cell responses to ER stress indicates that the relative dynamics of IRE1/XBP1 and PERK/ATF4 signalling rather than a switch between signalling branches determine cell fate. Cell Death Differ 22: 1502-1516, 2015.
17. Guo FJ, Liu Y, Zhou J, Luo S, Zhao W, Li X and Liu C: XBP1S protects cells from ER stress-induced apoptosis through Erk1/2 signaling pathway involving CHOP. Histochem Cell Biol 138: 447-460, 2012.
18. Miyake S, Makimura M, Kanegae Y, Harada S, Sato Y, Takamori K, Tokuda C and Saito I: Efficient generation of recombinant adenoviruses using adenovirus DNA-terminal protein as a transduction agent. Cell Death Differ 15: 1760-1771, 2008.
19. Cavener DR and Imaizumi K: Endoplasmic reticulum stress and disease. Am J Physiol Cell Physiol 308: C415-C425, 2015.
25. Lumley EC, Osborn AR, Scott JE, Scholl AG, Mercado V, McMahan YT, Coffman ZG and Brewster JL: Moderate endoplasmic reticulum stress activates a PERK and p38-dependent apoptosis. Cell Stress Chaperones: Oct 20, 2016 (Epub ahead of print).

26. Shah A and Kumar A: Methamphetamine-mediated endoplasmic reticulum (ER) stress induces type-1 programmed cell death in astrocytes via ATF6, IRE1α and PERK pathways. Oncotarget: Jun 14, 2016 (Epub ahead of print).

27. Guo FJ, Xiong Z, Han X, Liu C, Liu Y, Jiang R and Zhang P: XBP1S, a BMP2-inducible transcription factor, accelerates endochondral bone growth by activating GEP growth factor. J Cell Mol Med 18: 1157‑1171, 2014.

28. Han X, Zhou J, Zhang P, Song F, Jiang R, Li M, Xia F and Guo FJ: IRE1α dissociates with BiP and inhibits ER stress-mediated apoptosis in cartilage development. Cell Signal 25: 2136-2146, 2013.

29. Han D, Lerner AG, Vande Walle L, Upton JP, Xu W, Hagen A, Backes BJ, Oakes SA and Papa FR: IRE1alpha kinase activation modes control alternate endoribonuclease outputs to determine divergent cell fates. Cell 138: 562‑575, 2009.

30. Pincus D, Chevalier MW, Aragón T, van Anken E, Vidal SE, El-Samad H and Walter P: BiP binding to the ER-stress sensor Ire1 tunes the homeostatic behavior of the unfolded protein response. PLoS Biol 8: e1000415, 2010.

31. Degterev A, Boyce M and Yuan J: A decade of caspases. Oncogene 22: 8543‑8567, 2003.

32. Samali A, Zhivotovsky B, Jones D, Nagata S and Orrenius S: Apoptosis: Cell death defined by caspase activation. Cell Death Differ 6: 495‑496, 1999.

33. Hitomi J, Katayama T, Eguchi Y, Kudo T, Taniguchi M, Koyama Y, Manabe T, Yamagishi S, Bando Y, Imaizumi K, et al: Involvement of caspase-4 in endoplasmic reticulum stress-induced apoptosis and Abeta-induced cell death. J Cell Biol 165: 347‑356, 2004.

34. Nakagawa T and Yuan J: Cross-talk between two cysteine protease families. Activation of caspase-12 by calpain in apoptosis. J Cell Biol 150: 887‑894, 2000.

35. Szegedi E, Logue SE, Gorman AM and Samali A: Mediators of endoplasmic reticulum stress-induced apoptosis. EMBO Rep 7: 880‑885, 2006.

36. Nakagawa T, Zhu H, Morishima N, Li E, Xu J, Yankner BA and Yuan J: Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. Nature 403: 98‑103, 2000.

37. McCullough KD, Martinez DL, Klotz LO, Aw TY and Holbrook NJ: Gadd153 sensitizes cells to endoplasmic reticulum stress by down-regulating Bcl2 and perturbing the cellular redox state. Mol Cell Biol 21: 1249‑1259, 2001.

38. Nakashishi K, Sudo T and Morishima N: Endoplasmic reticulum stress signaling transmitted by ATF6 mediates apoptosis during muscle development. J Cell Biol 169: 555‑560, 2005.

39. Hassler J, Cao SS and Kaufman RJ: Ire1, a double-edged sword in pre-miRNA slicing and cell death. Dev Cell 23: 921‑923, 2012.

40. Ron D and Walter P: Signal integration in the endoplasmic reticulum unfolded protein response. Nat Rev Mol Cell Biol 8: 519‑529, 2007.