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MicroRNA-211-5p Overexpression Effect on Endoplasmic Reticulum Stress and Apoptotic Genes in Fibroblast-like Synoviocytes of Rheumatoid Arthritis

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ABSTRACT

Fibroblast-like synoviocytes (FLSs) play a major role in the pathogenesis of rheumatoid arthritis (RA). Endoplasmic reticulum (ER) stress and dysregulation of unfolded protein response are involved in the resistance to apoptosis of FLSs in RA (RA-FLSs). MicroRNA (MiR)-211 plays an important role in controlling ER stress and apoptotic genes in a PKR-like ER kinase (*PERK*)-activating transcription factor 4 (*ATF4*)-dependent manner. We investigated the effect of miR-211-5p overexpression on ER stress and apoptotic genes in RA-FLSs.

FLSs were isolated from synovial tissues of trauma (n=10) and RA (n=10) patients. MiR-211-5p and mRNA expression of the selected genes involved in the *PERK* pathway and apoptosis regulation were measured in RA, trauma, and thapsigargin (Tg)-treated RA-FLSs. Afterward, Tg-treated RA-FLSs following miR-211-5p overexpression were evaluated for miR-211-5p and mRNA levels of the study genes.

The expression of miR-211-5p, *PERK*, *BAX*, and *BCL2* showed no differences between RA and trauma. However, the expression of *ATF4* and *BCL-XL* showed a significant increase in trauma. In addition, the levels of C/EBP homologous protein (*CHOP*) and *MCL1* indicated a significant increase in RA-FLSs. Tg treatment significantly increased the expression of *PERK*, *ATF4*, and *CHOP* in RA-FLSs with no effect on miR-211-5p, *BAX*, *BCL2*, *BCL-XL*, and *MCL1*. Furthermore, Tg treatment following miR-211-5p overexpression in RA-FLSs showed a significant increase in levels of miR-211-5p with no changes in apoptotic genes.

MiR-211-5p overexpression in stimulated RA-FLSs did not alter the levels of selected genes involved in apoptosis regulation. However, more investigations are necessary to determine the ER stress role in apoptosis regulation in RA-FLSs.

Keywords: Endoplasmic reticulum stress; Fibroblast-like synoviocytes; MiR-211-5p; Rheumatoid arthritis

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INTRODUCTION

Rheumatoid arthritis (RA), a systemic autoimmune disorder, is the most prevalent inflammatory disease in the musculoskeletal system. It is characterized by hyperplastic synovium and chronic synovitis, which leads to the progressive destruction of articular structures.[1-7] One of the important pathophysiological features of RA is a significant increase in the number of synovium fibroblast-like synoviocytes (FLSs). FLSs, which are of mesenchymal origin, normally maintain synovial joint homeostasis. In RA, FLSs (termed RA-FLSs) display aggressive, tumor-like, and pathogenic behavior, and play a major role in both initiating and perpetuating synovial inflammation. These cells proliferate abnormally, show resistance to apoptosis, and mediate the destruction of articular cartilage/bone.[8-12] Therefore, induction of apoptosis in FLSs can be considered as an attractive therapeutic approach in RA.[13]

Some stressful stimuli, such as proinflammatory cytokines, autoantibodies, and hypoxia, can trigger endoplasmic reticulum (ER) stress in RA-FLSs.[14] ER stress results from the accumulation of unfolded/misfolded proteins in the ER lumen, leading to the activation of a set of intracellular signaling pathways, referred to the unfolded protein response (UPR), to restore homeostasis and maintain cell survival. However, under unresolvable ER stress, the UPR may switch into apoptosis. In mammalian cells, the UPR is initiated by three ER-resident transmembrane proteins: PKR-like ER kinase (*PERK*), inositol requiring 1 (*IRE1*), and activating transcription factor 6 (*ATF6*).[15,16] *PERK* is one of the important signaling pathways of ER stress.[17] Upon sensing ER stress, the activated *PERK* phosphorylates eukaryotic initiation factor2α (*eIF2α*) leading to the general halting of translation while simultaneously inducing the selective translation of a few transcripts, including the activating transcription factor 4 (*ATF4*). *ATF4* regulates the expression of genes involved in cell adaptation to ER stress. However, paradoxically, it may also stimulate the expression of genes involved in apoptosis, including C/EBP homologous protein (*CHOP*).[18-20] ER stress has been observed in a wide range of diseases, especially chronic autoimmune inflammatory diseases such as RA.[21-25]

MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression at the post-transcriptional level in response to environmental changes. Recent studies have shown a close link between UPR and microRNAs, such that microRNAs fine-tune the outcome of a cell to ER stress, promoting either cell survival or cell death.[26,27] Among ER stress-related microRNAs, miR-211 has been reported to act as a pro-survival microRNA and directly regulate *CHOP* expression in a *PERK-ATF*- dependent manner.[28] In the present study, considering the importance of ER stress in RA pathogenesis and resistance to apoptosis in RA-FLSs, as well as the possible role of miR-211 in the regulation of stress-related apoptosis, for the first time, we investigated the effect of miR-211-5p overexpression on ER stress-induced apoptotic genes of *PERK* dependent pathway in RA-FLSs.

MATERIALS AND METHODS

Collection of Synovial Tissues

The synovial tissues were obtained from 10 Iranian RA patients (mean age = 57.44 ± 11.45 years) during total knee replacement surgery. RA patients were diagnosed according to American College of Rheumatology (ACR) criteria;[29] were selected during 1 year (from 2019 to 2020) and referred to Shariati and Laleh hospitals (Tehran, Iran). In addition, the normal synovial specimens were taken from 10 non-arthritic individuals (mean age = 51.7 ± 12.73 years old) who had undergone knee arthroscopy following trauma and had no history of rheumatologic, inflammatory and autoimmune diseases. This case-control study was approved by the medical ethics committee of Tarbiat Modares University (IR.MODARES.REC.1398.137). All participants in the study signed the written informed consent.

Isolation and Primary Culture of FLSs

The extracted synovial tissues were rinsed with 70% ethanol and phosphate-buffered saline (PBS; Gibco, USA) containing 2% penicillin/streptomycin and amphotericin B (Sigma-Aldrich, USA), respectively. After removing the adipose tissue, the synovium was minced and digested in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) containing 1 mg/mL collagenase type VIII (Sigma-Aldrich, USA) shaking at 37°C for 80 min. Dissociated cells were centrifuged and then cultured in DMEM supplemented with 10% fetal bovine serum (FBS; Biosera, France) and 1%

penicillin/streptomycin at 37°C in a humidified incubator with 5% CO_2. The growth medium was replaced every 2 days, and the cells sub-cultured (split 1:3) at 80 to 90% confluency before characterization at passage 4. FLSs from passages 4 to 5 were used for all experiments.

Characterization of FLSs Using Morphology, Immunofluorescence Staining, and Flow Cytometry

The morphology of cells was examined by an inverted microscope (Nikon Eclipse TS100, Japan) daily. In addition, phenotype characterization of FLSs was accomplished by immunofluorescence staining and flow cytometry. To detect the fibroblast surface protein (FSP) in FLSs with immunofluorescence staining, the cells (5×10^4 cells/well) were seeded in 24-well plates and incubated at 37°C with 5% CO_2 for 24 h. After washing with PBS, FLSs were fixed with cold methanol for 5 min. The cells were rinsed with PBS again and incubated with PBS/Triton-X100 solution containing 1% bovine serum albumin (BSA; Sigma-Aldrich, USA) for 1 h. The cells were incubated with anti-FSP (Abcam Inc. Cambridge, UK) overnight at 4°C and then with sheep anti-mouse Ig-fluorescein isothiocyanate (FITC) conjugated secondary antibody (IbnSina, Iran) for 1 h at room temperature. After staining the nuclei with 4′, 6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, USA), the stained FLSs were evaluated using an inverted fluorescence microscope (Olympus, Japan).

In flow cytometric analysis, the cells were washed with PBS after the enzymatic treatment. FLSs were then stained with FITC conjugated anti-CD44, anti-CD13, anti-CD68, and anti-CD90 monoclonal antibodies (All from Abcam Inc. Cambridge, UK) for 30 min at 4°C in the dark. The cells were then washed and suspended in PBS on ice, and flow cytometric analysis was performed using a CyFlow ML Flow Cytometer (Partec GmbH, Germany) and the FlowJo software (Tree Star, Ashland, USA).

MTT Cytotoxicity Assay

RA-FLSs (7.5×10^3 cells/well) were seeded in 48-well plates and incubated at 37 °C with 5% CO_2 for 24 h. The cells were treated with thapsigargin (Tg; Sigma-Aldrich, USA) at increasing concentrations (10, 100, and 1000 nM) for 12 and 24 h. Then, MTT [3-(4, 5-Dimethylthiazol-2-yl)- 2, 5-diphenyltetrazolium bromide] solution (Sigma-Aldrich, USA) was added to each well at a final concentration of 5 mg/ml, and the cells were incubated for another 4 h. The formazan crystals were solubilized in dimethyl sulfoxide (DMSO; Sigma-Aldrich, USA), and then the absorbance was measured at 570 nm using a microplate reader (Biotek-ELx800, USA). Experiments were performed in triplicate.

FLSs Grouping and Cell Interventions

RA-FLSs (80%-90% confluence) were harvested and then seeded into 48-well plates at a final density of 1×10^5 cells/well. Each RA-FLS sample was divided into three experimental groups, including 1) negative control of miR-211-5p mimic with no Tg treatment, 2) Tg treated, 3) Tg treated and transfected with miR-211-5p mimic. In addition, the trauma-FLSs were used as the normal group.

ER Stress Stimulation

Tg, a specific sarcoplasmic/ER Ca2+-ATPase (SERCA) inhibitor that triggers the accumulation of unfolded/misfolded proteins in ER,[30] was selected to induce an ER stress response in RA-FLSs. Briefly, RA-FLSs (1×10^5 cells/well) were cultured in 24-well plates at 37°C with 5% CO_2 for 18–24 h. The cells were treated with Tg (10 nM) and then incubated for another 5 h at 37°C.

Transient Transfection of miR-211-5p Mimic

To evaluate the possible effect of miR-211-5p on ER stress-related apoptosis, RA-FLSs were transiently transfected with miR-211-5p mimic using HiPerFect transfection reagent (Qiagen, USA), according to the manufacturer's instructions. Briefly, RA-FLSs (1×10^5 cells/well) were seeded into 24-well plates and kept at 37°C for 18-24 h. After that, the cells were transfected with 10 nM synthetic miR-211-5p mimic as well as the negative control for microRNA mimic (10 nM) and cultured for 48 h, all obtained from Applied Biological Materials Inc. (ABM; Vancouver, Canada). The cells following Tg treatment were harvested 48 h after transfection and were subjected to the following downstream analyses.

Measurement of microRNA and mRNA Expression Levels Using Real-time PCR

Total RNA of FLSs was extracted using MiRJia Kit (ROJE Technologies, Iran) according to the manufacturer's instructions. The quality and quantity of

RNA were evaluated using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). Then, 300 ng total RNA was reverse transcribed using PrimeScript™ RT Reagent Kit (Takara Biotechnology, Japan) and RT-ROSET Kit (ROJE Technologies, Iran) for microRNA and mRNA detection, respectively, based on the manufacturer's protocols. To determine microRNA and mRNA levels, 75 ng cDNA was used as a template for a real-time PCR reaction using the RealQ Plus 2x Master Mix Green High ROX™ (Ampliqon, Denmark) on a StepOnePlus instrument (Applied Biosystems, Foster City, CA, USA). The relative expression levels of genes were calculated using the $2^{-\Delta\Delta CT}$ method. *U6-snRNA* and *GAPDH* were used as an internal control to normalize the levels of microRNA and mRNAs, respectively. The primer sequences used in this study are provided in the supplementary Table. The experiments were performed in duplicates.

Statistical Analysis

The results were presented as mean±standard deviation (SD). Statistical analysis was conducted using SPSS version 25.0 (IBM Corp., Armonk, NY, USA). The Friedman test was applied for comparison among multiple paired groups. The Mann–Whitney and Wilcoxon tests were used to compare two unpaired and paired groups, respectively. *p* values lower than 0.05 were considered statistically significant. GraphPad Prism version 9.0 (La Jolla, California, USA) was used to design the graphs.

RESULTS

Characterization of FLSs

In the microscopic evaluation, primary cultures of FLSs contained a mixed population of stellate, spindle-shaped, and large round cells. At the third passage, the cells were morphologically homogeneous and exhibited the typical spindle-like shape of fibroblasts (Figure 1). To characterize the phenotype of FLSs, the cells were investigated for FSP markers via immunofluorescence staining (Figure 2). The results indicated that the majority of the cells were positive for FSP. For further confirmation, the expression of some surface markers related to FLSs was measured by flow cytometry (Figure 3). The flow cytometric data showed that most cells expressed the surface markers CD90 (94.67%±3.7%), CD44 (99.12%±2.21%), CD13 (97.14%±2.06%), and were negative for the expression of CD68 (0.23%±4.01%).

MTT Cytotoxicity Assay of Tg

The cytotoxic effects of Tg (as a chemical ER stressor) on the viability of RA-FLSs were evaluated by MTT assay. The cells were treated with various concentrations of Tg at different times, and then an MTT assay was performed. The results showed no cytotoxic effect on FLS survival after Tg treatment with selected concentrations at different times (Figure 4). Although all doses studied in the MTT assay can be used for the treatment of the cells, our pilot assay of the gene expressions indicated that 10-nM Tg can induce ER stress without any cytotoxic effects (data are not shown).

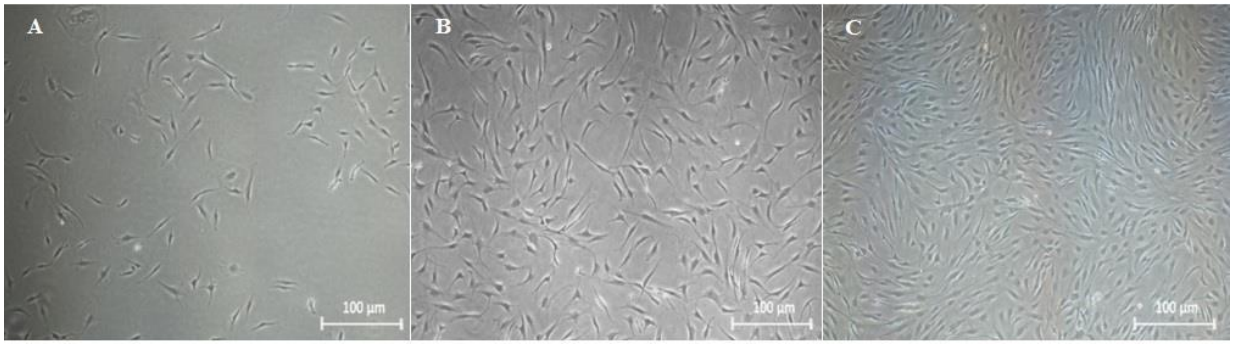

Figure 1. Light microscopic features of synovium-derived fibroblast-like synoviocytes (FLSs). (A) 4-5 days after isolation from tissue, (B) after the first passage, (C) at the third passage where homogeneous spindle-shaped cells covered the bottom of culture dishes; (scale bars=100 µm).

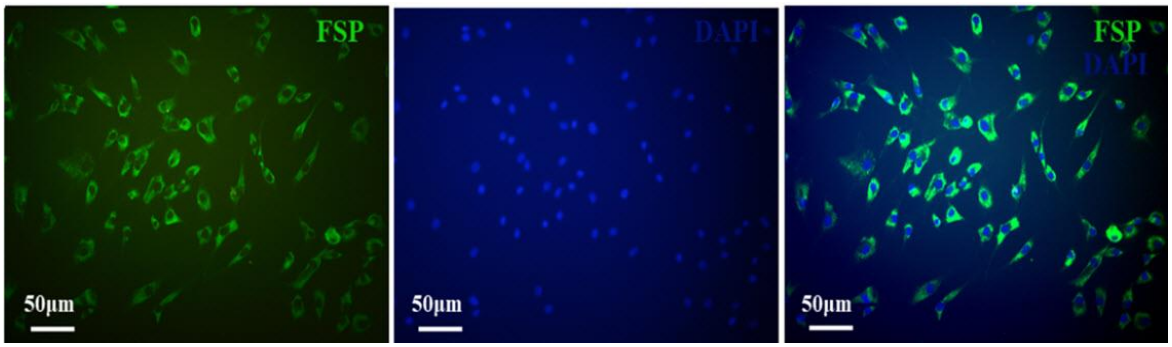

Figure 2. Characterization of fibroblast-like synoviocytes (FLSs) by immunofluorescence staining. Immunofluorescence staining of FLSs with specific antibody for fibroblast surface protein (FSP) marker (green), 4′, 6-diamidino-2-phenylindole (DAPI) color is shown in blue; positive staining was evident in the majority of the cells (scale bars=50 µm).

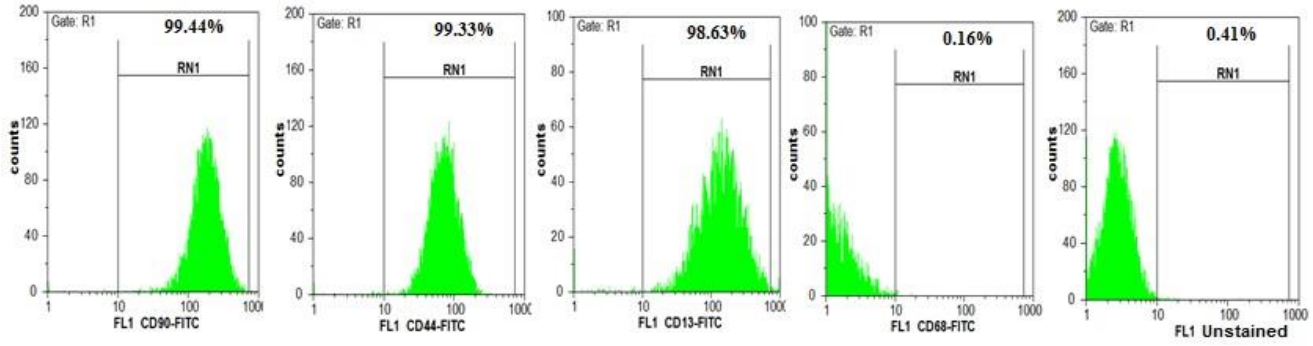

Figure 3. Characterization of fibroblast-like synoviocytes (FLSs) by flow cytometry. Flow cytometric analysis indicated high expression of CD90, CD44, and CD13 (94.67%±3.7%, 99.12%±2.21%, and 97.14%±2.06%, respectively), and negative or low expression of CD68 (0.23±4.01%) in FLSs.

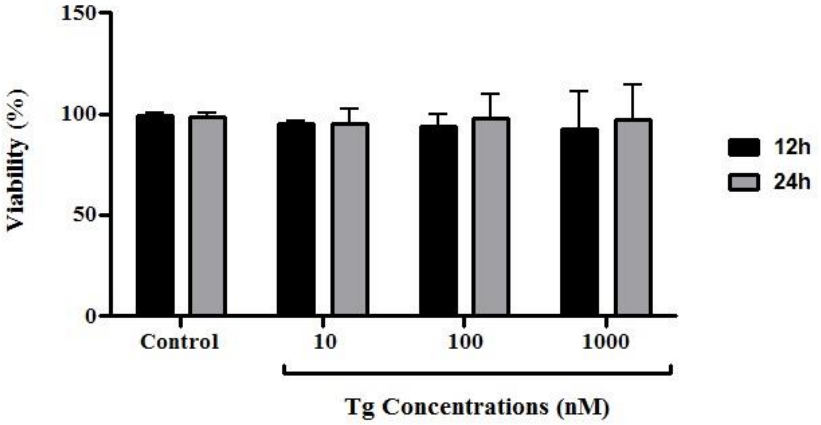

Figure 4. Effect of thapsigargin (Tg) on the viability of fibroblast-like synoviocytes in rheumatoid arthritis (RA-FLSs). The cytotoxic effect of Tg on RA-FLSs viability was evaluated with an 3- (4, 5-Dimethylthiazol-2-yl)- 2, 5-diphenyltetrazolium bromide (MTT) assay after treatment with different concentrations (10, 100, and 1000 nM) for 12 and 24 h. In addition, an untreated RA-FLSs group was used as a control group. There were no significant differences between goups. The data are presented as mean±SEM.

Baseline Gene Expression Patterns in RA-FLSs Compared to Trauma FLSs

MiR-211-5p and mRNA expression levels of the selected genes were compared between RA-FLSs and trauma FLSs (control group). As shown in Figure 5, there were no significant differences in miR-211-5p and mRNA levels of the study genes (*PERK*, *BAX*, and *BCL2*) between these two groups. In contrast, mRNA levels of *CHOP* and *MCL1* were significantly higher in RA-FLSs (p=0.015 and p=0.011, respectively). In addition, there was a significant increase in mRNA levels of *ATF4* and *BCL-XL* in trauma FLSs compared with RA-FLSs (p<0.0001 and p=0.023, respectively).

The Effect of Tg Treatment on the Expression levels of miR-211-5p and Selected Genes Involved in *PERK* Pathway and Apoptosis Regulation

As presented in Figure 6, Tg treatment significantly increased the mRNA expression level of *PERK* pathway-related genes (*PERK*, *ATF4* and *CHOP*) in RA-FLSs compared with the untreated group (p=0.012, p=0.008, and p=0.008, respectively). On the other hand, treatment with Tg had no significant effect on the expression of miR-211-5p and mRNA levels of the selected genes involved in apoptosis regulation (*BAX*, *BCL2*, *BCL-XL*, and *MCL1*) in RA-FLSs.

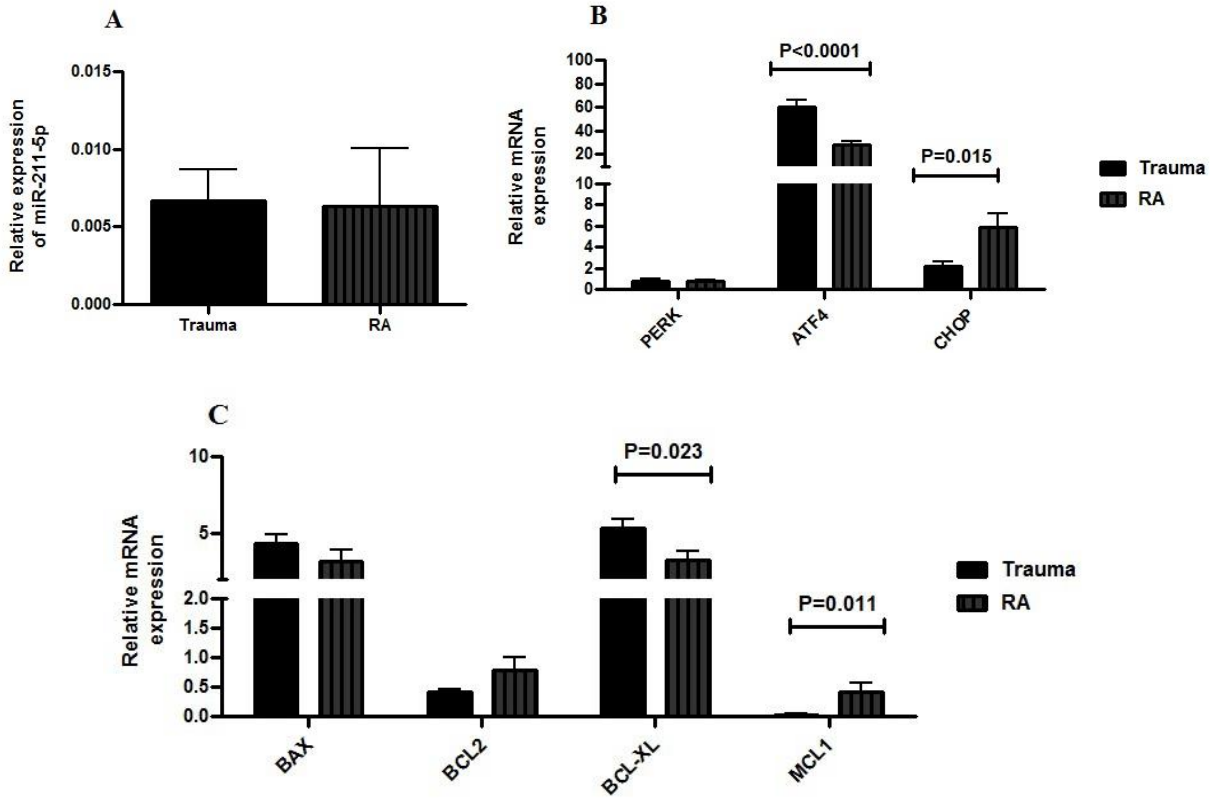

Figure 5. Baseline gene expression patterns in fibroblast-like synoviocytes in rheumatoid arthritis (RA-FLSs) compared with trauma fibroblast-like synoviocytes (FLSs). (A) The comparison of miR-211-5p expression levels in RA-FLSs versus the trauma FLS control group showed no significant difference. (B) There was no significant difference in mRNA levels of *PERK* between RA-FLSs and trauma FLSs. However, the results revealed a significant increase in mRNA levels of *CHOP* in RA-FLSs (p=0.015) compared with trauma FLSs. In addition, there was a significant increase in mRNA levels of *ATF4* in trauma FLSs (p<0.0001) compared with RA-FLSs. (C) A comparison of mRNA levels of *BAX* and *BCL2* in RA-FLSs versus trauma FLSs did not show any significant difference. In contrast, there was a significant increase in mRNA levels of *BCL-XL* in trauma FLSs (p=0.023) compared with RA-FLSs. Additionally, there was a significant increase in mRNA levels of *MCL1* in RA-FLSs (p=0.011) compared with trauma FLSs. The data are expressed as mean±SEM.

Effects of miR-211-5p Overexpression on mRNA Expression Levels of *CHOP* and Selected Genes Involved in Apoptosis Regulation

Transient transfection of miR-211-5p mimic led to a significant increase in miR-211-5p in Tg-treated RA-FLSs (p=0.005) (Figure 7). To confirm the inhibitory effect of miR-211-5p on *CHOP*, a well-known target gene of miR-211, the mRNA expression levels of *CHOP* were assessed. As shown in Figure 7, there was no significant change in Tg-treated RA-FLSs following miR-211-5p overexpression compared with the untransfected group. In addition, no differences were observed in the expression levels of the genes being studied related to apoptosis regulation between these two groups.

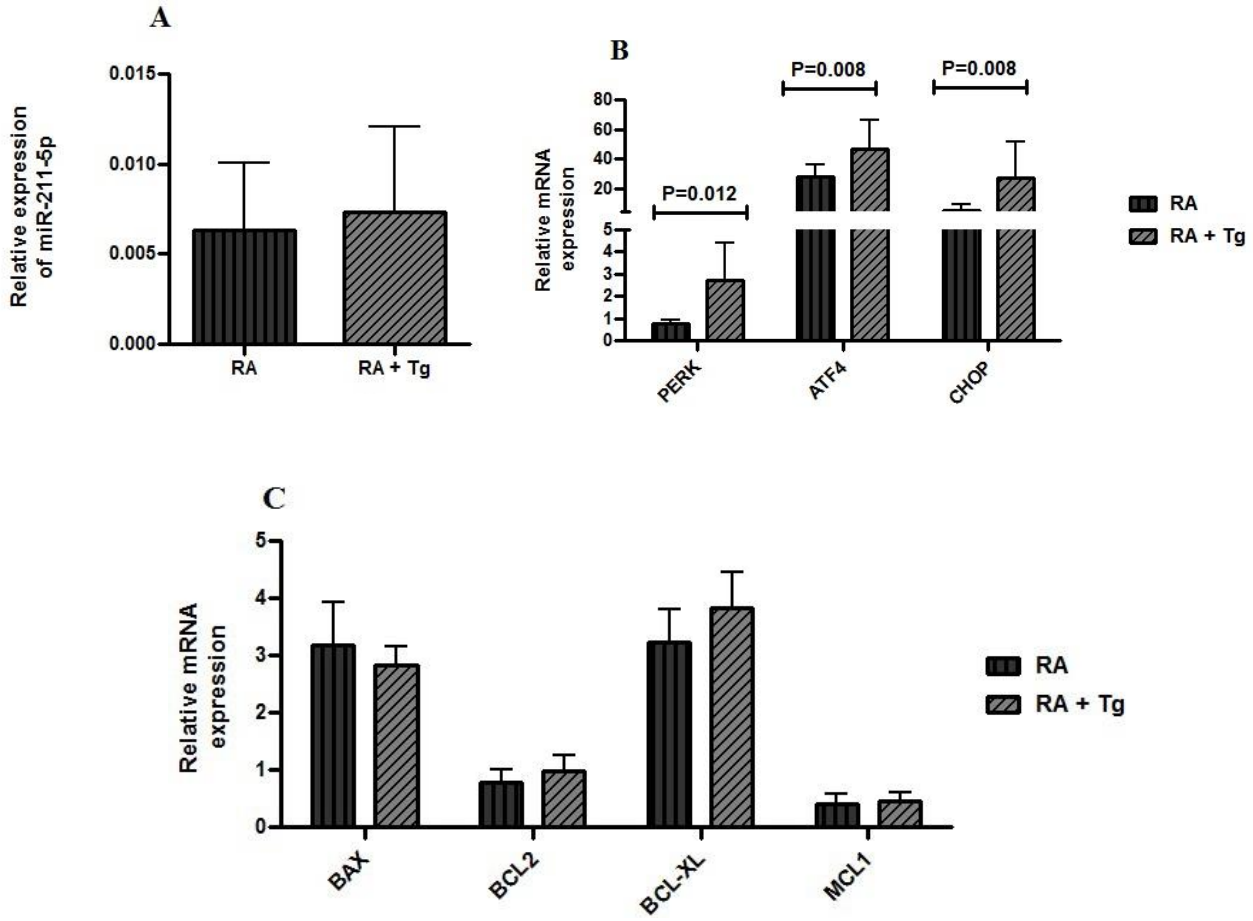

Figure 6. Effect of thapsigargin (Tg) treatment on the expression of miR-211-5p and selected genes involved in the *PERK* pathway and apoptosis regulation. Comparison of miR-211-5p expression levels in fibroblast-like synoviocytes in rheumatoid arthritis (RA-FLSs) treated with Tg versus untreated RA-FLS group did not show any significant difference (A). RA-FLS treatment with Tg revealed a significant increase in mRNA levels of *PERK*, *ATF4*, and *CHOP* (p=0.012, p=0.008, and p=0.008, respectively) compared with the untreated RA-FLS group (B). The mRNA levels of *BAX*, *BCL2*, *BCL-XL*, and *MCL1* in RA-FLSs treated with Tg versus untreated RA-FLS group showed no significant difference (C). The data are expressed as mean±SEM.

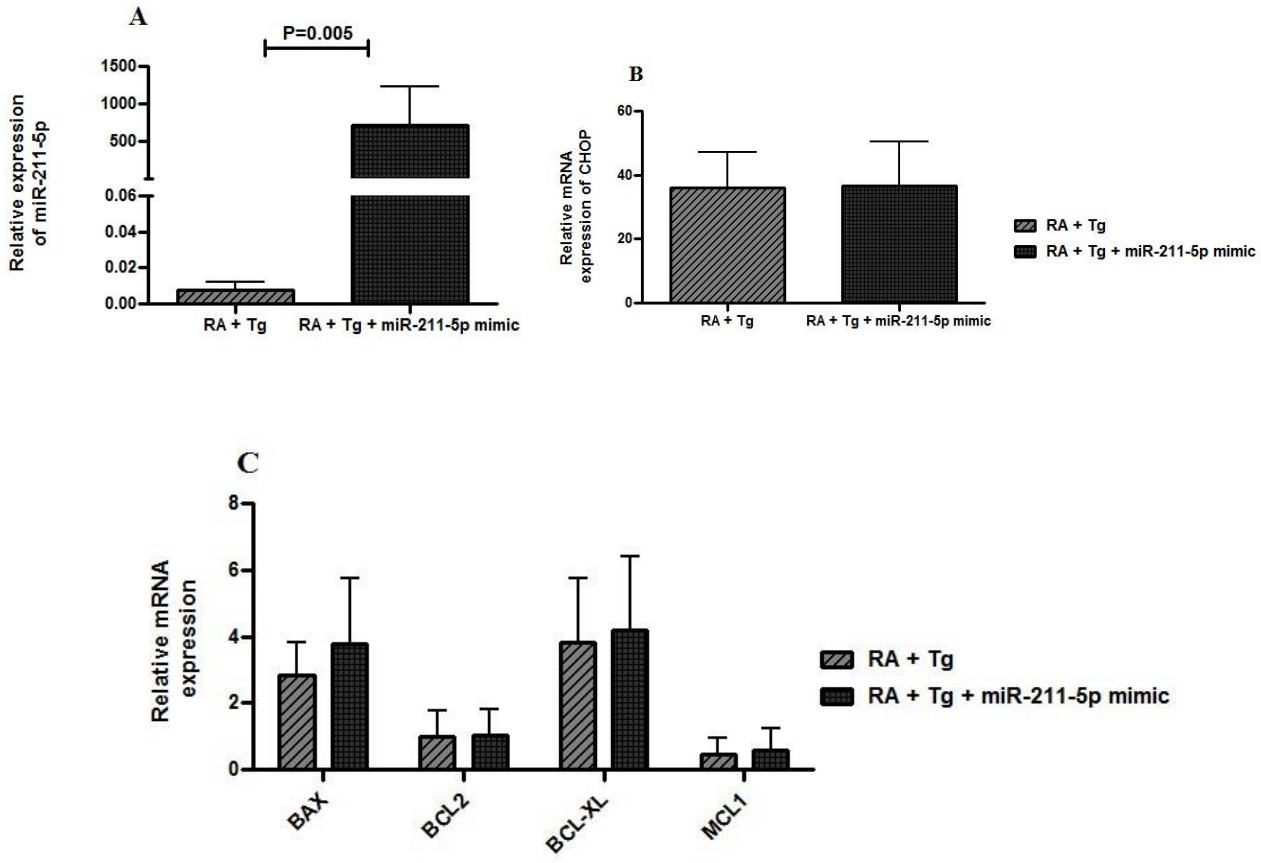

Figure 7. Effect of miR-211-5p overexpression on the mRNA expression levels of *CHOP* and selected genes involved in apoptosis regulation. (A) MiR-211-5p expression levels showed a significant increase in thapsigargin (Tg)-treated fibroblast-like synoviocytes in rheumatoid arthritis (RA-FLSs) (*p*=0.005) after miR-211-5p mimic transient transfection. (B and C) Comparison of expression levels of study genes (*CHOP*, *BAX*, *BCL2*, *BCL-XL*, and *MCL1*) in RA-FLSs treated with Tg following miR-211-5p overexpression versus untransfected RA-FLSs group did not indicate any significant change. The data are expressed as Mean±SEM.

DISCUSSION

The activated RA-FLSs with an altered aggressive phenotype play a vital role in the initiation and progression of the disease. On the other hand, RA-FLSs, similar to cancer cells, show abnormal proliferation and resistance to apoptosis, and cause the hyperplastic growth of the synovium and the progressive destruction of joint cartilage. Although the exact mechanism of increased survival and resistance to apoptosis in RA-FLSs has not yet been elucidated, recent studies have shown the possible involvement of ER stress and UPR dysregulation in FLSs. Thus, elevated levels of ER stress markers such as *BIP*, *IRE1*, *XPB1*, *ATF6*, and *eIF2α* have been reported in synovial tissues of RA patients.[21,31,32] Therefore, induction of apoptosis in RA-FLSs has been increasingly considered a therapeutic approach for RA.

In the UPR pathway, the activated *PERK* as a key sensor is essential in determining cell fate for survival or apoptosis. The *PERK*-dependent miR-211-5p has a pro-survival activity and controls ER stress-induced apoptosis by inhibiting the pro-apoptotic factor *CHOP*.

Here, we primarily investigated the expression of selected genes related to the *PERK* pathway, apoptosis regulation, and miR-211-5p in RA-FLSs compared with the trauma control group.

In this study, we did not find any differences in the expression of *PERK* between trauma and RA-FLSs. In contrast, *ATF4* expression showed a significant increase in trauma compared with RA-FLSs. According to a study by Andreas Lenz et al, tissue trauma activates local

inflammation in the affected area.[33] Furthermore, Tomomi Gotoh et al, have shown that inflammation can induce ER stress and subsequently activate the UPR pathway.[34] Also, the activation and induction of *ATF4* have been reported as the effector in ER stress-induced UPR pathway.[35] It seems that in our study, the inflammation following trauma has stimulated ER stress, activated UPR, and increased *ATF4* levels in trauma FLSs.

In this study, *CHOP* expression showed a significant increase in RA-FLSs compared with trauma FLSs. Previous studies have shown that in addition to *PERK*, two proteins, *ATF6* and *IRE1*, in the UPR pathway contribute to an increase in the expression of *CHOP* following ER stress.[21,24,36-40] Therefore, it seems that the enhanced expression of *CHOP* in RA-FLSs is due to ER stress and UPR activation in a *PERK* independent pathway.

Our findings did not show any differences in miR-211-5p expression between trauma and RA-FLSs which can be due to ER stress stimulation in both groups. In addition, we found no differences in expression levels of *BAX* and *BCL2* genes between trauma and RA-FLSs. However, the expression levels of *MCL1* in RA-FLSs were significantly higher than in the trauma group. Our results share similarities with a study by Hongtao Liu et al. that RA-FLSs had higher expression levels of *MCL1* compared with osteoarthritis (OA) patients and healthy individuals.[41] They also reported that the expression levels of *MCL1* are essential for the survival of RA-FLSs. Besides, it has been shown that ER stress can increase the expression of *MCL1*, which leads to cell survival and resistance to apoptosis.[42] In our study, it seems that the expression levels of *MCL1* in RA-FLSs have increased following ER stress, which can play a possible role in resistance to apoptosis. Our results also showed a significant difference in *BCL-XL* expression in trauma compared to RA-FLSs, which can be due to ER stress.

Our results showed that Tg treatment significantly increases the expression of selected genes related to the *PERK* pathway. These results had several similarities with other studies that indicated ER stress stimulation which could lead to elevated levels of the genes associated with the *PERK* pathway.[43-47] In addition, our results showed that Tg treatment did not affect miR-211-5p expression in RA-FLSs. Our results are inconsistent with other studies that showed ER stress induction results in increased expression of miR-211 in a *PERK*-dependent manner.[28,48] They have reported that overexpression of miR-211 following ER stress leads to a cumulative increase in *CHOP*, which acts as negative feedback and decreases miR-211 expression.[28] In our study, it seems that miR-211-5p expression is in the balance between the effects of *PERK* and *CHOP*, which are overexpressed upon Tg treatment (inducer and inhibitor of miR-211). In addition, in our study, no changes were observed in the expression of genes related to apoptosis in stimulated RA-FLSs. Previous findings have revealed that the role of *CHOP* in ER stress-dependent apoptosis and binding to known genes involved in apoptosis, such as *BCL2*, *BAX*, *BIM*, and *BAD*, depending on cell type.[49] In addition, it has been demonstrated that *CHOP* in interaction with *ATF4* can increase protein synthesis. Given the *CHOP* role in protein synthesis and cell survival and concerning no changes in the expression of genes involved in apoptosis, it seems that an increased level of *CHOP* in our study contributes to cell survival despite of apoptosis following ER stress. In addition, because of acute ER stress stimulation in RA-FLSs, in these cells, other pathways may play a crucial role in relieving ER stress and restoring homeostasis to ER to maintain cell survival.

Furthermore, the effect of miR-211-5p overexpression on genes involved in apoptosis in RA-FLSs was investigated. The present study showed that despite of a significant increase in miR-211-5p expression, no differences were seen in the expression of *CHOP* and selective genes related to apoptosis.

In our study, overexpression of miR-211-5p did not alter the levels of *CHOP*, which is a direct target of this microRNA. It has been indicated that overexpression of a circRNA (circular RNA)—hsa_circ_0001859—directly inhibited the miR-204/211 family in synovial tissues like a molecular sponge.[50] Therefore, our results may be due to the presence of circRNA in RA-FLSs, which acts as a miR-211 sponge and inhibits its regulatory function on its target gene(s).

In conclusion, the results of our study showed that despite of increasing ER stress markers, chemical stimulation of ER stress in RA-FLSs did not alter the expression levels of miR-211 and selected genes related to apoptosis. In addition, overexpression of miR-211-5p in stimulated RA-FLSs could not change the expression of *CHOP* and selected apoptotic genes. The ER stress induction in RA-FLSs seems to lead to cell survival and apoptosis resistance. Further studies are needed to

determine the ER stress role in apoptosis regulation in RA-FLSs.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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REFERENCES

1. Guo Q, Wang Y, Xu D, Nossent J, Pavlos NJ, Xu J. Rheumatoid arthritis: pathological mechanisms and modern pharmacologic therapies. Bone research. 2018;6.
2. Aletaha D, Smolen JS. Diagnosis and management of rheumatoid arthritis: a review. Jama. 2018;320(13):1360-72.
3. Firestein GS, McInnes IB. Immunopathogenesis of rheumatoid arthritis. Immunity. 2017;46(2):183-96.
4. Šenolt L, Grassi W, Szodoray P. Laboratory biomarkers or imaging in the diagnostics of rheumatoid arthritis? BMC medicine. 2014;12(1):49.
5. Alam J, Jantan I, Bukhari SNA. Rheumatoid arthritis: recent advances on its etiology, role of cytokines and pharmacotherapy. Biomedicine & Pharmacotherapy. 2017;92:615-33.
6. Smolen JS, Aletaha D, Barton A, Burmester GR, Emery P, Firestein GS, et al. Rheumatoid arthritis. Nature Reviews Disease Primers. 2018;4:18001.
7. Tanaka Y. Rheumatoid arthritis. Inflammation and regeneration. 2020;40:20. PubMed PMID: 32944095. Pubmed Central PMCID: PMC7487964. Epub 2020/09/19. eng.
8. Korb A, Pavenstädt H, Pap T. Cell death in rheumatoid arthritis. Apoptosis. 2009;14(4):447-54.
9. Bartok B, Firestein GS. Fibroblast-like synoviocytes: key effector cells in rheumatoid arthritis. Immunological reviews. 2010;233(1):233-55.
10. Bottini N, Firestein GS. Duality of fibroblast-like synoviocytes in RA: passive responders and imprinted aggressors. Nature Reviews Rheumatology. 2013;9(1):24.
11. Araki Y, Mimura T. The mechanisms underlying chronic inflammation in rheumatoid arthritis from the perspective of the epigenetic landscape. Journal of immunology research. 2016;2016.
12. Bustamante MF, Garcia-Carbonell R, Whisenant KD, Guma M. Fibroblast-like synoviocyte metabolism in the pathogenesis of rheumatoid arthritis. Arthritis research & therapy. 2017;19(1):110.
13. Nygaard G, Firestein GS. Restoring synovial homeostasis in rheumatoid arthritis by targeting fibroblast-like synoviocytes. Nature Reviews Rheumatology. 2020;16(6):316-33.
14. Morito D, Nagata K. ER stress proteins in autoimmune and inflammatory diseases. Frontiers in immunology. 2012;3:48.
15. Hetz C, Zhang K, Kaufman RJ. Mechanisms, regulation and functions of the unfolded protein response. Nature reviews Molecular cell biology. 2020;21(8):421-38.
16. Karagöz GE, Aragón T, Acosta-Alvear D. Recent advances in signal integration mechanisms in the unfolded protein response. F1000Research. 2019;8.
17. Liu Z, Lv Y, Zhao N, Guan G, Wang J. Protein kinase R-like ER kinase and its role in endoplasmic reticulum stress-decided cell fate. Cell death & disease. 2015;6(7):e1822-e.
18. Rozpedek W, Pytel D, Mucha B, Leszczynska H, Diehl JA, Majsterek I. The role of the PERK/eIF2α/ATF4/CHOP signaling pathway in tumor progression during endoplasmic reticulum stress. Current molecular medicine. 2016;16(6):533-44.
19. Hiramatsu N, Chiang W-C, Kurt TD, Sigurdson CJ, Lin JH. Multiple mechanisms of unfolded protein response–induced cell death. The American journal of pathology. 2015;185(7):1800-8.
20. Li Y, Guo Y, Tang J, Jiang J, Chen Z. New insights into the roles of CHOP-induced apoptosis in ER stress. Acta biochimica et biophysica Sinica. 2014;46(8):629-40.
21. Rahmati M, Moosavi MA, McDermott MF. ER Stress: A Therapeutic Target in Rheumatoid Arthritis? Trends in pharmacological sciences. 2018.
22. Junjappa RP, Patil P, Bhattarai KR, Kim H-R, Chae H-J. IRE1α implications in endoplasmic reticulum stress-mediated development and pathogenesis of autoimmune diseases. Frontiers in immunology. 2018;9.
23. Park Y-J, Yoo S-A, Kim W-U. Role of endoplasmic reticulum stress in rheumatoid arthritis pathogenesis. Journal of Korean medical science. 2014;29(1):2-11.
24. Todd DJ, Lee A-H, Glimcher LH. The endoplasmic reticulum stress response in immunity and autoimmunity. Nature Reviews Immunology. 2008;8(9):663.
25. Liu Q, Körner H, Wu H, Wei W. Endoplasmic reticulum stress in autoimmune diseases. Immunobiology. 2020;225(2):151881.

26. Jang JH, Lee T-J. The role of microRNAs in cell death pathways. Yeungnam University Journal of Medicine. 2021;38(2):107.
27. McMahon M, Samali A, Chevet E. Regulation of the unfolded protein response by noncoding RNA. American Journal of Physiology-Cell Physiology. 2017;313(3):C243-C54.
28. Chitnis NS, Pytel D, Bobrovnikova-Marjon E, Pant D, Zheng H, Maas NL, et al. miR-211 is a prosurvival microRNA that regulates chop expression in a PERK-dependent manner. Molecular cell. 2012;48(3):353-64.
29. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham III CO, et al. 2010 rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. Arthritis & Rheumatism. 2010;62(9):2569-81.
30. Oslowski CM, Urano F. Measuring ER stress and the unfolded protein response using mammalian tissue culture system. Methods in enzymology. 490: Elsevier; 2011. p. 71-92.
31. Yoo S-A, You S, Yoon H-J, Kim D-H, Kim H-S, Lee K, et al. A novel pathogenic role of the ER chaperone GRP78/BiP in rheumatoid arthritis. Journal of Experimental Medicine. 2012;209(4):871-86.
32. Connor AM, Mahomed N, Gandhi R, Keystone EC, Berger SA. TNFα modulates protein degradation pathways in rheumatoid arthritis synovial fibroblasts. Arthritis research & therapy. 2012;14(2):1-19.
33. Lenz A, Franklin GA, Cheadle WG. Systemic inflammation after trauma. Injury. 2007;38(12):1336-45.
34. Gotoh T, Endo M, Oike Y. Endoplasmic reticulum stress-related inflammation and cardiovascular diseases. International Journal of Inflammation. 2011;2011.
35. Wortel IM, van der Meer LT, Kilberg MS, van Leeuwen FN. Surviving stress: modulation of ATF4-mediated stress responses in normal and malignant cells. Trends in Endocrinology & Metabolism. 2017;28(11):794-806.
36. Oyadomari S, Mori M. Roles of CHOP/GADD153 in endoplasmic reticulum stress. Cell death and differentiation. 2004;11(4):381.
37. Hosoi T, Ozawa K. Endoplasmic reticulum stress in disease: mechanisms and therapeutic opportunities. Clinical Science. 2010;118(1):19-29.
38. Jäger R, Bertrand MJ, Gorman AM, Vandenabeele P, Samali A. The unfolded protein response at the crossroads of cellular life and death during endoplasmic reticulum stress. Biology of the Cell. 2012;104(5):259-70.
39. Lin JH, Walter P, Yen TB. Endoplasmic reticulum stress in disease pathogenesis. Annu Rev Pathol Mech Dis. 2008;3:399-425.
40. Hu H, Tian M, Ding C, Yu S. The C/EBP homologous protein (CHOP) transcription factor functions in endoplasmic reticulum stress-induced apoptosis and microbial infection. Frontiers in immunology. 2019:3083.
41. Liu H, Eksarko P, Temkin V, Haines GK, Perlman H, Koch AE, et al. Mcl-1 is essential for the survival of synovial fibroblasts in rheumatoid arthritis. The Journal of Immunology. 2005;175(12):8337-45.
42. Jiang CC, Lucas K, Avery-Kiejda KA, Wade M, debock CE, Thorne RF, et al. Up-regulation of Mcl-1 is critical for survival of human melanoma cells upon endoplasmic reticulum stress. Cancer Research. 2008;68(16):6708-17.
43. Kabala PA, Angiolilli C, Yeremenko N, Grabiec AM, Giovannone B, Pots D, et al. Endoplasmic reticulum stress cooperates with Toll-like receptor ligation in driving activation of rheumatoid arthritis fibroblast-like synoviocytes. Arthritis research & therapy. 2017;19(1):1-11.
44. Díaz-Martínez M, Benito-Jardón L, Alonso L, Koetz-Ploch L, Hernando E, Teixidó J. miR-204-5p and miR-211-5p contribute to BRAF inhibitor resistance in melanoma. Cancer research. 2018;78(4):1017-30.
45. Armstrong JL, Flockhart R, Veal GJ, Lovat PE, Redfern CP. Regulation of endoplasmic reticulum stress-induced cell death by ATF4 in neuroectodermal tumor cells. Journal of Biological Chemistry. 2010;285(9):6091-100.
46. Vandewynckel Y-P, Laukens D, Bogaerts E, Paridaens A, Van den Bussche A, Verhelst X, et al. Modulation of the unfolded protein response impedes tumor cell adaptation to proteotoxic stress: a PERK for hepatocellular carcinoma therapy. Hepatology international. 2015;9(1):93-104.
47. Ge X, Frank-Bertoncelj M, Klein K, McGovern A, Kuret T, Houtman M, et al. Functional genomics atlas of synovial fibroblasts defining rheumatoid arthritis heritability. Genome biology. 2021;22(1):1-39.
48. Bu Y, Yoshida A, Chitnis N, Altman BJ, Tameire F, Oran A, et al. A PERK–miR-211 axis suppresses circadian regulators and protein synthesis to promote cancer cell survival. Nature cell biology. 2018;20(1):104.
49. Han J, Back SH, Hur J, Lin Y-H, Gildersleeve R, Shan J, et al. ER-stress-induced transcriptional regulation increases protein synthesis leading to cell death. Nature cell biology. 2013;15(5):481-90.
50. Wang J, Yan S, Yang J, Lu H, Xu D, Wang Z. Non-coding RNAs in rheumatoid arthritis: from bench to bedside. Frontiers in immunology. 2020:3129.