

TXNDC5 protects synovial fibroblasts of rheumatoid arthritis from the detrimental effects of endoplasmic reticulum stress

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SUMMARY TXNDC5 is an endoplasmic reticulum (ER)-resident chaperone that protects the endothelium from secondary effects of ER stress. Previous studies by the current authors identified TXNDC5 as a key pathological factor in promoting the inflammatory phenotype of fibroblast-like synoviocytes (FLSs) from rheumatoid arthritis (RA). However, its activity in RA FLSs under ER stress remains unclear. The current study found that TXNDC5 is responsive to ER stress in RA FLSs since its expression was induced by ER stress at both the endogenous and secretory level. A functional study indicated that silencing TXNDC5 reduced the viability of RA FLSs more markedly in the presence of ER stressors. In contrast, rhTXNDC5 attenuated a decrease in cell viability as a result of ER stress. Moreover, silencing TXNDC5 attenuated the induction of IL-6 and IL-8 from RA FLSs in response to ER stress. In addition, rhTXNDC5 induced a greater increase in VEGF production during ER stress. These findings confirm the pro-survival and pro-inflammation roles of TXNDC5 under ER stress in RA FLSs. TXNDC5 appears to act as a mediator linking ER stress and inflammation of RA.

Keywords TXNDC5, endoplasmic reticulum stress, rheumatoid arthritis

1. Introduction

Rheumatoid arthritis (RA) is an inflammatory joint disease characterized by hyperplasia of synovial tissue and formation of pannus (1,2). Excessive inflammatory molecules, such as cytokines and matrix metalloproteinase, are secreted and promote the destruction of cartilage and bone (3,4). Analyses of hyperplastic synovial tissue from patients with RA have revealed features of transformed long-living cells, such as the presence of somatic mutations, expression of oncogenes, and resistance to apoptosis (5). Therefore, identification of the components that are sensitive to the stimuli in the microenvironment of an inflamed joint is crucial to understanding the pathogenesis of RA and may also provide new targets for diagnosis and treatment (6,7).

Endoplasmic reticulum (ER) stress is a cellular danger signal, and diverse conditions including hypoxia and inflammation, which are frequently observed in joints affected by RA, can be considered inducers of

ER stress (8). Fibroblast-like synoviocytes (FLSs) are predisposed to apoptotic resistance to ER stress and contribute to synovial hyperplasia of RA (9), but the mechanism of this pathology has yet to be fully clarified (10,11). Thioredoxin domain-containing protein 5 (TXNDC5) is a protein-disulfide isomerase located in the ER that functions as a chaperone. In vitro and in vivo studies by the current authors found that TXNDC5 was closely related to the pathological progression of RA and prostate cancer (12,13). Recently, the current authors found that TXNDC5 coordinated with HSC70 to exacerbate the inflammatory phenotype of RA at the endogenous level (14). More importantly, previous findings indicated that TXNDC5 is responsive to extracellular stimuli, such as hypoxia, pro-inflammatory factors, and androgen-deprivation, indicating that TXNDC5 may be required to adapt to extracellular changes in an inflamed joint (15). In addition, TXNDC5 expression was detected in synovial fluid from patients with RA, and its exact function in soluble form remains

unclear (16). Intriguingly, a previous study found that TXNDC5 protects endothelial cells from stress-induced cell death. However, the exact role of TXNDC5 under ER stress remains unclear (17).

The current study examined whether TXNDC5 expression is up-regulated when stimulated with an inducer of ER stress. TXNDC5 was knocked down to make the inhibitory effects of ER stress on the cell viability of RA FLSs more apparent, and RA FLSs were treated with recombinant human TXNDC5 (rhTXNDC5) to determine if those effects were attenuated. In addition, the expression of inflammatory factors, such as IL-6 and IL-8, was measured in response to rhTXNDC5 treatment plus ER stress. TXNDC5 was silenced to determine the response to an inducer of ER stress. This study examined whether TXNDC5 is sensitive to ER stress in RA FLSs and whether it functions as a link between ER stress and inflammation in RA.

2. Materials and Methods

2.1. Patients

Subjects were 12 patients with RA who had undergone knee joint replacement surgery. All of the patients fulfilled the 1987 American College of Rheumatology revised criteria for the diagnosis of RA. Written informed consent was obtained from every patient, and all samples were anonymized.

2.2. Stimulation assays

Primary RA FLSs were isolated and cultured as described previously (4), and cells passaged 3-7 times were used in this study. For stimulation, RA FLSs were plated on 24-well plates ($3-5 \times 10^5$ cells/well) in Dulbecco's modified Eagle's medium and stimulated for the indicated times with the following agents: recombinant human TXNDC5, thapsigargin, tunicamycin, LPS, and a hypoxia inducer (CoCl_2).

2.3. Small interfering RNA transfection in RA FLSs

RA FLSs (2×10^5 cells in 100-mm-diameter dishes or 8×10^4 cells on 6-well plates) were transiently transfected with siRNA targeting TXNDC5 (#1:SI00132440; #2: SI00132447, QIAGEN, Hilden, Germany) or a negative control (QIAGEN, SI03650318) with a HiPerfect transfection reagent (QIAGEN) in accordance with the manufacturer's instructions, and all experiments were performed 24-48 h after transfection. Non-specific negative control siRNAs were also designed and synthesized (sense strand: 5'-UUCUCCGAACGUGUCACG-3' and anti-sense strand: 5'-ACGUGACACGUUCGGAGAATT-3'). Two siRNAs targeting the same gene were mixed together in equal volumes to verify the reliability of silencing

efficiency and were designated siTXNDC5.

2.4. Cell viability

Briefly, 1×10^4 cells were seeded onto each well of 96-well plates for the indicated treatment. On the day of detection, 20 μL MTS (Promega) was added to each well. After incubation for 1 h at 37°C , the plates were shaken at room temperature for 10 min. The absorbance was measured at 495 nm, and three independent experiments were analyzed.

2.5. ELISA

Cells were cultured and stimulated as described above, and supernatants were collected as the indicated timepoints. After centrifugation to remove particulates, the release of VEGF (R&D Systems) was analyzed with ELISA in accordance with the manufacturer's instructions.

2.6. RNA extraction and real time RT-PCR (qRT-PCR)

RNA extraction and qRT-PCR were performed as previously described (18). Details on the primers are shown in Supplementary Table 1 (<http://www.irdrjournal.com/action/getSupplementalData.php?ID=54>). GAPDH was used as internal loading control. Relative mRNA levels were measured using the $2^{-\Delta}$ cycle threshold ($2^{-\Delta\text{CT}}$) method. Three independent experiments were completed, and each reaction was performed in triplicate.

2.7. Measurement of apoptosis using flow cytometry

Apoptosis was also assessed with Annexin V-APC/7-amino-actinomycin D staining (KeyGEN, Nanjing, China). Briefly, cells were harvested, washed with phosphate-buffered saline, resuspended in 500 μL of binding buffer, mixed with 5 μL of Annexin V-APC and 5 μL of 7-ADD, and incubated for 5-15 min in the dark. Fifty thousand cells were analyzed using a FACSCalibur cytometer. Annexin V-positive cells were considered apoptotic cells and analyzed using the software ModFit. The assays were carried out in triplicate in three experiments.

2.8. Statistical analyses

Data were statistically analyzed using the software SPSS V.16 (SPSS). The *t* test was used to assess statistical differences between two groups. A *P* value < 0.05 was considered significant. Data are expressed as the mean \pm standard deviation.

3. Results

3.1. TXNDC5 is responsive to ER stress in RA

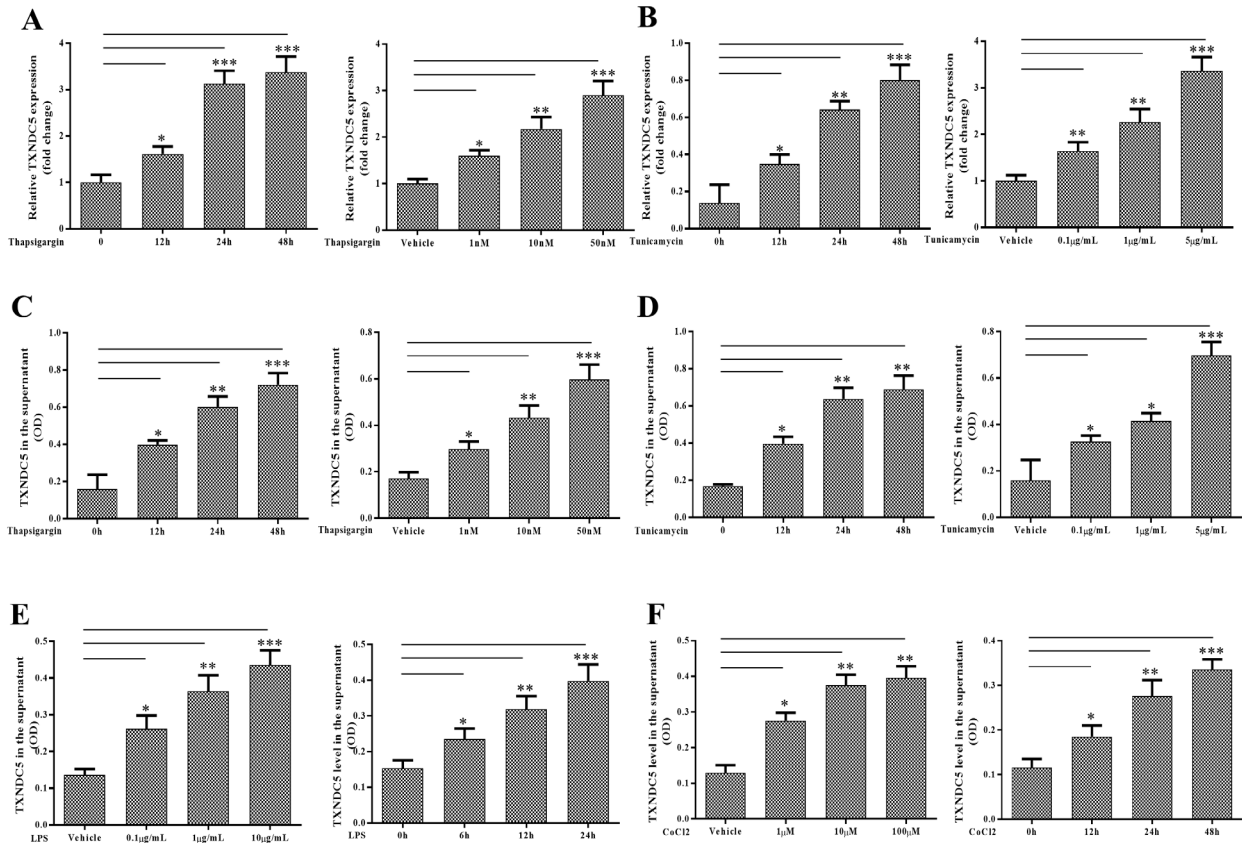


Figure 1. TXNDC5 expression is responsive to ER stress in RA. RA FLSs were exposed to inducers of ER stress, thapsigargin (A) and tunicamycin (B), at different doses and time periods, and qRT-PCR was performed to detect the expression of TXNDC5, which was normalized to GAPDH. The expression of TXNDC5 in the supernatant of RA FLSs after treatment with thapsigargin (C) and tunicamycin (D) was analyzed with ELISA. After stimulation with LPS (E) and CoCl₂ (F), the level of TXNDC5 protein in the supernatant of RA FLSs was determined with ELISA. Results are expressed as the mean ± SD of experiments performed in triplicate with culture supernatants from each of the three patients with RA. **p* < 0.05, ***p* < 0.01, and ****p* < 0.001.

TXNDC5 was localized in the ER, and its expression was induced under ER stress. However, its exact character in RA during ER stress remains unclear. In this study, RA FLSs were exposed to inducers of ER stress, thapsigargin and tunicamycin, and endogenous TXNDC5 expression was induced in a time- and dose-dependent manner (Figure 1A and 1B), indicating the potential involvement of TXNDC5 in RA FLSs in response to ER stress. Moreover, TXNDC5 was detected in serum from patients with RA (12). Interestingly, expression of TXNDC5 protein increased significantly after stimulation with thapsigargin and tunicamycin (Figure 1C and 1D). In addition, LPS and hypoxia are proven inducers of ER stress and induce endogenous TXNDC5 expression in RA FLSs (14). The current findings indicated that LPS or hypoxia up-regulated TXNDC5 expression in the supernatant of RA FLSs in a time- and dose-dependent manner (Figure 1E and 1F). Collectively, these results suggest that FLSs need TXNDC5 to cope with ER stress in RA, although the exact function needs to be studied in detail.

3.2. Down-regulation of endogenous TXNDC5 by siRNA increases ER-stress-induced apoptosis in RA FLSs

ER chaperones regulate cell survival, and TXNDC5 has been reported to protect endothelial cells from cell death induced by stress (19). However, the role of TXNDC5 in RA FLS survival under ER stress is not known. As shown in Figure 2A, tunicamycin and thapsigargin significantly increased the number of TUNEL-positive apoptotic cells among RA FLSs when TXNDC5 siRNA was introduced, in comparison to the control. Moreover, TXNDC5 siRNA-transfected cells had a more marked decrease in cell viability when treated with tunicamycin and thapsigargin (Figure 2B). These findings suggest that TXNDC5 played an important role in maintaining the survival of RA FLSs in response to proapoptotic ER stress in joints affected by RA.

3.3. Exogenous TXNDC5 protects cells from ER stress

A study has indicated that protein secreted in response to ER stress may in turn protect against ER stress-induced apoptosis (20). The current study investigated whether TXNDC5 in this soluble form had a similar effect on RA FLSs. Recombinant human TXNDC5 (rhTXNDC5) was used to mimic its extracellular form, and experimental doses of rhTXNDC5 used had no

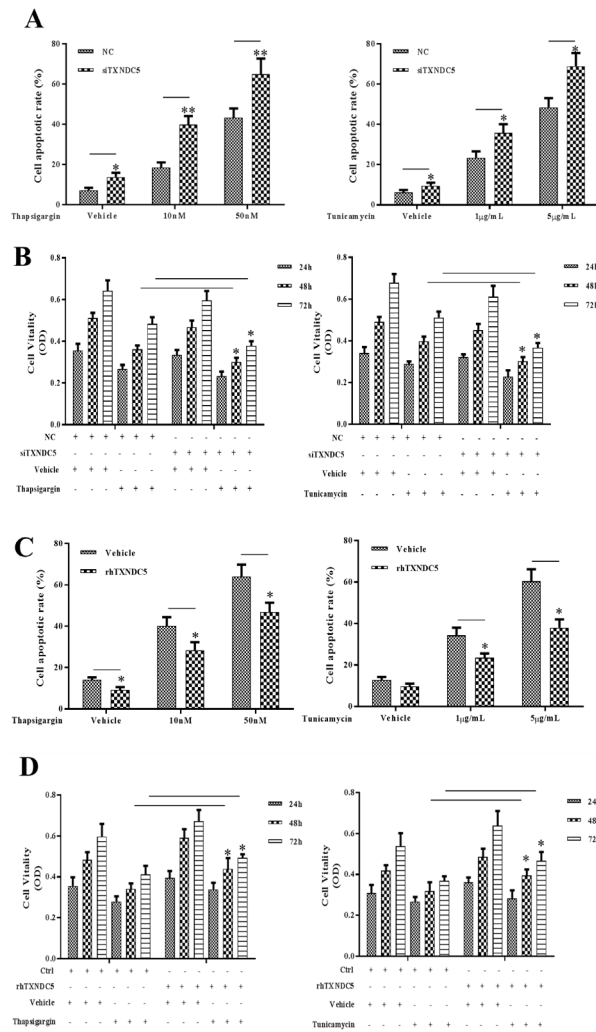


Figure 2. Effects of TXNDC5 on cell biological activity. (A) TUNEL-positive cells were analyzed in thapsigargin or tunicamycin-treated RA FLSs when TXNDC5 siRNA was introduced. Values are the mean and SD percent of TUNEL-positive cells in RA from 5 patients per group. (B) MTS was applied every 24 h to detect cell viability after RA FLSs were treated with thapsigargin or tunicamycin when TXNDC5 siRNA was introduced. (C) TUNEL-positive apoptotic cells were determined with flow cytometry in the presence of thapsigargin or tunicamycin plus rhTXNDC5. (D) MTS was applied every 24 h to detect cell viability after treatment with thapsigargin or tunicamycin plus rhTXNDC5. Data are presented from a single experiment, and each experiment was repeated three times. * $p < 0.05$ and ** $p < 0.01$.

cytotoxic effect on RA FLSs (results not shown). As expected, the number of TUNEL-positive apoptotic RA FLSs induced by tunicamycin and thapsigargin decreased significantly in a dose-dependent manner when RA FLSs were preincubated with rhTXNDC5 (Figure 2C). The effect of rhTXNDC5 on cell viability following ER stress was analyzed using the MTS assay. rhTXNDC5- and vehicle-treated cells were seeded and cultured for 6 h prior to addition of tunicamycin and thapsigargin for 24, 48 and 72 h. Cell viability at each time point was evaluated using the MTS assay and normalized to the respective cell type without ER stress. As shown in Figure 2D, the number of viable rhTXNDC5-treated cells was greater than the number

of control cells at all time points as a result of treatment with tunicamycin at a certain concentration (5 µg/mL). The number of cells differed significantly. Similar results were obtained when cells were treated with thapsigargin (50 nM, Figure 2D). Hence, rhTXNDC5-treated cells clearly have increased resistance to ER stress.

3.4. TXNDC5 facilitates cytokine expression and angiogenesis under ER stress

A previous study by the current authors found that cytokine production in response to pro-inflammatory stimuli was attenuated by silencing endogenous TXNDC5. A study has reported that tunicamycin can also induce cytokine production (21), so the current study investigated the effect of TXNDC5 on ER-stress mediated cytokine production by RA FLSs. Consistent with results of the previous study, levels of IL6 and IL-8 increased after stimulation with tunicamycin. However, silencing endogenous TXNDC5 suppressed the induction of cytokine expression in response to ER stress (Figure 3A and 3B).

The effect of rhTXNDC5 on cytokine expression in RA FLSs was examined. A qRT-PCR assay indicated that addition of rhTXNDC5 did not significantly induce the expression of IL-6 and IL-8 by RA FLSs. More interestingly, rhTXNDC5 plus LPS (Figure 3C and 3D) or hypoxia (Figure 3E and 3F) additively increased cytokine expression in a time- and concentration-dependent manner. These results indicate that TXNDC5 may act as a mediator to exacerbate inflammation under ER stress. An attempt was made to determine whether TXNDC5 affects the angiogenic process. Interestingly, the current findings indicated that rhTXNDC5 treatment induced VEGF production in RA FLSs in response to tunicamycin (Figure 3G) and thapsigargin (Figure 3H),.

4. Discussion

As a stress protein residing mainly in the ER, TXNDC5 is induced under ER stress and is involved in the regulation of ER homeostasis (22). RA is characterized by genes involved in ER stress (23,24). However, the relative contribution of TXNDC5 to RA in response to ER stress remains unclear. RA FLSs are exposed to diverse conditions including hypoxia and pro-inflammatory cytokines, and these factors act as ER stressors to exacerbate inflammation as well (25). A point worth noting is that previous findings indicated that TXNDC5 in RA FLSs is sensitive to proinflammatory cytokines (IL-1, TNF- α , or IL-6) and hypoxia, increasing the likelihood that TXNDC5 may be functional in RA when ER stress occurs. The current study characterized the expression of TXNDC5 in RA under ER stress, and results further confirmed that TXNDC5 expression both in the cytoplasm and

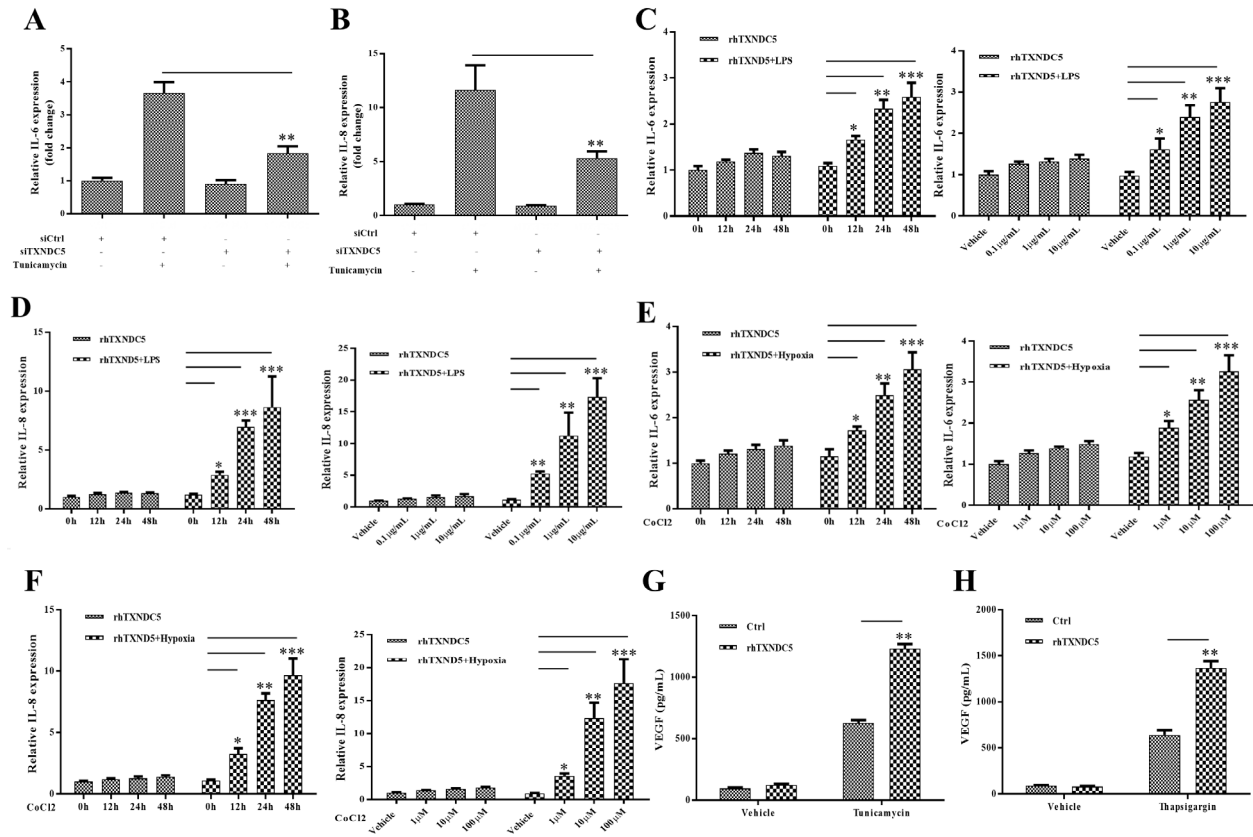


Figure 3. TXNDC5 modulates cytokine expression under ER stress. RA FLSs were exposed to tunicamycin when TXNDC5 siRNA was introduced. Then, IL-6 (A) and IL-8 (B) were analyzed with qRT-PCR. Each value represents the mean and S.D. of three independent experiments. IL-6 (C) and IL-8 (D) expression were evaluated with qRT-PCR after treatment with rhTXNDC5 and LPS at the indicated dosage or time period and normalized to GAPDH. In the presence of rhTXNDC5 and hypoxia, relative levels of IL-6 and IL-8 mRNA were evaluated with qRT-PCR (E and F). The secretion of VEGF (G and H) was measured with ELISA. The data shown are the mean \pm SD of experiments performed in triplicate. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

supernatant of RA FLSs is sensitive to an inducer of ER stress, such as thapsigargin and tunicamycin. Recently, the current authors found that silencing TXNDC5 attenuated the induction of IL6 and IL8 in the presence of proinflammatory factors, such as LPS and TNF- α (14). The current study provides evidence that silencing TXNDC5 in RA FLSs suppresses the stimulatory effects of ER stressors on inflammatory factors. These findings clearly corroborate TXNDC5's involvement in promoting inflammation under ER stress in RA.

A previous study indicated that TXNDC5 protects endothelial cells from stress-induced apoptosis (17). Similar effects were also observed in RA FLSs as evinced by both cell viability and inflammatory factor expression. TXNDC5 expression is higher in RA FLSs than in osteoarthritis (OA) FLSs (12), so the aforementioned finding may also explain why RA FLSs were more resistant to apoptosis than OA FLSs. Indeed, a growing body of evidence suggests that the signaling pathways in ER stress and inflammation are interconnected through various mechanisms, including the production of reactive oxygen species (ROS) (26), the release of calcium from the ER (27), the activation of nuclear transcription factor NF- κ B and the mitogen-

activated protein kinase (MAPK) known as JUN N-terminal kinase (JNK) (28), and the induction of the acute-phase response. A previous study by the current authors indicated that TXNDC5 directly interacts with heat shock cognate 70 protein (HSC70) to sequester it in the cytoplasm and that HSC70 activates NF- κ B signaling by destabilizing I κ B β protein (14). Importantly, RA has been found respond to TXNDC5 secretion by activating multiple inflammatory pathways and triggering the production of proinflammatory cytokines, suggesting that TXNDC5 is an important component of the micro-environment to mediate the pathological progression of RA. TXNDC5 may act as a mediator linking ER stress and the inflammatory response.

RA is the main source of many angiogenic factors, including VEGF, in the joints, and the level of VEGF has been proven to be highly correlated with RA disease activity (29). VEGF signaling is activated in response to ER stress, and activated VEGF signaling, in turn, buffers the levels of ER stress (30). A previous study by the current authors indicated that silencing TXNDC5 suppressed VEGF production in RA FLSs when stimulated with LPS and TNF- α . The current study

provides further evidence that rhTXNDC5 induces VEGF production during ER stress. These findings suggest that TXNDC5 may mediate angiogenesis under ER stress, and they also corroborate the close relationship between ER stress and angiogenesis during the progression of RA.

In summary, this study found that an increase in TXNDC5 as a cytoprotective response to ER stress may link ER stress and inflammation in RA. Specifically targeting TXNDC5 may be a potential treatment for RA.

Acknowledgements

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