CXCL10 and TRAIL Are Upregulated by TXNDC5 in Rheumatoid Arthritis Fibroblast-like Synoviocytes

Bing Xu, Jian Li, Changsun Wu, Chunyan Liu, Xinfeng Yan, and Xiaotian Chang

ABSTRACT. ***Objective.*** Thioredoxin domain containing 5 (TXNDC5) is highly expressed in synovial membranes of rheumatoid arthritis (RA). Our study aimed to investigate the pathogenic role of TXNDC5 in RA.

Methods. PCR arrays, CCK-8 assays, flow cytometry, and transwell migration assays were used to analyze cultured rheumatoid arthritis synovial fibroblasts (RASF).

Results. Increased CXCL10 and tumor necrosis factor-related apoptosis-inducing ligand levels were detected in RASF transfected with anti-TXNDC5 small interfering RNA (siRNA), and decreased expression was detected in RASF transfected with TXNDC5-expressing plasmids. Significantly attenuated RASF proliferation and migration, and increased RASF apoptosis, were observed in the siRNA-transfected RASF.

Conclusion. Downregulation of TXNDC5 could contribute to RASF antiangiogenic and proapoptotic features through the suppression of CXCL10 and TRAIL (tumor necrosis factor-related apoptosis-inducing ligand). (J Rheumatol First Release December 15 2017; doi:10.3899/jrheum.170170)

Key Indexing Terms:
TXNDC5 RHEUMATOID ARTHRITIS PATHWAY TRAIL CXCL10

Thioredoxin domain containing 5 (TXNDC5) is a member of the protein disulfide isomerase family[1,2]. In our previous study, we found that TXNDC5 expression was substantially increased in the blood, synovial fluid, and synovial tissues of rheumatoid arthritis (RA)[3,4]. Single-nucleotide polymorphism (SNP) in the genes encoding TXNDC5 are associated with increased susceptibility to RA[4]. TXNDC5 upregulates tumor necrosis factor (TNF)–α, interleukin (IL)-1α, IL-1β, and IL-17 in this disease[5]. Our current study aimed to investigate the involvement of the TXNDC5 regulatory pathway in RA.

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MATERIALS AND METHODS

Human tissue collection. Synovial tissue samples were collected from patients with RA (n = 13, all women, 28–78 yrs, mean age = 55 yrs) during knee-joint replacement surgery. All patients fulfilled the American College of Rheumatology criteria for the diagnosis of RA. We obtained all necessary consent from the patients involved in our study, including consent to participate in the study. Our study was approved by the Ethics Committee of Shandong Provincial Qianfoshan Hospital at Jinan, China (approval number: QYLL2015034).

Preparation of synovial fibroblasts. Rheumatoid arthritis synovial fibroblasts (RASF) were prepared based on the protocol described in our previous studies[4,5].

Inhibition of TXNDC5 expression with small interfering RNA (siRNA). SiRNA duplexes of the TXNDC5 gene (target sequence: 5′ AGG GCC CTA ACT AGA GTT CTA 3′) were designed and purchased from Qiagen. The cultured RASF samples (n = 8) were transfected with anti-TXNDC5 siRNA using HiPerFect transfection reagent (Qiagen). Parallel experiments were conducted with Allstars siRNA provided with the kit. Allstars siRNA has no inhibitory effect on gene expression and was used as the control siRNA.

TXNDC5-overexpressing plasmid construction and transfection. Total RNA was extracted from RASF and reverse-transcribed to first-strand cDNA (Qiagen). The full TXNDC5 coding sequence was amplified from the cDNA by real-time PCR. The PCR product was inserted into the pcDNA 3.1(+)-RFP expression vectors. The TXNDC5-expressing plasmid was transfected into the RASF (known as RA9-RA13) using GenMute siRNA transfection reagent (SignaGen).

Real-time PCR assay. Total RNA was isolated from RA1-RA13 synovial samples using an RNeasy Mini Kit (Qiagen). Real-time PCR was conducted as described in our previous studies[4,5]. The expression of each sample was normalized to the expression of GAPDH. The primer sequences are shown in Supplementary Table 1 (available with the online version of this article).

Western blotting analysis. Western blot analysis was conducted based on our previous studies[4,5]. The antibody against TXNDC5 was prepared by immunizing a rabbit with a recombinant fragment corresponding to amino acids 198-432 of human TXNDC5 (Abcam); the monoclonal antibody against CXCL10 was produced by immunizing a rabbit with a recombinant

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protein specific to human CXCL10 protein (Cell Signaling Technology); and the monoclonal antibody against TNFSF10 (tumor necrosis factor-related apoptosis-inducing ligand, or TRAIL) was produced by immunizing a rabbit with a synthetic peptide comprising the residues surrounding Lys60 within the extracellular region of human TRAIL (Cell Signaling Technology).

CCK-8 cell proliferation assay. RASF (n = 5) were seeded in 96-well culture plates. After the cells were incubated with anti-TXNDC5 siRNA, Cell Counting Kit-8 (CCK-8, Dojindo) reagent was added to each well and incubated for 4 h at 37°C. The absorbance was measured at 450 nm using a spectrophotometer (SpectraMax 190; Molecular Devices).

FITC-annexin V/PI apoptosis assay. Apoptosis was measured using an FITC-annexin V/PI apoptosis detection kit and flow cytometry (BioLegend). After transfection with anti-TXNDC5 siRNA, RASF (n = 5) were resuspended in Annexin V Binding Buffer and were then incubated with FITC Annexin V and propidium iodide solution. Cellular fluorescence was measured by a flow cytometer (FACSAria II, BD Biosciences).

Transwell migration assay. Transwell inserts (8.0-μm pore size) with polycarbonate filters (Costar Corning) were used to examine the effects of TXNDC5 on RASF cell migration. SiRNA-treated RASF (n = 5) in serum-free Dulbecco modified Eagle's medium were added to the upper compartment of the chamber. Following 12 h of incubation, the filter was immersed in methanol for 15 min and then treated with 0.25% crystal violet stain. The cells in the lower compartment were counted under a light microscope from 10 randomly selected visual fields.

PCR array analysis. We used the Signal Transduction PathwayFinder PCR Array, Inflammatory Response and Autoimmunity PCR Array, TNF Signaling Pathway PCR Array, and Angiogenesis PCR Array (Qiagen). PCR array analysis was performed in a ViiA7 DX (Life Technologies). Total RNA was isolated from RASF (n = 8) transfected with anti-TXNDC5 siRNA. The samples transfected with Allstars siRNA were used as controls. PCR array analysis was conducted based on our previous studies[6].

Statistical analysis. All statistical tests were performed using SPSS-19 software (IBM). The measured items and domain scores for the study groups were presented as the mean ± SD. The statistical significance of the differences was analyzed using a 2-tailed Student t test. In all cases, $p < 0.05$ was considered statistically significant.

RESULTS

Determining the effect of TXNDC5 expression on RASF proliferation, apoptosis, and migration. Following transfection with anti-TXNDC5 siRNA (n = 5), we detected significantly decreased TXNDC5 mRNA and protein levels by real-time PCR and Western blot analysis, respectively (Supplementary Figure 1, available with the online version of this article). CCK-8 assays demonstrated significantly decreased RASF proliferation in cells transfected with anti-TXNDC5 siRNA compared with cells transfected with Allstars siRNA and cells not transfected with siRNA (Figure 1A). Meanwhile, RASF transfected with anti-TXNDC5 siRNA (n = 5) showed a significant increase in apoptosis compared with Allstars siRNA-transfected RASF (Figure 1B). Additionally, anti-TXNDC5 siRNA-transfected RASF (n = 5) displayed a significantly decreased migratory capacity compared with Allstars siRNA-treated RASF (Figure 1C).

Elucidating the TXNDC5 pathway in RASF. We used a series of Qiagen PCR arrays to determine the involvement of the TXNDC5 pathway in RA pathogenesis. RASF (n = 8) were transfected with anti-TXNDC5 siRNA, and transfection efficiency was confirmed using real-time PCR (Supplementary Figure 2A, available with the online version of this article). RNA samples from different patients were pooled to conduct PCR arrays. We detected increased expression of C3AR1 (complement component 3a receptor 1), CCL5 (C-C motif chemokine ligand 5), CCL7 (C-C motif chemokine ligand 7), CCL8 (C-C motif chemokine ligand 8), CCR2 (C-C motif chemokine receptor 2), CXCL5 (C-X-C motif chemokine ligand 5), CXCL8 (C-X-C motif chemokine ligand 8), CXCL9 (C-X-C motif chemokine ligand 9), CXCL10 (C-X-C motif chemokine ligand 10), FLT1 (Fms related tyrosine kinase 1), PGLYRP1 (peptidoglycan recognition protein 1), TNFSF10 (tumor necrosis factor superfamily member 10/TNF-related apoptosis-inducing ligand, or TRAIL), and TNFSF13B [TNF (ligand) superfamily member 13b]. We also noticed decreased CCL11 (C-C motif chemokine ligand 11), IL-10 (interleukin 10), and TNFRSF10D expression (Figure 2 and Supplementary Table 2).

The PCR array results were verified by real-time PCR. RASF (RA1-RA8, n = 8) transfected with anti-TXNDC5 siRNA displayed significantly decreased TXNDC5 transcription compared with control cells (Supplementary Figure 2A, available with the online version of this article). C3AR1, CCL7, CCL8, CXCL10, PGLYRP1, TNFSF10, and TNFSF13B showed a significant increase in transcription; CCL11, IL10, and TNFRSF10D showed a significant decrease in transcription (Supplementary Figure 3). We also transfected RASF with TXNDC5-expressing plasmids. RASF (n = 5) transfected with TXNDC5-expressing plasmids showed significantly increased TXNDC5 expression compared with the controls (Supplementary Figure 2B). C3AR1, CXCL10, TNFSF10, and TNFSF13B showed a significant decrease in transcription; CCL11, IL-10, and TNFRSF10D showed a significant increase in transcription (Supplementary Figure 3).

We performed Western blotting to measure CXCL10 and TNFSF10 protein expression in RASF transfected with anti-TXNDC5 siRNA or TXNDC5-expressing plasmids. Compared with Allstars siRNA-transfected RASF, anti-TXNDC5 siRNA-transfected samples showed significantly increased 11-kDa CXCL10 protein expression and significantly increased 29-kDa TNFSF10 protein expression. Compared with RASF transfected with blank vectors, RASF samples transfected with TXNDC5-expressing plasmids showed significantly decreased 11-kDa CXCL10 protein expression and significantly decreased 29-kDa TNFSF10 protein expression (Figure 3).

DISCUSSION

In our present study, we used a series of PCR arrays to determine the involvement of TXNDC5 in the pathogenesis of RA in synovial tissues. RNA samples from different patients were pooled to conduct PCR arrays. The results of these arrays were verified with real-time PCR and Western blotting in RASF with downregulated or upregulated

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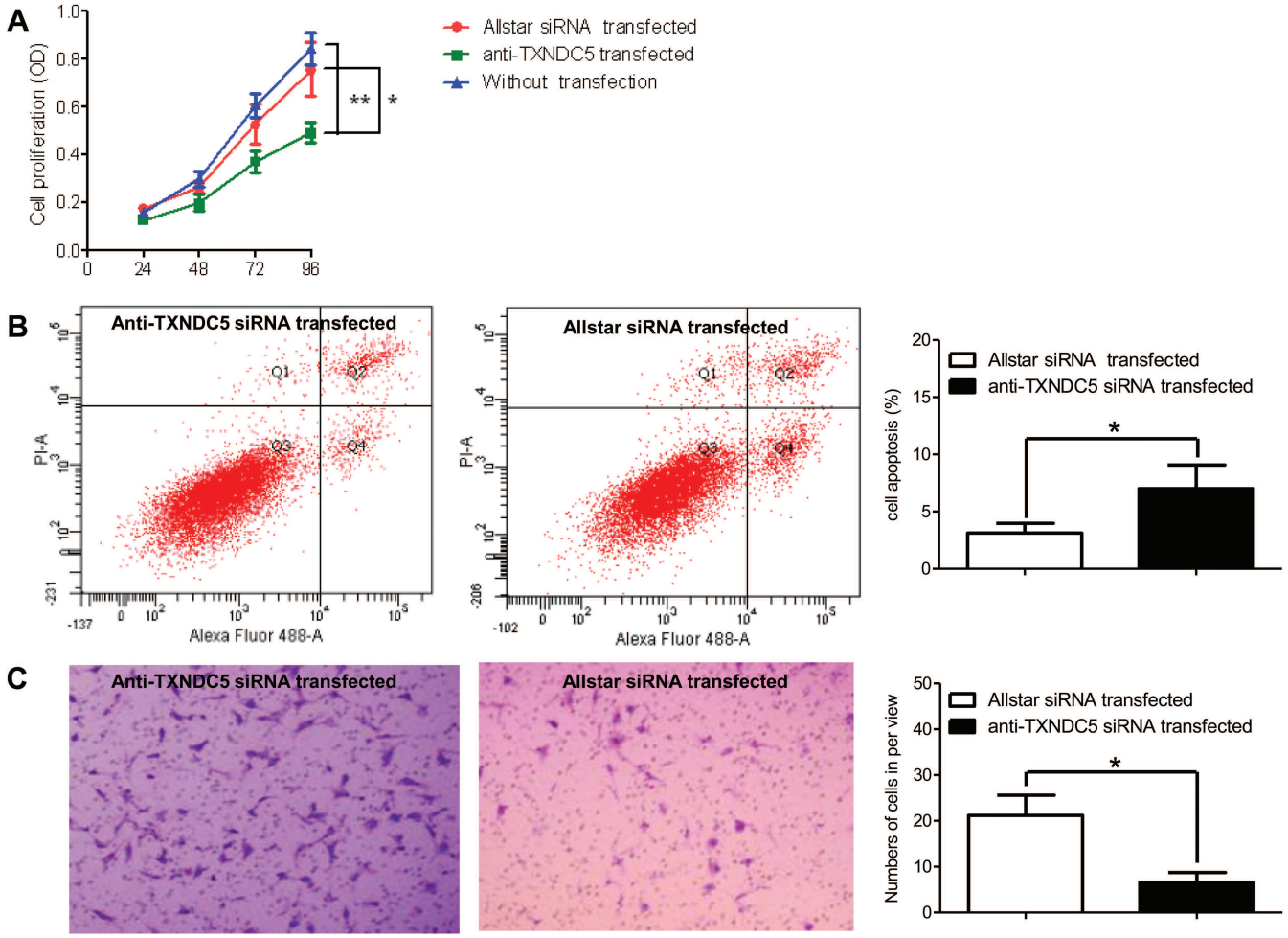

Figure 1. Effects of TXNDC5 siRNA on RASF proliferation, apoptosis, and migration. (A) RASF were either left untransfected or were transfected with anti-TXNDC5 siRNA or Allstars control siRNA. CCK-8 assays were performed to measure RASF cell proliferation (n = 5). (B) RASF were transfected with either anti-TXNDC5 siRNA or Allstars control siRNA. Flow cytometry was performed to measure RASF apoptosis (n = 5). (C) RASF were transfected as in (B), and Transwell assays performed to measure RASF migration (n = 5). Original magnification × 10. The data are presented as the mean ± SD; *p < 0.05; **p < 0.01. RASF: rheumatoid arthritis synovial fibroblasts; siRNA: small interfering RNA; TXNDC5: thioredoxin domain containing 5; OD: optical density; PI-A: propidium iodide axis.

TXNDC5 expression. Finally, we detected significantly decreased CXCL10 and TNFSF10 mRNA, and protein levels in RASF transfected with TXNDC5-expressing plasmids, whereas we observed significantly increased CXCL10 and TNFSF10 mRNA and protein levels in RASF transfected with anti-TXNDC5 siRNA. These results strongly suggest that high levels of TXNDC5 in RA synovial tissues suppress CXCL10 and TNFSF10 expression.

Many studies have demonstrated the importance of TRAIL and CXCL10 in RA. For example, dendritic cells that were pulsed with collagen II and transfected with an adenovirus-based vector expressing the TRAIL gene suppressed collagen-induced arthritis in rats[7]; TRAIL-deficient (-/-) mice showed defective thymocyte apoptosis and accelerated autoimmunity[8]; chronic TRAIL blockade in mice exacerbated autoimmune arthritis and led to substantial synovial cell and arthritogenic lymphocyte hyperproliferation[9]; and intraarticular TRAIL gene transfer induced apoptosis in cells within the synovial lining, reducing leukocytic infiltration and stimulating new matrix synthesis by cartilage in a rabbit model of arthritis[10]. Many studies have reported that CXCL10 is a potent inhibitor of angiogenesis, because CXCL10 can profoundly inhibit basic fibroblast growth factor-induced neovascularization and suppress endothelial cell differentiation into tubular capillary structures[11,12]. We recently reported that TXNDC5 overexpression promotes human umbilical vein endothelial cells to form tube structures, an experimental marker for angiogenesis[6]. Abnormal RASF proliferation and apoptosis, as well as angiogenesis, are main pathogenic features of RA synovial tissues. Our present study observed a significant decrease in RASF cell proliferation and migration, as well as a significant increase in RASF apoptosis following the transfection of anti-TXNDC5 siRNA. The results of our study and those of

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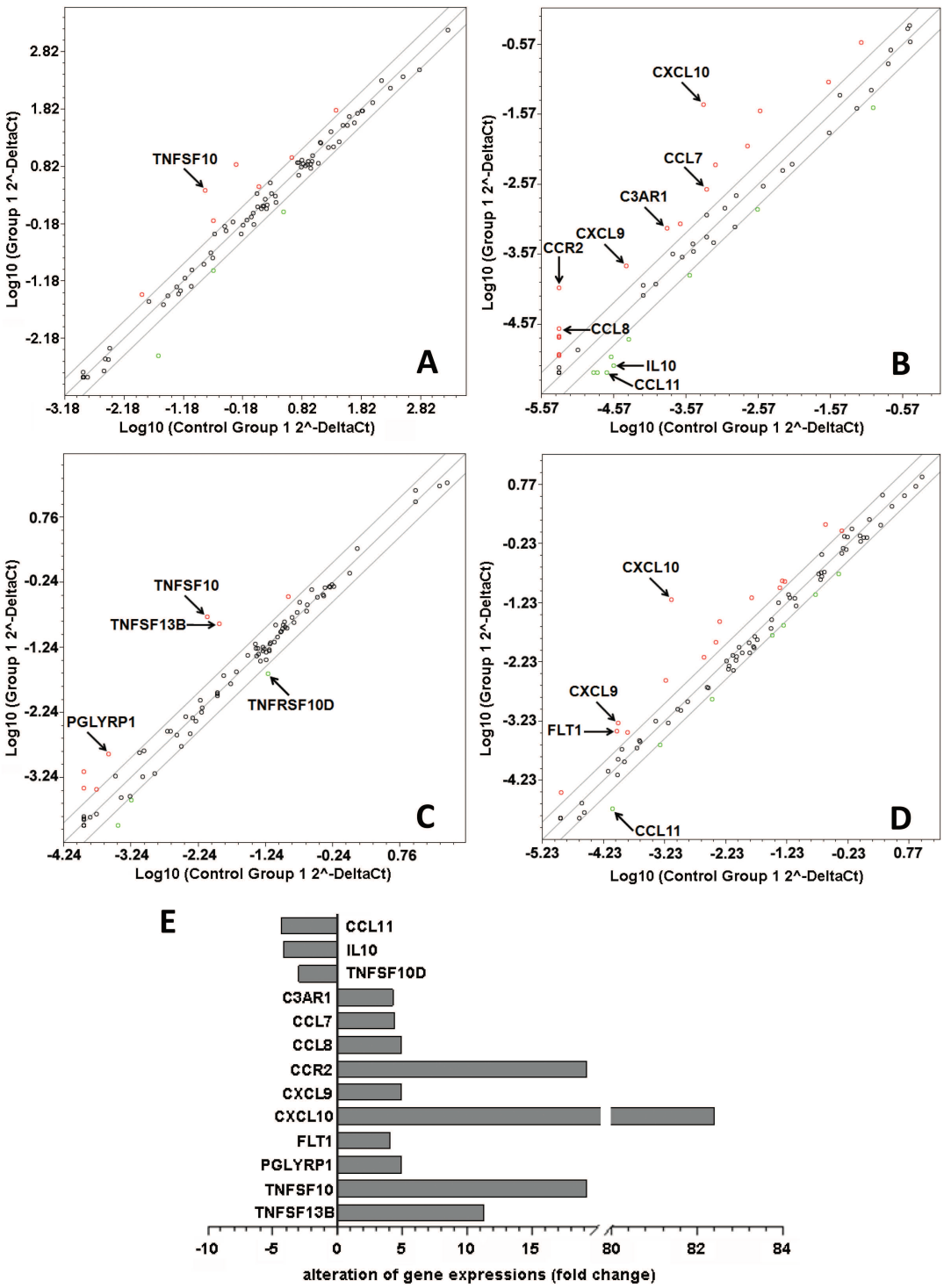

Figure 2. PCR array detection of significant changes in gene expression in anti-TXNDC5 siRNA-transfected RASF, using (A) Signal Transduction PathwayFinder PCR Array, (B) Inflammatory Response and Autoimmunity PCR Array, (C) TNF Signaling Pathway PCR Array, and (D) Angiogenesis PCR Array. Fold-change values > 1 indicate a positive change or upregulation. Fold-change values < 1 indicate a negative change or downregulation. Fold-change values > 2 are indicated in red, and fold-change values < 0.5 are indicated in green. (E) The above PCR array results are depicted. RASF: rheumatoid arthritis synovial fibroblasts; siRNA: small interfering RNA; TNF: tumor necrosis factor; TXNDC5: thioredoxin domain containing 5.

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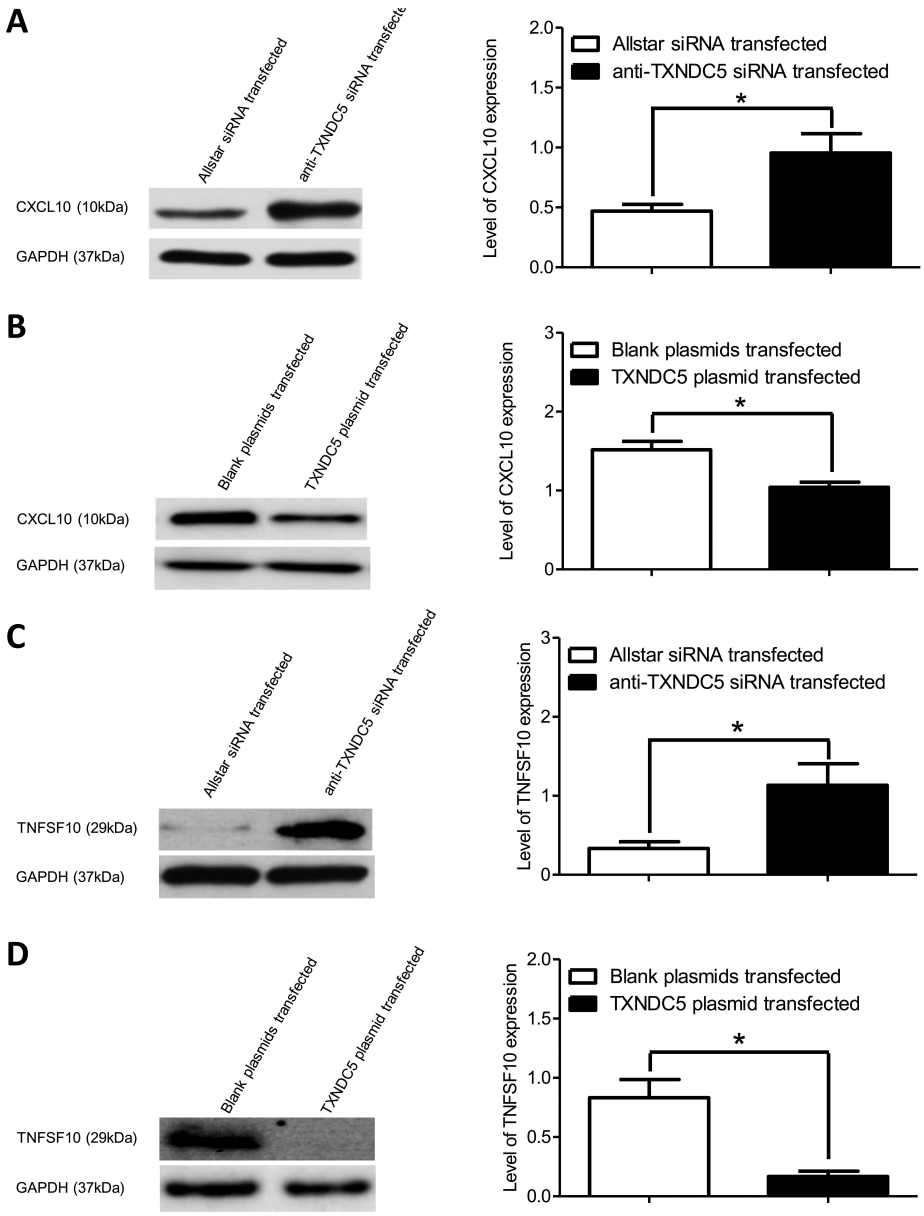

Figure 3. Western blot analysis of CXCL10 and TNFSF10 expression in RASF. (A) CXCL10 expression was detected in RASF after anti-TXNDC5 siRNA or Allstars siRNA transfection (left panel) and normalized to GAPDH expression (n = 8; right panel). (B) CXCL10 was detected in RASF after TXNDC5-expressing plasmids or the blank plasmids transfection (left panel) and normalized to GAPDH expression (n = 5; right panel). (C) TNFSF10 expression was detected in RASF transfected with anti-TXNDC5 siRNA or Allstars siRNA (left panel) and normalized to GAPDH expression (n = 8; right panel). (D) TNFSF10 was detected in RASF transfected with TXNDC5-expressing plasmids or blank plasmids (left panel) and normalized to GAPDH expression (n = 5, right panel). RASF: rheumatoid arthritis synovial fibroblasts; siRNA: small interfering RNA; TXNDC5: thioredoxin domain containing 5; TNFSF10 (tumor necrosis factor-related apoptosis-inducing ligand, or TRAIL).

others suggest that the downregulation of TXNDC5 could contribute to RASF antiangiogenic and proapoptotic features through suppression of CXCL10 and TRAIL.

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Supplementary material accompanies the online version of this article.

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