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RESEARCH ARTICLE

miR-573 is a negative regulator in the pathogenesis of rheumatoid arthritis

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Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by abnormal inflammation, angiogenesis, and cartilage destruction. Our previous study demonstrated an increased expression of thioredoxin domain containing 5 (TXNDC5) in the synovial tissues of RA, and its overexpression was implicated in RA pathology. Although TXNDC5 variation is linked to genetic susceptibility to RA, the regulation of its abnormal expression has not been well defined. Here, we show that TXNDC5 is directly targeted by microRNA (miR)-573, and TXNDC5, in turn, mediates the suppressive effect of miR-573 on the invasion of synovial fibroblasts of RA (RASFs). miR-573 overexpression suppressed the expression of interleukin 6 (IL-6) and cyclooxygenase 2 in RASFs, as well as the production of tumor necrosis factor-alpha and interleukin-1 beta by activated THP-1 cells in response to lipopolysaccharide (LPS) stimulation. Moreover, treatment with conditioned medium of RASFs transfected with miR-573 mimic inhibited the angiogenic ability of human umbilical vein endothelial cells (HUVECs). Of note, epidermal growth factor receptor and Toll-like receptor 2 were validated as new direct targets of miR-573, and mediate the regulation of miR-573 on IL-6 production as well as the angiogenesis of HUVECs. In addition, exogenous miR-573 expression suppressed the activation of mitogen-activated protein kinase (MAPK), signal transducer and activator of transcription 3, and phosphatidylinositol-3 kinase/activate protein kinase B in RASFs in response to LPS. Indeed, MAPK signaling was essential to ensure the function of miR-573. Taken together, our study points toward the protective roles of miR-573 in the pathological process of RA and suggests a potential target in the treatment of RA.
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INTRODUCTION

As a systemic inflammatory disease, rheumatoid arthritis (RA) is characterized by synovial inflammation and pronounced synovial hyperplasia, which produces several inflammatory cytokines and proteases to destroy the cartilage and bone.[1] In our previous study, proteomics experiments revealed increased thioredoxin domain containing 5 (TXNDC5) expression in the synovial tissues of RA patients.[2] Later studies showed that TXNDC5 levels are also higher in the synovial fluids and blood of RA patients, indicating its potential function in RA.[3] TXNDC5 belongs to the protein disulfide isomerase family and functions as a chaperone of endoplasmic reticulum.[4] Using a transgenic approach, we found that TXNDC5-overexpressing mice exhibit more susceptibility and severity to collagen-induced arthritis. Additionally, hypoxia-induced TXNDC5 expression promotes inflammation in RA through inducing cytokine production, cellular proliferation, and migration of fibroblasts derived from RA patients.[5] Hence, identification of the regulators modulating TXNDC5 expression and elucidation of the detailed mechanisms of regulation would pinpoint the mechanisms of RA pathology.

MicroRNAs (miRNAs) function through posttranscriptional regulation toward their targets. Emerging data have identified a close relationship between miRNA and the pathological progression of RA.[6] These data prompted us to look for miRNA directly targeting TXNDC5 and controlling inflammation in RA. In this study, miRNA prediction was utilized,

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identifying miR-573 as a miRNA that could bind to the 3′-untranslated region (UTR) of TXNDC5, thereby down-regulating its expression. We further evaluated the biological functions of miR-573 as well as its other target genes in synovial fibroblasts of RA (RASFs). The data suggest that restoring miR-573 expression might be useful for clinical management of RA.

MATERIALS AND METHODS

Cell culture

Human RASFs were isolated from the synovial tissues from RA ($n = 40$, 26 female, 27–72 years old, mean 52) and osteoarthritis (OA) patients ($n = 10$, 6 female, 42–76 years old, mean 62) at the time of knee joint arthroscopic synovectomy as described previously,[5] after informed consents were obtained. The diagnosis conformed to the revised criteria of the American College of Rheumatology.[7] RASFs were cultured as previously described and the cells, as a homogeneous population negative for CD16 were determined by fluorescence-activated cell sorting analysis, between passages three and nine were applied for further experiments.[5] RASFs (10^6 cells) were stimulated with lipopolysaccharides (LPS, 1 mg mL^{-1}, Sigma, St. Louis, MO, USA) or $CoCl_2$ (10 μM, Sigma, St. Louis, MO, USA) for a 6 h or 24 h. Then, total RNA and protein were extracted as described before.[6] HEK293T and THP-1 were purchased from the American Type Culture Collection and cultured according to the protocols. The Ethics Committee of Shandong Provincial Qianfoshan Hospital approved this study.

miRNA target analysis

MiRanda (http://www.microrna.org/microrna/home.do) was first applied to select the miRNA targeting the 3′-UTR of TXNDC5 mRNA. To predict the target genes of miR-573, TargetScan 6.1 (http://www.targetscan.org/) was further used.

Invasion and tube formation assays

The invasion assay was performed as described before.[5] To examine the effect of miR-573 on tube formation, the tube formation assay was performed.[8] Briefly, Matrigel (10 mg mL^{-1}, BD Biosciences, San Jose, CA, USA) was plated in 96-well culture plates and allowed to polymerise at 37 °C in 5% CO_2 humidified for 30 min. After treatment with conditioned medium of miR-573 mimic transfected RASFs for 24 h, human umbilical vein endothelial cells (HUVECs; 2×10^4 cells mL^{-1}) were trypsinized, resuspended, and added to each chamber in sterile medium (supplemented with 5% fetal bovine serum, 100 U mL^{-1} penicillin, and 80 U mL^{-1} streptomycin) with vascular endothelial growth factor (VEGF; 50 ng mL^{-1}, AF-100, Peprotech, Rocky Hill, NJ, USA), then incubated for 6 h at 37 °C in 5% CO_2. After that, the capillary-like tube formation of each well in the culture plates was photographed. All experiments were done in triplicate.

Western blotting

After various treatments, cells were collected for protein extraction in ice-cold lysis buffer. Western blot analysis was performed as previously described.[9] After probed with the TXNDC5 (ab13820, 1:1000, Abcam, Cambridge, MA, USA), epidermal growth factor receptor (EGFR; sc-03, 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), Toll-like receptor 2 (TLR2; ab108998, 1:1000, Abcam), matrix metalloproteinase 3 (MMP3; sc21732, 1:1000, Santa Cruz Biotechnology), pAKT (S473, 1:1000, Cell Signaling, Boston, MA, USA), AKT (C67E7, 1:1000, Cell Signaling), pERK1/2 mitogen-activated protein kinase (MAPK; T202/Y204, 1:1000, Cell Signaling), ERK1/2 MAPK(4370, 1:1000, Cell Signaling), p-STAT3 (Y705, 1:1000, Cell Signaling) and signal transducer and activator of transcription 3 (STAT3; 79D3, 1:1000, Cell Signaling) antibodies, membranes were visualized by enhanced chemiluminescence (Amersham, Piscataway, NJ, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; sc47724, 1:1000, Santa Cruz Biotechnology) was applied as the loading control.

Real time RT-PCR

Total RNA from RASFs and THP-1 was reverse transcripted as described before.[10] The primers for each gene were as listed in Supplementary Table 1. GAPDH was used as internal loading control. Three independent experiments were completed and each reaction was performed in triplicate. The primer sequences of miR-573 (HmiR-QP0627) were synthesized by GeneCopoeia, Inc. and its expression was measured using SYBR Primescript miRNA RT-PCR kit (TAKARA, Otsu, Japan). The universal small nuclear RNA U6 (HmiR-QP9001, GeneCopoeia, Inc., Rockville, MD, USA) was used as an endogenous control for miRNAs. Relative expression was calculated using the comparative threshold cycle method.[10] The specific product of the amplification was further confirmed by melting analysis.

Luciferase reporter constructs

The 3′-UTR of TXNDC5, EGFR, and TLR2 were amplified from HEK393T by PCR using primers as shown in Supplementary Table 1. After the confirmation by DNA sequencing, they were cloned into the pmirGLO dual luciferase reporter vector using SacI and XhoI restriction sites. Their mutant products were also generated on its recognition site (seed sequences) as shown in Figures 1a and 4a by site mutation according to the manufacture's instruction (D0206, Beyotime, Beijing, China).

Transfections and luciferase assay

Transient transfections of RASFs plated in 24-well plates (2×10^5 cells per well) with miR-573 mimic and inhibitor or their respective controls were performed using HiPerFect Transfection Reagent from QIAGEN as previously described.[5] All assays were performed at 48 h post-transfection.

Transfection of HEK293 cells with reporter constructs and miR-573 mimic or its inhibitor (100 nM) was performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 48 h, cells were collected and lysed with passive lysis buffer (Promega, Carlsbad, CA, USA). Then, the firefly luciferase (f-luc) and Renilla luciferase (r-luc) activities were assayed using the dual-luciferase reporter assay system (Promega) and a luminometer (Glomax, Promega). The relative reporter activity was obtained by normalization to r-luc activity.

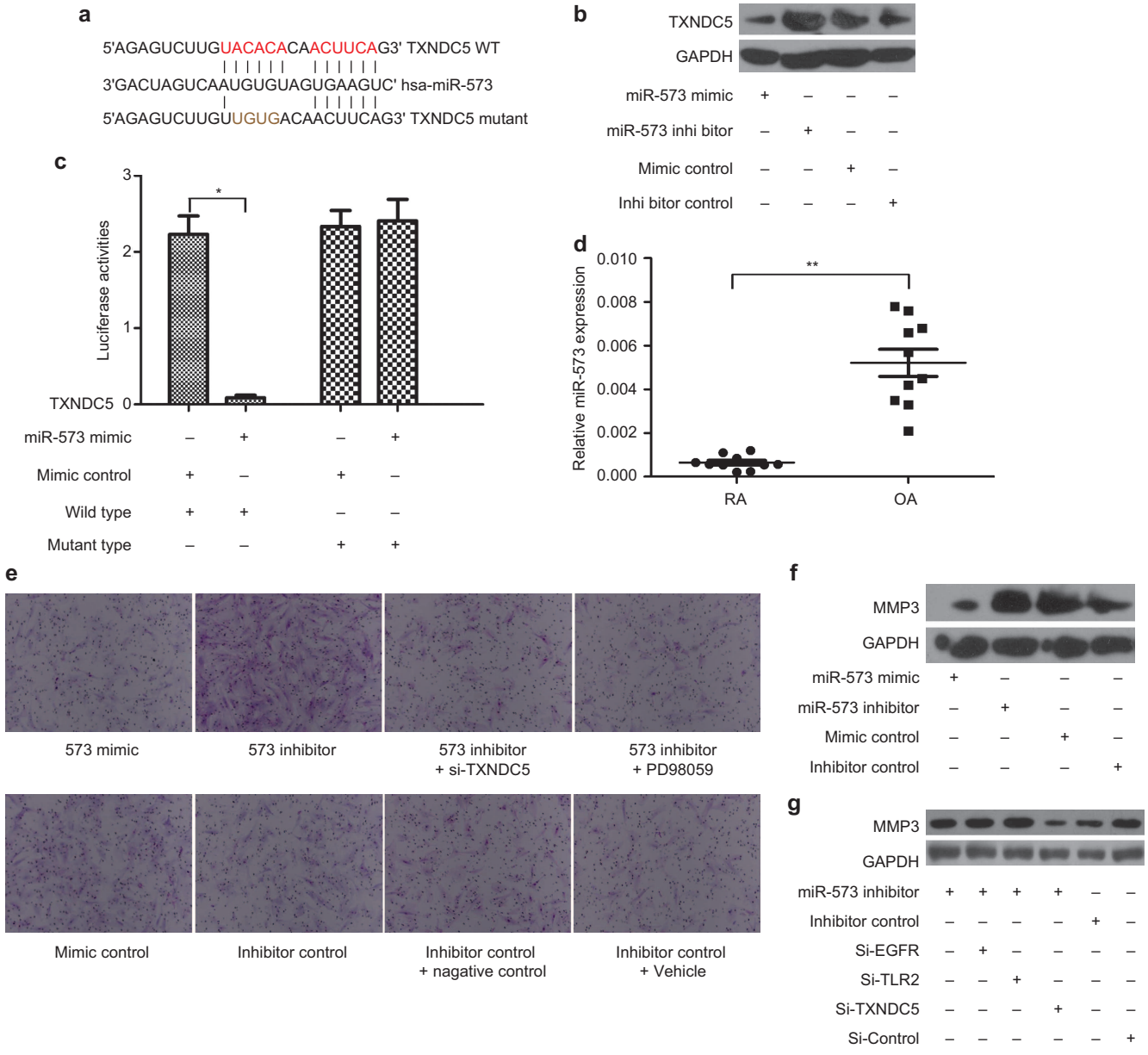

Figure 1 MiR-573 regulates the invasion of RASFs by directly targeting TXNDC5. (**a**) Sequence alignment of miR-573 and its target sites, as well as the mutant sites (indicate as brown) in the 3′-UTR of TXNDC5 mRNA. (**b**) miR-573 regulates TXNDC5 in RASFs at translational level. (**c**) miR-573 directly targets the 3′-UTR sequences of human TXNDC5. HEK293T cells were transiently co-transfected with Luciferase reporter constructs with wild-type or mutant-type (for miR-573-binding sites), together with miR-573 mimic. Then, the luciferase activities were measured and normalized to their Renilla luciferase control activity. (**d**) qRT-PCR validation of miR-573 expression in synovial tissues of RA and OA. (**e**) Transwell migration assays were applied to determine the effect of indicated treatments on the invasive ability of RASFs. MMP3 expression was detected by Western blot after transfection with miR-573 mimic/inhibitor (**f**) or the indicated siRNA (**g**) at the same time. All data are representative of three independent experiments. $*P<0.05$, $**P<0.01$.

Enzyme-linked immunosorbent assay (ELISA)

RASFs and THP-1 were treated with LPS at different concentrations following transfection with miR-573 mimic or its inhibitor. Then, the serum-free conditioned media was collected and centrifuged at 6000 rpm for 10 min to remove particulates at 4 °C. Tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), interleukin 6 (IL-6), and VEGF levels in culture supernatants were measured using ELISA kits provided by R&D Systems (Minneapolis, MD, USA).

Statistical analysis

Statistical analyses were carried out using the Statistical Package for Social Sciences, version 19.0. Statistical evaluation was performed by means of the Mann–Whitney *U* test. Differences were considered statistically significant at $P<0.05$.

RESULTS

Validation of miR-573 as a modulator of TXNDC5

Our laboratory reported that TXNDC5 is elevated in the synovial tissues and fluids from RA patients.[5] As miRNAs

could negatively regulate the expression of targets largely through binding to their 3′-UTR, miRanda was applied and, interestingly, miR-573 displayed double perfect base pairing of six nucleotides (Figure 1a).

To determine whether miR-573 regulates TXNDC5, we measured the effects of miR-573 on the expression of TXNDC5 in RASFs by Western blot. In contrast to mimic or inhibitor controls, the TXNDC5 protein level decreased in RASFs after transfection with miR-573 mimic (Figure 1b), but increased with the miR-573 inhibitor (Figure 1b). This evidence indicates that miR-573 may modulate TXNDC5 expression at the translational level by binding to the 3′-UTR of TXNDC5 mRNA. Further, we conducted a luciferase reporter assay, in which the 3′-UTR of TXNDC5 mRNA was cloned into the luciferase construct pmirGLO, in HEK293T cells. As shown in Figure 1c, after transfection with the luciferase construct TXNDC5-WT, the luciferase activity in TXNDC5-WT-transfected cells decreased by more than 40% in miR-573 mimic-cotransfected cells compared with that in mimic control-cotransfected cells. However, mutation of its putative binding sites, which disrupts the predicted binding site for miR-573 in the 3′-UTR of TXNDC5, abrogated luciferase responsiveness to the miR-573 mimic transfection. Next, we detected its expression levels in the synovial tissues from RA and OA, and miR-573 decreased significantly in RA but not in OA (Figure 1d), which further indicates its potential involvement in the pathogenesis of RA.

miR-573 regulates invasion of RASFs through TXNDC5

Next, we applied miR-573 mimic or inhibitor transfection for gain-of-function experiments to study its biological activities in RASFs. Using MTT and BrdU methods, no visible changes of the viability and proliferation of RASFs were detected after transfection with miR-573 mimic (100 nM) and its inhibitor (100 nM; data not shown). Next, an invasion assay was conducted using a transwell apparatus and, interestingly, showed that the percentage of cells invading was significantly less in the miR-573 mimic transfection group when compared with mimic control (Figure 1e). In contrast, an increased percentage of cells invading were found after transfection with miR-573 inhibitor (Figure 1e).

A major hallmark of RASFs is the production of matrix degrading enzymes, which contribute to cartilage degradation.[11] To gain deeper insight into the role of miR-573 in the invasion of RASFs, we also investigated the effects of miR-573 on MMP expression and, compared with their corresponding controls, MMP3 expression (Figure 1f), but not MMP1 and MMP13, in RASFs was suppressed after miR-573 mimic transfection but increased in the miR-573 inhibitor group. This may partly explain how miR-573 regulated the invasive phenotype of RASFs.

Taken with our previous study that hypoxia-induced TXNDC5 expression led to abnormal invasion of RASFs,[5] we further detected the effect of silencing TXNDC5 on the pro-metastasis effects of miR-573 inhibitor. Of note, silencing TXNDC5 could almost attenuate the effect of miR-573 inhibitor on the invasion (Figure 1e) and MMP3 expression (Figure 1g), which indicates miR-573 regulates the invasion of RASFs in a TXNDC5-dependent way.

miR-573 alleviates inflammation in LPS-activated RASFs

RASFs are characterized by secretion of various pro-inflammatory factors, including interleukin 1 alpha, IL-6, and cyclooxygenase 2 (COX2), in the inflamed synovium.[12] Next, we examined the regulation of miR-573 on their expression or release from RASFs. The results showed that delivery of miR-573 mimic for 48 h suppressed the expression of IL-6 (Figure 2a) and COX2 (Figure 2b) at the mRNA level. However, introduction of miR-573 inhibitor resulted in a dramatic increase compared with inhibitor control in RASFs (Figure 2a and b). IL-6 and COX2, with widespread activities, are central mediators of inflammation in RA and important therapeutic targets.[12,13] We also found that the secretion of IL-6 was suppressed in LPS-activated RASFs transfected with miR-573 mimic (Figure 2c). Taken together, these data demonstrate that miR-573 may protect from inflammation by inhibiting expression and secretion of inflammation mediators in RASFs.

miR-573 represses TNF-α and IL-1β expression and release by LPS-activated THP-1 cells

As RASFs do not secrete either TNF-α or IL-1β,[14] we next determined whether miR-573 could regulate the production of these cytokines by macrophages, which also play important roles in RA. We first treated the macrophage cell line THP-1 with miR-573 mimic for 24 h and then activated with LPS for 24 h. Compared with the control, we observed that transfection of miR-573 mimic repressed the expression (Figure 2d and e) and release (Figure 2f and g) of TNF-α and IL-1β in response to LPS, which suggests that miR-573 exerts its anti-inflammation activity by counteracting the functions of inflammatory cells in the microenvironment of RA.

miR-573 inhibits tube formation of HUVEC cells

Abnormal angiogenesis is also a hallmark of RA.[15] As such, to investigate the effects of miR-573 on angiogenic properties of endothelial cells, the tube network formation on Matrigel of conditioned medium-treated HUVECs was examined. The results showed that, after treatment with the medium of miR-573 mimic-transfected RASFs, the ability of HUVECs to form tube networks were impaired compared to mimic control (Figure 2h). Further study demonstrated that miR-573 inhibited the secretion of VEGF in RASFs (Figure 2i).

miR-573 modulates epidermal growth factor receptor (EGFR) and Toll-like receptor 2 (TLR2) expression in LPS-activated RASFs

Based on the above data, we wondered whether miR-573 regulates the production of inflammation mediators through TXNDC5. After co-transfection with miR-573 inhibitor and short interfering RNA (siRNA) targeting TXNDC5, no visible changes in IL-6 expression (Figure 3a) and secretion (Figure 3b) in RASFs, as well as TNF-α (Figure 3c) or IL-1β (Figure 3d)

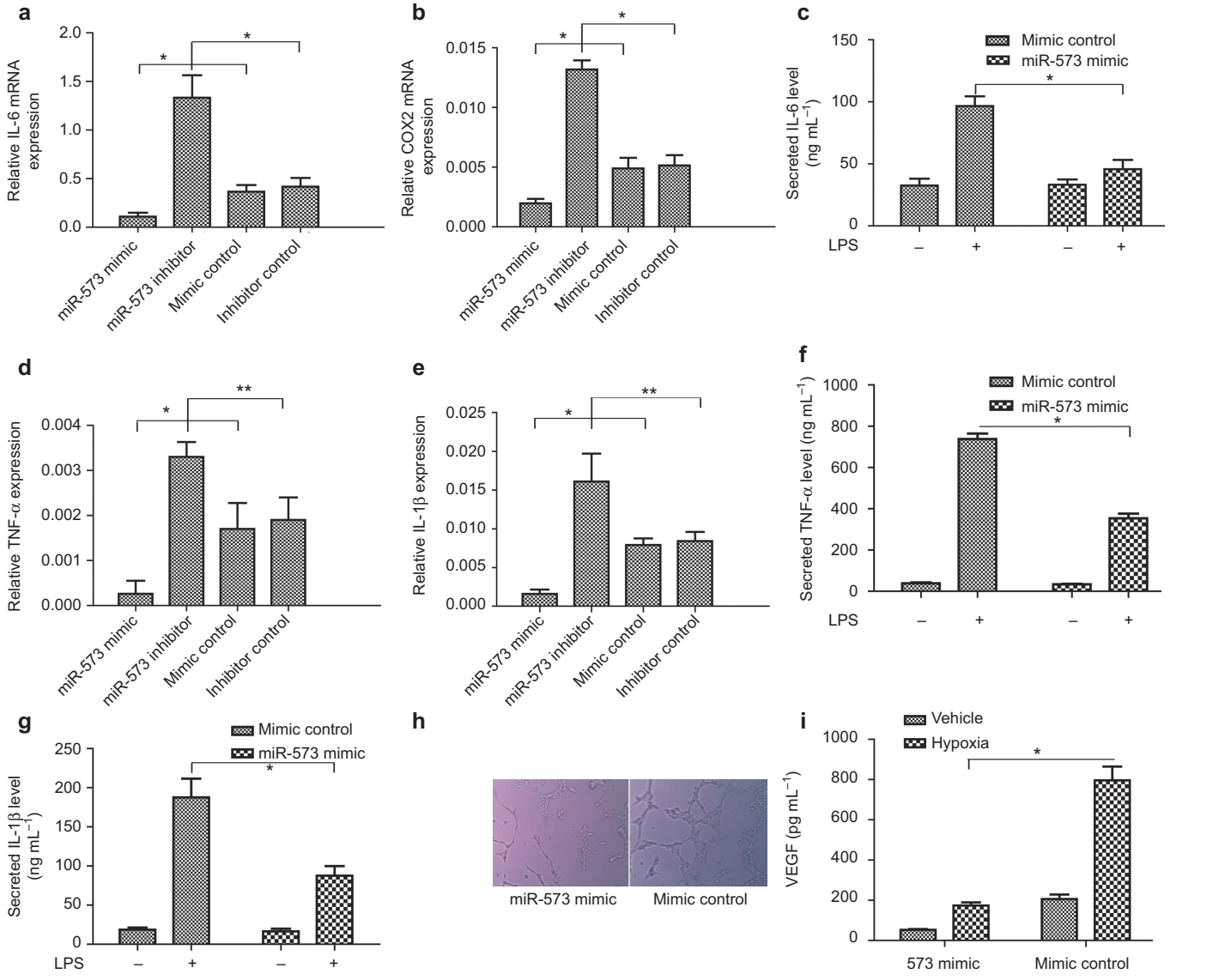

Figure 2 Anti-inflammation potential conferred by miR-573. Expression of IL-6 (**a**) and COX2 (**b**) mRNA in RASFs was detected by qRT-PCR. (**c**) Determination of IL-6 secretion by ELISA in LPS-activated RASFs. Effects of miR-573 on the expression of TNF-α (**d**) and IL-1β (**e**) at mRNA levels in THP-1 as determined by qRT-PCR. ELISA was used to determine the secretion of TNF-α (**f**) and IL-1β (**g**) in LPS-activated THP-1. (**h**) Tube formation assay was applied to determine the angiogenic ability of HUVEVs after treatment with conditioned medium of miR-573 transfected RASFs. (**i**) VEGF release was determined by ELISA in culture supernatants of RASFs after stimulation with hypoxia inducer, $CoCl_2$, stimulation. Data are expressed as the mean of triplicate samples ±SD and are representative of three independent experiments. $*P < 0.05$, $**P < 0.01$.

secretion in THP-1 cells, were detected, which prompted us to find other targets of miR-573 using different miRNA target gene prediction algorithms. In this list, we found TLR2 (Figure 3e) and EGFR (Figure 3e), whose down-regulation by miR-573 might lead to abnormal biological activities of RASFs. We next tested whether miR-573 regulated their protein levels in RASFs. As can be seen in Figure 3f, the Western blotting results indicated that transfection with miR-573 mimic led to a global decrease in their expression from RASFs, whereas its inhibitor transfection showed the opposite tendency. To verify whether the predicted binding sites for miR-573 within EGFR and TLR2 mRNA were functional (Figure 3e), we generated luciferase reporter constructs that contained the f-luc gene fused to the 3′-UTR of EGFR and TLR2. We also generated luciferase reporter constructs in which we inserted a mutant version of EGFR and TLR2 3′-UTR to disrupt the predicted binding site for miR-573. Therefore, we cotransfected the pmirGLO constructs with control or miR-573 mimic. In the presence of miR-573 mimic, we observed a decrease of the EGFR and TLR2 3′-UTR-controlled luciferase sensor (Figure 3g and h) but not of the luciferase sensor fused to the mutated EGFR and TLR2 3′-UTR (Figure 3g and h). Altogether, these data suggest that, in addition to TXNDC5, EGFR, and TLR2 are important targets of miR-573 in RA.

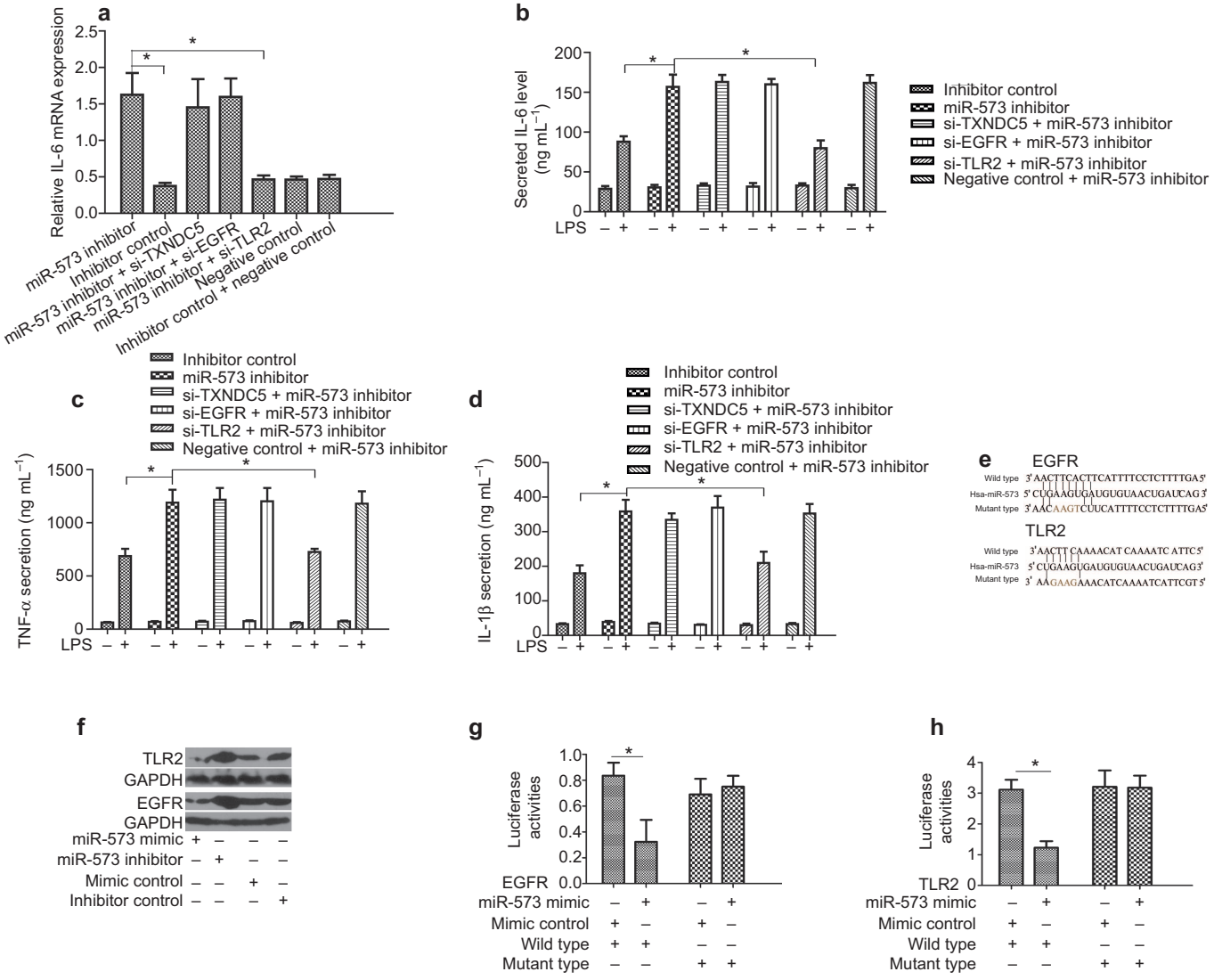

Figure 3 EGFR and TLR2 are the direct targets of miR-573 in RA. (**a**) Expression of IL-6 in RASFs was detected by qRT-PCR. (**b**) ELISA was used to quantify the secretion of IL-6 in RASFs after various treatments. The TNF-α (**c**) and IL-1β (**d**) release was measured by ELISA. (**e**) Analysis of the EGFR and TLR2 3′-UTR by targets can revealed putative miR-573-binding sites. Mutant types (indicated as brown) were also constructed. (**f**) Western blot was applied to detect the protein changes of EGFR and TLR2 in RASFs cells after transfection with miR-573 mimic, its inhibitor or their controls at the concentration of 100 nM. The effects of miR-573 on the EGFR (**g**) and TLR2 (**h**) 3′-UTR was confirmed by luciferase assay in HEK293T cells after the indicated treatments. Data represent the mean ± SD of at least three independent experiments. $*P < 0.01$.

Mimicking miR-573-mediated effects on the regulation of cytokine production and angiogenesis in RASFs by silencing of TLR-2 and EGFR

Having demonstrated that TLR-2 and EGFR are also direct targets of miR-573 in RASFs, we addressed whether silencing of either of them could mimic the effects of miR-573 on cytokine expression and angiogenesis. Therefore, we cotransfected RASFs with miR-573 inhibitor and siRNA targeting TLR2 or EGFR at the same time. Importantly, after transfection with miR-573 inhibitor, silencing TLR2 in RASFs could block the induction of IL-6 expression (Figure 3a) and secretion (Figure 3b) in RASFs, as well as TNF-α (Figure 3c) or IL-1β (Figure 3d) in THP-1 cells in response to LPS. In addition, miR-573 inhibitor transfection promoted angiogenesis of HUVECs (Figure 4a), as well as the VEGF secretion of RASFs (Figure 4b), but all these effects were blocked by co-delivery of siRNA targeting EGFR (Figure 4a and b).

miR-573 negatively regulates the activity of inflammation signaling pathways in RASFs

Dysfunctional intracellular signaling induced by pro-inflammatory stimulus is shown to be responsible for aberrant activity in RA.[16] We hypothesized that miR-573 might influence the activity of inflammation signaling pathways in RASFs. As expected, the phosphorylation of ERK1/2, MAPK, phosphatidylinositol-3 kinase/activate protein kinase B (PI3K/Akt), and STAT3 were activated after stimulation of RASFs with LPS in a time-dependent manner (Figure 4c, left panel), but transfection of miR-573

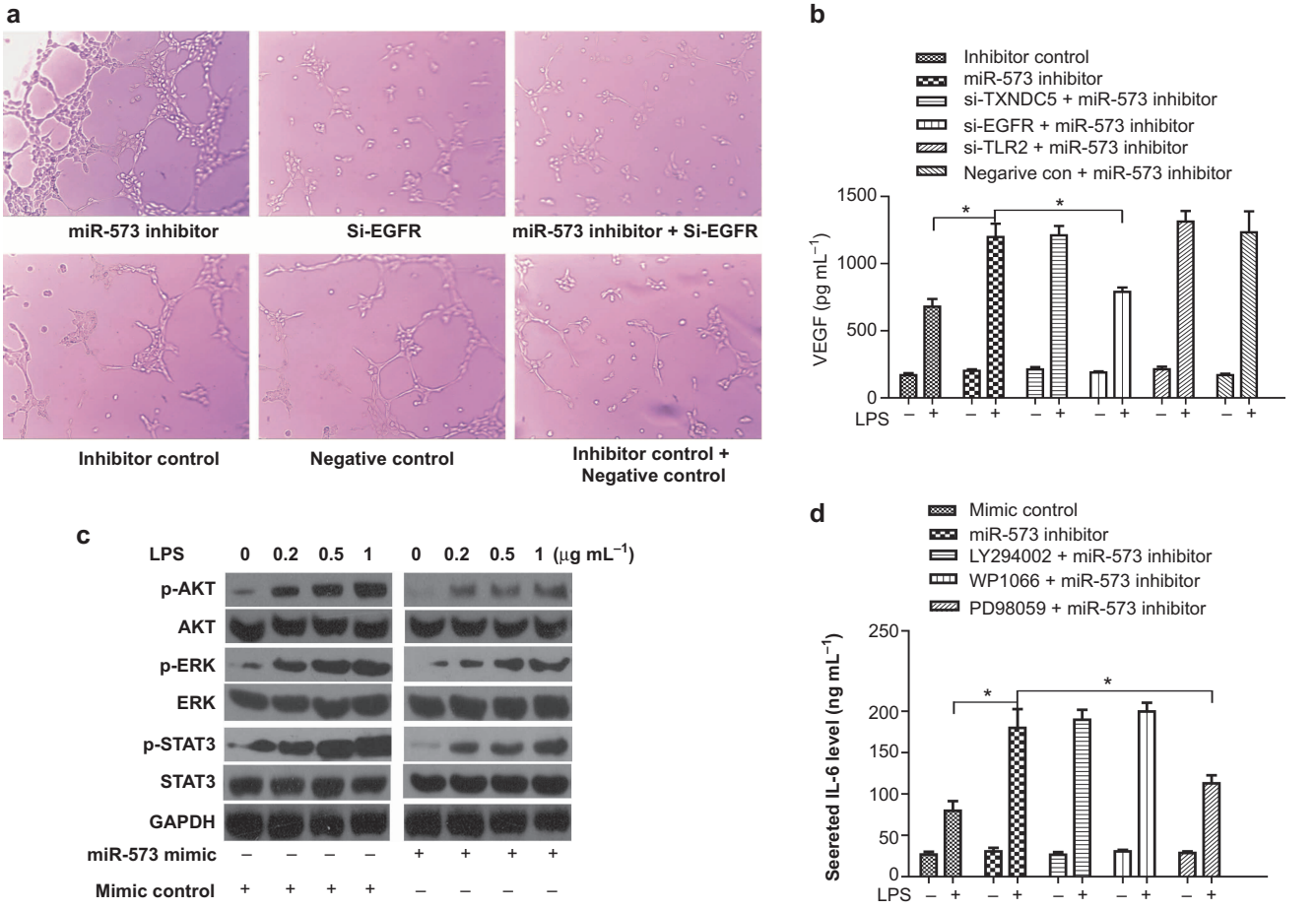

Figure 4 TLR2 and EGFR mediate the function of miR-573. Representative pictures of tube formation after treatment HUVECs with conditioned medium of RASFs are reported in **a**. (**b**) Secretion of VEGF in the supernatant of RASFs was analyzed by ELISA after the indicated treatments. (**c**) After miR-573 mimic transfection, activation of various signaling in RASFs in response to LPS was detected by Western blot. (**d**) ELISA was performed to detect the IL-6 release of RASFs after treatment with inhibitors of various signaling pathways.

mimic significantly suppressed the phosphorylation of these protein kinases (Figure 4c, right panel). In addition, the total expression levels of these proteins were not affected upon transfection with miR-573 inhibitor or mimic. Intriguingly, treatment with the MAPK inhibitor, PD98059, also blocked this alteration of invasion (Figure 1e) and IL-6 (Figure 4d) caused by transfection with miR-573 inhibitor, whereas treatments with the inhibitors of PI3K/Akt and STAT3 signaling had no such effects. All together, these results demonstrate that miR-573 is implicated in the negative regulation of LPS-induced immune response in RASFs.

Validation of the correlation of miR-573 with TXNDC5, TLR2, and EGFR in RA

We stimulated RASF and THP-1 with LPS at different concentrations and measured mRNA expression by qRT-PCR, and found that LPS treatment could efficiently suppress miR-573 expression (Figure 5a) but induce the expression of TXNDC5 (Figure 5b), EGFR (Figure 5c), and TLR2 (Figure 5d) levels in a dose-dependent manner. Further study showed that miR-573 mimic transfection could attenuate the induction of LPS on the expression of TXNDC5, EGFR, and TLR2 (Figure 5e). Consistent with these findings, basal expression levels of miR-573 in synovial fibroblasts were inversely correlated with basal TXNDC5 ($rs = 0.702$, $P = 0.002$, Figure 6f), TLR-2 ($rs = 0.737$, $P = 0.001$, Figure 6g), and EGFR levels ($rs = 0.769$, $P = 0.003$, Figure 6h). These findings further support important roles for miR-573, TXNDC5, EGFR, and TLR2 in the pathogenesis of RA.

DISCUSSION

Our previous study identified the increased expression of TXNDC5 in the synovial tissues and fluids from RA patients, and the *in vivo* and *in vitro* studies demonstrated that it is closely related with the pathogenesis of RA. In recent years, it has become clear that miRNAs are dysregulated in RA and contribute to autoimmunity and joint destruction.[17] As such, this study was aimed at identifying whether miRNAs might be involved in the control of TXNDC5 expression.

miR-573 has been reported to exhibit decreased expression in various tumors, and reintroduction with its mimic in tumor

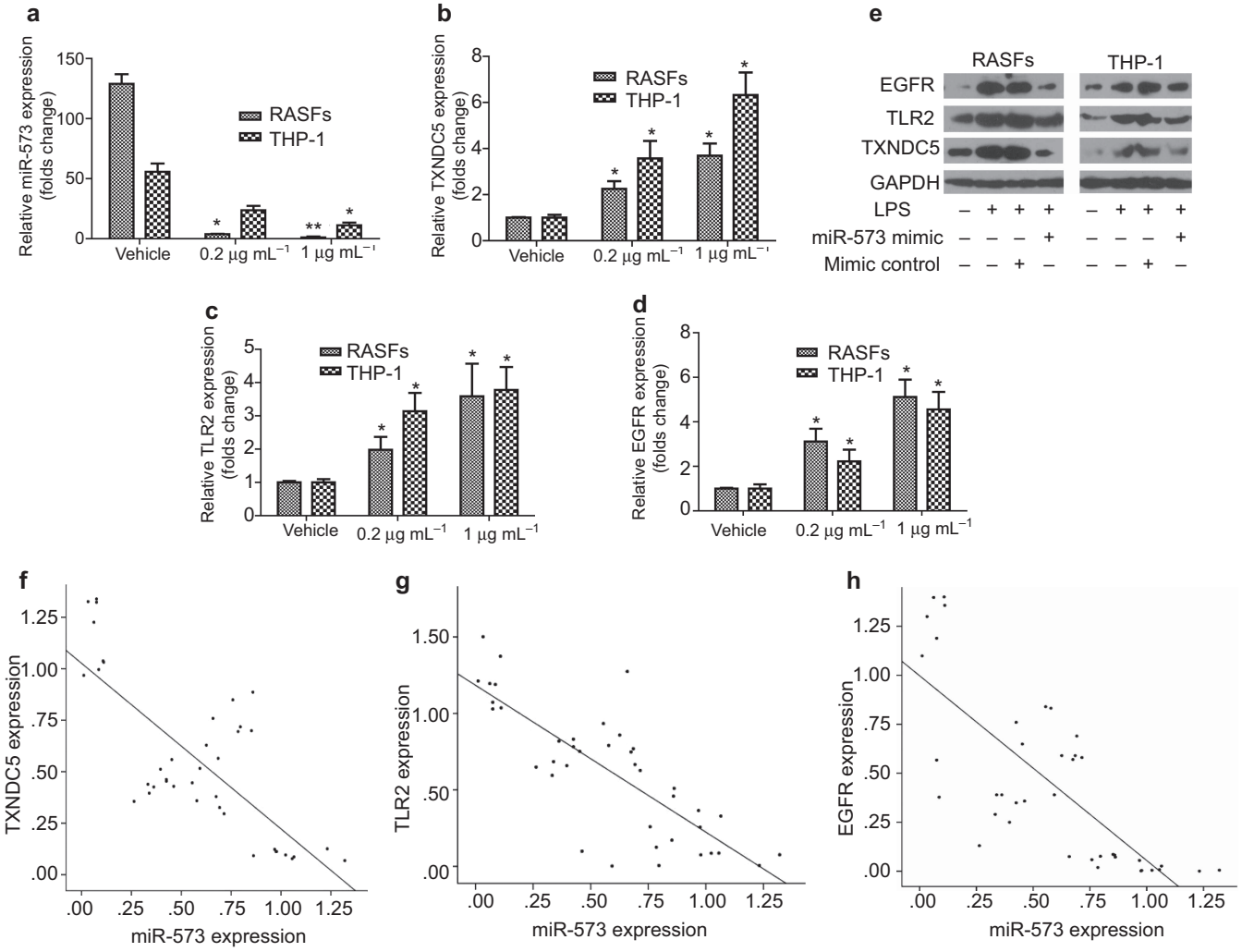

Figure 5 Association of miR-573 with TXNDC5, TLR2, and EGFR in RA. (**a**) After stimulation with LPS for 6h, expression levels of miR-573 in RASFs and THP-1 were determined by qRT-PCR. Results were normalized to small nuclear RNA U6 as folds change. Expression levels of TXNDC5 (**b**), TLR2 (**c**), and EGFR (**d**) in the RASFs and THP-1, 24 h after stimulation with LPS, were determined by qRT-PCR. LPS *vs.* Vehicle, $*P < 0.05$, $**P < 0.01$. (**e**) Western blotting was performed to detect the expression of TXNDC5, TLR2, and EGFR in RASFs and THP-1 after the indicated treatments. The correlation between the basal expression of miR-573 and TXNDC5 ($r_S = 0.702$, $P = 0.002$, f), TLR2 ($r_S = 0.737$, $P = 0.001$, g), or EGFR ($r_S = 0.769$, $P = 0.003$, h) in RASFs was determined by Spearman's rank correlation test, respectively. Data are expressed as the mean of triplicate samples ±SD and are representative of three independent experiments.

cells inhibits the onset and progression of melanoma.[18] In this study, we found that decreased miR-573 expression in the synovial tissues of RA may contribute to the abnormal TXNDC5 expression as evidenced by Western blot and luciferase activity assay. Our previous study reported that silencing TXNDC5 could inhibit the hypoxia-induced invasion of RASFs. As expected, the invasive ability was also hindered by miR-573 overexpression in RASFs, but silencing TXNDC5 abrogated the pro-invasive effect of miR-573 inhibitor treatment, indicating the essential mediation of TXNDC5 to the effect of miR-573 on the invasion of RASFs. Furthermore, the expression of MMP-3, which was one of the major MMPs involved in invasion and cartilage destruction in RA,[19] was regulated by miR-573 in RASFs through a TXNDC5-dependent manner, which further confirmed the protective role of miR-573 during the progression of RA.

RASFs are implicated in the inflammatory response essentially by synthesizing cytokines, chemokines and proangiogenic factors.[20] Among them, IL-6 is a major cytokine implicated in RA and a therapeutic target[21] and was recently shown to be targeted by miRNAs such as miR-365 and miR-203.[22,23] Further, COX2 is highly inducible by growth factors, LPS, and cytokines in certain cell types involved in inflammatory processes, e.g., fibroblasts and macrophages, and selective COX2 inhibitors are being used as anti-inflammatory agents to treat patients with RA.[24] Our study also demonstrated that miR-573 negatively regulates the synthesis of IL-6 and COX2 and leads to a strong reduction in IL-6 release in LPS-activated RASFs. Moreover, since transfection of

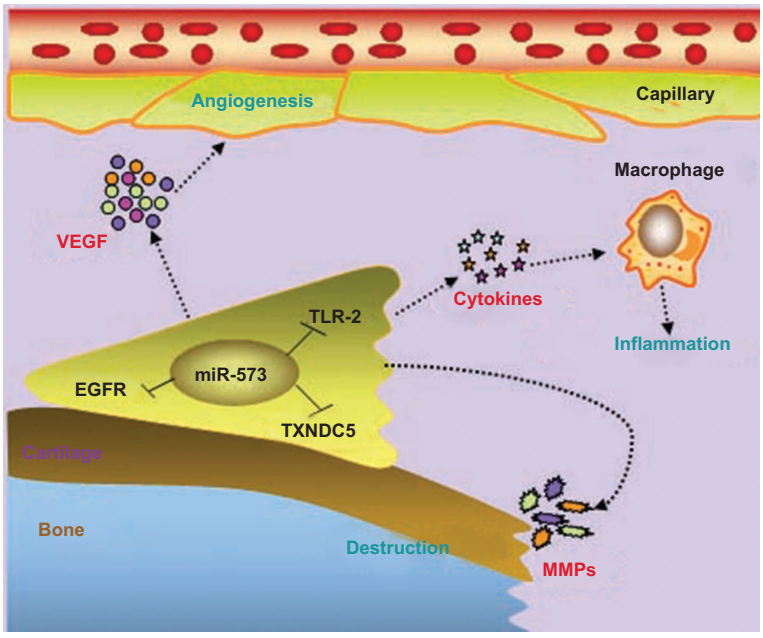

Figure 6 Schematic model of the functions of miR-573 in the pathogenesis of RA.

miR-573 mimic in macrophages inhibited the release of TNF-α induced by LPS, these data indicate that miR-573 played a global role in anti-inflammatory response by regulating the expression or release of inflammation mediators in RA.

LPS could promote pro-inflammatory cytokine secretion of RASFs and macrophages by activating TLR2/4.[25] We found that LPS stimulation led to decreased miR-573 expression, with a concomitant increase in TXNDC5 by an unidentified mechanism. Such a decrease was also observed for other miRNA, e.g., let-7i, miR-125b, and miR-98, in response to LPS treatment.[26–28] This phenomenon shows a proinflammatory phenotype as demonstrated by the increase in TLR2 expression, which could exacerbate inflammation by promoting secretion of various cytokines (IL-6 and IL-8) and MMPs release.[29] In particular, TLR2 was confirmed as a direct target of miR-573, and silencing TLR2, but not TXNDC5, could duplicate the miR-573 transfection, as shown strikingly by experiments involving IL-6, TNF-α, and IL-1β, supporting the existence of TLR-2 dependent manner during miR-573 functioned to negatively regulate inflammation in RA.

In addition to TXNDC5 and TLR2, we identified EGFR as another target of miR-573 by various validation experiments. Increasing evidence shows that EGFR contributes to the pathogenesis of RA by modifying the pathological environment, such as cytokine production or angiogenensis, and inhibition of EGFR tyrosine kinase activity ameliorates collagen-induced arthritis.[30,31] Of interest, treatment with conditioned medium of miR-573 mimic transfected RASFs could inhibit angiogenic ability of HUVECs, indicating that miR-573 also mediated the interaction between RASFs and its surrounding cells. In this study, silencing EGFR did not reduce the production of cytokines or invasion of RASFs, but down-regulated VEGF secretion in the conditioned medium, thereby inhibiting the pro-angiogeneic ability of HUVECs after concurrent transfection with miR-573 inhibitor. All the above further confirms that miRNAs function by regulating different targets, resulting in a finely balanced activation pattern that becomes disturbed under pathologic conditions such as RA. Besides, TXNDC5, TLR2, and EGFR were all reported to be able to activate PI3K/Akt, ERK1/2, MAPK, and STAT3 signaling.[16,32] Our results revealed that miR-573 could attenuate the activities of PI3K/Akt, ERK1/2, MAPK, and STAT3 in response to LPS in RASFs. Further, the ERK1/2-MAPK pathway is essential for its function, as demonstrated by the fact that its inhibitor (PD98059) treatment could block the pro-metastatic and inflammation effects of miR-573 inhibitor treatment, which suggests that the activity repression of inflammatory signaling may be another indirect way that miR-573 protects against RA.

The relationship between miR-573 and its targets was also confirmed in that LPS treatment of RASFs led to a decreased expression of miR-573 but increased the expressions of TXNDC5, TLR2, and EGFR in a miR-573-dependent way. More important, inverse correlations were noted in this study by characterizing their expression in the synovial tissues from RA. All these support the "coordinating complexity" in the pathological mechanism of RA and elicit our interest to study further the regulation of miR-573.

Taken together, our data point toward miR-573 as a protective regulator of inflammation in synovial tissues in RA by controlling TXNDC5, TLR2, and EGFR (Figure 6). Although the absence of a miR-573 gene in mouse species limits our ability to further validate its pathological function in RA *in vivo*, this study offers a better understanding of the complex nature of miRNA regulatory interactions with multiple targets, which is

bound to prove important for identifying future targets and developing therapeutic strategies in RA.

COMPETING INTERESTS

The authors declare no competing financial interests.

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