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RESEARCH ARTICLE

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Th17 cell-derived *miR-155-5p* modulates interleukin-17 and suppressor of cytokines signaling 1 expression during the progression of systemic sclerosis

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Abstract

Background: *miR-155-5p* is associated with autoimmune diseases. T helper 17 (Th17) cells, interleukin (IL)-17, and suppressor of cytokines signaling 1 (SOCS1) have important roles in the pathogenesis of systemic sclerosis (SSc). The purpose of this study was to explore the role of *miR-155-5p* in the regulation of IL-17 and SOCS1 expression in Th17 cells and the subsequent effect on SSc disease progression.

Methods: Th17 cells were isolated from peripheral blood mononuclear cells of SSc patients and healthy controls (HCs). RT-qPCR and western blotting were used to examine the expression patterns of *miR-155-5p*, IL-17, and SOCS1. Luciferase reporter assays were performed to confirm SOCS1 as a target of *miR-155-5p*. RNA pull-down assays were performed to detect the interaction of IL-17 and SOCS1 with *miR-155-5p*. *In situ* hybridization was performed to analyze the co-expression pattern of *miR-155-5p* and IL17A in Th17 cells.

Results: The levels of Th17 cell-derived *miR-155-5p* were significantly up-regulated in SSc patients compared with HCs, and its levels were negatively correlated with SOCS1 levels. Meanwhile, *miR-155-5p* positively regulated IL-17 expression levels in Th17 cells isolated from SSc patients as the disease progressed. Using pmirGLO vectors, *SOCS1* was confirmed as a target of *miR-155-5p*. The binding status of *IL-17* and *SOCS1* to *miR-155-5p* was related to SSc progression. An increase in the co-localization of *miR-155-5p* and IL-17 was associated with greater SSc progression.

Conclusions: IL-17 and SOCS1 expression modulated by Th17 cell-derived *miR-155-5p* are critical for SSc progression, which may provide novel insights into the pathogenesis of SSc.

KEYWORDS
interleukin-17, *miR-155-5p*, suppressor of cytokines signaling 1, systemic sclerosis, T helper 17 cell

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1 | INTRODUCTION

Systemic sclerosis (SSc) is an autoimmune disease characterized by thickening and fibrosis of the skin and the involvement of internal organs, including the lungs, kidneys, and digestive tract. The overall pooled incidence rate of SSc is 1.4 per 100,000 person-years,[1] and the 10 years cumulative survival rate has been reported to be approximately 71.7% and 87.8% in French and Chinese cohorts, respectively.[2,3] Immunological abnormalities, vascular disease, abnormal connective tissue metabolism, and the interaction between these factors are thought to be the main mechanisms of SSc.[4,5] Of these mechanisms, immunological abnormalities, especially the cell-mediated immune response, play a leading role in the development of SSc and associated fibrosis.[6]

T helper 17 (Th17) cells are a subset of $CD4^+$ pro-inflammatory T helper cells that secrete the cytokine, interleukin 17 (IL-17). Initial $CD4^+$ T cells differentiate into Th17 cells and secrete cytokines, such as IL-17, IL-2l, and IL-23, after stimulation by an antigen and co-induction by transforming growth factor-β (TGF-β) and IL-6. Th17 cells mediate chronic inflammation and autoimmune diseases.[7] Micro-RNAs (miRNAs) are small endogenous non-coding RNAs that function in RNA silencing and the post-transcriptional regulation of gene expression. They participate in the regulation of various biological processes, including the promotion of immune cell differentiation and maturation, the production of antibodies, and the release of inflammatory mediators to participate in inflammatory signaling pathways.[8] The abnormal expression of miRNAs is related to many human diseases, including inflammation and autoimmune diseases. Recent evidence suggests that *miR-155* plays an important role in immune regulation and the maintenance of immune system balance, especially by regulating T cells. Previous studies have found that *miR-155* mediates the inflammatory response by regulating the proliferation, differentiation, and development of Th17 cells and the secretion of cytokines by Th17 cells.[9] Mature *miR-155* can be processed from either the 5′ or 3′ end of the pre-miRNA to form *miR-155-5p* and *miR-155-3p*, of which *miR-155-5p* is the more abundant and functionally dominant form.[10] Recent findings suggest a role of *miR-155-5p* in valvular damage and inflammation in certain autoimmune diseases, such as autoimmune encephalomyelitis and rheumatic heart disease.[11,12]

Suppressor of cytokines signaling 1 (SOCS1) is an important negative feedback factor that regulates signal transduction in Th17 cells. The role of SOCS1 in Th17 cells has also been confirmed using an SOCS1 mimic (Tkip) that inhibits the IL-6-induced differentiation of Th17 cells and the secretion of IL-17.[13] Therefore, an analysis of SOCS1, IL-17, and *miR-155* levels in the Th17 cells of SSc patients is warranted to elucidate the role of Th17 cells in the pathogenesis of SSc. However, recent studies of the role of *miR-155* have used peripheral blood mononuclear cells (PBMCs) or $CD4^+$ cells from patients with SSc,[14–16] which may result in an inaccurate assessment of the disease. In addition, no study has investigated the regulatory effect of Th17 cell-derived *miR-155-5p* on the downstream regulator, SOCS1, and IL-17 in the pathogenesis of SSc. Therefore, the role of *miR-155-5p* and related gene expression patterns and their relationship to Th17 cells during the development of SSc are worth investigating.

In the present study, we sought to investigate the direct involvement of SOCS1, IL-17, and *miR-155-5p* in the pathogenesis of SSc by isolating Th17 cells from patients with different SSc statuses and analyzing the expression patterns of SOCS1, IL-17, and *miR-155-5p*. Moreover, we aimed to explore the underlying mechanisms whereby Th17 cell-derived *miR-155-5p* affects the development of SSc.

2 | MATERIALS AND METHODS

2.1 | Human subjects

Anticoagulated blood samples were obtained from 23 SSc patients who were diagnosed according to the 2013 American College of Rheumatology/European Alliance of Associations for Rheumatology criteria[17] and from 12 age- and sex-matched healthy controls (HCs) who visited the Department of Rheumatism Immunity of the Nanyang Central Hospital and the Nanyang Traditional Chinese Medicine Hospital from March, 2018 to July, 2019. The revised European Scleroderma Study Group/European Scleroderma Trials and Research Group activity score[18,19] was used to define disease progression in SSc patients. Patients scoring ≤3 and >3 (0 represents no disease and 10 represents maximum disease severity) were classified as early and late SSc groups, respectively. The characteristics of the enrolled patients with SSc are shown in Table 1. Informed consent was obtained from all patients and healthy subjects involved in the study.

2.2 | Th17 cell isolation and culture conditions

PBMCs were isolated from anticoagulated blood samples by density-gradient centrifugation through Ficoll-Paque PLUS (GE Healthcare Life Sciences,). The EasySep™ Human Th17 Cell Enrichment Kit II (Stemcell Technologies Inc.,) was used to isolate Th17 cells according to the manufacturer's instructions. Briefly, PBMCs were harvested from the interface and their concentration was adjusted to 1×10^8 cells/ml. $CCR6^+$ cells were isolated using antibody complexes and EasySep™ Releasable RapidSpheres™ (Stemcell Technologies Inc.). The bound magnetic particles were then removed from the EasySep™-isolated $CCR6^+$ cells, and unwanted non-$CD4^+$ T cells, $CD45RA^+$ cells, and $CXCR3^+$ cells were removed using EasySep™ Dextran RapidSpheres™. The final isolated fraction was enriched for IL-17-producing Th17 cells.

The isolated Th17 cells were cultivated in ImmunoCult-XF T Cell Exp Medium (Stemcell Technologies Inc.) with penicillin-streptomycin at 37°C and 5% CO_2. At day 0, Th17 cells were stimulated by adding ImmunoCult™ Human CD3/CD28 T Cell Activator

TABLE 1 Characteristics of enrolled patients with SSc

	Early SSc ($n = 10$)	Late SSc ($n = 13$)	HCs ($n = 12$)
Female/male (*n*)	9/1	11/2	10/2
Age (years)	45.6 ± 10.1	51.0 ± 13.9	50.2 ± 10.6
Disease duration (year)	1.5 (0.5–3)	5 (4–15)	–
Raynaud's phenomenon (*n*)	10	13	–
Active digital ulcers	3	9	–
mRSS	7 ± 5	22 ± 4	–
Lung fibrosis (*n*)	1	9	–
Kidney involvement (*n*)	1	8	–
Gastro-intestinal involvement (n)	3	7	–
Antinuclear antibodies (ANA) + (*n*)	10	13	–
Anti-Scl–70 antibody + (*n*)	9	12	–

Abbreviation: *mRSS, modified Rodnan skin scores.

(Stemcell Technologies Inc.) at a final concentration of 1 μg/ml for each antibody and 10 U/ml IL-2. Cells were washed on day 2 and split as needed in the presence of IL-2 for future use.

2.3 | Flow cytometric assay

To determine the proportion of Th17 cells, aliquots of PBMCs were incubated for 4 h with 4 μl of BD Leukocyte Activation Cocktail containing BD GolgiPlug (BD Biosciences, Franklin Lakes,) in an incubator at 37°C. Surface staining was performed using an Alexa Fluor®488-conjugated mouse anti-human CD4 antibody (clone RPA-T4; BD Biosciences), phycoerythrin-conjugated mouse anti-human CD25 antibody (clone M-A251; BD Biosciences). Cells were incubated with the antibodies for 20 min in the dark and then resuspended in BD Transcription Factor Buffer (BD Biosciences). Intracellular staining was performed using the Brilliant Violet 421-conjugated mouse anti-human IL-17 antibody (clone N49-653; BD Biosciences) or an isotype control. Flow cytometric analysis was performed to analyze $CD25^{+}IL\text{-}17^{+}$ T cells using a $CD4^{+}$ gate, using a previously described method with some modifications.[20] The purity of the isolated Th17 cells was determined using the same procedure described above.

2.4 | Reverse transcription-quantitative polymerase chain reaction

To analyze mRNA and miRNA expression levels, Th17 cells were seeded into 12-well plates at a density of 2.0×10^5 cells per well. Total RNA was extracted from the cells using a UNIQ-10 Column TRIzol Total RNA Isolation Kit (Sangon Biotech,). A RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific,) was used to synthesize cDNA. mRNA expression levels were determined using a FastStart Universal SYBR Green qPCR kit (Roche Applied Science,) on an ABI ViiA 7 system (Applied Biosystems,). miRNA extraction, cDNA synthesis, and amplification were performed using an All-in-One™ miRNA qRT-PCR Kit (GeneCopoeia, Inc.,). The following primer pairs were used: *SOCS1* forward, 5'-CACTTCCGCACATTCCGTTC-3' and reverse, 5'-GAGGCCATCTTCACGCTAAG-3'; *IL-17* forward, 5'-GGATGCCCAAATTCTGAGGAC-3' and reverse, 5'-ACTTTG CCTCCCAGATCACA-3'; *GAPDH* forward, 5'-GAGTCAACGGAT TTGGTCGT-3' and reverse, 5'-GACAAGCTTCCCGTTCTCAG-3'; *hsa-miR-155-5p* forward, 5'-GGGGGGTTAATGCTAATCGTG-3' and reverse, 5'-TATGGTTGTTCACGACTCCTTCAC-3'; *RNU6B* forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCT TCACGAATTTGCGT-3'. The relative expression levels of *SOCS1*, *IL-17*, and *miR-155-5p* were normalized against *GAPDH* or the small nuclear RNA, *RNU6B*, using the $2^{-\Delta\Delta Ct}$ method.

2.5 | Western blotting assay

Western blotting analyses were performed to determine the expression levels of SOCS1 and IL-17 proteins. Th17 cells were treated as above for 48 h, and then total protein was extracted using a Minute™ Total Protein Extraction Kit (Invent Biotechnologies,). The protein concentrations were quantitated using a Pierce Rapid Gold BCA Protein Assay Kit (Thermo Fisher Scientific). The samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore,), which were then blocked with 5% non-fat milk for 1 h at room temperature. The membranes were then incubated with a rabbit monoclonal anti-GAPDH antibody (1:8,000 dilution, clone EPR16891; Abcam,), a rabbit monoclonal anti-SOCS1 antibody (1:1,000 dilution, clone EPR24290-356, Abcam), or a mouse monoclonal anti-IL-17 antibody (1:3,000 dilution, clone 4K5F6, Abcam), at 4°C overnight. After washing three times with Tris-buffered saline containing 0.1% Tween 20, the PVDF membranes were incubated with horseradish peroxidase-linked secondary antibodies (Proteintech Group,) for 1.5 h at room temperature. The membranes were then developed using Pierce ECL Western Blotting Substrate

(Thermo Fisher Scientific), and images were captured using a Tanon 5200 Imaging System (Tanon Science & Technology Co., Ltd.,). The gray intensity value of each protein band was analyzed using ImageJ software (version 1.53; National Institutes of Health, Bethesda). Relative protein expression levels were calculated as the ratio of the intensity values of the target protein band to the intensity value of the GAPDH protein band.

To verify whether *miR-155-5P* can reduce the level of *SOCS1* protein in Th17 cells and simulate the role of *miR-155-5P* targeting *SOCS1* in SSC disease progression, the Th17 cells from HCs were transfected with an *miR-155-5p* agomir, antagomir, or a scramble negative control (SNC) oligo (15 nM) (Guangzhou RiboBio Co., Ltd,). After 48 h, the lysed cells were analyzed by western blotting as above.

2.6 | Luciferase reporter gene assay

Luciferase reporter gene assays were performed as previously described with some modifications.[21] The online tool, TargetScanHuman,[22] was used to predict possible *miR-155-5p*-binding sites in *SOCS1*. Fragments of the wild-type 3′ untranslated

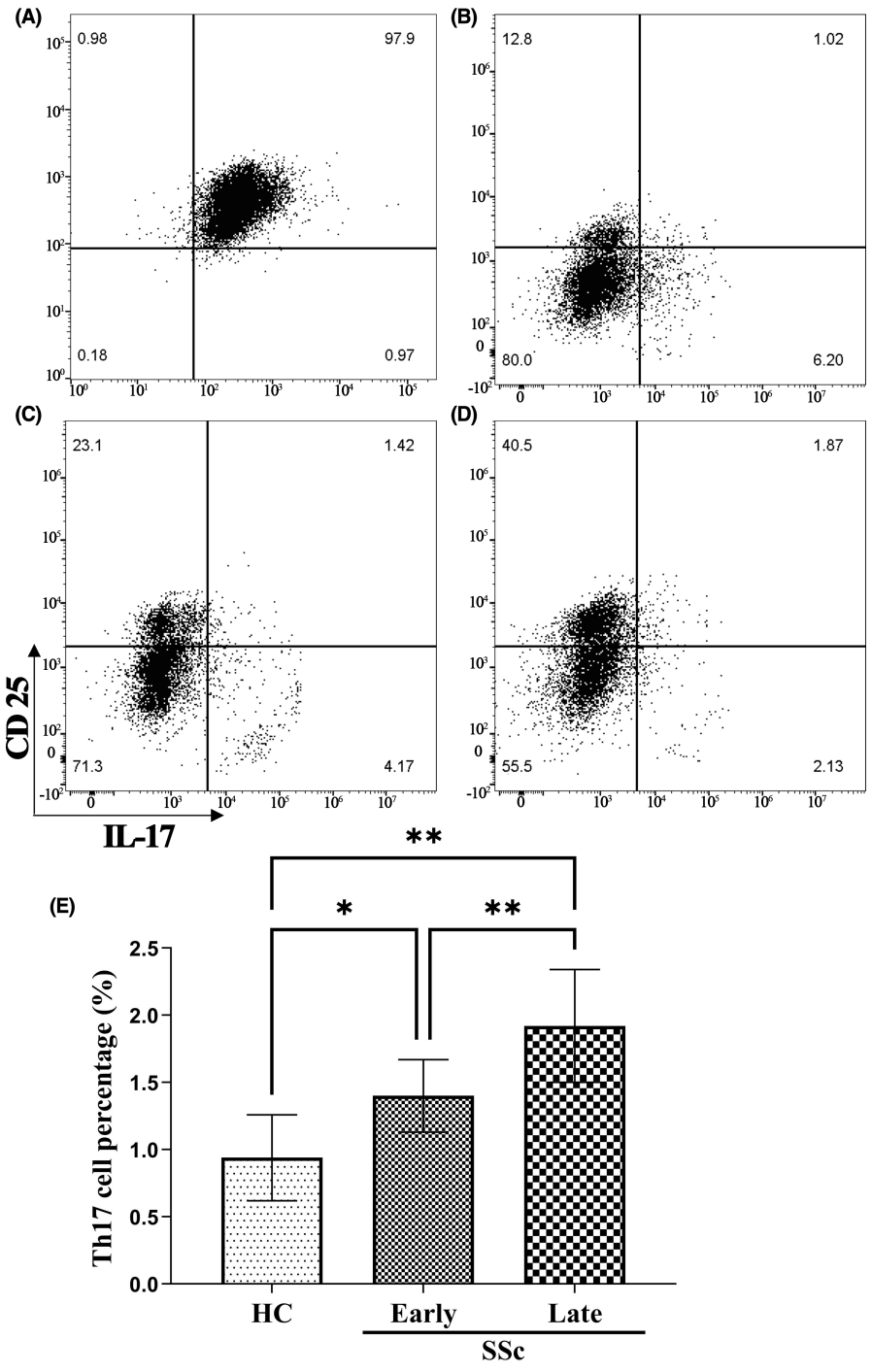

FIGURE 1 Flow cytometric assays of Th17 cells. (A) The purity of the isolated Th17 cells. Representative flow cytometric charts of Th17 percentage in PBMCs from HCs (B), early-stage (C) and late-stage (D) SSc patients. (E) A comparison of the percentage of Th17 cells in PMBCs samples. * $p < 0.05$, ** $p < 0.01$

region (UTR) of *SOCS1* containing the predicted target sites of *miR-155-5p* were cloned into the pmirGLO dual-luciferase reporter (Promega Corporation,). Mutant constructs were generated with the target sites mutated, as specified in the "Results" section. Firefly luciferase was used as the primary reporter to investigate miRNA binding to the 3′-UTR and subsequent regulation of gene expression. *Renilla* luciferase was used as an internal control for normalization. Human embryonic kidney 293T cells were seeded in 96-well plates and co-transfected with luciferase reporters (0.05 μg/well) and an *miR-155-5p* agomir or a SNC oligo (15 nM). Luciferase activities were measured 72 hours later using the Promega GloMax 20/20 system. Firefly luciferase activity was normalized to *Renilla* luciferase activity to assess the regulatory effect of *miR-155-5p* on its putative targets.

2.7 | RNA pull-down

A Pierce™ Magnetic RNA-Protein Pull-Down Kit (Thermo Fisher Scientific) was used to perform RNA pull-down assays to detect proteins specifically interacting with *miR-155-5p*, according to the manufacturer's instructions. A DNA probe complementary to *miR-155-5p* was synthesized and biotinylated by GenePharma. Biotinylated *miR-155-5p* was incubated with streptavidin-coated magnetic beads at 4°C overnight. Th17 cells were lysed using the Minute™ Total Protein Extraction Ki and the lysate was incubated with the labeled beads for 30–60 min at 4°C with agitation. The *miR-155-5p*-protein complexes were analyzed by western blotting.

2.8 | miRNA fluorescence *in situ* hybridization

FISH analysis of *miR-155-5p* and *IL17A* in Th17 cells was performed using the ViewRNA® Cell Plus Assay (Thermo Fisher Scientific) according to the manufacturer's guidelines. Briefly, Th17 cells were applied to adhesion microscope slides using a cytospin (Maxim Biotechnologies,), and were then fixed and permeabilized using ViewRNA Cell Plus Fixation/Permeabilization Solution with RNase inhibitor. The cells were then overlayed with a mouse monoclonal anti-IL-17 antibody for 1 h and stained with Alexa Fluor 488-conjugated goat anti-mouse secondary antibody (Proteintech Group) for 1 h in the dark at room temperature. After washing with viewRNA Cell Plus RNA Wash Buffer Solution, the cells were hybridized with an Alexa Fluor 647-conjugated *miR-155-5p* probe (Thermo Fisher Scientific). The cells were then washed with phosphate-buffered saline, mounted with Fluoromount-G containing 4′,6-diamidino-2-phenylindole (Thermo Fisher Scientific), and coverslipped. Images were acquired using a Zeiss LSM 800 confocal microscope and Zen software (blue edition, version 2.6; Zeiss,). The co-localization coefficients of *miR-155-5p/IL-17* were determined using the Confocal Topography module of Zen software.

2.9 | Data analysis

Statistical evaluation was performed using a one-way analysis of variance with Tukey's multiple comparisons test. The co-localization and correlation between genes or proteins of interest were investigated using Pearson's correlation coefficient. Statistical analysis

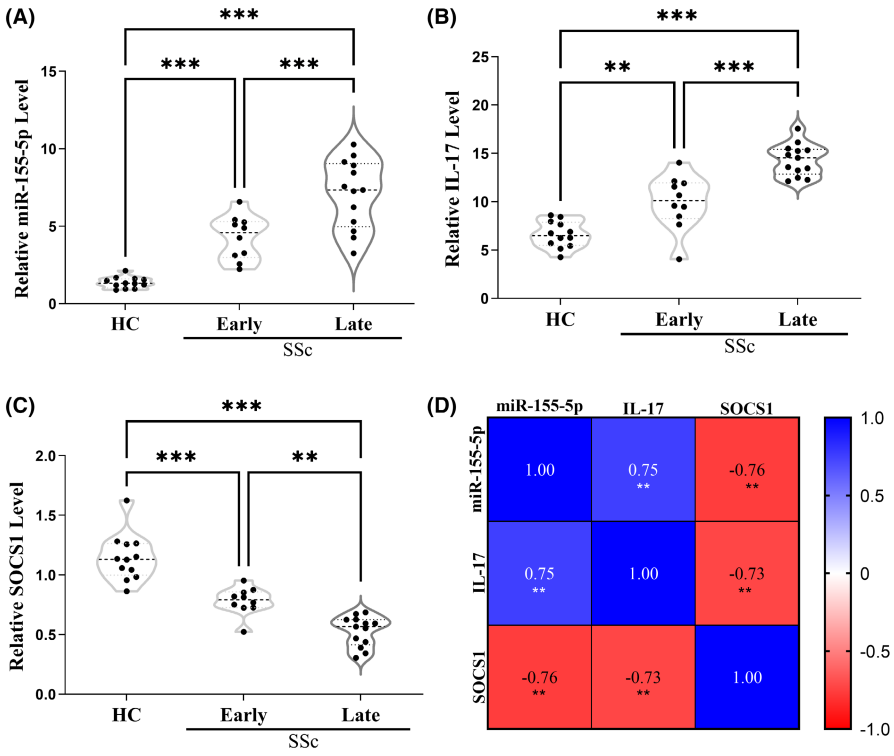

FIGURE 2 Relative quantification of *IL-17* and *SOCS1* mRNA and *miR-155-5p* levels in Th17 cells isolated from SSc patients. Comparison of the expression levels of *miR-155-5p* (A), *IL-17* (B), and *SOCS1* (C) in HCs and SSc patients. (D) Correlation between the expression levels of *miR-155-5p*, *IL-17*, and *SOCS1*. The $2^{-\Delta\Delta Ct}$ method was used to analyze changes in the mRNA and miRNA expression levels compared to their levels in HCs. *GAPDH* and *RNU6B* were used as internal standards for mRNA and miRNA expression levels, respectively. $^{**}p < 0.01$, $^{***}p < 0.001$

was performed using GraphPad Prism version 6.01 for Windows (GraphPad,). Differences were considered to be statistically significant when $p < 0.05$.

3 | RESULTS

3.1 | Th17-derived *miR-155-5p* levels were correlated with IL-17 and SOCS1 expression levels and SSc disease progression

In this study, 10 and 13 SSc patients were classified as having early- and late-stage disease, respectively. The purity of the isolated Th17 cells was 96%–98%, with no difference in purity between samples from different patient and control groups (Figure 1A). Flow cytometric assays were further performed to investigate the Th17 subgroups in PBMCs of SSc patients. The results showed that the percentage of $CD25^{+}IL\text{-}17^{+}$ Th17 cells was significantly increased in patients with SSc compared with HCs and was associated with disease progression (Figure 1B–E).

Compared to HCs, *miR-155-5p* expression levels were up-regulated and were positively correlated with disease progression, with late-stage SSc patients displaying the highest *miR-155-5p* expression levels in Th17 cells. Similarly, the up-regulation of *IL-17* mRNA levels was observed in Th17 cells as disease progressed, and *IL-17* mRNA levels were positively correlated with *miR-155-5p* expression levels. On the contrary, the expression levels of *SOCS1* mRNA in Th17 cells were negatively correlated with *miR-155-5p* expression levels (Figure 2A–D).

3.2 | Changes in IL-17 and SOCS1 protein levels and binding activities with *miR-155-5p*

Western blotting analysis directly confirmed the marked expression differences of IL-17 and SOCS1 proteins in the Th17 cells isolated from SSc patients compared to those isolated from HCs. Similar to the mRNA expression results, the highest IL-17 protein levels and the lowest SOCS1 protein levels were observed in Th17 cells from late-stage SSc patients. Again, a trend of increased IL-17 levels and decreased SOCS1 levels was seen as the disease progressed (Figure 3A–B).

RNA-protein pull-down assays confirmed that IL-17 and SOCS1 bind to *miR-155-5p*. The binding of IL-17 to *miR-155-5p* was stronger and the binding of SOCS1 to *miR-155-5p* was weaker in Th17 cells from SSc patients compared to those from HCs (Figure 3C), indicating that the binding status of IL-17 and SOCS1 to *miR-155-5p* was affected by the SSc disease status.

3.3 | *miR-155-5p* directly targets *SOCS1* to promote disease progression

To determine the mechanisms underlying the role of *miR-155-5p* in regulating disease progression, the potential targets of *miR-155-5p*

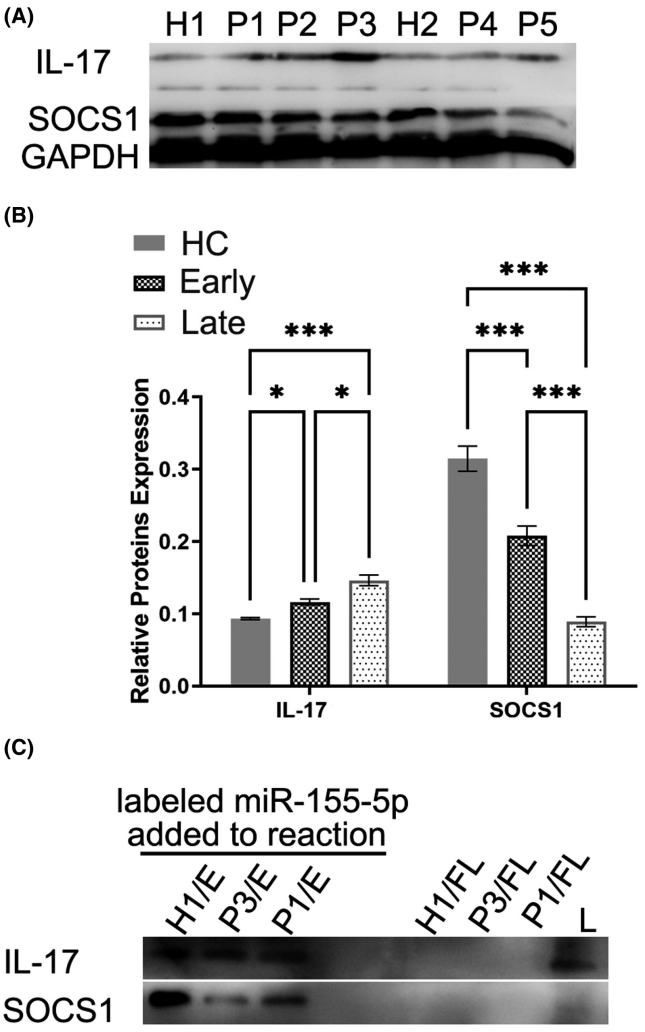

FIGURE 3 Interaction between *miR-155-5p* and the protein expression levels of IL-17 and SOCS1 in Th17 cells isolated from SSc patients and HCs. A western blotting assay was used to measure IL-17 and SOCS1 protein expression levels in Th17 cells from HCs (H1, H2) and early-stage (P1, P2, P4) and late-stage SSc patients (P3, P5) (A). The band intensities were analyzed using Image J software (version 1.53) (B). An RNA-protein pull down assay was used to analyze the interactions between *miR-155-5p*, IL-17, and SOCS1 (C). Representative data for HCs (H1) and early- (P1) and late-stage SSc patients (P3) are shown. L = lysate load; FT =flow-through; E = eluate. * $p < 0.05$, ***$p < 0.001$

were explored using TargetScanHuman. *SOCS1* was predicted as a potential target of *miR-155-5p* as it contained binding sites in its 3′-UTR (Figure 4A). pmirGLO vectors were constructed with *SOCS1* containing wild-type (WT) and mutated (MUT) *miR-155-5p*-binding sites and the constructs were confirmed by sequencing (Figure 4B,C) and then used in luciferase reporter gene assays. The results showed that an *miR-155-5p* agomir significantly inhibited the luciferase reporter activity of the pmirGLO-*SOCS1*-WT construct ($p < 0.001$ vs. control group). However, the *miR-155-5p* agomir lost its inhibitory effect upon transfection of the pmirGLO-SOCS1-MUT construct (Figure 4D). Western blotting assay further confirmed the SOCS1 protein level reduced by *miR-155-5p* agomir and increased by *miR-155-5p* antagomir in the Th17 cells from HCs (Figure 4E). These

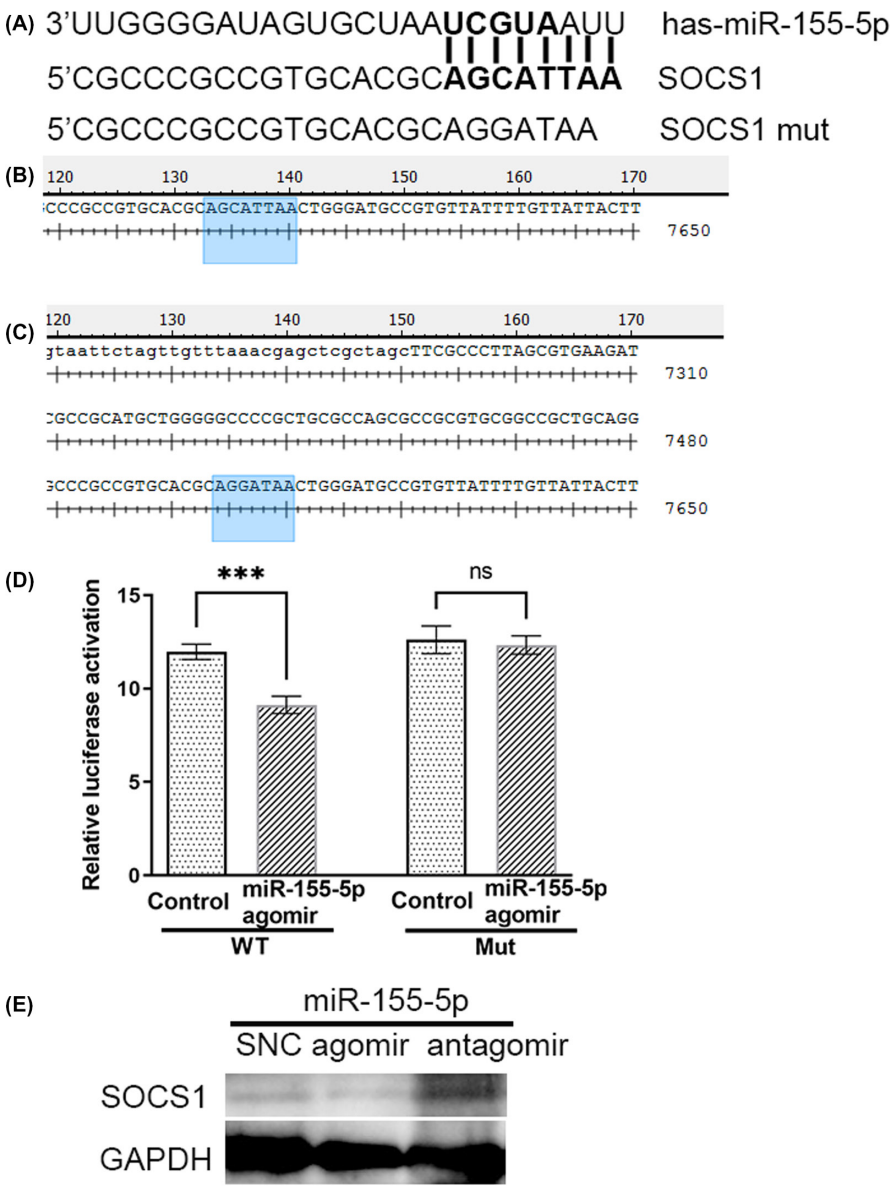

FIGURE 4 *miR-155-5p* directly targets *SOCS1*. (A) Predicted binding sites of *miR-155-5p* in the 3′-UTR of *SOCS1*. Partial sequencing results of the pmirGLO vector containing the wild-type (WT) (B) and mutated (MUT) (C) *miR-155-5p*-binding sites in *SOCS1*. (D) Luciferase activity of pmirGLO vectors containing WT or MUT *SOCS1* 3′-UTRs after co-transfection with an *miR-155-5p* agomir or a control miRNA in the 293T cells. (E) Western blotting assay was used to measure the effect of *miR-155-5p* on *SOCS1* protein in the Th17 cells from HCs. ***$p < 0.001$. NS =No significant difference

results indicate that *SOCS1* is a direct target of *miR-155-5p*, suggesting a role of this targeting effect in SSc disease progression.

3.4 | Co-expression of *miR-155-5p* and IL-17 in Th17 cells

To analyze the co-expression status of *miR-155-5p* and *IL-17* in Th17 cells from SSc patients at different stages of disease, we performed FISH assays. Combined staining of *miR-155-5p* and *IL-17* showed a simultaneous increase in the expression of *miR-155-5p* and *IL-17*, as reflected by the mean fluorescence intensities (MFIs) of the dyes in the Th17 cells of SSc patients compared to those from HCs. Analyses of the images using Mander's co-localization coefficient showed that *IL-17* completely co-localized with *miR-155-5p* in Th17 cells. Augmented co-localization was associated with greater disease progression, indicating that the co-expression of *miR-155-5p* and *IL-17* plays an important role in the pathogenesis of SSc (Figure 5A–C).

4 | DISCUSSION

Immunological abnormalities are crucial for the development of skin and lung fibrosis and vasculopathy in SSc. Th17 cells have been shown to have a considerable role in the pathogenesis of many inflammatory and autoimmune diseases, including SSc.[23] Th17 cells are involved in the pathogenesis of skin and lung fibrosis by enhancing fibroblast proliferation and IL-17 production in a bleomycin (BLM)-induced mice model of SSc,[24] indicating that Th17 cells and IL-17 play a critical role in the development of pulmonary fibrosis. IL-17 is also involved in lung and skin fibrosis in another animal model of SSc. IL-17 deficiency in TSK-1/+ mice reduces skin thickness and recombinant IL-17 increases *TGF-β* and *CTGF* mRNA expression levels in skin fibroblasts, suggesting that IL-17 contributes to the development of skin fibrosis.[25]

The number of Th17 cells was notably increased in SSc patients and was positively correlated with disease progression. Th17 cells promote fibroblast proliferation and increase collagen-secreting

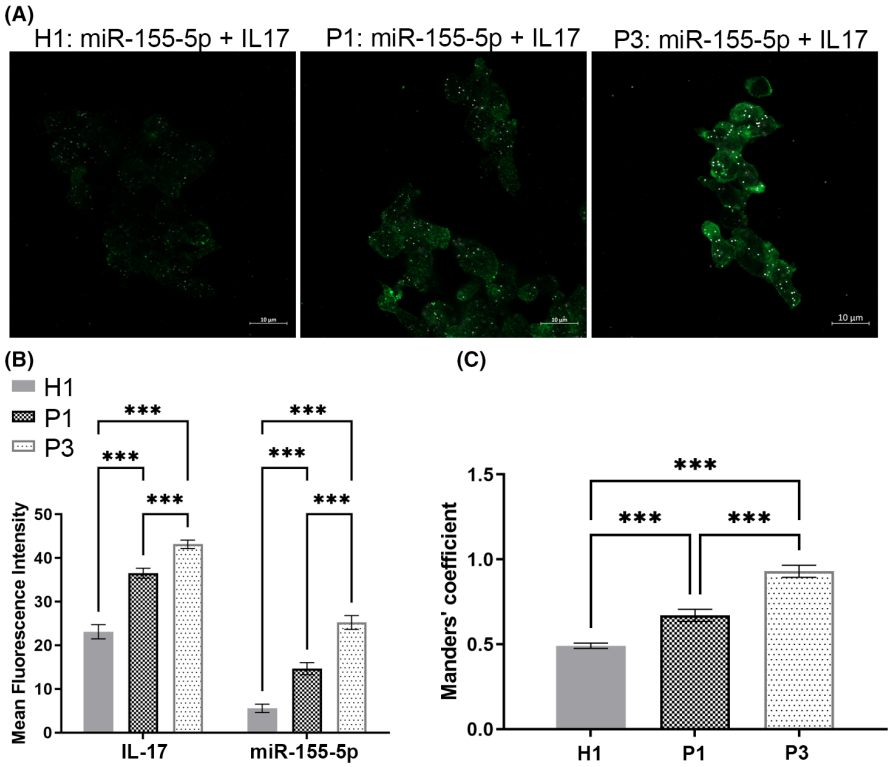

FIGURE 5 The co-expression of *miR-155-5p* and *IL-17*. (A) Representative confocal microscopy images of RNA-FISH analysis of HCs (H1) and early- (P1) and late-stage (P3) SSc patients; 630× magnification, bar =10 μm. All the images are merged pictures of Alexa Fluor-488-tagged *IL-17* (green) and Alexa Fluor-647-tagged *miR-155-5p* (white). (B) The mean fluorescence intensities (MFIs) of *IL-17* and *miR-155-5p* in Th17 cells, as measured using the graphics module of Zeiss Zen software. Error bars represent the standard deviation. (C) Degree of *IL-17* co-expression with *miR-155-5p* in Th17 cells. ***$p < 0.001$

ability by producing IL-21, thus contributing to fibrosis in SSc.[26] IL-17 and serum isolated from patients with SSc have been shown to promote the proliferation and migration of and collagen synthesis and secretion by dermal vascular smooth muscle cells.[27] In another study, IL-17 isolated from SSc patient's blood induced endothelial cell inflammation by increasing the production of chemokines and adhesion molecules.[28] In the present study, we first reported that the expression levels of IL-17 were greater in Th17 cells isolated from late-stage SSc patients than those isolated from early-stage SSc patients and HCs. These results are similar to previous results showing that late-stage SSc patients show markedly higher levels of serum IL-17 compared with early-stage SSc patients.[29]

miR-155 has been identified as a critical regulator of immune responses and it is involved in various autoimmune diseases, including SSc, multiple sclerosis (MS), rheumatoid arthritis (RA), systemic lupus erythematosus, and lupus nephritis (LN).[30] The expression levels of *miR-155* are higher in PMBCs and skin lesions of SSc patients compared with those of HCs and the severity of skin lesions is positively correlated with *miR-155* levels.[31] In the BLM-induced mouse model of SSc, the expression levels of *miR-155* are also increased, and skin fibrosis is relieved after treatment with an *miR-155* inhibitor. Conversely, *miR-155*(-/-) SSc model mice are resistant to skin fibrosis.[32] The expression levels of *miR-155* in serum PBMCs isolated from SSc patients with pulmonary fibrosis are highly related to the progression of high-resolution computed tomography scores and lung function.[15] In our cohort, we observed a strong correlation between *miR-155-5p* expression levels in Th17 cells from patients with SSc and disease progression. As far as we know, there is no previous report on the correlation between *miR-155-5p* expression levels in Th17 cells and SSc disease progression. However, a correlation between *miR-155* levels in PBMCs and disease progression has been demonstrated in RA and LN patients.[33,34]

Cumulative evidence shows that miRNAs are involved in various autoimmune disease pathologies by targeting cytokines or the expression of relevant genes. miRNAs regulate immune function by binding to the 3′- or 5′-UTR of target mRNAs to promote their degradation or inhibit their translation. *miR-384* regulates Th17 cell differentiation during the pathogenesis of MS by targeting *SOCS3* in an animal model of MS.[35] Increased *miR-155* levels increase the expression levels of the pro-inflammatory cytokines, TNF-α, and IL-1β, by targeting *SOCS1* in PMBCs isolated from RA patients.[36] *miR-155* negatively regulates the expression of SOCS1 and maintains the homeostasis of regulatory T cells.[37] Extensive studies have demonstrated that *miR-155* and *SOCS1* are part of a complex recruited by argonaute 2 (AGO2), which promotes the assembly of the miRNA-induced silencing complex (miRISC).[38] As a key protein of miRISC, AGO2 is dependent on *miR-155* as shown by the fact that it is eliminated in *miR-155*-deficient T cells.[39] In this study, the level of *SOCS1* decreased, leading to increased IL-17 levels as the disease progressed. Using an miRNA-specific pmirGLO vector, *SOCS1* was confirmed as a direct target of *miR-155-5p*, which is in accordance with the findings of a previous study.[20] The present study is the first to demonstrate the co-expression of Th17-derived *miR-155-5p* and IL-17, and that the co-expression status changed with the progression of SSc. Although there is no direct evidence that *IL-17* is a target of *miR-155-5p*, SOCS1 has been reported to modulate Th17 cell function by suppressing IL-17 production.[40] Furthermore, IL-17 receptor (IL-17R) B is a direct target of *miR-155*,[41] and therefore,

the co-expression of *miR-155-5p* and *IL-17* may reflect the role of the receptor complex consisting of IL-17RA, IL-17RB, IL-17RC, and IL-17.[42,43]

In conclusion, this study fills a gap in the literature by demonstrating the regulatory role of *miR-155-5p* by targeting *IL-17* and *SOCS1* in Th17 cells and the relationship between these interactions and the development of SSc. The status of the interaction between *miR-155-5p* and *IL-17* or *SOCS1* in Th17 cells may be used to evaluate the efficacy of immunotherapy in SSc patients. To further develop these findings, additional investigation is required in more clinical SSc cases.

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CONFLICT OF INTEREST

No potential conflict of interest was reported by the author(s).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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