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The role of microRNA-5196 in the pathogenesis of systemic sclerosis

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Abstract

Background Systemic sclerosis (SSc) is a chronic autoimmune disease characterised by tissue fibrosis and immune abnormalities. Recent evidence suggests that activated circulating monocytes from SSc patients play an important role in early stages of SSc pathogenesis due to enhanced expression of tissue inhibitor of metalloproteinases 1 (TIMP-1), IL-8 and reactive oxygen species (ROS) induction. However, the exact factors that contribute to chronic inflammation and subsequently fibrosis progression are still unknown.

Material and methods The expression pattern of IL-8, TIMP-1, AP-1 transcription factor-Fra2 and ROS induction in peripheral blood monocytes following DZNep (histone methyltransferase inhibitor) and TLR8 agonist stimulation was investigated. Exogenous microRNA-5196, which is predicted to bind 3'UTR of Fra-2 gene, was delivered in order to reverse profibrotic phenotype in monocytes. Expression of circulating microRNA-5196 was correlated with SSc parameters.

Results DZNep+TLR8 agonist stimulation enhanced profibrotic TIMP-1, IL-8 and ROS generation in HC and SSc monocytes. As opposed by the decrease of miRNA-5196 and antioxidant SOD1 expression in SSc monocytes. Exogenous delivery of microRNA-5196 reduced Fra2 and TIMP-1 expression suggesting that it may be used as a potential modulator of fibrogenesis in SSc. Circulating microRNA-5196 was significantly increased in SSc and positively correlated with CRP level but not with Rodnan skin score or ESR.

Conclusions These results suggest that microRNA-5196 can be used as a potential biomarker characterising SSc. Overall, this study may open new possibilities for the development of microRNA-5196-based diagnostics and therapy in early phases of SSc.

Introduction

Systemic sclerosis (SSc) is a chronic multisystem autoimmune disease characterised by skin and internal organs fibrosis and immune abnormalities. Recent evidence suggests that activated circulating monocytes from SSc patients play an important role in SSc pathogenesis due to enhanced expression of tissue inhibitor of metalloproteinases 1 (TIMP-1) which likely contributes to accumulation of extracellular matrix protein (ECM) [1]. Also, the level of TIMP-1 is elevated in SSc sera and in affected skin tissue, as opposed to reduced level of matrix proteinases (MMPs) which directly contribute to fibrosis development [2, 3].

Another factor promoting SSc progression during early phases of disease is inflammation initiated by the Toll-like receptors (TLRs) signaling pathway. It has been reported that TLR stimulated monocytes are involved in SSc development through increased production of proinflammatory cytokines such as IL-6, TNF- α or by driving profibrotic phenotype [4-6]. Furthermore, reactive oxygen species (ROS) has been suggested to contribute to scleroderma pathogenesis. In particular, it has been shown that skin fibroblasts from SSc patients release more ROS compared to normal fibroblasts. This consequently induces enhanced proliferation and production of collagen in SSc fibroblasts [7]. Similarly, blood phagocytes from SSc patients, especially from those with interstitial lung disease, release more ROS than phagocytes from healthy individuals [8]. Also, scleroderma-associated autoantigens, that are involved in breaking the tolerance, are specifically susceptible to ROS-mediated fragmentation and processing, which supports an important role of ROS production in SSc pathogenesis [9]. The effect of ROS induction is mostly limited by antioxidant enzyme superoxide dismutase 1 (SOD1) in order to restore cellular redox homeostasis [10]. Moreover, AP-1 transcription factor family which is composed of proteins including Fos-related antigen 2 (Fra2) play an important role in SSc development. Indeed, Fra2-transgenic mice are a widely used mouse model of SSc mimicking systemic manifestations [11]. Also,

in bleomycin-treated mice and in skin biopsies from SSc patients the level of Fra2 is increased [12]. It has been demonstrated that Fra2 regulates the production of matrix protein, including collagen, both in patients and animal models for SSc [13]. Our recent studies have also shown that stimulation with TLR8 ligand enhanced Fra2 and its target gene TIMP-1 expression in SSc monocytes compared to healthy controls. Furthermore, increased production of TIMP-1 in monocytes leads to trans-differentiation of fibroblast towards pathogenic myofibroblasts phenotype suggesting that monocytes have impact on the pathogenesis of SSc [12]. However, precise mechanisms involved in the contribution of monocytes in inflammation and fibrotic progression remain largely unknown.

A growing body of evidence suggests that epigenetic mechanisms, including microRNAs (miRNAs), mediate in SSc development. MiRNAs are small noncoding RNA molecules that negatively regulate gene expression via degradation or translational repression of their targeted mRNA transcripts. It is known that expression of miRNAs can be altered under conditions of pathophysiological stress or disease, allowing miRNAs to be important biomarkers and attractive candidates for therapeutic manipulation. Indeed, our recent studies have shown reduced level of miRNA-135b in sera, monocytes and fibroblasts from SSc patients [14]. In addition, IL-13-mediated collagen induction was inhibited in fibroblasts transfected with miRNA-135b targeting STAT6.

The aim of this study was to investigate the expression pattern of AP-1 transcription factor - Fra2, TIMP-1, IL-8 and ROS induction in peripheral blood monocytes from HC and SSc patients following DZNep (histone methyltransferase inhibitor) and TLR8 agonist (ssRNA) stimulation. In addition, the effect of miRNA-5196 targeting Fra2 was evaluated in order to reverse fibrotic properties of monocytes. The level of circulating miRNA-5196 was measured

in sera from SSc patients for diagnostic purposes. Thus, identification whether miRNA-5196 can both modulate profibrotic genes and be used as a biomarker may open up new therapeutic approaches and diagnostic tools in SSc.

Materials and methods

Sample collection and cell purification

Twenty two patients who fulfilled the American College of Rheumatology criteria according to LeRoy for the classification of SSc [15] were obtained from Wroclaw Medical University. This study was approved by the local ethics committee (approval no. 335/2014) SSc patients provided fully informed written consent. Their clinical characteristics and treatment are summarised in Table 1 and 2, respectively. Twenty one donors with no history of autoimmune disease were included as healthy control. The blood from healthy controls (HC) was collected from a local blood donor centre or directly from healthy volunteers. The blood was collected in EDTA-coated tubes from HC and SSc patients. Peripheral blood mononuclear cells (PBMC) were separated from whole red blood cells as described elsewhere [1]. To isolate monocytes fraction, PBMC were incubated with anti-CD14+ beads (Miltenyi-Biotec) according to the manufacturer's protocol. Serum samples from HC, RA and SSc patients were collected in serum separation tubes (BD Vacutainer® SST II Plus), aliquoted and frozen at -20°C.

Compounds, reagents and *in vitro* cell cultures

Monocytes from HC, SS patients and U937 monocytic cell line were seeded in 24-well plates at the concentration of $0.5-1 \times 10^6$ cells/ml and cultured for 24 h in 500 μ l of RPMI (Laboratory of General Chemistry, IITD Poland) supplemented with penicillin (100 U/ml), streptomycin (100 μ g/ml), L-glutamine (2 mM) and 10% FBS (all from Life Technologies) at

37°C in 5% CO₂. Cells were stimulated for 24 h with 2 µg/ml TLR8 agonist (ssRNA40/LyoVec, InvivoGen) and 20 µM DZNep (4703, Tocris) or 100 ng/ml apicidin (A8851, Sigma).

Gene expression study

RNA from freshly isolated HC or SSc monocytes or U937 monocytic cell line was obtained using the RNA mini kit (Genoplast), according to the manufacturer's protocol. RNA (200–500 ng) was reverse transcribed to cDNA with the use of random primers and reverse transcriptase enzyme (Life Technologies) according to the manufacturer's protocol. Samples (20 ng of cDNA) were analysed in triplicate using the Viia 7 qRT-PCR machine (Thermo Fisher Scientific). Expression levels relative to the average healthy control (arbitrarily set at 1) were calculated using the following equation: $(2^{-\Delta\Delta CT}) - 1$, all normalised to 18S housekeeping gene (Table 3).

ELISA and nuclear fraction isolation for Fra2 activity assay measurement

TIMP-1 protein concentrations in culture supernatants were measured by ELISA, according to the manufacturer's protocol (Human TIMP-1 DuoSet, DY970, R&D Systems). To identify Fra2 transcription factor activities, Fra2-TFactTM DNA binding ELISA Kit (TFE-7014 antibodies) was used according to the manufacturer's protocol. Nuclear fraction was isolated using centrifugation at 3000 x g at 4°C for 30 min according to the manufacturer's protocol.

Oxidative stress measurement

To determine oxidative stress induction in monocytes, bioluminescent ROS-GloTM H₂O₂ Assay (Promega) was used according to the manufacturer's protocol. Luminescence was measured using detection system GloMax® 96 Microplate Luminometer (Promega).

MiRNA experiments and computational analysis

Computational prediction analysis of miRNA-5196 targeting Fra2 was performed using computational prediction algorithms. Pre-miRNA-5196 (Thermo scientific, UK) was transfected into U937 monocytic cell line at 75 nM concentration using Viromer transfection reagent (Lipocalyx GmbH) in antibiotic and FBS-free medium along with the control miRNA (75 nM) for 48 h. The sequence of the control non-targeting miRNA was designed to be complementary to the nematode worm *C. elegans*. The volume of 300 μ l of serum was used to isolate circulating miRNA-5196 using NucleoSpin® miRNA plasma/serum (Macherey-Nagel) according to the manufacturer's protocol. TaqMan® microRNA RT Kit (Thermo Fisher Scientific) was used to reverse transcribe to cDNA with the use of TaqMan® MicroRNA Assays for hsa-miR-5196-5q (471527_mat), and hsa-let-7a (000377) all from Thermo Fisher Scientific was used as a stable control.

Statistical analysis

All data are presented as mean \pm SEM. The differences between the groups were tested for their statistical significance by non-parametric two tailed T-test using GraphPad Prism (GraphPad Software). A p value of less than 0.05 was considered statistically significant; p values are expressed as follows: ns for not significant; $0.05 > p > 0.01$ as*; $0.01 > p > 0.001$ as**; $p < 0.001$ as***.

Results

Enhanced TIMP-1 secretion and reduced expression of MMPs in SSc monocytes

We have previously demonstrated that HC monocytes increased expression of Fra2 and TIMP-1 following DZNep+TLR8 treatment [12], therefore we wanted to measure Fra2 and TIMP-1 levels in patients monocytes and to test if stimulation even further enhances profibrotic genes in SSc monocytes. In order to do that, HC and SSc monocytes were

stimulated as previously with TLR8 ligand (ssRNA) and DZNep for 24 h. It can be seen that the level of Fra2 was increased in the presence of DZNep+TLR8 in both HC ($p=0.001$, 2.1-fold) and SSc ($p=0.024$, 1.9-fold) monocytes, however there was no difference between HC and SSc monocytes population (Fig. 1a). In contrast, secreted level of TIMP-1 was elevated in both unstimulated and stimulated SSc monocytes and was significantly higher ($p=0.016$, 2.1-fold) in SSc than in HC monocytes (Fig. 1b).

Furthermore, we also looked on Fra2-transcriptional activity upon DZNep+TLR8 stimulation. It is well known that Fra2 regulates TIMP-1 expression and plays an important role in SSc pathogenesis [13, 16]. In order to test that, we used the colorimetric DNA-binding assay in which we measured only the translocated Fra2 into the nucleus of U937 monocytic cell line. Although the Fra2 DNA-binding activity did not reach statistical significance ($p=0.412$) upon stimulation, the tendency mirrored upregulated Fra2 gene expression as seen in primary monocytes (Fig. 1c).

We also examined the morphology of DZNep+TLR8 treated monocytes. It can be seen in Fig. 1d that DZNep+TLR8 treatment led to cell elongation and aggregation as compared to monocytes treated with DZNep, apicidin, TLR8 alone or apicidin plus TLR8 agonist. Apicidin is another histone modifier inducing histones hyperacetylation. We have previously validated that DZNep and apicidin concentrations, which were used to stimulate monocytes, did not affect cells viability [12]. This suggests that only specific epigenetic agent (DZNep, but not apicidin) plus TLR8 stimulation can activate monocytes to produce profibrotic genes and changes cell morphology. We have also measured the expression levels of MMP-1 and MMP-15 following stimulation. The balance between TIMP-1 and MMPs is altered in SSc leading to abnormal ECM formation, thus we wanted to test if profibrotic stimulation can result in even further TIMP-1/MMPs balance disruption. It can be seen that the basal gene expression levels of MMP-1 (Fig. 1e) and MMP-15 (Fig. 1f) were significantly decreased

($p=0.034$, 2.4-fold, $p=0.018$, 2.6-fold, respectively) in SSc monocytes. However, DZNep+TLR8 stimulation did not affect MMPs expression. Although MMP1 and MMP-15 promoters have similar AP-1 binding sites as TIMP-1 [17], these data suggest that stimulation with DZNep+TLR8 specifically induces only profibrotic TIMP-1 but not MMPs expression.

DZNep+TLR8-dependent induction of IL-8 and oxidative stress in HC and SSc monocytes

It is well known that inflammation during early phases of disease play an important role in fibrogenesis and subsequent SSc progression. Therefore, we tested if DZNep+TLR8 can also induce production of proinflammatory mediators in monocytes. As previously, HC and SSc monocytes were stimulated with DZNep+TLR8 agonist for 24 h and the expression of IL-8 and ROS was measured.

It can be seen in Fig. 2a that the expression of IL-8 was strongly elevated following stimulation in both HC and SSc monocytes ($p=0.003$, 4.6-fold and $p=0.0008$, 7.8-fold, respectively). Similarly, DZNep+TLR8-induced ROS secretion was significantly increased ($p=0.039$, 2.3-fold) compared to untreated HC monocytes (Fig. 2b). Due to the limitation of SSc patients' material, we tested the DZNep+TLR8-mediated ROS induction only in HC monocytes. In order to measure ROS release, we used ROS-Glo H₂O₂ assay. This bioluminescent assay measures generated ROS directly in cell cultures.

To better understand changes in redox balance induced by DZNep+TLR8, we also analysed the SOD1 gene expression. SOD1 is a major anti-oxidative enzyme. As expected, the level of SOD1 was significantly ($p=0.025$, 2.4-fold) decreased in HC monocytes (Fig. 2c). Although the SOD1 expression was significantly attenuated in HC monocytes only, the tendency of

reduced (1.77-fold) SOD1 expression was also seen in SSc monocytes, implying that redox homeostasis is disturbed upon the DZNep+TLR8 treatment.

Exogenous transfection of miRNA-5196 reduced profibrotic properties in DZNep+TLR8-treated monocytes

We then used a computational algorithm to identify possible miRNAs that target the downstream signal molecule Fra2. Based on bioinformatics analysis, we have selected miRNA-5196 because it hybridises to 5 seed regions of 5'UTR of Fra2 transcript in the position of 45-51, 361-367, 404-410, 592-458, 1055-1061 (Fig. 3a). Formation of such duplexes lead to miRNA-mediated transcriptional repression. Subsequently, to evaluate the functional effect of miRNA-5196 repression on profibrotic genes, HC monocytes were transfected with exogenous miRNA-5196 following DZNep+TLR8 stimulation and both Fra2 and TIMP-1 expression levels were measured. As shown in Fig. 3b, the expression level of Fra2 was significantly reduced ($p=0.0001$, 4.1-fold) upon miRNA-5196 transfection in stimulated monocytes. In contrast, the Fra2 expression was only slightly reduced in monocytes transfected with non-targeting miRNA (scramble miRNA), suggesting specific binding of miRNA-5196 to Fra2 target gene (Fig. 3b). Accordingly, TIMP-1 was significantly attenuated ($p=0.046$, 2.1-fold) in miRNA-5196 transfected monocytes, but not in scramble treated monocytes. These findings imply that miRNA-5196 negatively regulates its target gene Fra2 and indirectly TIMP-1 following stimulation.

Elevated level of miRNA-5196 in monocytes and sera from SSc patients

Since Fra2 plays an important role in SSc pathogenesis, we have analysed whether the expression of miRNA-5195 targeting Fra2 is abrogated in monocytes and sera from SSc patients. As shown in Fig. 4a, miRNA-5196 was significantly ($p=0.014$) elevated in SSc monocytes compared to HC, however treatment with DZNep+TLR8 resulted in further reduction of miRNA-5196 expression in SSc monocytes ($p=0.003$, 1.8-fold). This implies that the DZNep+TLR8 treatment not only induces profibrotic (Fra2, TIMP-1) and proinflammatory mediators (IL-8 and ROS), but it also reduces antifibrotic miRNA5196. Next, we measured the expression level of circulating miRNA-5196 in sera in order to determine whether miRNA-5196 can be used as a potential biomarker for SSc. The level of miRNA-5196 was significantly elevated in all SSc patients ($p=0.001$, 3-fold) (Fig. 4b). Interestingly, the patients with limited SSc (lSSc) had 2.7-times higher level of miRNA-5196 compared to patients with diffused SSc and 3.79-fold increased level of miRNA-5196 ($p=0.0001$) compared to HC sera. In addition, there was a positive correlation ($p=0.03$) based on Spearman analysis between the miRNA-5196 expression and the CRP level in SSc sera (Fig. 4c). However, no correlation was seen between miRNA-5196 and Rodnan skin score (Fig. 4d) and between miRNA-5196 and ESR (Figure 4e), probably due to a limited number of samples. CRP is a general inflammatory parameter, suggesting that miRNA-5196 can help to monitor disease severity and to understand its pathogenesis. Indeed, it has been shown that the increased level of CRP correlates with disease activity, severity, poor pulmonary function and shorter survival of SSc patients [18]. Therefore, miRNA-5196 along with CRP, but not with ESR, level may be used for better SSc diagnosis and to provide information of the

disease outcome. We also measured the expression level of circulating miRNA-5196 in sera of other rheumatic disease including RA. The level of miRNA-5196 was significantly elevated (data not shown), suggesting that miRNA-5196 can be used as a potential biomarker for other rheumatic disease including RA.

Discussion

Systemic sclerosis (SSc) is a complex autoimmune connective tissue disease characterised by vascular injury, inflammation and tissue fibrosis. Inflammation in SSc is mediated mostly by the tissue infiltrating CD14⁺ monocytes where they can differentiate into macrophages or dendritic cells during early phases of the disease [19]. SSc monocytes also display the elevated level of collagen and α -SMA, which indicates their maturation towards myofibroblasts and their important role in fibrogenesis [20].

To date, much evidence underscores the effect of environmental factors, including viral infection activating the TLR signaling pathway, smoking or pollution on SSc pathogenesis [21-23]. It has been reported that environmental factors are associated with epigenetic abnormalities contributing to inflammation and fibrosis development in SSc [23-25]. This suggests that abnormal SSc monocytes, responsible for initiation of inflammation and subsequently fibrosis, may develop from a subset of cells that have escaped from a normal control mechanisms following exposure to environmental agents. Furthermore, there is a strong correlation between inflammation and fibrosis by upregulation of proinflammatory and profibrotic markers in SSc sera and in affected skin tissue [26, 27]. Thus, finding the mechanisms which will modulate inflammation and fibrosis mediated by circulating monocytes may provide therapeutic opportunities to treat SSc in early stages of disease.

Our findings demonstrate that epigenetic modification induced by the histone methylation inhibitor (DZNep) and the activation of TLR8 signaling pathway lead to the increased expression TIMP-1 and its upstream mediator Fra2 both in HC and SSc monocytes (Fig. 1). The increased profibrotic gene expression was compatible with morphological changes in activated monocytes. Fig. 1d illustrates that only DZNep+TLR8 stimulated monocytes had elongated shapes and form spheres, but not in DZNep or TLR8 alone or in apicidin+TLR8 treated cells. Apicidin is another epigenetic modifier inducing histone hyperacetylation by inhibition of histone deacetylase. This is consistent with our previous results showing the synergistic effect of DZNep+TLR8 stimulation on TIMP-1 expression and the induction of fibroblasts transdifferentiating by HC monocytes, but not in apicidin+TLR8 treated cells [1]. These data imply that only a specific epigenetic modification along with TLR8-activation is able to induce profibrotic properties in monocytes.

Since the balance between TIMPs/MMPs is altered in SSc leading to abnormal ECM formation, we have also measured the expression levels of MMP-1 and MMP-15, which catalytic activity is blocked by TIMPs. We have noted that the basal levels of MMP-1 and MMP-15 were significantly reduced ($p=0.034$, $p=0.018$, respectively) in SSc monocytes compared to HC. Further stimulation did not affect MMPs expression (Fig. 1h, i) suggesting that DZNep+TLR8 treatment changes TIMP-1/MMPs ratio only by enhanced TIMP-1 production.

Next, we have shown the induction of proinflammatory mediators including IL-8 and ROS and the reduced expression of antioxidant enzyme SOD1 in HC and SSc monocytes, suggesting the disturb redox homeostasis upon treatment (Fig. 2). Indeed, it has been demonstrated that ROS promotes a profibrotic phenotype in SSc fibroblasts by inducing the collagen and autoantigens development [9, 28]. Another study has reported that direct injection of ROS induces local formation of DNA-topoisomerase autoantibodies and

development of lung and skin fibrosis in mouse model [29]. Similarly, the increased level of IL-8 in SSc sera correlates with the severity of a disease [30, 31]. This suggests that proinflammatory IL-8 and ROS play an important role in SSc pathogenesis.

In order to reduce DZNep+TLR8-mediated profibrotic phenotype of monocytes, we used the miRNA specifically targeting Fra2 transcription factor and subsequently TIMP-1 expression (Fig. 3). Based on prediction algorithms we selected miRNA-5196 which binds to 5 seed regions within 3'UTR of Fra2. Next, we tested if a computational analysis can be applicable in experimental approaches. Indeed, the exogenous transfection of miRNA-5196 significantly reduced the expression of Fra2 and TIMP-1. In contrast, transfection with non-targeting miRNA derived from *C. elegans* did not reduce expression of Fra2 and TIMP-1 following DZNep+TLR8 stimulation. This suggests that only a specific binding within 3'UTR resulted in direct Fra2 reduction and indirect TIMP-1 inhibition. Similarly, we have previously found that exogenous transfection with miRNA-29a targeting TAB1 reversed profibrotic phenotype of SSc fibroblasts by indirect inhibition of TIMP-1 [32]. TAB1 is the TGF- β -dependent upstream mediator regulating TIMP-1. Another study also showed therapeutic role of miRNA in SSc fibroblasts. Transfection with miRNA-29 targeting collagen reversed profibrotic phenotype of SSc fibroblast, which implies that specific miRNAs targeting profibrotic genes could be used in SSc therapy. [33]. Next, we compared the level of miRNA-5196 between HC and SSc monocytes (Fig. 4). The basal level of miR-5196 was elevated in SSc monocytes compared to HC, however stimulation with DZNep+TLR8 significantly reduced miRNA-5196 expression in SSc monocytes. This demonstrates that DZNep+TLR8 stimulation not only induces the secretion of TIMP-1 and ROS but also reduces the expression of antifibrotic miRNA-5196 and antioxidant SOD1, which subsequently develops pathogenic properties of monocytes.

Although miRNAs are already tested in gene therapies, numerous studies have also explored that miRNA could be used as a potential biomarker due to their specificity, stability and release under conditions of pathophysiological stress or disease. Moreover, miRNA can be easily available from circulatory biofluids eliminating painful biopsies. Some miRNAs have been already validated as versatile biomarkers of rheumatic diseases. In particular, abnormal expression of miRNA-146a has been described in several rheumatic diseases including RA, Systemic lupus erythematosus or Sjögren's syndrome [34-36]. Thus, finding circulating biomarkers reflecting ongoing inflammatory or fibrotic processes may provide a promising new direction for future diagnosis of SSc and probably other rheumatic diseases. We have shown that circulating miRNA-5196 was significantly elevated in SSc sera and monocytes compared to healthy controls (Fig. 4). This suggests that increased level of miRNA-5196 may serve as a compensatory mechanism to restore proper expression its indirect target gene TIMP-1 which is regulated by Fra2. Also, upregulated serum expression of miRNA-5196 positively correlated with the CRP level in SSc patients. These findings indicate that miRNA-5196 can be used as a biomarker of inflammation suggesting of SSc activity and severity. Our data are similar to other findings showing that miRNA-29c was readily detected from urinary exosomes and correlated with progression of renal fibrosis in patients with lupus nephritis [37]. Also, circulating miRNA-223 positively correlated with CRP and DAS28 score in patients with early RA [38].

In conclusion, we have shown for the first time that the epigenetic agent (DZNep) and TLR8 agonist mimicking viral infections upregulate TIMP-1 and inflammatory markers including ROS and IL-8 in HC and SSc monocytes. Fra2-mediated TIMP-1 production was blocked by

miRNA-5196 in profibrotic monocytes. Furthermore, the increased expression of miRNA-5196 in SSc sera positively correlated with the CRP level, but not with ESR. Overall, our study may open new possibilities for the development of miRNA-5196-based diagnostics and therapy for SSc.

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Figure legends

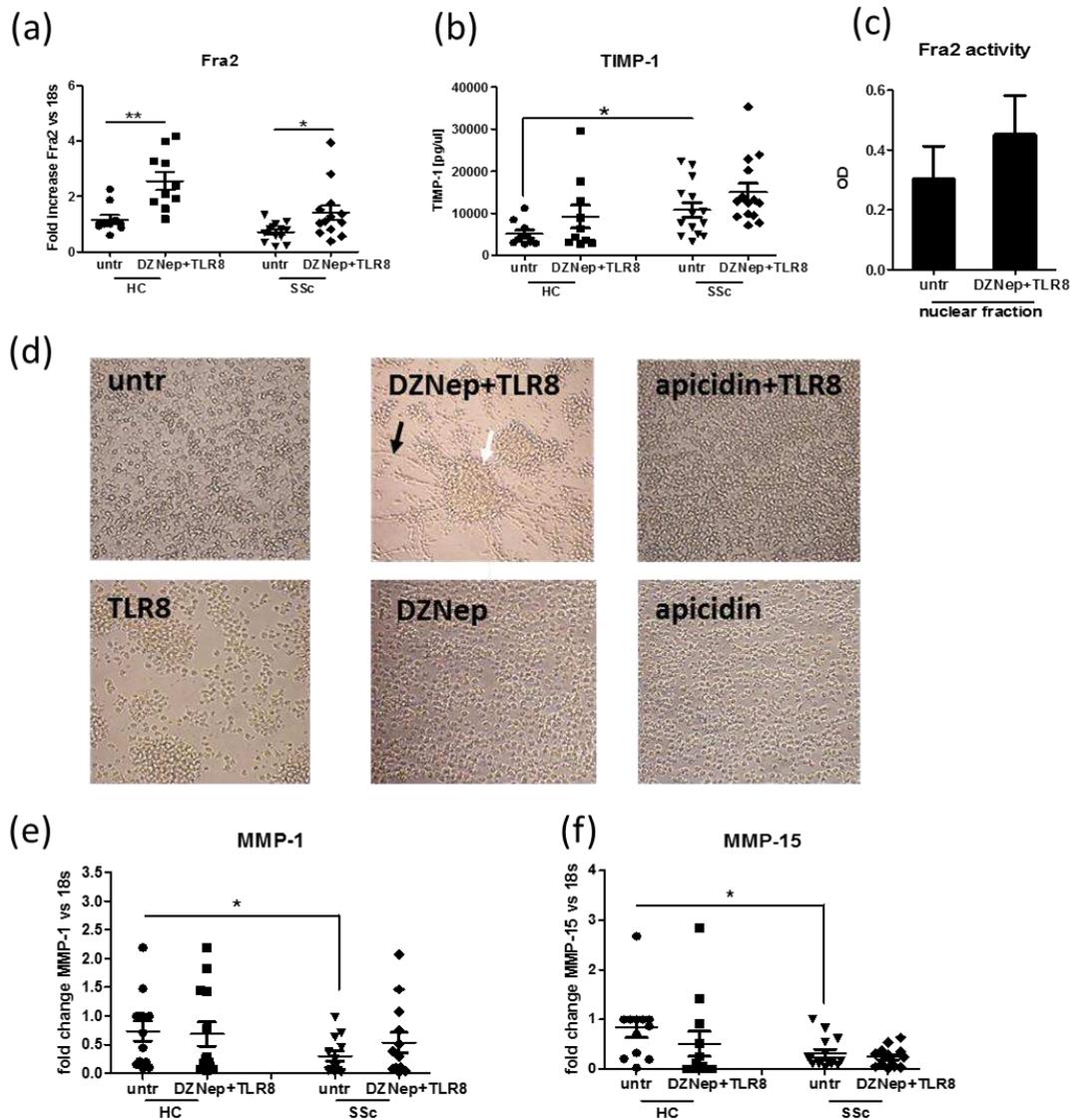


Figure 1. The effect of DZNeP+TLR8 stimulation on profibrotic properties induction in monocytes. HC and SSc monocytes were treated for 24 h with TLR8 ligand+DZNeP and expression of Fra2 (a) and TIMP-1 synthesis (b) were measured. Nuclear fraction (n=5) was isolated from 24 h DZNeP+TLR8 treated or untreated U937 monocytic cell line (c).

Morphological changes in HC monocytes following treatment were observed using light microscope (d). White arrows indicate cells aggregation and black arrow indicates elongated shape. Magnification used 20x. HC and SSc monocytes were DZNep+TLR8 treated for 24 h and MMP-1 (e) or MMP-15 (f) expression were measured.

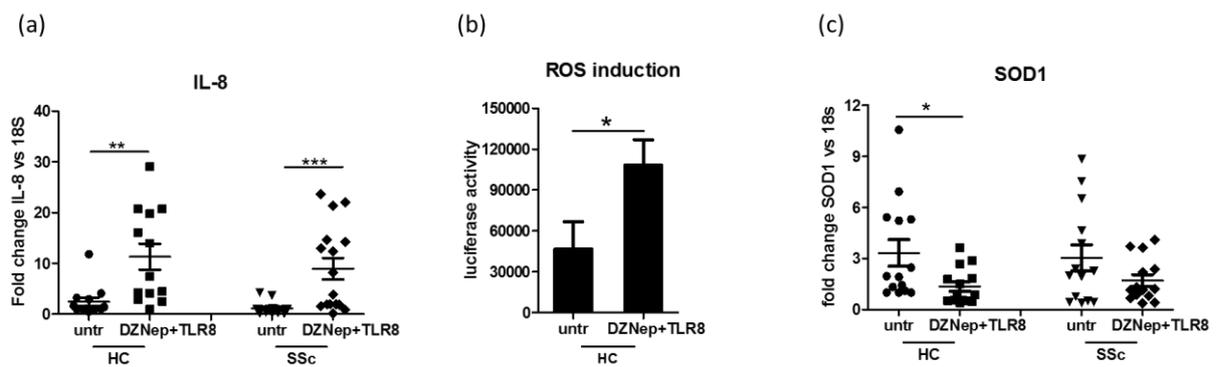


Figure 2. DZNep+TLR8-mediated IL-8, ROS, SOD1 expression. HC and SSc monocytes were DZNep+TLR8 treated for 24 h and IL-8 (a) and SOD1 (c) expression were measured. ROS induction was measured in HC monocytes (n=9) (b).

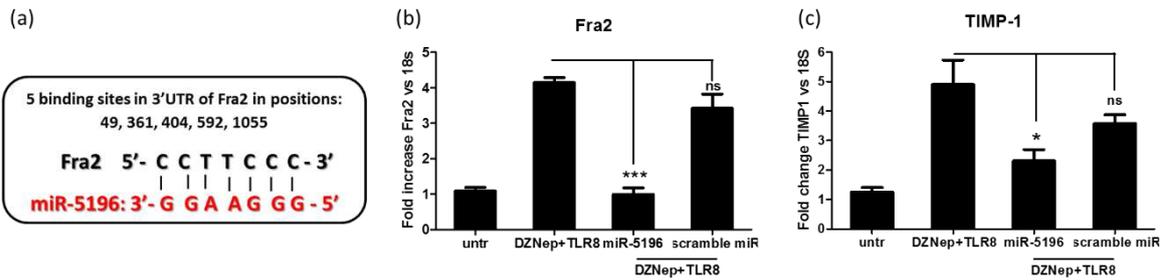


Figure 3. MiRNA-5196 reverses profibrotic phenotype in monocytes. Based on prediction algorithms, miRNA-5196 binds into 5 regions of 3'UTR of Fra2 in positions 49, 361, 404, 592, 1055 (a). HC monocytes were transfected with exogenous miRNA-5196 or scramble miRNA prior to DZNep+TLR8 stimulation and Fra2 (b) and TIMP-1 (c) were measured.

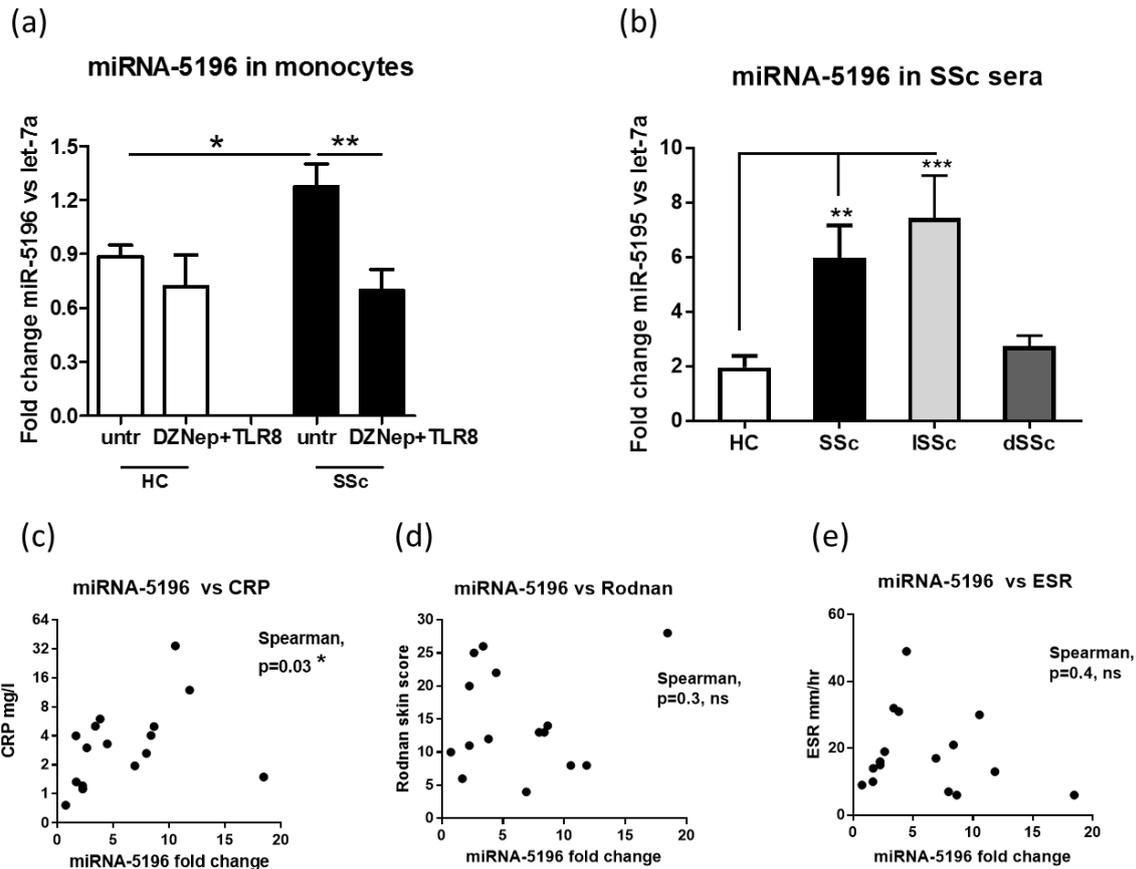


Figure 4. Increased level of miRNA-5196 in monocytes and sera from SSc patients. HC (n=9) and SSc (n=10) monocytes were DZNep+TLR8 treated for 24 h and miRNA-5196 was measured (a). Circulating miRNA-5196 was measured in HC (n=21), SSc sera (n=16) including ISSc (n=11) and dSSc (n=5) (b) and correlated with CRP level (c), Rodnan skin score (d) and ESR (e).

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Table 1. Clinical and laboratory data of SSc patients.

Parameters of SSc patients (n = 22)	SSc Patients
Age, years, median (range)	65 (27–86)
Sex F/M	18/4
Disease duration, years, average (range)	4 (0–12)
diffuse SSc, % (n)	36 % (n = 8)
limited SSc, % (n)	64 % (n = 14)
Raynaud's phenomenon, % (n)	100 % (n = 22)
modified Rodman skin score in 17 body surfaces 0-3, median, (range)	13 (6-29)
pulmonary hypertension, % (n)	13 % (n = 3)
lung fibrosis, % (n)	41 % (n=9)
Smoker, % (n)	32 % (n=7)

Parameters of SSc patients (n = 22)	SSc Patients
CRP, mg/L, median (range)	4.64 (1.12–34.49)
ESR, mm/h (range)	17.94 (6-49)
Active SSc	24% (n=5)
ACA, % (n)	22 % (n=5)
ANA, % (n)	95 % (n=21)
SCL-70, % (n)	50 % (n=11)
Anti-RNP, % (n)	9 % (n=2)

Table 2. Treatment of SSc patients.

<i>Treatment</i>	
Prednisone, % (dose of treated patients)	9 % (5 mg)
Methylprednisolone, % (dose of treated patients)	13 % (2-4 mg)
Azathioprine, % (n)	18 % (n=4)
Cyclophosphamide, % (n)	23 % (n=5)

Table 3. Sequence of primers and numbers of probes used in gene expression study.

Gene	Forward primer	Reverse primer	TaqMan Gene Expression Assays (Thermo Fisher Scientific)
Fra2	5'-ACG CCG AGT CCT CTC CA-3',	5'-TGA GCC AGG CAT ATC TAC CC-3'	
TIMP-1	5'-GAC GGC CTTC TGC AAT TCC-3'	5'-GTG GTC TGG TTG ACT TCT G-3'	
18S	5'-GAA TGG CTC ATT AAATCAGTTATGG- 3'	5'-TAT TAG CTC TAG AAT TACCACAGTTATCC-3'	
MMP-1	5'-AGT GAC TGG GAA ACC AGATGCTGA-3'	5'-GCT CTT GGC AAATCTGGCCTGTAA-3'	
SOD1			Hs00916176_m1, Amplicon Length 138
IL-8			Hs00174103_m1, Amplicon Length 101
MMP-15			Hs00233997_m1, Amplicon Length 68
18S			Hs99999901_s1, Amplicon Length 187