

Histone demethylation and TLR8-dependent crosstalk in monocytes promotes trans-differentiation of fibroblasts in systemic sclerosis via Fra2

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ABSTRACT

Objectives: To investigate whether epigenetic changes can modulate monocytes to produce tissue-inhibitor of metalloproteinase-1 (TIMP-1) via Fra2 (AP-1 family member), a novel downstream mediator promoting fibrogenesis.

Methods: AP-1 transcription factors and TIMP-1 expression was measured in monocytes from systemic sclerosis (SSc) patients and healthy controls (HC). Involvement of Fra2 in the regulation of TIMP-1 following TLR8 agonist treatment was investigated using luciferase activity assay and ChIP analysis. Expression of TIMP-1 and Fra2 was determined in response to TLR8 treatment and different histone modifications including 3'deazaneplanocin (DZNep) and apicidin. HC fibroblasts were co-cultured with DZNep plus TLR8-treated HC monocytes.

Results: Upregulation of Fra2 was detected in bleomycin-challenged mice and SSc skin biopsies. Enhanced expression of Fra2 and TIMP-1 was correlated in SSc monocytes ($p=0.021$). The expression of Fra1 was significantly ($p=0.037$) reduced in SSc monocytes. Inhibiting AP-1 activity reduced TIMP-1 production in TLR8 stimulated HC and SSc monocytes. ChIP experiments revealed binding of Fra-2 to the TIMP-1 promoter. Combination of DZNep plus TLR8 enhanced Fra2 and TIMP-1 expression in HC monocytes, whereas TLR8 plus apicidin repressed Fra2 and TIMP-1 expression. Finally, DZNep plus TLR8-treated HC monocytes induced strong production of α -SMA in dermal fibroblasts, which was inhibited by TIMP-1 blocking antibody.

Conclusions: These data demonstrate a novel role of histone demethylation induced by DZNep on Fra2-mediated TIMP-1 production by monocytes in the presence of TLR8 agonist. This consequently orchestrates fibroblasts' trans-differentiation, a key event in the pathogenesis of SSc.

Introduction

Systemic sclerosis (SSc) is an autoimmune connective tissue disease characterised by accumulation of extra cellular matrix (ECM) proteins in skin and visceral organs, resulting in organ dysfunction and premature death (1). This ECM deposition, predominantly composed of collagen, is observed when the homeostasis between matrix metalloproteinases (MMPs) and their natural inhibitors is lost. Tissue inhibitor of metalloproteinases-1 (TIMP-1) is a specific inhibitor of MMPs regulating the balance of ECM turnover, which is mostly produced by fibroblasts, hepatic stellate cells (HSC) and monocytes. Serum concentrations of TIMP-1 are increased in SSc patients, thereby contributing to fibrosis development (2). Furthermore it has been shown that overexpression TIMP-1 is important for growth, proliferation and pathogenic differentiation of fibroblasts, which demonstrates an additional role of TIMP-1 independent of its inhibition of MMP activity (3, 4).

Another hallmark of SSc is perivascular infiltration of immune cells, mainly monocytes, which are the first immune cells to infiltrate SSc skin (5). Skin infiltrating CD14⁺ monocytes can secrete profibrotic TIMP-1 in SSc patients and TIMP-1 production is enhanced upon TLR8 stimulation by ssRNA (6). This raises the possibility that circulating monocytes expressing TIMP-1 migrate into skin and contribute to increased deposition of ECM proteins. The important role of monocytes in the pathogenesis of fibrotic conditions is further supported by preclinical evidence in mice where depletion of monocytes attenuated lung fibrosis after bleomycin challenge, a fibrosis model (7, 8). This clearly indicates that monocytes play an important role in the pathogenesis of SSc, however the factors and molecular mechanisms promoting TIMP-1 expression are not yet fully defined.

It has been shown that AP-1 (activator protein 1) family transcription factors, including Fra2 and JunD, play a prominent role in pathological ECM production. Studies in an animal model of liver fibrosis showed that the AP-1 complex plays a key role in the transcriptional regulation of TIMP-1 and IL-6 activity. This AP-1 complex contributes to the matrix protein deposition by HSC via their trans-differentiation into myofibroblasts-like HSC (9). Removal of specific AP-1 binding sites located in the TIMP-1 promoter caused significant reduction of promoter activity in HSCs (10). Another study demonstrated that SSc dermal fibroblasts treated with the AP-1 inhibitor T-5224 decreased the

expression of collagen and fibronectin in a dose-dependent manner (11). Furthermore, Fra2 transgenic mice developed a proliferative vasculopathy of the lung and skin fibrosis, resembling similar disease manifestations seen in SSc patients (12). On the other hand, in Fra2 knockout mice, collagen expression upon bleomycin administration was strongly reduced, demonstrating that Fra2 is a novel downstream mediator for fibrosis development in SSc (13).

Recently epigenetic modifications including histone acetylation or methylation have been implicated in SSc development. Histone demethylation, induced by 3'deazaneplanocin (DZNep), is involved in collagen induction (14). It has been shown that DZNep inhibits, whereas Methyl-CpG binding protein 2 (MeCP2) upregulates expression of histone 3 methyltransferase – Enhancer of zeste homologue-2 (Ezh2) (15, 16). Ezh2 catalyzes trimethylation of lysine 27 on histone 3 (H3K27me3). Inhibition of H3K27me3 by DZNep induced strong expression of Fra2, CTGF and collagen *in vivo* and *in vitro* models (14). In contrast, inhibition of histone deacetylases (HDACs) by Trichostatin A (TSA) decreased collagen expression in bleomycin-treated mice (17, 18). Similarly, murine fibroblasts from distal pulmonary arteries treated with apicidin (selective class I HDAC inhibitor) reduced the expression of monocyte-attracting chemokine (MCP-1) (19). MCP-1 is a crucial chemokine for the recruitment of monocytes and supports a cytokine network maintaining tissue inflammation and fibrosis progression, since MCP-1 deficient mice have reduced carbon tetrachloride (CCL4)-induced fibrosis (20). Overall, this suggests that histone modifications affect fibrogenesis, which may have therapeutic implications. However, a clear understanding of how histone demethylation or acetylation can modulate TIMP-1 production in monocytes is still lacking.

We hypothesised that Fra2 plays a key role in TLR8-mediated TIMP-1 production by SSc monocytes and that epigenetic changes affect TIMP-1 production by monocytes. This enhanced production of TIMP-1 may initiate myofibroblasts differentiation and consequently fibrosis progression.

Materials and methods

Patients, controls and bleomycin-induced mouse model

Blood donors: thirteen patients with SSc (mean age 61.7 years) and 18 healthy controls (HC) were included in the study. All patients with SSc fulfilled the American College of Rheumatology criteria according to LeRoy (21). Monocytes from an IRAK4-deficient patient characterised by absent cytokine responses to TLR ligands were also used in this study (6). Dermal HC fibroblasts were cultured from skin biopsies as described before (22). Skin biopsies from 5 SSc and 5 HC that had been formalin fixed and paraffin embedded and sectioned at 10µm were used for qRT-PCR. Briefly tissue was deparaffinised using xylene and then rehydrated through descending ethanols (100%-70%) and RNA was extracted after proteinase K digestion using the Allprep RNA FFPE kit (Qiagen). 250 ng of RNA was then reverse transcribed to cDNA. We used bleomycin-induced skin fibrosis in mice, to mimic the early-inflammatory stage of SSc. Mice were anaesthetised with isoflurane, their backs shaved and 100 µl 0.5 mg/ml bleomycin or saline (vehicle) administered via subcutaneous injection to an area approximately 1 cm². Injections were repeated every other day for 4 weeks at which point mice were sacrificed.

Sample collection and cell purification

Blood was collected in EDTA-coated tubes from HC and SSc patients. Peripheral blood mononuclear cells were separated from whole red blood cells as described elsewhere (6).

Compounds, reagents and *in vitro* cell cultures

CD14⁺ monocytes from HC and SS patients, U937 monocytic cell line or HC fibroblasts were seeded in 24-well Costar plates at a concentration of 5×10^5 cells/ml and cultured for 24 h in 500 µl of RPMI (Invitrogen) supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), L-glutamine (2 mM) (all Sigma) and 10% FSC at 37°C in 5% CO₂. TLR8 agonist (ssRNA40/LyoVec, InvivoGen), DZNep (4703, Tocris), apicidin (A8851, Sigma), TSA (T8552, Sigma), AP-1 inhibitor (SR11302, Tocris), recombinant TIMP-1 (PHC8024, Invitrogen), recombinant TGF-β (R&D Systems) and anti-TIMP-1 antibody (AF970, R&D Systems) were kept in sterile conditions in -20°C.

Microscopic analysis of mouse skin and HC fibroblasts

For immunohistochemical analysis, deparaffinised, ethanol dehydrated skin sections from bleomycin or saline treated mice were incubated with 20 µg/ml of proteinase K (P2308, Sigma-Aldrich) for 20 min. at 37°C and stained with rat anti-mouse pan macrophage marker F4/80 (14-4801, eBioscience,) and anti-mouse Fra2 (ab1827, Abcam,) primary antibodies overnight at 4°C. Incubation with secondary antibodies (goat anti-rabbit-PE, Santa Cruz, sc-3739 and donkey anti-rat-FITC, Jackson ImmunoResearch, 712-096-153) was performed for 1 h at RT. For immunocytochemical analysis, HC fibroblasts were seeded in 24-well glass coverslips, fixed with 4% PFA and permeabilized with 0.1% Triton X-100/PBS for 4 min. Following intense washing, coverslips were incubated with mouse anti- α -SMA-FITC (A2547, Sigma-Aldrich) for 1 h at RT. Both mouse skin sections and coverslips with HC fibroblasts were further analysed by fluorescent microscopy Leica DM4000, using LASAF v4 software.

Semiquantitative gene expression study

RNA from freshly isolated monocytes was obtained using TRIzol method. RNA (1000 ng) was reverse transcribed to cDNA with the use of random hexamers and the Moloney murine leukaemia virus reverse transcriptase enzyme (Invitrogen), according to the manufacturer's protocol. cDNA (20 ng), forward and reverse primers were used for Fra2 - For-5'-ACGCCGAGTCCTACTCCA-3', Rev-5'-TGAGCCAGGCATATCTACCC-3'; TIMP-1 - For-5'-GACGGCCTTCTGCAATTCC-3', Rev-5'-GTGGTCTGGTTGACTTCTG-3'; Fra1 - For-5'-ACAGATCAGCCCGGAGGAAG-3', Rev-5'-CTCCAGTTTGTGAGTCTCCGC-3'; JunD - For-5'-CAGCGAGGAGCAGGAGTT-3', Rev-5'-GAGCTGGTTCTGCTTGTGTAAT-3'; JunB - For-5'-CACAAGATGAACCACGTGACA-3', Rev-5'-GGAGTAGCTGCTGAGGTTGG-3'; c-Jun - For-5'-CCAAGGATAGTGCGATGTTT-3', Rev -5'-CTGTCCCTCTCCACTGCAAC-3'; Ezh2 - For-5'-TGTGGATACTCCTCCAAGGAA-3', Rev -5'-GAGGAGCCGTCCTTTTCA-3'; HDAC3 - For-5'-GGGTGGTGGTGGTTATACTGTC-3', Rev-5'-ATGAAACGGGGTCTGAAGTGTGGAGTA-3'; coll 1A1 - For-5'-CAAGAGGAAGGCCAAGTCGAGG-3', Rev-5'-CGTTGTCGCAGACGCAGAT-3'; α -SMA - For-5'-CAGGGAGTAATGGTTGGAAT-3', Rev-5'-TCTCAAACATAATCTGGGTCA-3'; 18S - For-5'-CGAATGGCTCATTAATCAGTTATGG-3', Rev-5'-TATTAGCTCTAGAATTACCACAGTTATCC-3', MeCP2 - For-5'

GATCAATCCCCAGGGAAAAGC-3', Rev-5'-CCTCTCCCAGTTACCGTGAAG-3'. Samples were analysed in triplicate and normalised to the 18S housekeeping gene using the AB7500 (Applied Biosystems) qRT-PCR machine and programme. 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.

Western Blot and ELISA analysis

Lysates of 5×10^5 U937 cells were run on 12% SDS PAGE and transferred to membranes and probed using rabbit anti-H3K27me3 (SAB4800025, Sigma) or anti-H3 (SAB4500352, Sigma-Aldrich) followed by anti-rabbit HRP (DAKO). 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). Signal development was performed using horseradish peroxidase/streptavidin and o-phenylenediamine dihydrochloride substrate (Sigma-Aldrich) at RT. Fluorescence was measured with a plate reader (Tecan, Sunrise). Samples were run in duplicate and serial dilution was performed to fall within the detection limits of the assay (0–40 ng/ml).

Transfection experiment

HC monocytes were transfected with siFra2 (100 μ M), siEzh2 (100 μ M) or scramble siRNA (100 μ M) (On-target plus-SMART pool, Dharmacon, UK) using Viromer (Lipocalyx GmbH) for 48 h. U937 cells were transfected with DNA fragments of the TIMP-1 promoter cloned downstream of luciferase plasmid in a pGL2-basic vector or empty pGL2 at the final concentration of 300 ng using 1.5 μ l of Fugene HD (Promega) transfection reagent per well of a 24 well plate. Renilla plasmid at the final concentration of 30 ng was used to normalise transfection efficiency. 24 h after transfection, cells were stimulated with TLR8 agonist for an additional 6 hours. Following stimulation, assays for reporter gene activity were performed according to Dual-luciferase Promega's protocol and analysed using Glomax multi detection system (Promega, UK).

Chromatin immunoprecipitation experiments

U937 monocytic cell were cultured and stimulated with ssRNA Iyovec (Invivogen) at the appropriate concentration and after 4 hours cells were fixed in 1% formaldehyde to cross-link chromatin, then lysed by sonication, cleared chromatin was then incubated with 4 µg anti Fra2 antibody (sc-604) or 4 µg control matched isotype antibody, the complexes precipitated, washed and eluted. Crosslinks were reversed and cDNA was isolated. qPCR was performed for TIMP-1 promoter region using primers proximal For' ATTTGAGACCCTGGCTTTGG Rev' GCAGCAGTGGAGGGAGATAA (162bp), and distal promoter region For' CACGCCTGTAATCCCAACAC Rev' CCTCCGGGGTTCAAGAGATT (194bp) the values were normalised to average values of control antibody and expressed as fold enrichment above isotype control.

Results

Marked Fra2 expression in skin-infiltrating macrophages in bleomycin-treated mice and skin biopsies from SSc patients

To confirm that skin-infiltrating macrophages express Fra2 in bleomycin-induced dermal fibrosis, immunohistochemical staining of mouse skin sections was performed. It can be seen that macrophages (defined by F4/80 antigen expression) accumulating in skin of bleomycin-treated mice (Figure 1B) expressed more Fra2 compared to control saline-treated animals (Figure 1A). The white arrows indicate the overlay images between F4/80 and Fra2 expression. Fra2 expression was also seen in the perivascular area, since vasculopathy is one of the manifestations of SSc progression (23). This is consistent with previously published data showing a positive staining of c-Jun, c-Fos (other AP-1 family members) in fibroblasts, keratinocytes and in endothelial cells of bleomycin-challenged mice (11, 24). Specificity of antibodies staining was validated using negative control (Supplemental Figure1). Increased expression of Fra2 ($p=0.0034$) was also seen in skin biopsies from SSc patients compared to healthy controls by qPCR (Figure 1C).

Different expression of AP-1 transcription factors in HC and SSc monocytes

It has been shown that Fra2, which is AP-1 family member, plays an important role in organ fibrosis therefore we also analysed the expression pattern of all AP-1 members and TIMP-1 in monocytes following TLR8 stimulation. HC and SSc monocytes were treated with TLR8 agonist (ssRNA) for 24 h and gene expression was measured. It can be seen (Figure 2A, B) that both Fra2 ($p=0.002$, 2.4-fold) and TIMP-1 (1.6-fold) increased in TLR8-treated SSc monocytes compared to HC monocytes. In contrast, Fra2 and TIMP-1 were repressed in monocytes from an IRAK4-deficient patient upon TLR8 stimulation, indicating the crucial role of the TLR signaling pathway in Fra2 and TIMP-1 induction. This patient has a genetic lesion in IRAK-4 that leads to no IRAK-4 protein production. Interestingly, there was also a positive correlation between Fra2 and TIMP-1 expression in SSc monocytes ($p=0.02$) upon TLR8 stimulation (Supplemental Figure 2), but not in HC ($p=0.38$), which corroborates Fra2-mediated TIMP-1 expression in diseased monocytes. Surprisingly, the basal level of TLR8 was 2.1-fold lower ($p=0.037$) in SSc monocytes compared to HC monocytes (Figure 2C). The same tendency was observed following ssRNA stimulation showing 1.6-fold lower expression of TLR8 in SSc monocytes compared to HC. This suggests that diseased monocytes may have an altered downstream signaling pathway maintaining strong Fra2 and TIMP-1 expression despite reduced expression of TLR8.

We also analysed the expression of other AP-1 members in HC and SSc monocytes. As expected, Fra1 was significantly downregulated in TLR8-treated ($p=0.03$) and untreated ($p=0.01$) SSc monocytes compared to HC (Figure 2D). The expression of other AP-1 family members including JunD (Figure 2G) and JunB (Figure 2E) did not differ significantly between HC and SSc monocytes apart from the basal level of c-Jun, which was significantly downregulated ($p=0.04$) in SSc monocytes (Figure 2F). This indicates that only Fra2 plays a pivotal role during TLR8 stimulation in SSc monocytes.

TLR8 agonist induces TIMP-1 expression via AP-1 family member - Fra2

To test that TIMP-1 production is Fra2 dependent, HC and SSc monocytes were pre-treated with an AP-1 inhibitor prior to TLR8 stimulation. It can be seen (Figure 3A) that pre-treatment for 2 h with an AP-1 chemical inhibitor (SR 11302) significantly reduced TIMP-1 production in HC ($p=0.03$) and

SSc ($p=0.02$) monocytes stimulated with the TLR8 ligand. We used a different dilutions of AP-1 inhibitor (data not shown) and the concentration of 20 μM of AP-1 inhibitor was the most optimal for TIMP-1 inhibition and also did not affect cell viability, as determined by MTS assay (Figure 3B). In order to exclude off-target effects from AP-1 chemical inhibitor we performed Fra2 silencing in TLR8 stimulated primary HC monocytes. It can be seen in Figure 3C that genetic inactivation of Fra2 significantly ($p=0.0068$) reduced TIMP-1 expression. In contrast, TLR8 stimulated monocytes transfected with scramble siRNA did not display reduced TIMP-1 expression. To confirm Fra2 knockdown, Fra2 expression also was measured in primary monocytes (Supplemental Figure 3). We next measured the TIMP-1 promoter activity in the presence of TLR8 agonist in the human monocytic U397 cell line, since primary monocytes are difficult to transfect. U397 cells were transfected with a plasmid where TIMP-1 promoter was cloned downstream of firefly luciferase gene. We used renilla plasmid to normalise TIMP-1 plasmid expression in untreated, TLR8-treated or empty pGL-2 vector transfected cells. As seen in Figure 3D, TIMP-1 promoter activity was significantly increased in TLR8-treated cells compared to untreated ($p=0.01$) and cells transfected with empty vector ($p=0.001$), respectively. This suggests that both gene expression and promoter activity of TIMP-1 are increased upon TLR8 stimulation in monocytes. In order to determine whether TIMP-1 could be transcriptionally regulated by Fra-2, we performed ChIP analysis in TLR8 stimulated U937 cells (Figure 3E). We found that Fra2 was able to bind the TIMP-1 promotor in the proximal rather than a distal region (7-fold enrichment, $p < 0.0001$) suggesting a direct transcriptional activation of TIMP-1 by Fra-2.

TLR8 -mediated TIMP-1 production and its regulation by histone modification in U937 cells

Since epigenetic modifications are important in regulating fibrosis, we investigated the role of histone modification on Fra2 and TIMP-1 expression upon 24 h DZNep or TSA-treatment in U937 cells, a monocytoïd cell line. It can be seen that DZNep (reducing H3K27me3) treatment significantly increased Fra2 expression (Figure 4A, $p=0.01$), while TIMP-1 expression was only moderately elevated (Figure 4B). In contrast, TSA (inducing histone acetylation) treatment significantly reduced the expression of Fra2 ($p<0.001$) and TIMP-1 ($p=0.01$). Western blot analysis confirmed the specific

activity of DZNep, because the level of H3K27me3 was reduced in DZNep-treated compared to untreated cells (Figure 4C). The total histone 3 (H3) was used as a loading control across groups. The MTS test showed no significant changes in U937 cell viability upon TSA or DZNep treatment (Figure 4D).

Synergistic effect of TLR8 agonist and DZNep on Fra2 and TIMP-1 expression in HC monocytes

As seen before (Figure 4A), DZNep alone induces only a 2-fold upregulation of Fra2 in U937 cells, therefore we asked if combination of TLR8 agonist and DZNep can synergistically enhance Fra2 and TIMP-1 expression in primary HC monocytes. To test this hypothesis, HC monocytes were pre-treated with DZNep for 4 h and then stimulated with TLR8 agonist. The level of Fra2 and TIMP-1 was significantly increased in DZNep+TLR8-treated monocytes compared to monocytes stimulated with TLR8 or DZNep alone (Figure 5A, B). Furthermore, combined DZNep+TLR8 activation altered monocytes morphology, displayed as cell aggregations and elongated shape formation (Supplemental Figure 4). This suggests that DZNep+TLR8 synergistically induce strong Fra2 and TIMP-1 expression, which mirrors the changes seen in cell morphology. In contrast, pre-treatment with apicidin, which is more specific HDAC inhibitor than TSA, repressed the expression of Fra2 and TIMP-1 upon TLR8 stimulation. Also the morphology of apicidin+TLR8-treated monocytes remained as untreated (Supplemental Figure 4). The results from ELISA showed a similar pattern of DZNep+TLR8-mediated upregulation and apicidin+TLR8-mediated reduction of secreted TIMP-1 (Figure 5C). The MTS test showed no changes in cell viability in DZNep or apicidin-treated HC monocytes indicating that these concentrations are not toxic (Figure 5D).

We then investigated if the expression level of Ezh2, the enzyme involved in H3K27me3 modification and part of the polycomb repressive complex 2, is altered in HC and SSc monocytes upon TLR8 stimulation. DZNep is also involved in Ezh2 inhibition. The constitutive basal level of Ezh2 was significantly higher in SSc monocytes compared to HC monocytes ($p=0.01$) and was even more increased upon TLR8 stimulation in both HC and SSc monocytes (Figure 5E). We also measured the expression level of HDAC3, which can be specifically inhibited by apicidin. Although the basal level

of HDAC3 was higher in SSc monocytes than in HC ($p=0.01$), TLR8 stimulation did not change the HDAC3 expression in SSc monocytes. Only in TLR8-stimulated HC monocytes, HDAC3 expression was significantly increased compared to untreated (Figure 5F).

TLR8 agonist and DZNep treatment of HC monocytes induces fibroblasts trans-differentiation via TIMP-1

Finally, to evaluate the functional effect of monocytes-derived TIMP-1 on HC fibroblasts trans-differentiation, HC monocytes were stimulated with TLR8 agonist and different histone modifiers. To test this, HC monocytes were pre-treated for 4 h with DZNep or apicidin or transfected with siEzh2 for 24 h and vigorously washed in order to remove residual histone modifiers, since they could have a direct effect on collagen production in fibroblasts. Next, HC monocytes were additionally treated with TLR8 agonist to induce enhanced TIMP-1 production and inserted into trans-wells physically separating monocytes population from adherent HC fibroblasts. 48 h later, profibrotic markers including α -SMA (Figure 6A) and collagen (Figure 6B) were determined in HC fibroblasts co-cultured with TIMP-1-producing monocytes. DZNep+TLR8 or siEzh2+TLR8 treatment of HC monocytes induced significant upregulation of collagen and α -SMA genes in HC fibroblasts compared to untreated or apicidin+TLR8-treated HC monocytes. In addition using immunocytochemistry, HC fibroblasts showed a positive staining for α -SMA in the presence of monocytes stimulated with DZNep+TLR8 (Figure 6C). To confirm that α -SMA expression is induced by TIMP-1-producing monocytes, HC fibroblasts were also directly stimulated with human recombinant TIMP-1. Figures 6D and 6E have shown that HC fibroblasts stimulated with TIMP-1 significantly upregulated the expression α -SMA but not collagen. However, pre-treatment HC fibroblasts with a TIMP-1 neutralising antibody 2 h prior TIMP-1 treatment significantly reduced the expression level of α -SMA compared to fibroblasts treated only with TIMP-1 (Figure 6F). Isotype control antibody did not have any effect on the α -SMA production. As a positive control for α -SMA induction, HC fibroblasts were stimulated with TGF- β a known inducer.

Discussion

Previous results from our group have shown that monocytes from SSc patients contribute to the imbalance between TIMP-1 and MMPs upon TLR8 agonist stimulation (ssRNA) (6), but the role of the Fra2 transcription factor in TIMP-1 regulation is unknown. Fra2 is involved in several biological processes including differentiation, proliferation and oncogene transformation, but also is a downstream mediator promoting fibrogenesis (13, 24).

In this study we demonstrate that TLR8-mediated overexpression of TIMP-1 in SSc monocytes is mediated via Fra2. In addition, histone demethylation due to DZNep treatment enhances Fra2 and TIMP-1 expression in monocytes in the presence of TLR8 agonist and consequently induces fibroblasts trans-differentiation to myofibroblasts; the cell type responsible for excess ECM. In particular, we showed that Fra2 was upregulated in skin infiltrating macrophages in bleomycin-induced skin fibrosis, mimicking the early-inflammatory stage of SSc progression (25) and in skin biopsies from SSc patients. Furthermore, we showed a positive correlation between Fra2 and TIMP-1 expression induced by TLR8 stimulation in SSc monocytes, but not in HC monocytes, whereas TLR8 stimulation of an IRAK-4-deficient patient did not induce neither Fra2 nor TIMP-1 expression. Also, activation of Fra2 via TLR8 agonist was crucial for TIMP-1 induction, since an AP-1 inhibitor prevented TIMP-1 secretion in SSc monocytes. Similarly ChIP analysis confirmed direct *in vivo* activation of the TIMP-1 promoter by Fra2 in TLR8 stimulated U937 cells. Taken together these results confirm that Fra2 acts as a positive regulator of TIMP-1 production upon TLR8 stimulation by transactivating TIMP-1. In contrast, Fra1 expression was significantly reduced in SSc monocytes. The effect of Fra1 has been previously reported in Fra1 knockout mice, where Fra1 depletion resulted in exaggerated pulmonary fibrosis due to increased TIMP-1, collagen and TGF- β expression (26), while overexpression of Fra-1 in mice reduced proinflammatory cytokine production (27). On the other hand some studies have shown that Fra1 transgenic mice spontaneously developed biliary fibrosis (28). Further studies are needed to confirm whether Fra1 is a faithful biomarker in SSc.

To further investigate the role of TLR8 in TIMP-1 induction, U937 monocytic cells were transfected with a plasmid encoding the TIMP-1 promoter, which was cloned downstream of firefly luciferase

gene. The luciferase activity was significantly increased in the presence of TLR8 agonists, but not in untreated cells, confirming TIMP-1 activity is governed by TLR8 signaling.

Furthermore, DZNep-treated U937 cells increased the expression of Fra2 and TIMP-1, in contrast to TSA-treated cells. Western blot analysis corroborates the specificity of DZNep treatment, as the activity of H3K27me3 was reduced in DZNep-stimulated U937 cells. Histones in the chromatin undergo multiple post translational modifications that regulate gene expression and include acetylation and deacetylation by HDACs. A similar result of DZNep-dependent Fra2 induction has been previously shown in dermal HC fibroblasts (14). Interestingly, administration of DZNep to bleomycin-treated mice (inducing skin fibrosis) even further increased the number of fibroblasts expressing Fra2, a transcription factor regulating TIMP-1 (14). In contrast, administration of DZNep to mice during acute CCL4-induced injury (inducing liver fibrosis) decreased the expression of collagen and TIMP-1 (15). This suggests that DZNep may have the opposite effect on profibrotic genes expression due to organ specificity.

To further elucidate the role of histone modification(s) and TLR8 agonist on Fra2 and TIMP-1 expression, HC monocytes were stimulated with DZNep or apicidin alone or in combination with TLR8 agonist. Enhanced expression of Fra2 and TIMP-1 was observed when HC monocytes were stimulated with both DZNep and TLR8, than DZNep or TLR8 alone. This suggests that initial epigenetic changes included by DZNep dynamically modifies chromatin architecture allowing the TLR8 agonist to be a powerful stimulator of Fra2 transcription factor, which consequently enhances TIMP-1 production. In contrast chromatin alterations induced by apicidin may prevent TLR8 stimulation to activate Fra2, thereby repressing TIMP-1. Interestingly, blockade of HDACs through HDAC class I inhibitors suppresses angiotension II-mediated cardiac fibrosis, a model of heart fibrosis in the mouse. This was demonstrated by inhibiting 'activation' of cardiac fibroblasts and also by differentiation of fibrocytes from bone marrow cells (29). This is important as fibrocytes can develop from bone marrow-originated monocytes (30).

Surprisingly, the level of Ezh2 was 4.8-fold higher in SSc monocytes than HC basally and TLR8 stimulation even further enhanced Ezh2 expression. Ezh2 is part of the polycomb repressive complex

important in mediating chromatin modifications. Ezh2 is overexpressed in many cancers (31, 32). Similar data were previously published by Kramer et al, showing the upregulation of H3K27me3 due to Ezh2 activity in fibroblasts from SSc patients compared to HC fibroblasts. These results were explained by potential compensatory mechanism that regulates the profibrotic genes in SSc patients (14). In addition, MeCP2 has been shown to upregulate Ezh2 expression (24). We found high expression of MeCP2 in SSc skin biopsies compared to HC (data not shown). Therefore, the higher expression of Ezh2 in our system may be due to the elevated MeCP2 levels in SSc patients. Interestingly MeCP2 has been identified as a SNP conferring risk in SSc in a genome wide association study [27].

Although the expression of HDAC3, an example of class I HDACs repressed by apicidin, was significantly upregulated in HC monocytes upon TLR8 stimulation, it was not changed in TLR8-treated SSc monocytes. This suggests that HDAC3-dependent deacetylation does not play a significant role in SSc pathogenesis, or at least during TLR8 stimulation. Finally, we assessed the functional effect of DZNep+TLR8-treated monocytes on fibroblasts differentiation. Co-culture of DZNep+TLR8-treated or siEzh2+TLR8-treated monocytes with HC fibroblasts significantly upregulated the expression of α -SMA and collagen genes in fibroblasts. Furthermore, HC fibroblasts exhibited a positive staining for α -SMA, which is considered to be the marker for myofibroblasts (the effector cells in fibrosis). This confirms that DZNep+TLR8, but not apicidin+TLR8-treated monocytes induce production of TIMP-1, which has the ability to trans-differentiate fibroblasts toward pathogenic myofibroblasts. We have also demonstrated that HC fibroblasts directly stimulated with recombinant TIMP-1 upregulate α -SMA but not collagen expression. Neutralising anti-TIMP-1 antibody inhibited α -SMA expression, confirming an additional role of TIMP-1 as a growth factor regulating fibroblasts differentiation. This further shows the link between inflammatory cells and fibrosis in SSc and underpins a complex sequence of events beginning with innate immune activation. Our observations are consistent with previous findings showing that adenovirus-mediated overexpression of TIMP-1 significantly increased α -SMA in cardiac fibroblasts but had no effect on collagen production (3). Although in this study we demonstrated DZNep+TLR8-dependent TIMP-1

production, we cannot exclude the possibility that other factors promoting fibroblasts differentiation may also be induced following DZNep+TLR8 stimulation including TGF- β . This needs to be tested in the further experiments.

Overall, these results showed that selective, epigenetic modification of monocytes can either promote or repress myofibrogenic differentiation, and hence impact on the pathogenesis of SSc. A growing appreciation of the role of epigenetics in SSc and fibrosis is garnered and currently there are epigenetic drugs in use clinically for other indications (33).

Study Approval: The study was approved by the ethics committee (ethical approval no. 13/NE/0089 and 10/H0906/22 for the IRAK4-deficient patient) and written informed consent was obtained from all patients. Animal experiments were approved by local ethical review committee and performed under a UK Home Office license.

Competing interests: There are no competing interests.

Contributorship: Study Design: MC, JvL. Acquisition of Data and Analysis: MC. Interpretation of Data: MC, JvL, SO'R, SP, FO, KBK. Manuscript Preparation: MC, JvL, SOR.

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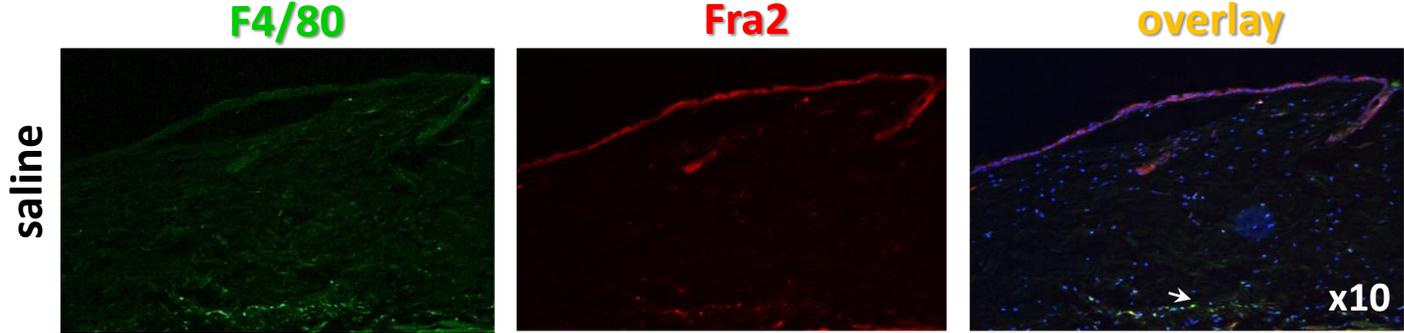
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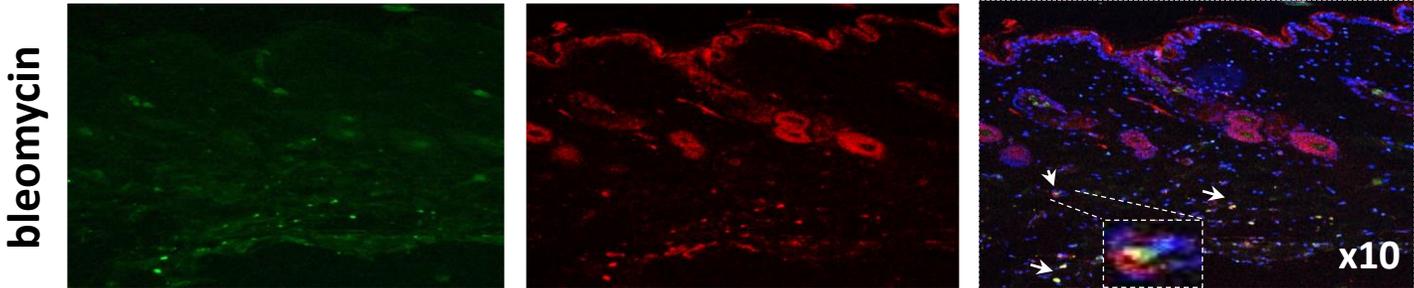
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Figure 1

A



B



C

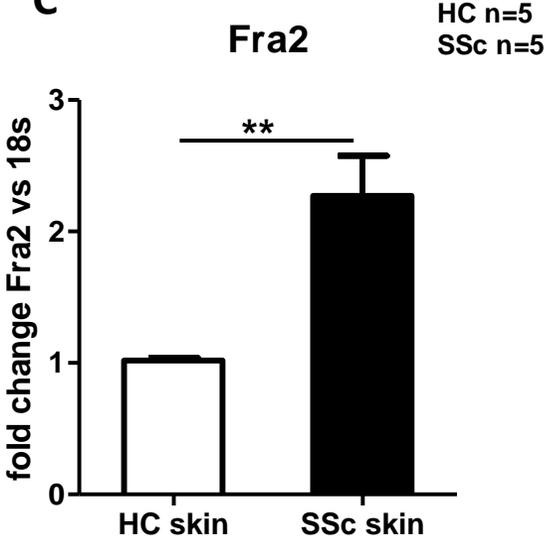


Figure 2

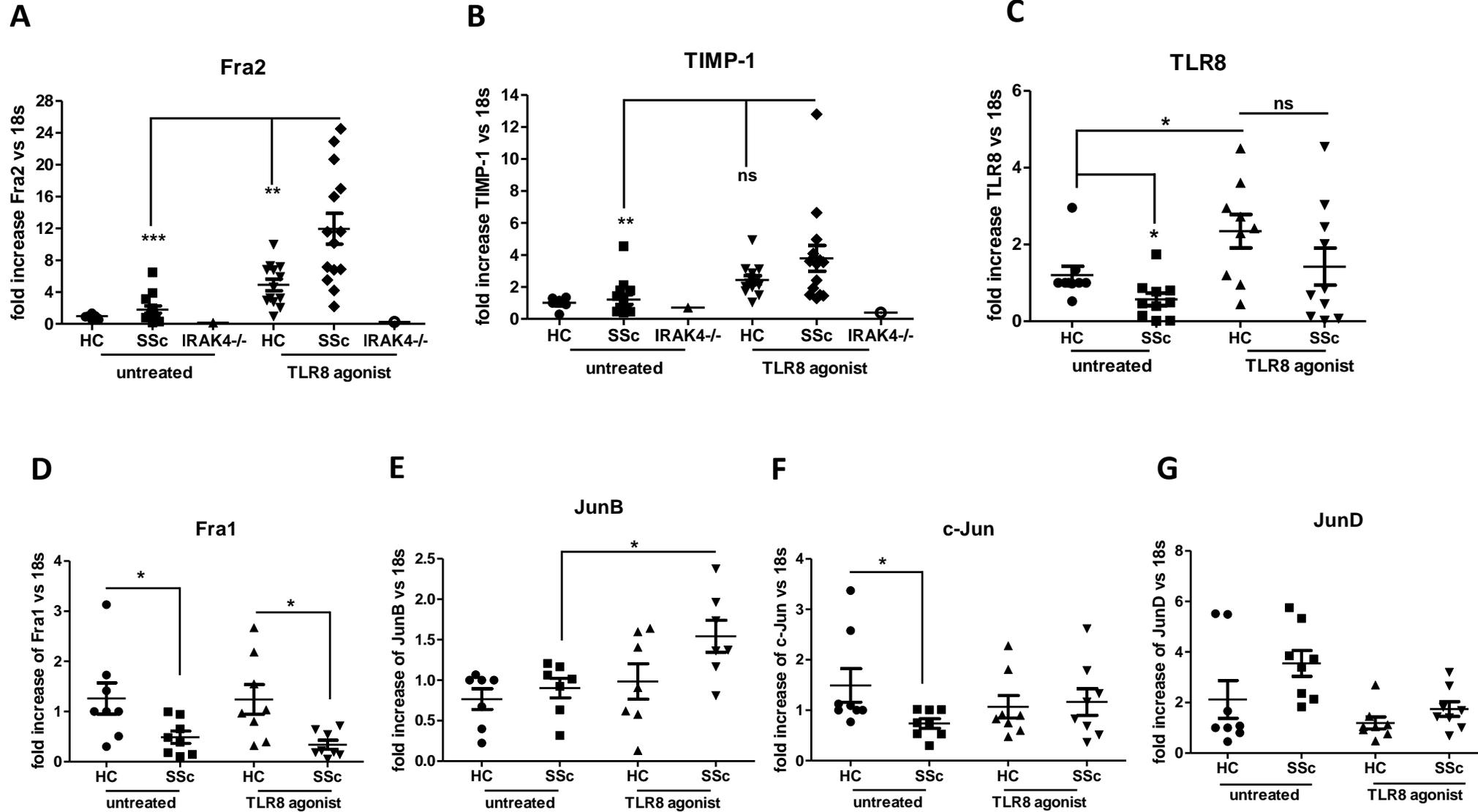


Figure 3

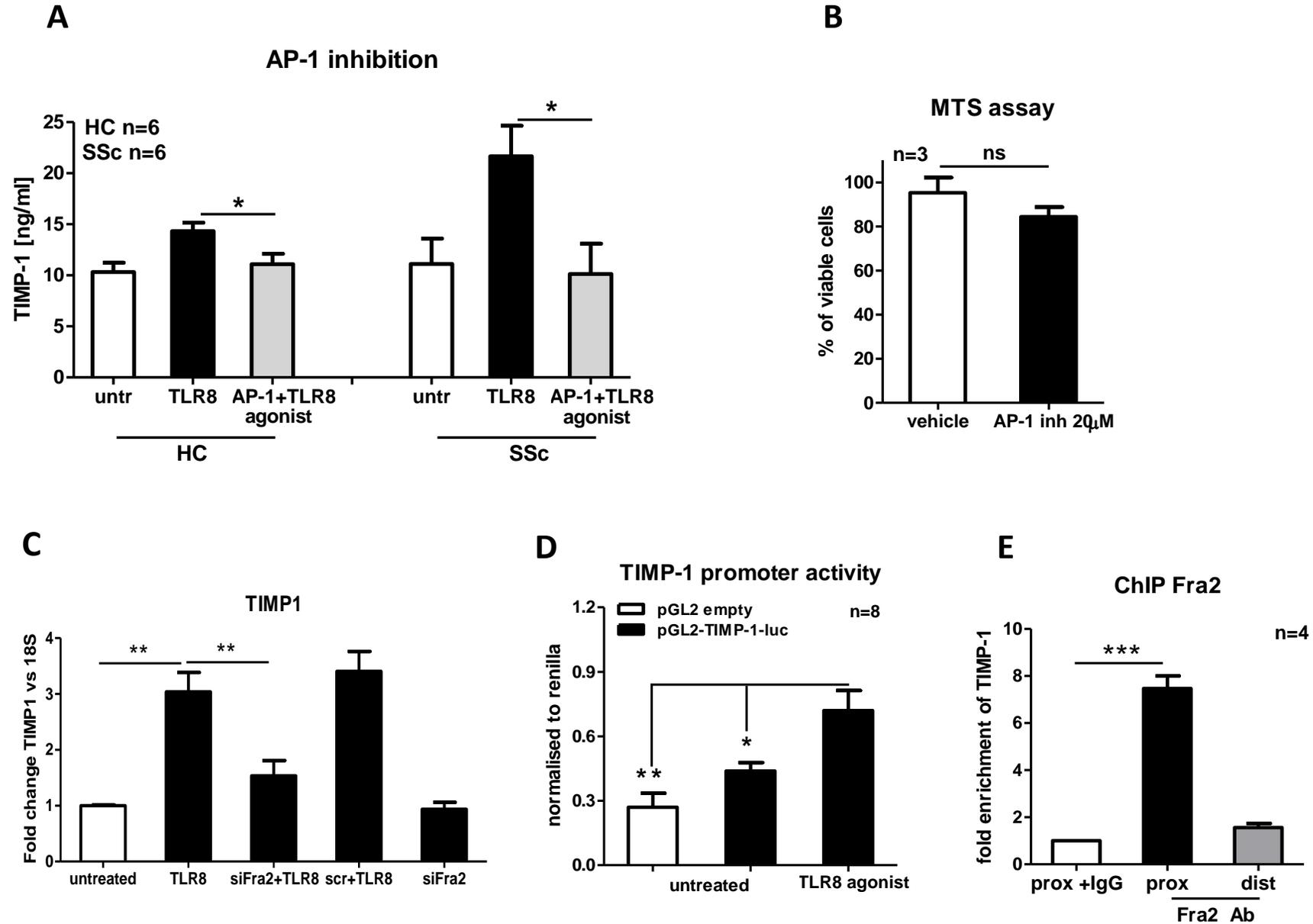
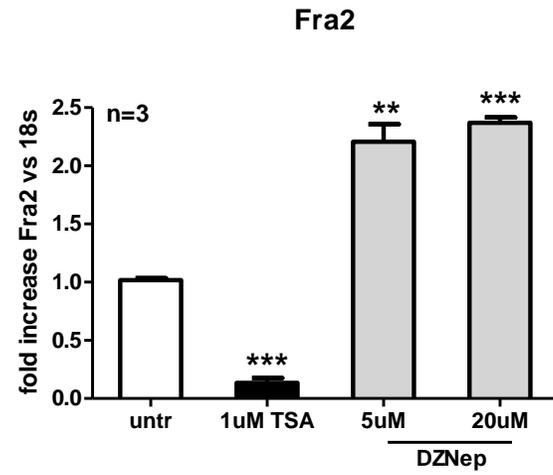
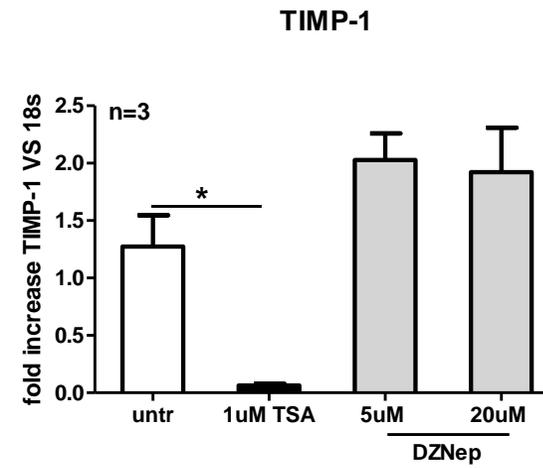


Figure 4

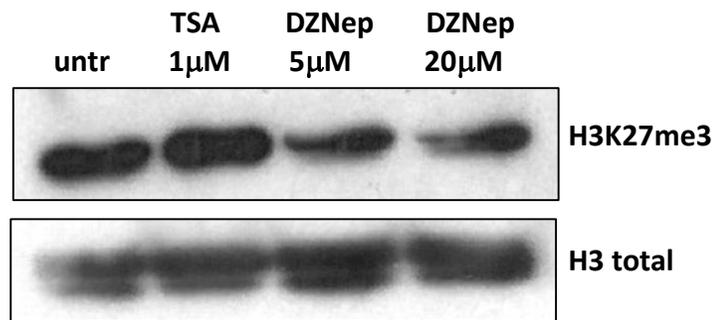
A



B



C



D

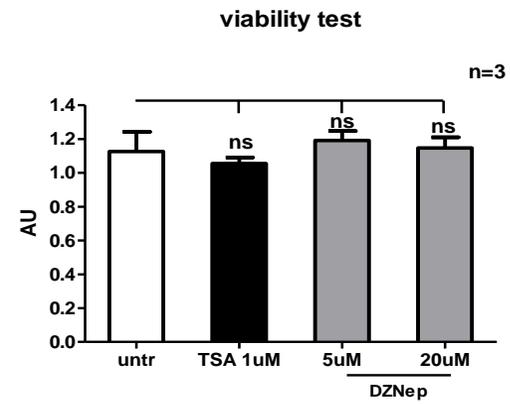


Figure 5

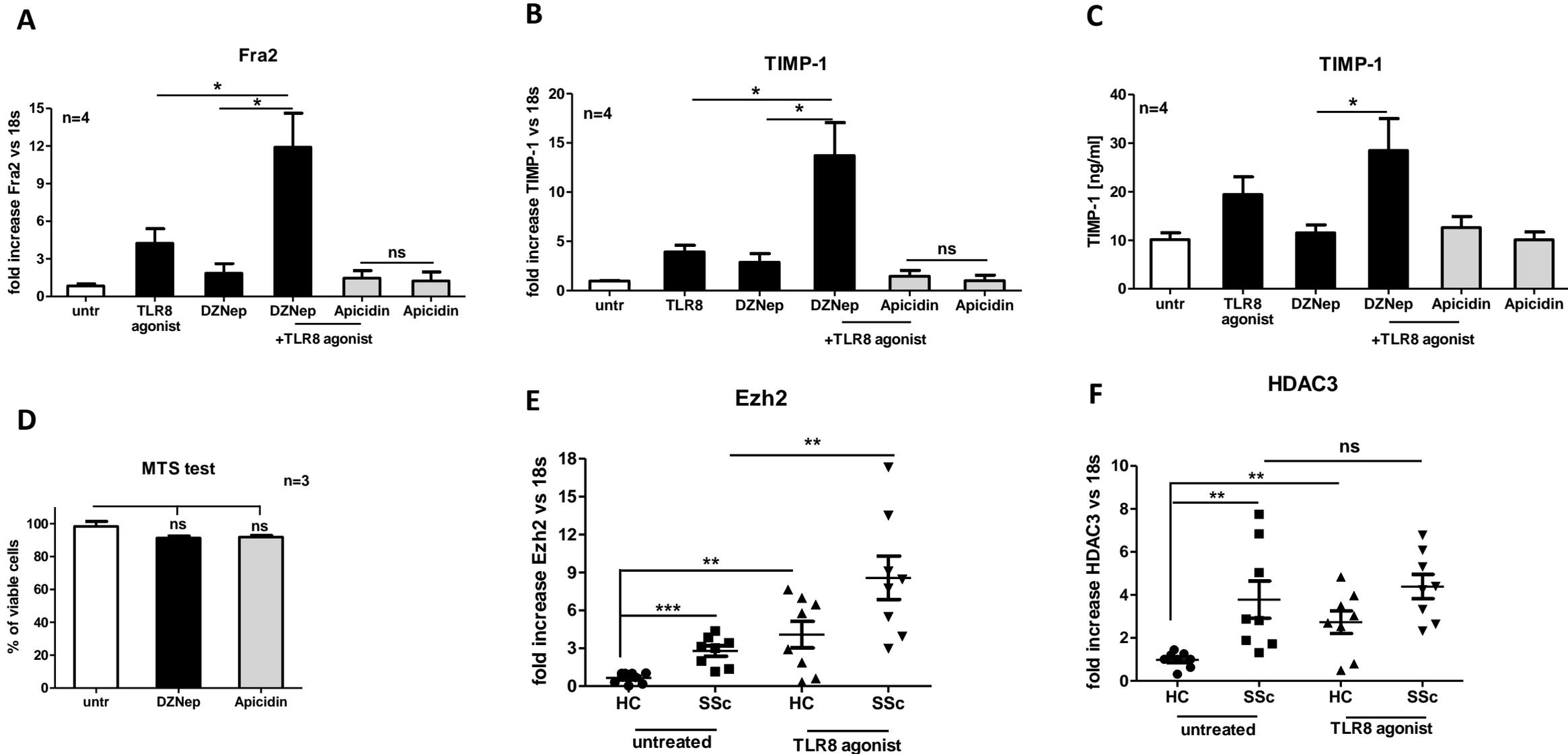
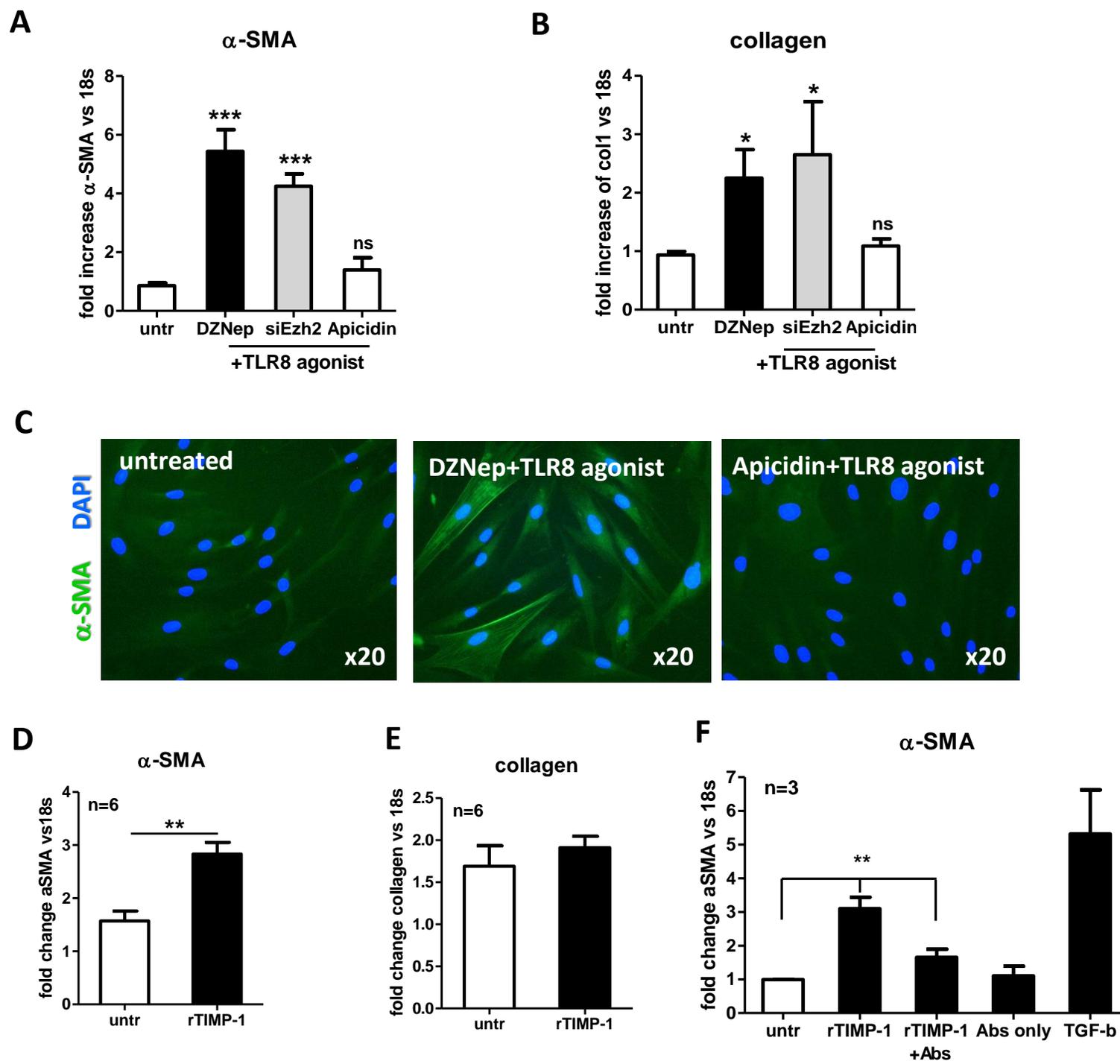
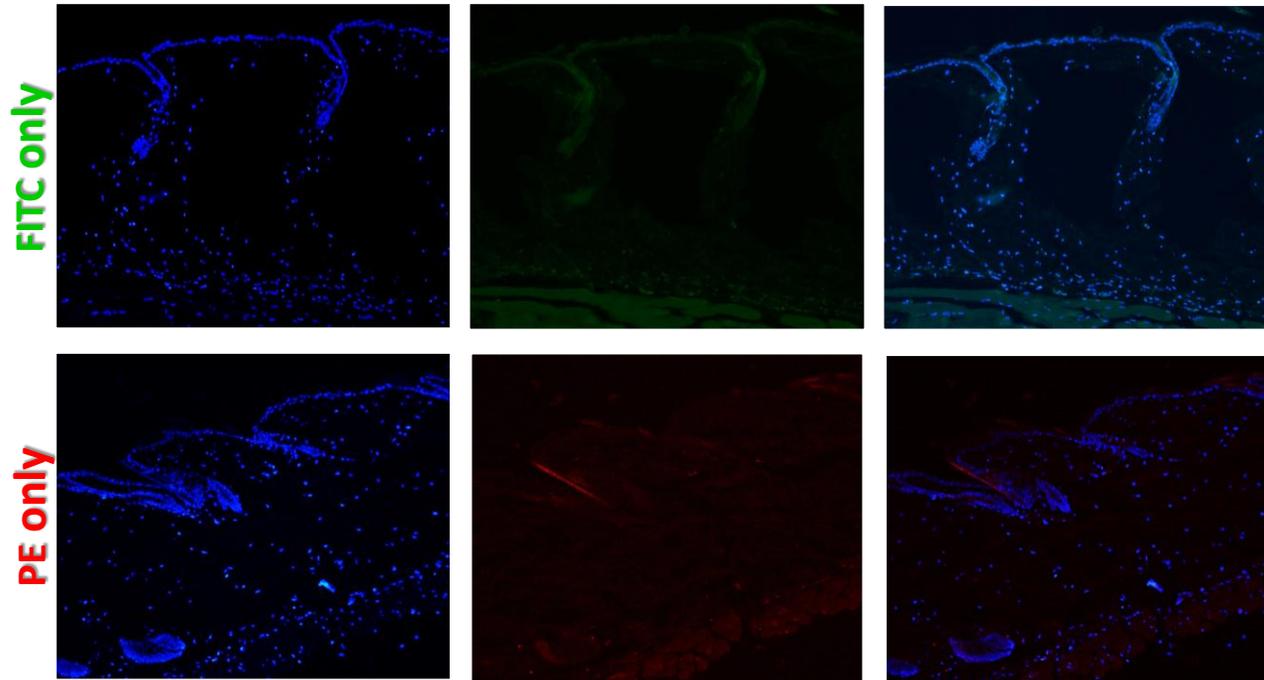
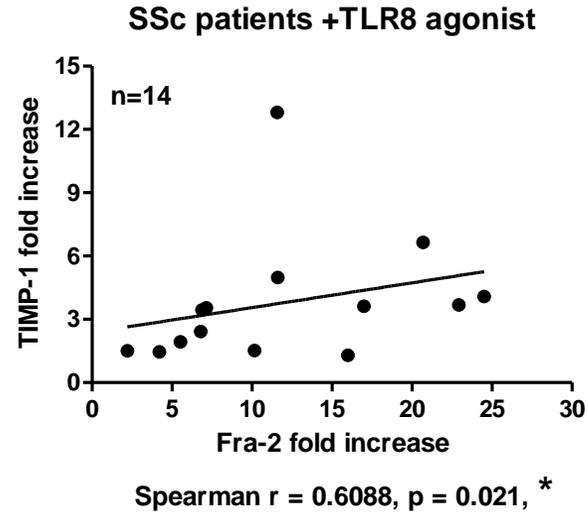
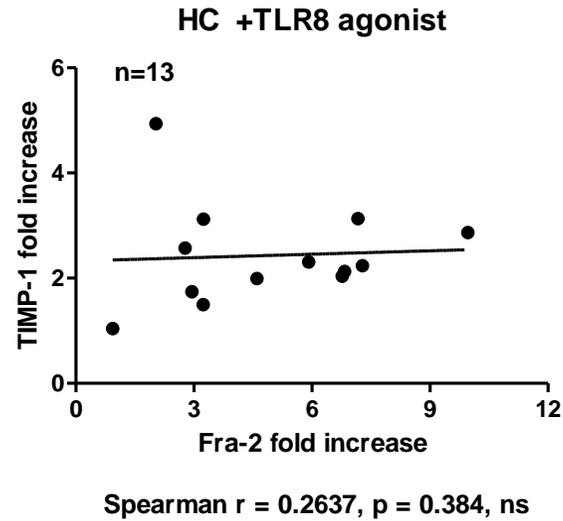


Figure 6

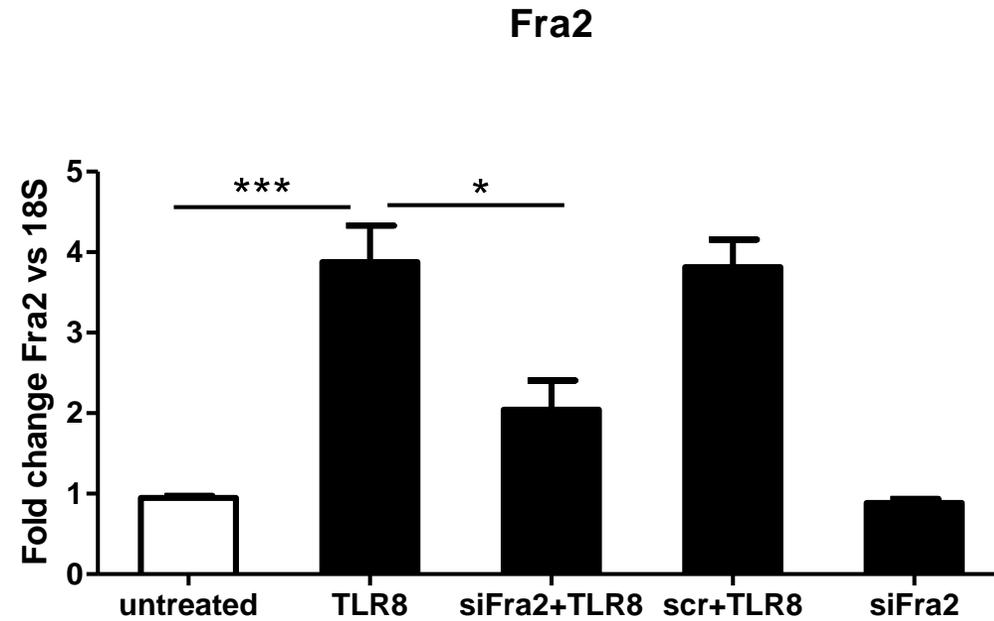




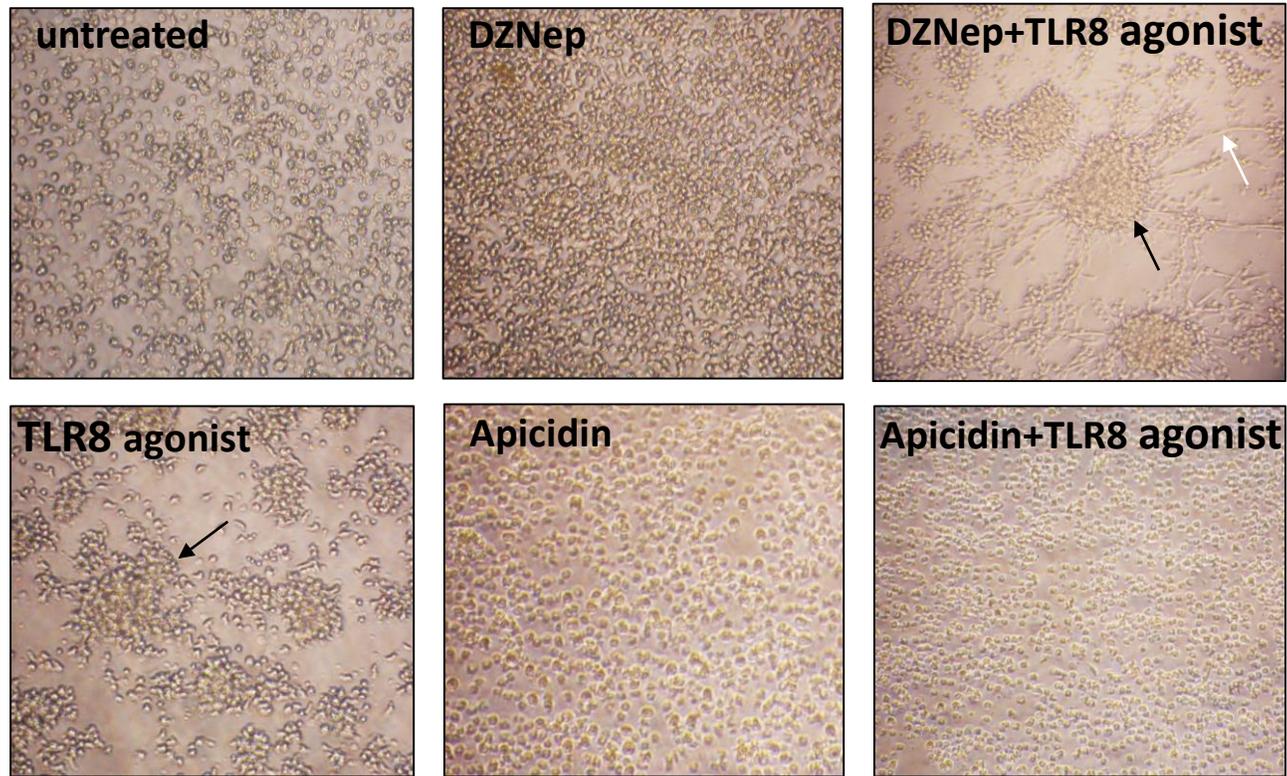
Supplemental Figure 1. Negative control (no primary) of Fra2 and F4/80 staining of mice skin section.



Supplemental Figure 2. HC (n=13) and SSc monocytes (n=14) were stimulated with 2 μ g/ml TLR8 agonist (ssRNA) for 24 h and correlation between TIMP-1 and Fra2 gene expression in HC and SSc monocytes was measured.



Supplemental Figure 3. HC monocytes (n=4) were transfected with Fra2 siRNA or scramble siRNA and 48 h later Fra2 expression was measured.



Supplemental Figure 4. The effect of TLR8 stimulation and histone modifications on morphological changes in HC monocytes. HC monocytes were treated with TLR8 alone or in combination with DZNep or Apicidin for 24 h and morphological changes were observed using light microscope. Black arrows indicate cells aggregation and white arrow indicates elongated shape.