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Basic science

Long non-coding RNA H19X as a regulator of mononuclear cell adhesion to the endothelium in systemic sclerosis

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Abstract

Objective: To define the functional relevance of H19 X-linked (H19X) co-expressed long non-coding RNA (lncRNA) in endothelial cell (EC) activation as a key process in SSc vasculopathy.

Methods: H19X expression in SSc skin biopsies was analysed from single-cell RNA sequencing (scRNA-seq) data. Differential expression and pathway enrichment analysis between cells expressing ($H19X^{pos}$) and non-expressing H19X ($H19X^{neg}$) cells was performed. H19X function was investigated in human dermal microvascular ECs (HDMECs) by silencing. H19X and EC adhesion molecule levels were analysed by real-time quantitative PCR and western blot after stimulation with pro-inflammatory cytokines. Cytoskeletal rearrangements were analysed by fluorescent staining. Endothelial adhesion was evaluated by co-culture of HDMECs and fluorescent-labelled peripheral blood mononuclear cells (PBMCs). Shedding vascular cell adhesion protein 1 (VCAM1) was evaluated by ELISA on HDMEC supernatant.

Results: The scRNA-seq data showed significant upregulation of H19X in SSc compared with healthy ECs. In HDMECs, H19X was consistently induced by IFN type I and II. H19X knockdown lead to a significant decrease in the mRNA of several adhesion molecules. In particular, VCAM1 was significantly reduced at the protein and mRNA levels. Co-expression analysis of the scRNA-seq data confirmed higher expression of VCAM1 in $H19X^{pos}$ ECs. ECs were also strongly associated with the 'cell adhesion molecule' pathway. Moreover, the VCAM1 downstream pathway displayed less activation following H19X knockdown. Contractility of HDMECs, PBMC adhesion to HDMECs and VCAM1 shedding were also reduced following H19X knockdown.

Conclusions: lncRNA H19X may contribute to EC activation in SSc vasculopathy, acting as a regulator of expression of adhesion molecules in ECs.

Keywords: systemic sclerosis, long non-coding RNA, H19X, vasculopathy, interferons, adhesion molecules, VCAM1, scRNA-seq, microvascular endothelial cells, activated endothelium.

Rheumatology key messages

- Interferon-regulated long non-coding RNA H19X is upregulated in SSc endothelial cells.
- H19X silencing in endothelial cells leads to downregulation of adhesion molecules and reduces cytoskeletal rearrangements and cell adhesion.
- H19X exhibits functional significance in microvascular endothelial cells contributing to endothelial activation in SSc vasculopathy.

Introduction

SSc is a chronic multisystem disease characterized by immune dysregulation, vasculopathy and tissue fibrosis. Vascular manifestations, including RP, digital ulcers and pulmonary hypertension, are prominent clinical features of SSc [1]. Microvascular changes, such as a loss of capillaries, vessel wall thickening of small arteries and perivascular mononuclear infiltration, might be detected even before the onset of overt symptoms [1]. Therefore the endothelium seems a very early target in SSc pathogenesis, and endothelial activation and dysfunction are believed to play a crucial role in disease onset and progression [2, 3]. Microvascular endothelial cell (EC) injury in SSc is well described and is characterized by impairment of numerous functions, including increased adhesion and interaction with immune cells, increased apoptosis, disturbed angiogenesis, dysfunctional vasodilation and others [3]. These alterations lead to structural changes resulting in vascular remodelling that causes progressive obliterative

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vasculopathy, as well as functional changes contributing to tissue damage, inflammation and eventually fibrosis [4].

Long non-coding RNAs (lncRNAs) are transcripts longer than 200 nucleotides, which are not translated into proteins. They exert a regulatory effect on gene expression at multiple levels, inducing transcriptional and post-transcriptional modifications [5–7]. As growing evidence supports their relevance in many biologic processes, lncRNAs are being extensively investigated as pathogenic targets across many conditions, including autoimmune and rheumatic disorders [6, 7]. However, the exact mechanisms underlying their functions in disease development remain largely to be understood.

Recently, our group showed that lncRNA H19 X-linked co-expressed lncRNA (H19X) plays a pivotal role in the regulation of TGF-β-driven fibrosis in SSc and other fibrotic conditions [8]. Herein we investigated whether lncRNA H19X is involved in endothelial dysfunction and its potential role in the development of SSc vasculopathy. We can show that H19X is upregulated in ECs by IFN and that it acts as a regulator of adhesion molecules and their functions.

Methods

Single-cell RNA sequencing (scRNA-seq)

scRNA-seq performed on skin biopsies has been described recently [9]. Full-thickness 3-mm skin biopsies were obtained from 27 patients with active dcSSc and from 10 healthy controls (HCs) [10, 11]. Clinical characteristics have been reported previously [9]. HC skin samples were obtained from age- and sex-matched donors [9]. All participants gave written informed consent, in accordance with the Declaration of Helsinki. Analysis of differentially expressed genes was conducted between ECs expressing and not expressing H19X ($H19X^{pos}$ and $H19X^{neg}$). In the analysis, genes expressed by >25% of cells within each population were included. The study was approved by the University of Pittsburgh Medical Center Institutional Review Board (Pittsburgh, PA, USA).

Cell culture

Human dermal microvascular endothelial cells (HDMECs) were purchased from Lonza Pharma & Biotech (Basel, Switzerland) and grown in Lonza Endothelial Cell Growth Basal Medium-2 supplemented with the Microvascular Endothelial Cell Growth Medium-2 SingleQuots Kit at 37°C in 5% carbon dioxide. HDMECs were stimulated with 10 ng/ml of TNF-α, IFN-α, IFN-β, IFN-γ, IL-1β, IL-4 and TGF-β and 50 ng/ml of IL-6. Blood samples were collected in EDTA tubes (BD Vacutainer, Becton, Dickinson, Franklin Lakes, NJ, USA) from HCs and processed within 24 h. Peripheral blood mononuclear cells (PBMCs) were isolated by gradient centrifugation on cell separation medium (Lymphoprep, Stemcell Technologies, Vancouver, BC, Canada).

Transient transfection of HDMECs

HDMECs were transfected with 5 nM antisense oligonucleotide (ASO; Antisense LNA GapmeRs, Qiagen, Venlo, Netherlands) targeting H19X (knockdown 5′-CGCGGGCTTGGTCTTT-3′; Scrambled 5′-AACACGTCTATACGC-3′) using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) at a final concentration of 0.83 µl/ml. Cells were harvested for gene and protein expression analysis 48 and 72 h after transfection or prepared for functional assays.

Real-time quantitative PCR (qPCR) analysis

Total RNA from cells was extracted with the Zymo Quick-RNA MicroPrep RNA isolation kit. A total of 200 ng of total RNA was reverse transcribed with random hexamers using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Subsequent real-time qPCR was performed with 2x SYBR Green Master Mix (Promega, Madison, WI, USA) on an Stratagene Mx3005P real-time qPCR system (Agilent Technologies, Santa Clara, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ribosomal protein lateral stalk subunit P0 (RPLP0) were used as housekeeping genes. Primer sequences are listed in Supplementary Table S1, available at *Rheumatology* online.

Western blot

Cells were lysed with radioimmunoprecipitation assay buffer (MilliporeSigma, Burlington, MA, USA) supplemented with phosphatase inhibitors (PhosphoStop, Roche, Basel, Switzerland) and a protease inhibitor cocktail (cOmplete ULTRA Tablets, Roche). Protein concentration was measured by the bicinchoninic acid method according to the manufacturer's protocol (Thermo Fisher Scientific). To maximize nucleic acid disruption, three cycles of 20 s sonication in ice-cold water followed by 20 s of cooling in ice were performed. Insoluble material was removed with 16 000 *g* centrifugation for 20 min at 4°C. Proteins were separated on 10% SDS with polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane overnight at 4°C. Membranes were blocked for 1 h at room temperature in Tris-buffered saline and Tween-20 (Thermo Fisher Scientific) containing 5% milk and probed overnight with the following antibodies: VCAM1 (1:1000; Cell Signaling Technology, Danvers, MA, USA), P-selectin (1:1000, Abcam, Cambridge, UK), E-selectin (1:500; R&D Systems, Minneapolis, MN, USA), p21 (RAC1) activated kinase 1/2/3 (PAK 1/2/3, 1:1000; Cell Signaling Technology), Phospho-PAK1 (Thr423)/PAK2 (Thr402) (1:1000; Cell Signaling Technology), Phospho-PAK1 (Ser144)/PAK2 (Ser141) (1:1000; Cell Signaling Technology) and α-tubulin as a loading control (1:5000; Abcam). Horseradish peroxidase–conjugated secondary antibodies were used for detection with enhanced chemiluminescence substrate (SuperSignal West Pico Plus, Thermo Fisher Scientific). Semi-quantitative determination of protein expression was performed with ImageJ software.

RAC1 pulldown assay

The RAC family small GTPase 1 (RAC1) pulldown assay was performed by using the Active RAC1 Pull-Down and Detection Kit (16118, Thermo Fisher Scientific) following the manufacturer's protocol as described in the Supplementary Methods, available at *Rheumatology* online.

Phalloidin staining

Cells were fixed with 4% paraformaldehyde (PFA, MilliporeSigma) for 10 min at room temperature, permeabilized with 0.1% Triton X-100 (MilliporeSigma) and blocked with 10% foetal bovine serum for 30 min at room temperature. Cells were then stained with 4 U/ml of fluorescent-labelled phalloidin (BioLegend, San Diego, CA, USA) for 40 min at room temperature. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) solution (1 µg/ml;

Roche). Images were acquired with an BX53 microscope equipped with a DP80 camera (Olympus, Tokyo, Japan).

Adhesion assay

The adhesion assay is extensively described in the Supplementary Methods, available at *Rheumatology* online. HDMECs were transfected as described above and stimulated with TNF-α. Carboxyfluorescein succinimidyl ester (CFSE, Thermo Fisher Scientific)–stained PBMCs were added on top of the endothelial monolayer. Following overnight co-culture, a solution of 5 μM Hoechst 33342 (Thermo Fisher Scientific) in 4% PFA was used to fix the cells and counterstain endothelial nuclei. Imaging was performed using the CellInsight CX7 high-content platform (Thermo Fisher Scientific) as described in the Supplementary Methods, available at *Rheumatology* online.

Statistical analysis

All data are presented as mean (S.D.). Normality was assumed using the Shapiro–Wilk W test. Normally distributed data were analysed by two-tailed paired *t*-test. Comparison of multiple groups was performed by one-way analysis of variance with Dunnett's multiple comparisons test. *P*-values <0.05 were considered statistically significant. All statistics tests were performed with GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA).

Results

lncRNA H19X is upregulated in the endothelium from skin biopsies of SSc patients

To evaluate its relevance for the SSc endothelium, we analysed the expression of H19X using the scRNA-seq data. Comparisons of average expression levels of H19X showed that H19X expression was significantly upregulated in SSc endothelium compared with HCs ($P < 0.01$) (Fig. 1).

IFN type I and II regulate H19X expression in ECs

In SSc, microvascular endothelial injury manifests as an early physiopathological event triggered by inflammation. We hypothesized that H19X expression in ECs might be regulated by pro-inflammatory cytokines. Therefore, we analysed H19X expression after stimulation with TNF-α, IFN-α, IFN-β, IFN-γ, IL-1β, IL-4, IL-6 and TGF-β at several time points (3, 6, 24, 48 and 72 h). Initial screening experiments did not show consistent effects or showed downregulation of H19X after stimulation with TNF-α, IL-1β, IL-4 and IL-6 (not shown). Interestingly, opposite to what we previously observed in fibroblasts [8], H19X expression in HDMECs was downregulated 3–24 h after TGF-β stimulation (not shown). However, H19X expression was significantly upregulated after prolonged stimulation (48–72 h) with IFN-α. Although not significant, a similar trend was observed with IFN-β and IFN-γ starting 24 h after stimulation (Fig. 2).

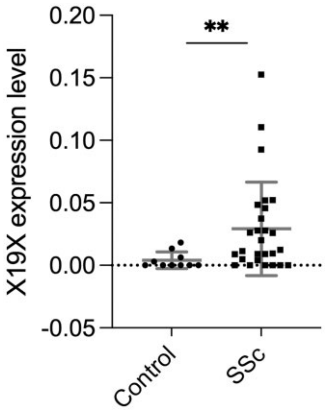

Figure 1. Analysis of scRNA sequencing data for H19X expression in skin ECs. Comparison of expression levels of H19X in HCs ($n = 10$) *vs* SSc ($n = 27$) ECs (Mann–Whitney test). $^{**}P < 0.01$

These findings show that H19X expression in the endothelium might be regulated by both type I (IFN-α and IFN-β) and type II IFN (IFN-γ). Additionally, VCAM1 and E-selectin expression was rapidly induced by IFNs, peaking at 3–6 h after stimulation (Supplementary Fig. S1, available at *Rheumatology* online).

H19X knockdown decreases the expression of adhesion molecules in HDMECs

Endothelial activation in SSc is a complex process that encompasses numerous alterations of endothelial functions, including adhesion, angiogenesis, inflammation and production of vasoactive molecules. We identified several markers of endothelial activation and analysed their expression in HDMECs following H19X knockdown with or without IFN stimulation. Among several adhesion molecules tested, the expression of VCAM1, E-selectin and P-selectin was significantly downregulated at the mRNA level after transfection (Fig. 3A). However, we did not observe a significant downregulation of VCAM1 and E-selectin after H19X knockdown followed by stimulation with IFNs (Supplementary Fig. S2, available at *Rheumatology* online). VCAM1, but not E-selectin and P-selectin, was also downregulated after H19X knockdown at the protein level (Fig. 3B). These data suggest that H19X expression in ECs, which is induced by IFN, might regulate VCAM1 expression and thus contribute to EC activation.

Less consistent results were noted for other endothelial activation markers. Specifically, a slight significant upregulation was detected for VEGFA at the mRNA level after H19X knockdown. An upregulation was also observed for VEGF receptor 2 (KDR), TEK receptor tyrosine kinase [Tie2 (TEK)] and fractalkine (CX3CL1), however, without statistical significance. No changes were noted for other adhesion molecules [ICAM, PECAM1, junctional adhesion molecules 2/3 (*JAM2 and JAM3*)], C-X-C motif chemokine receptor 1 (CXCR1) and apoptosis-inducing factor (AIF). Endothelin-1 (EDN1) and thrombospondin 1 (THBS1) displayed a slight but not significant downregulation (Supplementary Fig. S3, available at *Rheumatology* online).

H19X expressing ECs present with higher levels of adhesion molecules

The role of H19X on cell adhesion was further confirmed by Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Database analysis on differentially expressed genes between $H19X^{pos}$ and $H19X^{neg}$ ECs [12]. This analysis revealed that genes expressed by $H19X^{pos}$ cells were strongly associated with the 'cell adhesion molecule' pathway ($P = 2.209e\text{-}7$; Fig. 4A), with integrin subunit beta 1 (*ITGB1*), *VCAM1*, intercellular adhesion molecule 2 (*ICAM2*), E-selectin (*SELE*), V-set immunoregulatory receptor (*VSIR*), endothelial cell adhesion molecule (*ESAM*), integrin subunit alpha 6

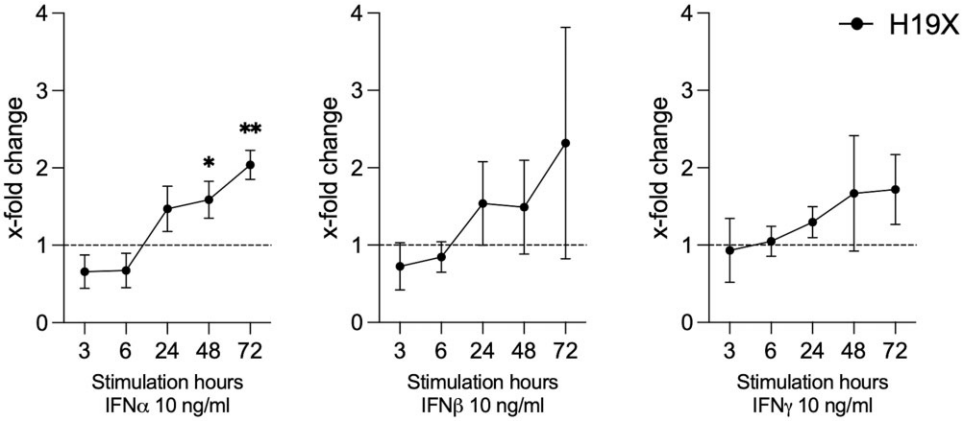

Figure 2. Time course analysis of the expression of H19X in HDMECs after IFN stimulation. Time-dependent induction of H19X by IFN type I and II. HDMECs were stimulated for 3–72 h with 10 ng/ml IFN-α, IFN-β and IFN-γ. Expression levels were measured by qPCR, normalized by GAPDH and RPLP0 and compared with non-stimulated dermal HDMECs. $n = 5$ biological replicates. $^{*}P < 0.05$, $^{**}P < 0.01$

(*ITGA6*), cluster of differentiation 99 (*CD99*), nectin cell adhesion molecule 2 (*NECTIN2*) and MHCs class I C (*HLA-C*) and class II DP alpha 1 (*HLA-DPA1*), DQ alpha 1 (*HLA-DQA1*), DQ beta 1 (*HLA-DQB1*), DR alpha (*HLA-DRA*) and DR beta 1 (*HLA-DRB1*) among the top deregulated genes (Fig. 4B). Next, we analysed our scRNA-seq data to evaluate whether there was correlation between the expression of H19X and adhesion molecules. Interestingly, in skin ECs from SSc and HC samples, VCAM1 resulted in higher EC expression of H19X (Fig. 4C). A higher expression was noted also for other adhesion markers analysed in the scRNA-seq dataset, such as ICAM1, 2, 3 and 4 (Fig. 4C). These skin biopsy data further support our *in vitro* data suggesting a role of H19X in the regulation of adhesion in the SSc endothelium.

H19X knockdown affects VCAM1 shedding

To address whether H19X could also regulate VCAM1 shedding, we knocked down H19X in HDMECs and carried out ELISAs on the supernatants. We also isolated proteins from the respective cells and performed western blot analysis. We observed a significant reduction of the soluble form of VCAM1 in the supernatant of H19X knockdown cells at 48 and 72 h after transfection. When we compared the cellular protein fold change to the fold change of the soluble protein, we observed a much stronger reduction of the soluble VCAM1 following H19X knockdown. Additionally, in this set of experiments, cellular VCAM1 levels were reduced 72 h after transfection while the soluble form was decreased 48 h after transfection (Supplementary Fig. S4, available at *Rheumatology* online). These data might indicate different mechanisms of regulation of protein expression and VCAM1 shedding.

H19X knockdown decreases the activation of VCAM1 downstream signalling

The activation of RAC1 is a characteristic VCAM1 downstream event promoting the phosphorylation of PAK, which in turn activates myosin light chains leading to cytoskeletal changes and leucocyte transmigration into the tissue [13]. To analyse whether H19X influences VCAM1 downstream activation, we analysed the activation of RAC1 after TNF-α stimulation to induce VCAM1 expression (Supplementary Fig. S5, available at *Rheumatology* online) and H19X knockdown. Activation of RAC1 was reduced by 50% in H19X knockdown HDMECs as revealed by GTP-RAC1 pulldown followed by western blot (Fig. 5A). Total protein levels of PAK and the phosphorylation of the PAK Thr^{402} site were not significantly changed after H19X knockdown, neither in basal conditions nor after TNF-α stimulation. However, phosphorylation levels at Ser^{141} of PAK were reduced following H19X knockdown both in basal conditions and in TNF-α-stimulated cells (Fig. 5B). These data suggest that H19X not only induces VCAM1 expression, but also affects its downstream signalling in ECs.

H19X knockdown decreases EC contractility and PBMC adhesion to ECs

To further confirm the role of H19X in VCAM1-mediated microvascular activation, we analysed cytoskeletal rearrangements following H19X knockdown in untreated and TNF-α-stimulated HDMECs. As revealed by F-actin staining with phalloidin, HDMECs had a larger cytoplasmic area after H19X knockdown as compared with control cells, indicating reduced cell contractility after H19X knockdown required for leucocyte transmigration. Consistently, a significant reduction in the area not covered by cells was detected after H19X knockdown in basal conditions. Similar morphological changes after H19X knockdown with TNF-α stimulation were observed, but without reaching statistical significance (Fig. 6A).

Following our observations on the effects of H19X on VCAM1 expression and pathway activation, we evaluated adhesion of PBMCs to HDMECs as a functional effect of EC and adhesion molecule activation in SSc. Under basal conditions, without specific pro-inflammatory stimuli, no difference was observed. However, when HDEMCs where stimulated with TNF-α, H19X silencing induced a significant reduction of 21.2% (95% CI 6.2, 36.2; $P = 0.0156$) in the number of PBMCs binding to HDMECs (Fig. 6B).

These results provided additional functional evidence that H19X upregulation in SSc ECs leads to VCAM1 expression and VCAM1 pathway activation, resulting in increased cytoskeletal rearrangements and adhesion of PBMCs to the activated endothelium.

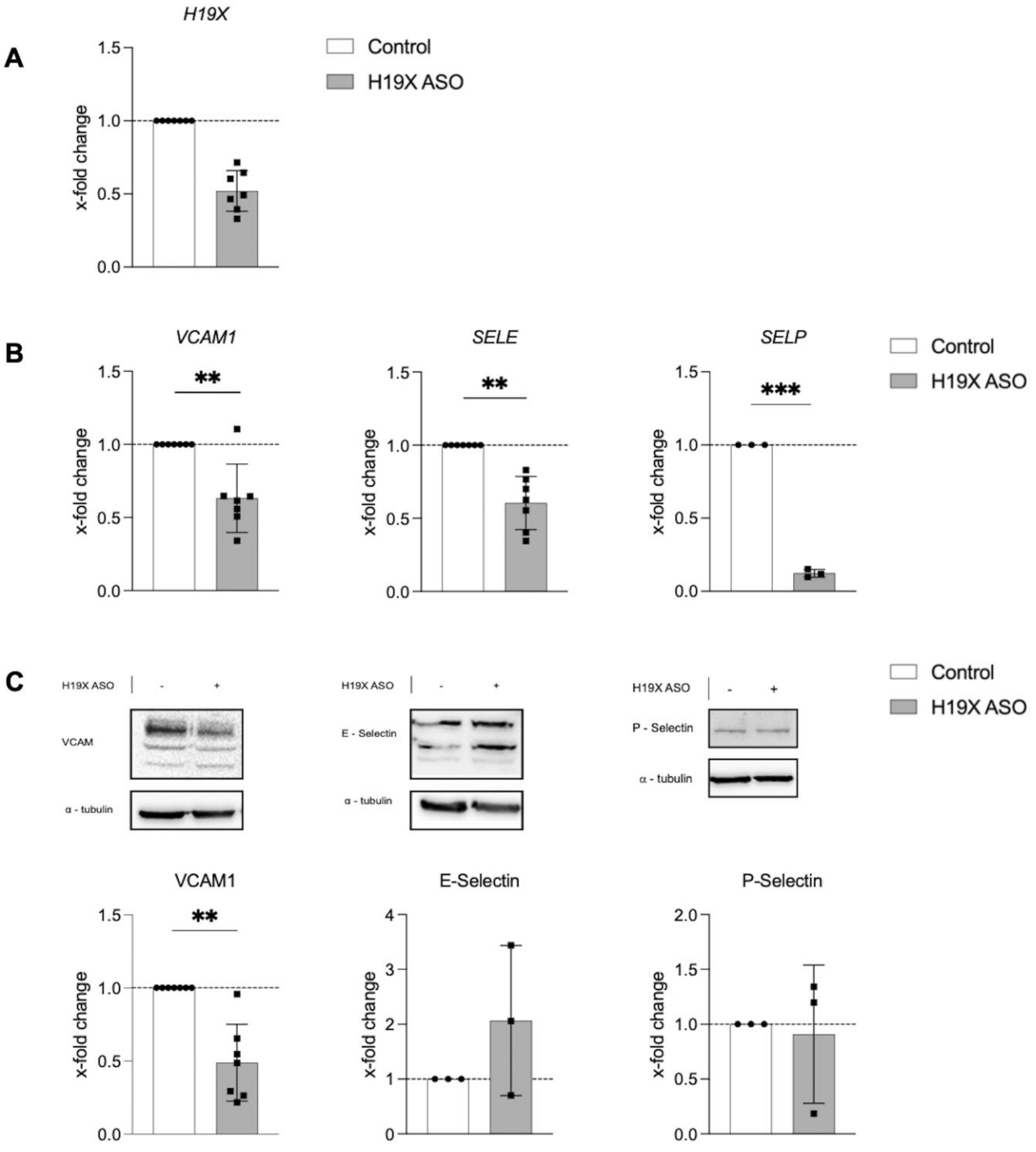

Figure 3. Adhesion molecules expression after H19X knockdown. HDMECs were transfected with locked nucleic acid ASO-negative control or ASO targeting H19X for a total of 48 h. **(A)** mRNA levels of H19X following knockdown as measured by qPCR. mRNA expression was normalized by GAPDH and RPLP0. **(B)** mRNA levels of VCAM1, E-selectin and P-selectin were measured by qPCR. mRNA expression was normalized by GAPDH and RPLP0. **(C)** Protein levels of VCAM1, E-selectin and P-selectin were measured by western blot analysis. Protein expression was normalized by α-tubulin. Pictures are representative of $n = 3$–7 biological replicates. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$

Discussion

In this study, we characterized the functions of H19X in SSc endothelium. We demonstrated that H19X is involved in the regulation of endothelial adhesion molecules, especially VCAM1, and that its expression is regulated by IFNs.

The role of lncRNA H19X, also known as MIR503 hosting gene (*MIR503HG*), has been described in a few diseases, especially in cancer [14–17]. Recently, H19X depletion has been associated with endothelial–mesenchymal transition in vascular remodelling and pulmonary artery hypertension [18]. In a previous study, we showed that H19X is upregulated across several TGF-β-driven fibrotic disorders [8]. Here, we analysed scRNA-seq data from skin biopsies of a dcSSc cohort and demonstrated that H19X is upregulated in the

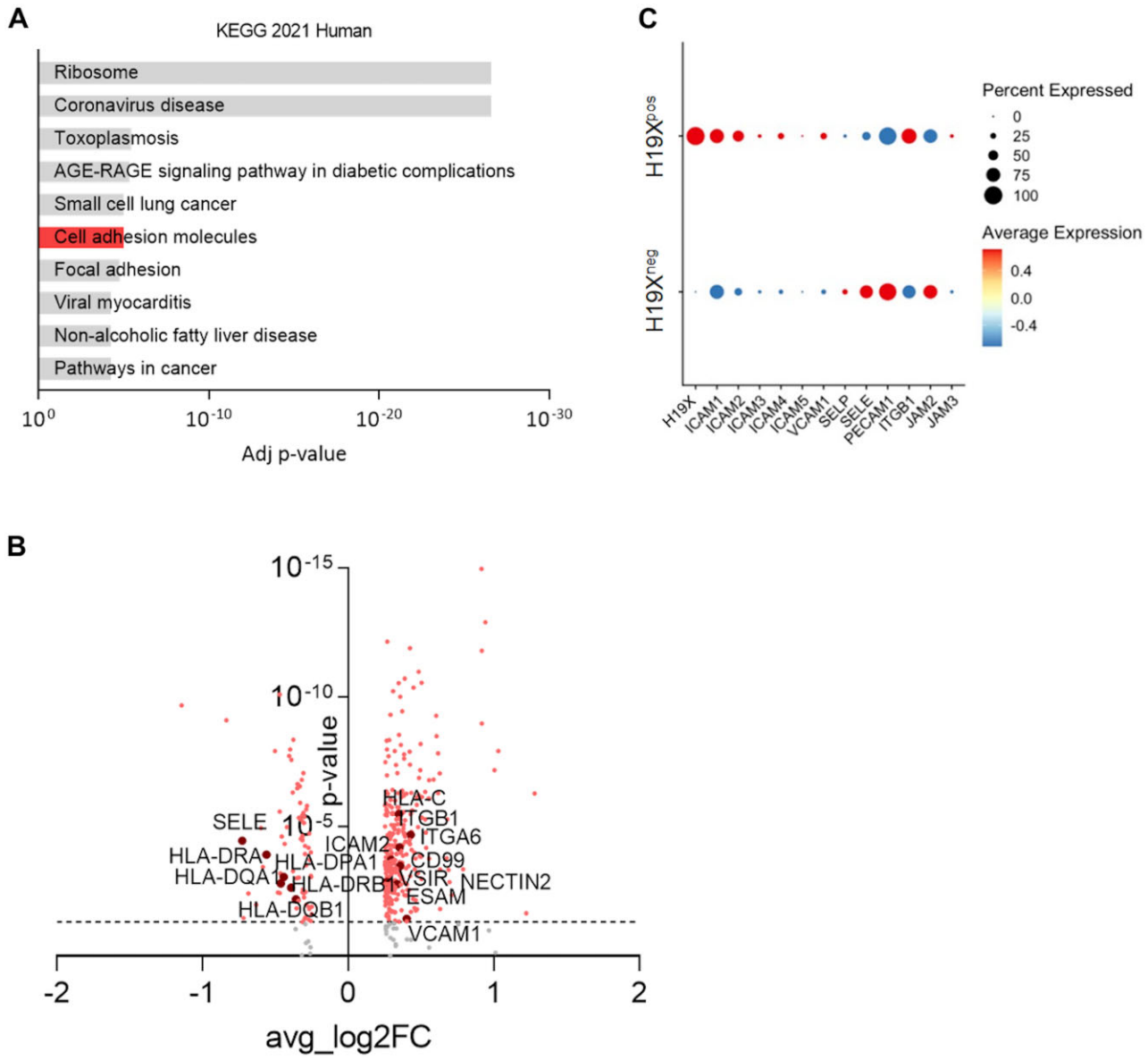

Figure 4. H19X correlates with cell adhesion molecular pathways in ECs. **(A)** Pathway enrichment analysis (KEGG Pathway Database) performed on significantly deregulated genes ($P < 0.05$) in scRNA-seq data between ECs expressing high levels of H19X and ECs expressing low levels of H19X **(B)** Differentially expressed genes between ECs expressing high levels of H19X and ECs expressing low levels of H19X. Significant correlations are highlighted in pink (Pearson $P < 0.05$). Genes that belong to the 'cell adhesion molecule' pathway are marked in red. **(C)** scRNA-seq data from skin biopsies were analysed for the expression of adhesion molecules and compared between H19X-expressing ($H19X^{pos}$) and non-expressing ($H19X^{neg}$) ECs

endothelium of the skin of SSc patients. This finding suggests that H19X might play a relevant role in endothelial injury in SSc.

SSc is a heterogeneous disease with variable clinical manifestations; the extent of skin involvement can range from none to puffy fingers to severe fibrosis. This is inevitably reflected in the different level of activity of molecular effectors and pathways. In our scRNA-seq analysis, we detected a larger distribution of H19X expression in SSc patients as compared with HCs, with a considerable overlap between the two groups. This observation can be explained by variations in the extent of skin involvement and vasculopathy and the heterogeneous molecular profiles at the site of biopsy in SSc patients. As a consequence, as for many other molecular factors, H19X and its associated VCAM1 expression might not play a role in the pathophysiology of all patients. It should also be noted that in this study we specifically looked into very early diffuse SSc patients, and these results cannot be simply extrapolated into other disease stages or subgroups. Importantly, we must underline that the scRNA-seq assay used is designed to detect 3′-polyadenylated [poly(A)] transcripts. Of the 10 transcript variants currently reported in the Ensembl database for H19X [19], not all present a poly(A) tail, meaning that our analysis likely lacks data on these variants. Although we recognize this as a relevant limitation of our study, we might also hypothesize an even higher expression of H19X in ECs from our samples, which might at least partially explain the lower number of cells expressing our target.

We demonstrated that H19X is upregulated by IFN types I and II. The expression of IFN type I–inducible genes is increased in peripheral blood of SSc patients and it is detected

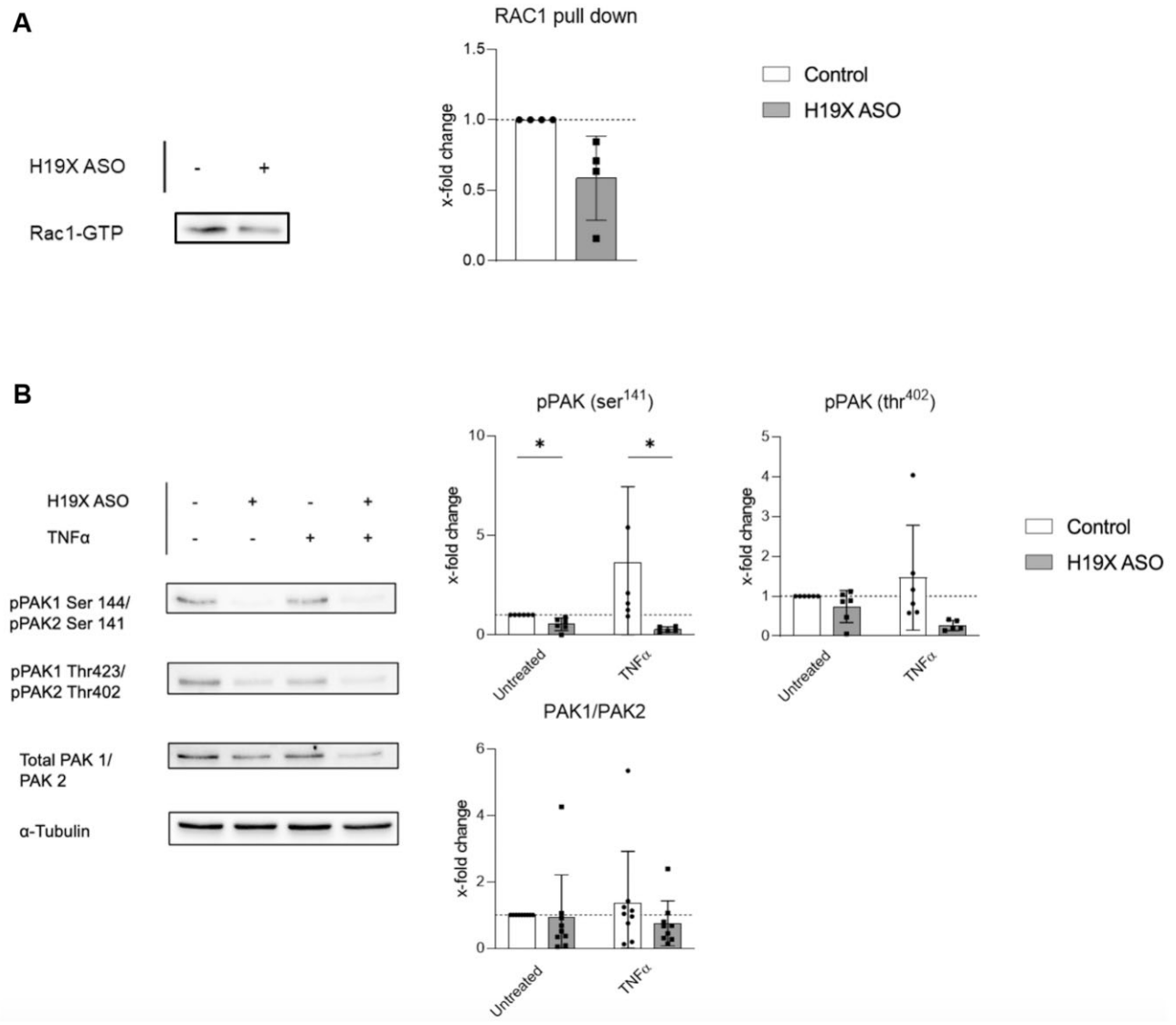

Figure 5. H19X knockdown silencing decreases activation of the VCAM1 downstream pathway. HDMECs were transfected with locked nucleic acid ASO-negative control or ASO targeting H19X for a total of 48 h. HDMECs were also stimulated with TNF-α (10 ng/ml) for 3 h in order to enhance pathway activation. **(A)** Protein levels of RAC1 were analysed by pull-down assay. **(B)** Protein levels of phosphorylated PAK at site Ser^{141} and Thr^{402} were analysed by western blot, compared with total PAK levels and normalized to α-tubulin. Pictures are representative of $n = 6$–7 biological replicates. $^*P < 0.05$

in very early SSc, even before the occurrence of fibrotic manifestations [20–22]. In SSc skin, an altered expression is observed both in lesional and non-lesional areas [22]. Higher circulating levels of IFN-γ are also described in SSc, and a higher expression of IFN-γ has been shown in RP, an early vascular manifestation of SSc [23]. Moreover, it seems to modulate vascular permeability and influence transmigration of PBMCs through the endothelium [24]. Considering the effects of IFN in the pathogenesis of SSc and the effects on vascular and endothelial functions, we might hypothesize that H19X, since the earliest phases of the disease, is involved in IFN-mediated pathogenic mechanisms leading to endothelial injury.

We then wanted to characterize the possible functions of H19X in the endothelium. In a hypothesis-driven manner, we selected EC injury and EC activation biomarkers relevant for SSc [3] and performed H19X knockdown experiments on HDMECs. Several markers were tested and adhesion molecules were identified as H19X targets. Furthermore, H19X expression was closely associated with deregulated expression of adhesion molecules, suggesting that H19X might be a regulator of the cell adhesion process. Exposure of surface adhesion molecules allows recruitment and transmigration of immune cells to the tissues. This process is crucial in the initiation of immune and inflammatory responses. Adhesion molecule expression is typically altered in case of endothelial injury and is well characterized in SSc [3]. Among the identified adhesion molecules, VCAM1 was the most consistently downregulated following H19X silencing. Moreover, VCAM1 upregulation was confirmed in SSc endothelium by scRNA-seq data analysis. VCAM1 is a surface glycoprotein mainly expressed by ECs in response to pro-inflammatory cytokines TNF-α and IL-1β. The role of these cytokines in SSc pathogenesis and vasculopathy remains elusive. Both TNF-α (sTNF-α) and IL-1α/IL-1β have been found to be upregulated in SSc compared with HCs [25, 26]. In our experiments, TNF-α and IL-1β could not induce H19X expression.

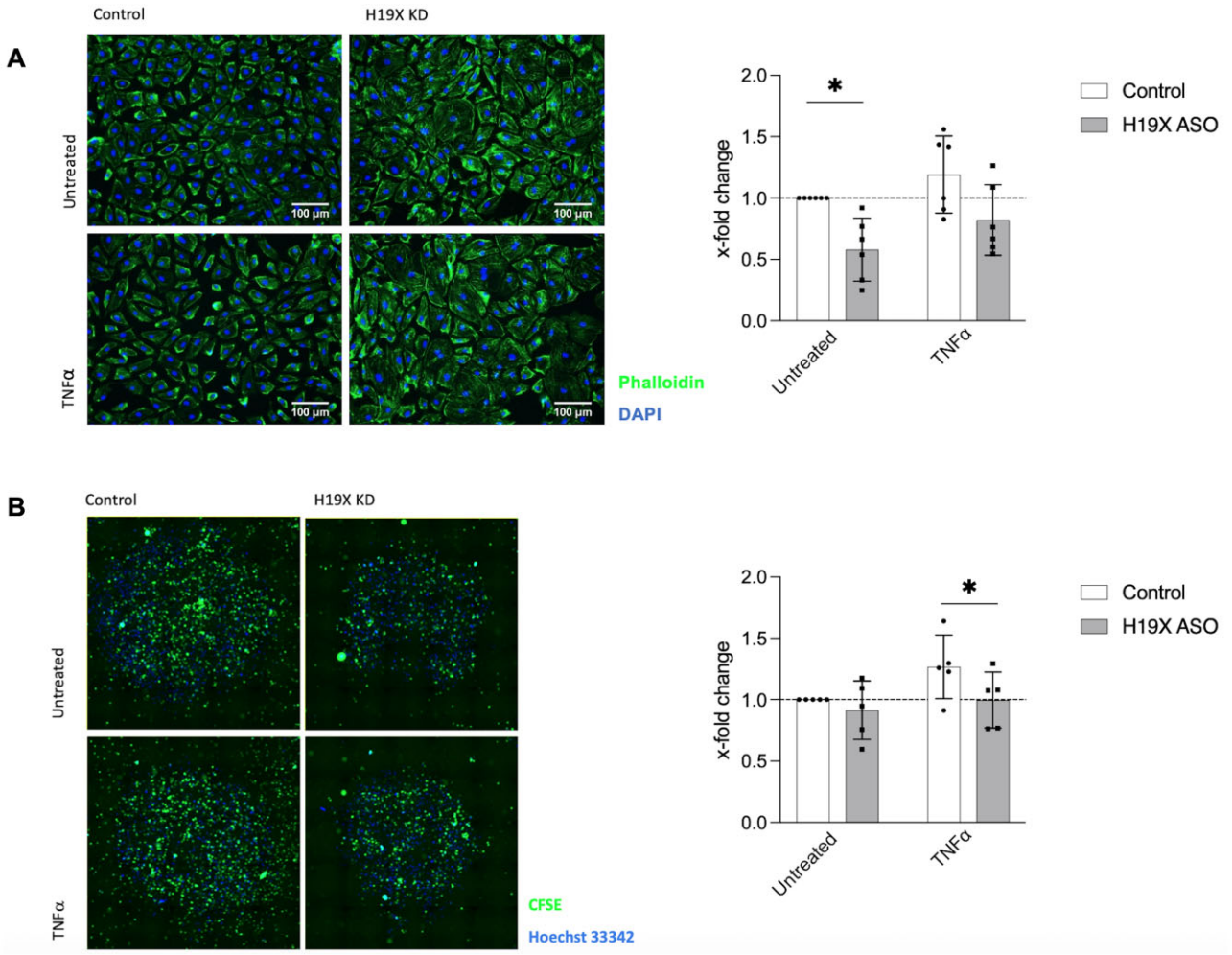

Figure 6. H19X mediates EC activation. HDMECs were transfected with locked nucleic acid ASO-negative control or ASO targeting H19X for a total of 48 h. HDMECs were also stimulated with TNF-α (10 ng/ml) for 3 h in order to enhance pathway activation. **(A)** Stress fibres were stained using fluorescent-labelled phalloidin (green). Uncovered area was normalized on cell nuclei (blue) and compared with untreated controls. Magnification 20×. **(B)** PBMCs were added to an HDMEC monolayer and co-incubated overnight. HDMECs and PBMCs were counted using the CellInsight CX7 high-content imaging platform. The PBMC count (green) was normalized on the HDMEC count (blue) and compared with the untreated control

As already discussed, there is strong evidence for IFNs playing a major role in the context of SSc. Therefore, the action of the IFN–H19X axis on the expression of VCAM1 could be more relevant in SSc vasculopathy. VCAM1 binds integrin-α4β1 on leucocyte surfaces, allowing leucocyte recruitment and, upon activation, it mediates intracellular mechanisms that cause an increase in endothelial permeability, allowing leucocyte trans-endothelial migration [27, 28]. VCAM1, together with other adhesion molecules, is upregulated in affected skin of SSc patients [29]. Additionally, various articles [30–32] have described elevated circulating VCAM1 levels in SSc patient sera, suggesting that VCAM1-mediated adhesion is impaired in the disease and might contribute to its pathogenesis. H19X silencing leads to a significant reduction in the soluble form of VCAM1. We observed a strong decrease of VCAM1 shedding even before reduction of the cellular protein levels. These data indicate that H19X might regulate VCAM1 function at different levels. Additional experiments are needed to comprehensively explore H19X involvement in VCAM1 shedding.

We demonstrated that the VCAM1 signalling pathway is less active following H19X knockdown. Specifically, we observed less GTP-RAC1 and reduced levels of $pSer^{141}$ PAK. It was reported that the majority of $pSer^{141}$ PAK is localized to areas of cell–cell contact and that displacement of active PAK from sites of cell–cell contact prevented increases in vascular permeability [33]. H19X knockdown also reduced the growing area occupied by the cells, restoring the integrity of the monolayer of ECs. These data support our hypothesis that H19X-mediated expression of VCAM1 is crucial for EC cytoskeletal rearrangements and in turn infiltrate transmigration into the tissue.

To further confirm the involvement of H19X in vascular injury, we explored its functional relevance for PBMC adhesion. When performing endothelial and PBMC co-cultures, H19X silencing determined a significant reduction in adhesion of PBMCs to HDMECs. Not surprisingly, the reduction of PBMC adhesion, though significant, was modest and ≈20%. This can be explained by the fact that the function of VCAM1 is redundant and partially overlaps with other adhesion molecules likely not affected by H19X. Thus a partial reduction of the adhesion capacity was expected.

For the first time, we provide evidence for a functional role of the lncRNA H19X in microvascular ECs, a cell type that

plays a major role in SSc. We showed that H19X had a higher expression in ECs of SSc skin and that in HDMECs it was upregulated by both type I and type II IFNs. We demonstrated that H19X silencing significantly affected the expression of adhesion molecules, predominantly VCAM1, with consequent EC cytoskeletal rearrangements and reduction of adhesion of PBMCs that might favour infiltrate transmigration. Altogether, our results suggest that the upregulation of H19X contributes to endothelial activation, a crucial mechanism in the pathophysiology of SSc (Supplementary Fig. S6, available at *Rheumatology* online).

Supplementary material

Supplementary material is available at *Rheumatology* online.

Data availability

Data are available upon reasonable request to the corresponding author.

Authors' contributions

O.D. directed, supervised and obtained funding for the project. O.D., F.T. and E.P. designed, analyzed and interpreted experiments. O.D., F.T. and E.P. wrote the manuscript. F.T. and E.P. performed the experiments with the support of G.S. and E.C.R. R.L. and M.H. performed, analyzed and helped with the interpretation of the scRNA-seq data. G.K. and F.Z. participated in the design, analysis and interpretation of the experiments.

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