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SCLERODERMA (J VARGA, SECTION EDITOR)

Epigenetic Control of Scleroderma: Current Knowledge and Future Perspectives

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Abstract

Purpose of Review Epigenetics has been implicated in the pathogenesis of systemic sclerosis (SSc). In this review, the involvement of the three epigenetic mechanisms in SSc development and progression—DNA methylation, histone modifications, and non-coding RNAs—will be discussed.

Recent Findings Alteration in epigenetics was observed in immune cells, dermal fibroblasts, and endothelial cells derived from SSc patients. Genes that are affected include those involved in immune cell function and differentiation, TGFβ and Wnt pathways, extracellular matrix accumulation, transcription factors, and angiogenesis. All the studies remain in the pre-clinical stage.

Summary Extensive research provides evidence that epigenetic alterations are critical for SSc pathogenesis. Future epigenomic studies will undoubtedly continue to broaden our understanding of disease pathogenesis and clinical heterogeneity. They will also provide the scientific basis for repurposing epigenetic-modifying agents for SSc patients.

Keywords Scleroderma · Epigenetics · DNA methylation · Histone modifications · Non-coding RNAs

Introduction

Over the past decade, sufficient evidence has been generated which supports the fact that both genetic and environmental factors are critical for systemic sclerosis (SSc) susceptibility [1, 2]. Indeed, although having a family member with SSc is one of the highest risk factors among all autoimmune diseases, SSc has the lowest concordance rate in monozygotic twins [3, 4]. In individuals that are genetically susceptible to SSc, exposure to environmental factors, such as silica or solvents [5, 6], heavy metals [7], viral infection [8], and certain drugs [9], could lead to autoimmunity, possibly through epigenetic mechanisms. These dynamic and reversible modifications, including DNA methylation, histone modifications, and non-coding RNAs, regulate chromatin structure and accessibility of transcriptional machinery, thereby governing gene transcription (Table 1). This new line of research, coupled with the availability of next-generation sequencing technology, has pushed the SSc field into uncharted territories in recent years. In this review, the current knowledge of epigenetics in SSc (published in the past 5 years; summarized in Table 2), as well as future perspectives and where the field is leading us, will be discussed.

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Epigenetic Involvement in SSc Pathogenesis

The three major events in SSc pathogenesis include immune activation, microvascular dysfunction, and tissue fibrosis. The cells that play prominent roles in SSc, including immune cells, endothelial cells (ECs), and fibroblasts (both lung and skin), retain their diseased phenotype in culture for days, suggesting an epigenetic mechanism might be involved. Indeed, various studies have provided substantial evidence, and they will be discussed in this section.

Immune Cells

Altered immunological processes are involved in the development of SSc pathogenesis. Epigenetic mechanisms in immune cells are responsible, at least in part, for the dysregulation of the immune system in this disease. Indeed, aberrant DNA

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Table 1 Epigenetic modifications

Mechanisms	Descriptions	Key mediators	Effect on gene transcription
DNA methylation	Addition of a methyl group from s-adenosylmethionine to the C5 position of cytosine nucleotides occurring at cytosine-phosphate-guanine dinucleotides	DNMTs; TETs; MBDs; MeCP2	Hypermethylation➔downregulation Hypomethylation➔upregulation
Histone modifications	Post-translational modifications on amino acid residues on histones that alter chromatin structure	HATs; HDACs; HMTs; HDMs; histone readers	Histone acetylation➔upregulation Histone methylation➔up- or down-regulation
Non-coding RNAs	Functional RNAs that regulate gene expression	lncRNAs; miRNAs	Up- or down-regulation

methylation was reported in peripheral blood mononuclear cells (PBMCs), CD4+ T cells, CD8+ T cells, and Tregs [10, 11•, 14, 15–17, 18••, 19, 20, 65]. Histone changes were observed in T cells and B cells [21, 22, 23•], whereas non-coding RNAs varied in PBMCs, plasmacytoid dendritic cells (pDCs), and monocytes [13, 24, 25, 26••].

DNA Methylation

The impact of DNA methylation in CD4+ T cells from SSc patients has been studied extensively over the years. These cells were found to be globally demethylated, possibly due to significantly lower levels of DNA methyltransferase 1 (DNMT1) and methyl-CpG-binding domain proteins (MBDs) [14]. Co-stimulatory molecules, including CD40L, CD70, and CD11a, in SSc CD4+ T cells were upregulated due to hypomethylation at their promoter regions [15–17]. Genome-wide DNA methylation profiling in CD4+ and CD8+ T cells from SSc patients revealed that interferon (IFN)-associated genes were globally hypomethylated in both cell types [18••]. However, strong correlation was found among the methylation status, serum levels of type I IFNα/β, and gene expression of these IFN-associated genes only in CD4+ T cells. In Tregs, the methylation status of *FOXP3* is less clear in SSc. Hypermethylation and downregulation of *FOXP3*, accompanied with lower number of Tregs in SSc patients, were observed in one study, whereas hypomethylation and upregulation of *FOXP3* with expanded Tregs were shown in another [19, 20]. The cell surface markers used to define Tregs were different in these two studies, which might explain the discrepancy. However, these studies do imply the role of DNA methylation in the imbalance of Tregs in this disease.

Aberrant DNA methylation was also observed in PBMCs in SSc patients. Hypomethylation of the *IRF7* promotor might be the reason for its increased expression in PBMCs in limited cutaneous SSc (lcSSc) patients [10]. In contrast, the upregulation of *ITGB2* in PBMCs from SSc patients appeared to be independent of DNA methylation, as there was no correlation between *ITGB2* expression and the methylation status at its promoter region [65]. Simultaneous genome-wide analysis of the transcriptome and methylome in PBMCs from healthy controls and SSc patients identified 453 differentially expressed genes and 618 differentially methylated genes that were significantly altered in SSc PBMCs [11•]. Among them, only 20 genes were potential methylation-regulated genes because their expression and methylation status were inversely correlated. These genes, such as *F2R*, *CXCR6*, *FYN*, *LTBR*, *CTSG*, and *ELANE*, were enriched in pathways including cell proliferation, immune cell migration, and inflammation.

Histone Modifications

Changes in histone modifications have been suggested in PBMCs, CD4+ T cells, and B cells in SSc patients [12, 21, 22]. In CD4+ T cells, global reduction of H3K27me3, a repressor mark, was observed in SSc patients [21]. This might be due to the elevation of the histone demethylase JMJD3. B cells isolated from SSc patients showed elevated levels of H4 acetylation and reduction of H3K9 methylation, both of which lead to active transcription [22]. Utilizing ChiP-seq and RNA-seq techniques, van der Kroef et al. recently showed that genome-wide distribution of H3K4me3 and H3K27ac marks were altered in monocytes from SSc patients compared with healthy controls [23••]. Associated with active promoters or active enhancers, variations in these histone marks were further shown to be associated with increased expression of immune-, IFN-, and antiviral response-related genes, which had overlapping binding sites for transcription factors of the IRF and STAT family. Interestingly, these alterations in H3K27ac and H3K4me3, as well as a transcription signature that resulted in increased histone acetylation, were observed in all SSc subsets, including early SSc patients (defined as patients presenting with Raynaud's phenomenon with either nailfold abnormalities or SSc-specific autoantibodies). This suggests that the altered epigenetic landscape in SSc monocytes might be priming these cells in the early phase of the disease and is critical in initiating and sustaining the SSc phenotype.

Non-Coding RNAs

It was not until recently that the field began to examine the impact of non-coding RNAs in immune cells. Microarray

Table 2 Key epigenetic changes in SSc

Cell types	DNA methylation	Histone modifications	Non-coding RNAs
PBMCs	Hypomethylation of *IRF7* [10] Identification of 20 potential methylation-regulated genes after genome-wide transcriptome and methylome analysis [11•]	Downregulation of SIRT1 in SSc-related pulmonary fibrosis patients [12]	Downregulation of HNRNPU-AS1 [13]
T cells	Global hypomethylation [14] Hypomethylation of CD40L, CD70, and CD11a [15–17] Hypomethylation of IFN-associated genes [18] Methylation of *FOXP3* [19, 20]	Decrease in H3K27me3 [21]	
B cells		Increase in global H4 acetylation [22] Decrease in global H3K9 methylation [22]	
Monocytes		Altered genome-wide distribution of H3K4me3 and H3K27ac marks [23••]	Upregulation of NRIR [24] Upregulation of miR-5196 [25]
pDCs			Upregulation of miR-618 [26•]
Dermal fibroblasts	Global hypomethylation and increase in 5-hydroxymethylcytosine [27] Genome-wide changes in DNA methylation differ in disease subtype [28•] Hypermethylation of *FLI1* and *KLF5* [29, 30] Hypermethylation of *PARP1* [31] Upregulation of MeCP2 [32, 33•]	Increase in H3K27me3 [34, 35••] Inhibition of overexpressed JMJD3 inhibits fibrosis [36] Inhibition of overexpressed EZH2 inhibits fibrosis [35••] Upregulation of p300 is involved in TGFβ-mediated fibrosis [37] Novel *GFB2* enhancer identified [38••] Downregulation of SIRT1 [39, 40] Downregulation of SIRT3 [41]	Downregulation of miR-29, miR-196a, and let-7a directly target collagen [42–44] miR-29a targets *TAB1* and Bcl-2 family genes [45, 46] Downregulation of miR-150 [47] Upregulation of miR-21 and downregulation of miR-145 affect Smads [48, 49] Upregulation of miR-155 [50, 51] Upregulation of miR-202 and miR-92a downregulate *MMP1* [52, 53] Downregulation of miR-135b targets *STAT6* [54] Upregulation of miR-130b targets *PPARG* [55] Downregulation of miR-126 targets *EGFL7* in early-onset dcSSc patients [56] Downregulation of miR-30a-3p targets *BAFF* [57] Downregulation of miR-193b targets *PLAU* [58] Increased miR-142-3p and decreased miR-150/miR-196a in exosomes [59] Upregulation of TSIX [60] Deregulation of CTBP1-AS2, OTUD6B-AS1, and AGAP2-AS1 [61•]
Lung fibroblasts		Downregulation of SIRT7 [62]	Upregulation of miR-155 [51]
Dermal endothelial cells	Hypermethylation of *BMPR2* [63]	Upregulation of HDAC5 [64••] Increase in EZH2 and H3K27me3 [35••]	

analysis with PBMCs isolated from patients with SSc detected more than one hundred long non-coding RNAs (lncRNAs); however, among them, only heterogeneous nuclear ribonucleoprotein U antisense RNA 1 (HNRNPU-AS1 or ncRNA00201) was significantly downregulated compared with PBMCs from healthy controls [13]. Although the functional relevance of this lncRNA in SSc is not known, its gene target, *HNRNPC*, encodes for a known SSc autoantigen. Pathway and gene ontology enrichment analysis also revealed the potential involvement of this lncRNA in inflammation, vasculitis, and fibrosis. In addition, ncRNA00201 was predicted to modulate 26 miRNAs that affect genes involved in SSc pathogenesis. The role of epigenetic influence on pDCs in SSc has been shown for the first time by Rossato et al. [26•]. Through miRNA profiling via microarray technology, miR-618 was identified to be upregulated in pDCs from SSc patients, which led to downregulation of *IRF8* in these cells. Overexpression of miR-618 in pDCs inhibited pDC development and enhanced IFNα secretion; therefore, it is possible that dysregulation of this miRNA contributes to the type I IFN signature seen in SSc patients.

In addition to pDCs, monocytes are also affected by non-coding RNAs in SSc. Transcriptomic analysis of LPS-stimulated normal and SSc monocytes revealed 1278 upregulated and 534 downregulated lncRNAs [24]. Through a series of gene ontology enrichment and correlation analysis, the authors identified a list of IFN/viral-related lncRNAs. Among them, Negative Regulator of the IFN Response (NRIR) was significantly upregulated in SSc monocytes and correlated strongly with the patients' IFN score (calculated based on the expression of 6 IFN-related genes). The functional relevance of this lncRNA was validated in NRIR-silenced monocytes; downregulation in NRIR resulted in decreased expression of 15 in silico-predicted NRIR target genes, some of which were relevant in SSc pathogenesis. MiR-5196, which is elevated in monocytes and sera from SSc patients compared with controls, was shown to be critical in modulating the pro-fibrotic phenotype of monocytes [25]. Monocytes treated with the histone methyltransferase inhibitor DZNep and TLR8 agonist ssRNA exhibited a pro-fibrotic phenotype, including increased TIMP-1 and Fra2 expression. As miR-5196 directly targets *FRA2*, transfection of miR-5196 in DZNep/ssRNA-treated monocytes significantly reduced both *FRA2* and *TIMP1* expression. These studies suggest that epigenetic dysregulation in monocytes is responsible, at least in part, for the inflammatory response as well as subsequent fibrotic manifestations in SSc.

Dermal Fibroblasts

The epigenetic mechanisms in SSc fibroblasts have been studied extensively. Dysregulation of DNA methylation, histone changes, and non-coding RNAs have been reported to affect the transforming growth factor β (TGFβ) pathway [31, 39–41, 47, 48, 62], Wnt pathway [32, 50, 66, 67], transcription factors [29, 30, 34, 35••, 36], and extracellular matrix synthesis (ECM)/turnover [32, 42–44, 52, 60, 68].

DNA Methylation

Global hypomethylation was observed in SSc dermal fibroblasts [27, 28], as aberrant changes in DNMT1 [29, 69], methyl-CpG-binding protein 2 (MeCP2) [29, 32, 33•, 54], and Ten-eleven translocation methylcytosine dioxygenase 1 (TET1) [27] have been documented. By examining genome-wide changes in DNA methylation in diffuse cutaneous SSc (dcSSc or lcSSc) patients that are matched with healthy controls, Altorok et al. revealed the distinct differences in methylation patterns in the two disease subtypes; there were only 6% differentially methylated CpG sites that were shared between the two, with the majority of them being hypomethylated compared with controls [28•]. These common hypomethylated genes include ones that encode integrins, matrix metalloproteinases, collagen, and genes relevant to the TGFβ and Wnt pathways. These genes were enriched in gene ontologies, such as negative regulation of MAP kinase activity, cell projection morphogenesis, and pathways that involve ECM–receptor interaction and focal cell adhesion, to name a few.

In addition to global DNA methylation changes in SSc dermal fibroblasts, the effect of DNA methylation on transcription factors and the TGFβ pathway has also been implicated [30, 31]. The critical role of transcription factor Friend leukemia integration 1 (FLI1) in SSc pathogenesis has been well-documented over the years [70, 71]. This collagen suppressor gene, which is epigenetically regulated by DNA methylation and histone acetylation [29], works synergistically with Kruppel-like factor 5 (KLF5) to suppress connective tissue growth factor (CTGF) [30]. Interestingly, similar to *FLI1*, *KLF5* is also epigenetically suppressed in dcSSc fibroblasts via hypermethylation at its promoter region and hypoacetylation. As the $Klf5^{+/-}$;$Fli1^{+/-}$ mice show autoimmunity, vascular complications followed by skin and lung fibrosis that mimic the natural course of SSc pathogenesis, epigenetic reprogramming of *KLF5* and *FLI1* appears to be a pivotal event for SSc pathogenesis. Recently, the downregulation of *PARP1*, which codes for poly(ADP-ribose) polymerase-1 (PARP-1) in SSc fibroblasts and TGFβ-treated normal fibroblasts, was reported to be due to increased DNA methylation at its promoter region [31]. TGFβ recruits PARP-1 to bind and poly(ADP-ribosyl)ate Smad3, which in turn inactivates Smad3 and stops Smad-dependent gene transcription. This negative feedback loop is dysfunctional in SSc due to the PARP-1 deficiency, which might contribute to the heightened TGFβ response in this disease.

To repress gene transcription, methylated CpGs serve as docking sites for MBDs to recruit corepressors. The

expression of MBDs in SSc fibroblasts has been documented [29, 54]; however, two recent studies examined the functional relevance of the upregulated MeCP2 in SSc fibrosis [32, 33•]. He et al. showed that MeCP2 overexpression in dermal fibroblasts inhibited myofibroblast differentiation, proliferation, and migration, as well as decreased contractile properties [33•]. Several genes, including *PLAU*, *NID2*, and *ADA,* were in part mediating the anti-fibrotic effect of MeCP2 in dcSSc fibroblasts, identified using RNA-seq in MeCP2-deficient dcSSc fibroblasts and validation in functional assays. *NID2* and *PLAU* were indeed MeCP2-regulated genes, as significant MeCP2-binding enrichments were observed in regulatory sequences in *NID2* and *PLAU* gene loci via ChIP-seq. In contrast, Henderson et al. suggested that MeCP2 is involved in an epigenetic loop to promote collagen synthesis in dcSSc fibroblasts [32]. They showed that elevated MeCP2 in dcSSc fibroblasts, which is possibly mediated by downregulation of miR-132, decreased Wnt antagonist sFRP-1, thereby activating the Wnt signaling pathway and collagen production in these cells. The reason for the discrepancies between the two studies is not known. Two very different approaches were adopted in these studies; one used an unbiased genome-wide sequencing approach in identifying downstream targets whereas the other was hypothesis-based. In addition, cells were treated with TGFβ in all experimental conditions in the study by Henderson et al., and this could alter the transcriptome and fibrotic phenotype in dermal fibroblasts. Interestingly, significant enrichment of MeCP2 in regulatory regions of *SFRP1* was absent in the ChIP-seq data generated by He et al. [33•]. In addition, *SFRP1* was also not significantly altered in dcSSc fibroblasts with deficient of MeCP2.

In addition to the involvement of DNMTs in SSc, active demethylation by TETs has also been suggested in this disease. Hattori et al. showed that TET1 wa upregulated in SSc fibroblasts compared with control fibroblasts, accompanied with elevated 5-hydroxymethylcytosine levels in the skin [27]. The expression of TET1, however, is not altered in lc SSc fibroblasts compared with controls in another report [54]. More studies with different disease subtypes and larger sample sizes will be needed to further dissect the involvement of TETs in SSc.

Histone Modifications

Histone H3 trimethylation of lysine (H3K27me3) has been studied extensively in SSc fibrosis [34, 35••, 36]. This histone mark, which is catalyzed by histone methyltransferase EZH2 and demethylated by histone demethylases JMJD3 or UTX, is associated with gene repression. In dcSSc fibroblasts, elevated H3K27me3 has been reported [34, 35••]. Interestingly, increased levels of EZH2 and JMJD3 were also reported in these cells [35••, 36]. Fra2, a pro-fibrotic transcription factor, appears to be controlled by this epigenetic mechanism in SSc. A recent study by Bergmann et al. showed that pharmacological inactivation of JMJD3 inhibited dcSSc fibroblast activation and ameliorated fibrosis in two animal models in a Fra2-dependent manner [36]. The overexpression of Fra2 in dcSSc fibroblasts was accompanied with reduced levels of H3K27me3 in its promoter region. When JMJD3 was inhibited in these cells, *FRA2* expression was downregulated via induction of H3K27me3 marks at the promoter. These results were supported by an earlier study from the same group showing that inhibition of EZH2 exacerbated SSc fibrosis both in vitro and in vivo via inducing *FRA2* [34]. However, our recent study suggested otherwise [35••]. Using two EZH2 inhibitors, DZNep and GSK126, inactivation of EZH2 appeared to be anti-fibrotic in dcSSc fibroblasts, as shown by downregulation of pro-fibrotic gene expression and inhibition of gel contraction and cell migration. Both inhibitors also ameliorated fibrosis in the bleomycin-induced skin fibrosis mouse model. By examining the genome-wide changes of DNA methylation after EZH2 inhibition in dcSSc fibroblasts, we found that epigenetic dysregulation and overexpression of the cell membrane-cytoskeleton protein LRRC16A accounted for EZH2-mediated fibroblast migration in these cells. Given that DZNep affects both EZH2 expression and activity while GSK126 inactivates methyltransferase activity of EZH2 without altering its expression, we believe that the pro-fibrotic effect of EZH2 in SSc stems from both its expression and enzymatic activity. It has been suggested that, in addition to its gene-repressing activities, EZH2 itself promotes gene transcription acting as a transcription factor [72]. Indeed, we speculate that EZH2-mediated gene repression via H3K27me3 might play a minor role in its pro-fibrotic effect, as a majority of the genes that were examined were down-regulated after EZH2 inhibition in dcSSc fibroblasts. Taken together, results from these studies suggested that examining the global changes in histone marks might not be a good indication for monitoring SSc fibrosis, as alteration in the histone marks might be context- and gene-specific. In addition, this also emphasizes the importance of considering individual properties and mechanisms involved in histone proteins, as they themselves might have opposing effects on gene transcription. In-depth examination is required when conducting epigenetic studies.

In addition to histone methylation, histone acetylation has also been implicated in SSc fibrosis. One such example includes histone acetyltransferase p300, which was shown to regulate myofibroblast transformation as a transcriptional activator [73]. Upregulation of p300 in SSc skin and fibroblasts appears to play a critical role in TGFβ-mediated fibrosis [37, 74]. In dermal fibroblasts, p300 was induced by TGFβ via upregulation of early growth response-1 (Egr-1). This in turn enhanced p300 recruitment at the promoter of *COL1A2*, thereby increasing histone H4 acetylation and facilitating the transcriptional activities of Smad2/3, and ultimately leading to increased collagen expression.

The involvement of p300 was further demonstrated in a study by Shin et al., where they found that this enzyme contributes to the elevation of TGFβ2 in dcSSc fibroblasts [38••]. The authors identified an enhancer for *TGFB2* that was enriched with H3K27ac marks and occupied by p300. Targeted histone acetylation at this *TGFB2* enhancer by co-transfecting *TGFB2* enhancer guide RNA and dCas9-p300 in dcSSc fibroblasts significantly induced *TGFB2*, *COL1A1*, and expression whereas it had no effect on *TGFB1* and *TGFB3*. Surprisingly, inactivation of p300 by inhibitor SGC-CBP30 only transiently decreased *TGFB2*, p300 occupancy, and H3K27ac levels at the *TGFB2* enhancer. Instead targeting BRD4, a member of the bromodomain and extraterminal domain family member which binds to acetylated histones, was able to downregulate *TGFB2* in dcSSc fibroblasts for at least 5 days after removing the inhibitor from culture. The BRD inhibitor JQ1 also repressed collagen synthesis and promoted collagen turnover in skin explants from SSc patients.

As early studies focused on the anti-fibrotic effect of general class I and II HDAC inhibitors in SSc [67, 75, 76], recent studies suggest that class III HDACs, specifically SIRT1, SIRT3, and SIRT7, protect against SSc skin and lung fibrosis [12, 41, 62, 77, 78]. Downregulation of SIRT1, SIRT3, and SIRT7 was observed in SSc dermal and lung fibroblasts, as well as PBMCs from SSc-related pulmonary fibrosis patients. Pharmacological activation of SIRT1 via resveratrol or selective SIRT1 activators, such as SRT1720, disrupts TGFβ-induced Smad signaling and ECM turnover in dcSSc fibroblasts [39]. Moreover, the protective effect of SIRT1 in skin and lung fibroblasts appeared to be mediated through mTOR inactivation [12, 78]. Similarly, SIRT3 activation via resveratrol or hexafluoro attenuated SSc skin and lung fibrosis [41, 77]. Given that SIRT3 is located predominantly in the mitochondria, it is critical for mitochondrial homeostasis. The protective effect of SIRT3 in pulmonary fibrosis could stem from preventing ROS-mediated mitochondrial DNA damage in lung fibroblasts [77, 79].

Non-Coding RNAs

Increasing evidence has highlighted the critical involvement of non-coding RNAs, including lncRNAs and miRNAs, in SSc fibrosis. This class of epigenetic modifier contributes to various fibrinogenesis processes in this disease, including ECM accumulation and turnover [42–44, 52, 60], as well as myofibroblast activation via the TGFβ and Wnt pathways [47, 48, 50]. In addition, miRNAs are also involved in SSc fibroblast apoptosis [45], immune activation [57], and vasculopathy [58].

Several studies have demonstrated the contribution of miRNAs to the excessive production of collagen in SSc [42–44, 52]. These miRNAs, such as let-7a, miR-196a, and miR-29, directly target collagen and thereby suppress the production of these proteins in SSc dermal fibroblasts [42–44]. Among these miRNAs, the miR-29 family has been studied most extensively as an anti-fibrotic in SSc. Downregulated miR-29a in SSc fibroblasts led to increased collagen deposition [42]. In addition to directly targeting collagen, reduction of miR-29a in SSc upregulated TGFβ activated kinase 1 binding protein 1 (TAB1), thereby triggering TIMP-1 and collagen production [46]. MiR-29a is also involved in inducing apoptosis in dcSSc fibroblasts via regulating the anti-apoptotic Bcl-2 family genes [45]. Given that miR-29a can be downregulated by TGFβ, PDGF, or IL-4, treatment with the tyrosine kinase inhibitor imatinib restored miR-29a levels in cells as well as in the bleomycin fibrosis mouse model [42].

Instead of directly targeting collagen, several dysregulated miRNAs have been reported to affect ECM accumulation via other mechanisms. Multiple miRNAs regulate the TGFβ pathway in SSc. Downregulation of miR-150 in dcSSc fibroblasts upregulated *ITGB3*, which subsequently resulted in activation of the TGFβ pathway [47]. Interestingly, the authors showed that the downregulation of miR-150 might be due to hypermethylation at its promoter. Other miRNAs target signaling molecules in the TGFβ pathway. Two examples are upregulation of miR-21 in SSc fibroblasts that downregulates the negative modulator *SMAD7* and downregulation of miR-145 that elevates *SMAD3* [48, 49]. In addition to the TGFβ pathway, other signaling pathways are also affected by the miRNAs in SSc. One example is miR-155, which is upregulated in SSc skin and lung [50, 51]. MiR-155 overexpression in skin fibroblasts resulted in activation of the Wnt/β-catenin and Akt signaling pathways, whereas miR-155 inhibition led to the opposite. Using a luciferase reporter assay, the authors showed that miR-155 directly targets CK1α, a negative regulator for β-catenin signaling, and SHIP-1, a phosphatase that turns off AKT activation [50]. Of note, miR-155 elevation in SSc fibroblasts depended on NLRP3 inflammasome activation and played a key role in inflammasome-mediated collagen production in SSc [51].

Upregulated miR-202-3p and miR-92a are responsible for the downregulated *MMP1* levels in SSc dermal fibroblasts compared with controls [52, 53]. Elevated *STAT6* in lcSSc fibroblasts was due to IL-13-mediated downregulation of miR-135b, which resulted in excessive collagen production in these cells [54]. Downregulation of peroxisome proliferator-activated receptor γ (PPARγ) in skin from SSc patients or bleomycin-treated mice was accompanied by elevated miR-130b levels [55]. The authors further showed that manipulation of miR-130b expression in healthy fibroblasts led to changes in *PPARG*, which resulted in alteration of *COL1A1, COL1A2, ACTA2*, and fibronectin levels, confirming the role of miR-130b on SSc fibrosis via modulating PPARγ expression. In fibroblasts isolated from early-onset dcSSc patients (defined as patients diagnosed less than

one year from Raynaud's onset), elevated epidermal growth factor like-domain 7 (EGFL7), possibly due to downregulated miR-126, downregulated collagen and induced angiogenesis [56]. However, in late-onset dcSSc fibroblasts, the elevated *EGFL7* levels returned back to baseline, accompanied with increased miR-126 in these cells. The author suggested that EGFL7 and miR-126 might contribute to SSc vasculopathy and fibrosis.

Dysregulation of certain miRNAs in SSc patients has been implicated in other processes, such as apoptosis and immune activation. In addition to its collagen-modulation effects in SSc, miR-29a induced cell apoptosis by affecting the Bax:Bcl-2 ratio in dcSSc fibroblasts [45]. Similar findings were reported in miR-21 [80]. Negative regulation of *BAFF* production in dcSSc fibroblasts by miR-30a-3p has been shown by Alsaleh et al. [57]. This process, which is Poly(I:C)- or IFNγ-dependent, could potentially have an impact on autoimmune activation in SSc. Iwamoto et al. showed that downregulation of miR-193b in SSc fibroblasts and skin contributes to elevated urokinase-type plasminogen activator (uPA) [58]. Given that uPA induced vascular smooth muscle cell proliferation and suppressed apoptosis, the authors suggested that this miRNA might contribute to the proliferative vasculopathy in SSc.

Extracellular vesicles can be released from cells to promote diverse cellular functions through intercellular communication. These vesicles can be taken up by neighboring cells and subsequently modulate the functions of the recipient cells. Exosomes, one type of extracellular vesicles, are small vesicles in the nanometer range that are originated from endocytosis, therefore they are enriched in endosomal proteins. In addition to proteins, various nucleic acids are found in them, including non-coding RNAs. Nakamura et al. showed that exosomes from dcSSc fibroblasts contain elevated miR-142-3p and decreased miR-150 and miR-196a [59]. Moreover, these dcSSc fibroblast-derived exosomes, when cultured with healthy dermal fibroblasts, were able to induce *COL1A1* and *COL1A2* in these cells. Similarly, exosomes isolated from SSc patient serum stimulated pro-fibrotic gene expression and collagen production in normal dermal fibroblasts in a dose-dependent manner [81]. Six pro-fibrotic miRNAs were elevated and 10 anti-fibrotic miRNAs were downregulated in these dcSSc serum exosomes compared with healthy controls, supporting their pro-fibrotic properties. These studies provide a novel mechanism for SSc fibroblasts to extend and propagate the pro-fibrotic signal to unaffected tissue.

The exact role of lncRNAs in SSc is still poorly understood. The study by Wang et al. showed that TSIX, a lncRNA that binds to *XIST* during X-chromosome inactivation, was upregulated in dcSSc fibroblasts compared with healthy controls [60]. Instead of *XIST*, which was not altered in dcSSc fibroblasts, the authors found that *COL1A1* and *COL1A2* were novel targets of TSIX in these cells. Recently, a transcriptomic analysis of whole skin tissue from healthy controls and SSc patients was performed, and 676 differentially expressed lncRNAs were identified [61•]. After careful validation and in-depth analysis, the authors identified three antisense lncRNAs, CTBP1-AS2, OTUD6B-AS1, and AGAP2-AS1, as most significantly deregulated. In addition, the expression of these lncRNAs strongly correlated with the levels of their paired-sense genes. The functional relevance of these identified lncRNAs in SSc fibrosis remained to be examined.

In addition to the skin, non-coding RNAs in serum have also been measured [82, 83]. Chouri et al. profiled miRNAs in a microarray platform and, after further validation, identified miR-483-5p to be upregulated in both lcSSc and dcSSc patients, as well as patients with pre-clinical symptoms [83]. Overexpression of miR-483-5p in dermal fibroblasts and ECs affected several genes involved in myofibroblast differentiation and collagen disposition.

Endothelial Cells

There are only a few studies that examined the epigenetic changes in SSc dermal ECs, partly due to the difficulties in isolating and growing these cells in culture. The pioneer study that examined DNA methylation in SSc dermal ECs was conducted by Wang et al. [63]. They found that SSc ECs are more prone to apoptosis compared with healthy ECs, and this might be due to the lower expression of the bone morphogenetic protein type II receptor (BMPRII) in SSc ECs. The downregulation of *BMPR2* was due to hypermethylation at its promoter region.

Aberrant histone changes have also been reported in dcSSc ECs [35••, 64••]. We showed that overexpression of HDAC5 in dcSSc ECs renders these cells into an anti-angiogenic state, as knockdown of HDAC5 in these cells restored angiogenesis [64••]. By utilizing an assay for transposase-accessible chromatin using sequencing (ATAC-seq) in HDAC5 knockdown cells, we identified three genes, *FSTL1*, *CYR61*, and *PVRL2*, that appeared to be repressed by HDAC5 and played functional roles in SSc EC angiogenesis. We followed up on CYR61 in a subsequent study and showed that this matricellular protein not only promotes angiogenesis in dcSSc ECs but also inhibits fibrosis by inducing senescence in dcSSc fibroblasts [84].

In our recent study, we reported an upregulation of the histone methyltransferase EZH2 and H3K27me3 in dcSSc ECs compared with healthy ECs [35••]. EZH2 is the catalytic component of the polycomb repressive complex 2 that represses gene transcription through catalyzing H3K27me3. We found that EZH2 inhibits dermal EC angiogenesis, as knocking down EZH2 by siRNA or inhibiting EZH2 by DZNep, an EZH2 inhibitor, in dcSSc ECs resulted in increased tube formation on Matrigel. The ability of EZH2 to inhibit angiogenesis in dcSSc ECs was through activating the

Notch pathway, as inhibition of EZH2 led to upregulation of several Notch-related genes. Among them, *DLL4*, which is a Notch ligand, was critical in promoting angiogenesis when cells were treated with DZNep.

Epigenetic-Modifying Drugs in SSc

Unlike genetic mutations, epigenetic changes are dynamic and reversible, therefore optimal for therapeutic targeting. The U.S. Food and Drug Administration (FDA) was supportive in targeting the epigenome as a therapeutic strategy and approved 5-azacytidine (Vidaza), a DNMT inhibitor, for the treatment of myelodysplastic syndrome in 2004. In addition, a HDAC inhibitor, suberolyanilidehydroxamic acid (SAHA) or vorinostat (Zolinza), was approved later on for treating cutaneous T-cell lymphoma. Since then, many epigenetic-modifying agents have been developed for preclinical studies, and several have entered clinical trials for their antineoplastic effects. Despite the effort put in to decipher the role of epigenetics in SSc in the past decade or so, we are still far from translating these epigenetic findings to clinical applications. Indeed, as summarized in Fig. 1, all studies utilizing epigenetic compounds were in the preclinical stage, i.e., in cells isolated from SSc skin or animal models of SSc.

DNMT Inhibitors

Azacytidine (5-azaC) and its deoxy derivative, 5-aza-2'-deoxycytidine (5-aza), are pan-DNMT inhibitors that incorporate into DNA during replication and sequester DNMTs via forming covalent bonds between azacytosine-containing DNA and DNMTs. These enzymes are eventually depleted through replication, leading to loss of methylation marks and activation of gene transcription. These inhibitors have been tested extensively in SSc. The BMPRII levels in SSc ECs were restored after treated with 5-aza and HDAC inhibitor trichostatin A (TSA), which could potentially decrease EC apoptosis [63]. In dermal fibroblasts, inhibition of DNMTs showed prominent anti-fibrotic effects by restoring anti-fibrotic transcription factors *FLI1* and *KLF5* [29, 30], Wnt antagonists *DKK1* and *SFRP1* [66], and SMAD3 modulator *PARP1* [31], but downregulating miR-135b [54]. These DNMT inhibitors also had immune-modulatory effects, as 5-azaC was able to enhance *FOXP3* expression and Treg production in CD4+ T cells from SSc patients [19]. Moreover, inhibition of DNMTs prevented bleomycin-induced skin fibrosis in mice [66].

HDAC Inhibitors

The beneficial effect of targeting HDACs in SSc has been well-documented. One such example is TSA. Trichostatin A is a selective inhibitor for class I and II HDACs. This drug blocks the catalytic activity of HDACs by chelating its cofactor zinc ion with its hydroxamic acid group. Administration of TSA in bleomycin-treated mice prevented ECM accumulation in the skin [75]. It was also efficacious in downregulating fibrotic-related genes, including *COL* and Wnt inhibitor *WIF1* in dermal fibroblasts isolated from SSc patients [67, 75, 76]. In addition, combining TSA and 5-aza appears to be efficacious in mitigating the pro-apoptotic and pro-fibrotic phenotypes seen in SSc EC and fibroblasts [29, 30, 63]. Class II HDACs appear to be more effective in inhibiting fibrosis, as MC1568, a selective class IIa HDAC inhibitor, downregulated collagen, whereas the selective class I inhibitor PD106 had minimal effect [85]. In SSc monocytes, blockade of histone acetylation via inhibition of histone reader BET bromodomain inhibitor JQ1 suppressed expression of several IFNα-related genes [23••]. In dcSSc fibroblasts, JQ1 efficiently blocked the activation of the *TGFB2* enhancer thereby downregulated *TGFB2* and *COL1A1* in these cells [38••]. It

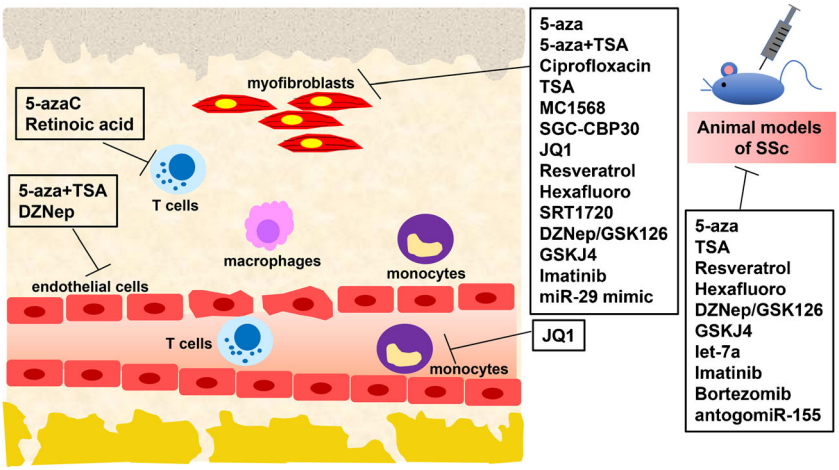

Fig. 1. The application of epigenetic-modifying drugs in SSc is summarized. Agents include DNMT inhibitors (5-azaC and 5-aza), HDAC inhibitors (TSA and MC1568), HAT inhibitor (SGC-CBP30), SIRT activators (resveratrol, SRT1720, and hexafluoro), EZH2 inhibitors (DZNep and GSK126), JMJD3 inhibitor (GSKJ4), BET bromodomain inhibitor (JQ1), and miRNAs. Other drugs include ciprofloxacin and retinoic acid (target DNMTs), as well as imatinib and bortezomib (target miRNAs)

also reduced collagen and increased MMP-1 in SSc skin explants.

In contrast to class I and II HDACs, class III HDACs are NAD-dependent. Resveratrol and SIRT1720, both of which activate SIRT1, abrogated fibrotic responses in SSc dermal fibroblasts and bleomycin-induced skin fibrosis in mice [39, 78]. Similarly, SIRT3 activator hexafluoro attenuated SSc skin and lung fibrosis in vitro and in vivo [41]. Conflicting results for resveratrol were reported by Zerr et al. in vitro [40]. Mice null of Sirt1 in fibroblasts were also less susceptible to fibrosis induced by bleomycin, or in ones that had active Tgfbr1 constitutively expressed. This discrepancy might stem from different animal models used in these studies. The off-target effects of resveratrol might be the culprit too. Studies using selective SIRT1 activators would likely provide more insights on the role of SIRT1 in SSc fibrosis. This is worth exploring considering the prominent anti-fibrotic properties of class III HDACs reported recently in SSc-related lung fibrosis [12, 62, 77].

Targeting histone methylation is also an attractive way to treat SSc. Inactivation of H3K27me3 demethylase JMJD3 by GSKJ4 ameliorated fibroblast activation and murine SSc models in a Fra2-dependent manner [36]. Interestingly, inhibition of EZH2, a histone methyltransferase that facilitates H3K27me3, also alleviated SSc fibrosis in vitro and in vivo [35••]. Targeting EZH2 also benefits SSc ECs by restoring their angiogenic activity. The anti-fibrotic effect in this study was shown by two EZH2 inhibitors, DZNep and GSK126. DZNep is not specific to EZH2 as it inhibits the hydrolysis of S-adenosyl-L-homocysteine (SAH), resulting in accumulation of SAH which in turn inhibits EZH2 and other histone methyltransferases. In contrast, GSK126 is a potent, S-adenosylmethionine-competitive EZH2 inhibitor that is highly selective towards EZH2. As conflicting results for the effect of DZNep were shown in an earlier study [34], specific EZH2 inhibitors should be used for more extensive studies for SSc. In addition, co-treatment of DZNep and TLR8 agonist appears to enhance a pro-fibrotic phenotype in SSc monocytes [25]; therefore, the immunomodulating effect of EZH2 inhibition requires more examination as well.

MiRNAs

Certain miRNAs, such as let-7a and topical antagomir-155, have been implicated in treating SSc fibrosis [44, 50]. In a recent study, the safety, tolerability, pharmacokinetics, and pharmacodynamics of miR-29 mimic Remlarsen were examined in healthy controls and showed promising anti-fibrotic effects by repressing collagen expression and fibroplasia development in incisional skin wounds [86•]. These results suggest that miRNAs may be an effective therapeutic option for cutaneous fibrosis.

Miscellaneous

Many compounds originally not designed as epigenetic-modifying drugs were later found to modify epigenetic mechanisms. Retinoic acid, which is the active metabolite of vitamin A, demethylated the promoter region of *FOXP3*, thereby increasing *FOXP3* expression in SSc CD4+ T cells [87]. The antibiotic ciprofloxacin showed anti-fibrotic properties by downregulating DNMT1 and upregulating FLI1 in SSc dermal fibroblasts [88]. The anti-fibrotic effects of imatinib and bortezomib were in part due to restoring miR-29a and miR-21 levels in animal models of SSc [42, 48].

Future Perspectives

In the past two decades, our understanding of the etiology of SSc has progressed tremendously, especially with the rapid development in epigenetic involvement, which we highlighted in this review. It is clear that epigenetics is critical in SSc pathogenesis and that the use of the so-called "epidrugs" would be potential effective therapeutics, either used alone or in combinatorial therapy. Repositioning epigenetic-modifying drugs currently in clinical trials for cancer to treat SSc would be an attractive approach to potentially lower overall development costs and shorten development timelines. However, one of the major limitations using epigenetic-modifying agents is the lack of specificity. As the epigenome is complex and dynamic, it is critical to minimize the off-target effects of these drugs. Targeted drug delivery systems might be an effective way to minimize side effects and achieve high therapeutic index. Nanoscale carrier systems and prodrug approaches can also overcome the low solubility/permeability and the poor pharmacokinetic properties of epigenetic drugs. Indeed, the second-generation of epidrugs has already been designed and tested in cancer [89, 90]. Another approach to enable epigenetic precision is to target specific loci using the CRISPR/Cas9 system. This epigenetic editing approach, although still in its early phase in development in autoimmune diseases, has been successfully applied to demethylate and upregulate *FOXP3* in Jurkat cells after transfecting the cells with a dCas9 epigenetic machinery containing TET1 [91].

Another complexity for epigenetics studies in SSc is the discordance of reports. This could be attributed to the patient demographics and environmental differences, as studies conducted in the U.S. often contradict those done in Europe. Scleroderma itself is a complex disease with diverse heterogeneity. It is important to recruit large numbers of patients with similar disease classification and duration with strict exclusion criteria for any confounding diseases or medications that affect the epigenome. Age-, ethnicity-, and gender-matched healthy

controls should be recruited. As epigenetic modifications are cell type specific, it is critical to obtain a pure cell population for these studies. This could be challenging in sample collection, particularly for biomarker studies. For instance, although DNA from blood or PBMCs is easily accessible, it is often difficult to predict whether DNA methylation status in blood or PBMCs is reflective of methylation changes in other tissues.

With the advancement in medicine, the population is aging. Ironically, age is one of the strongest risk factors for many diseases, including cancer and cardiovascular diseases. There is therefore a great interest in identifying molecular targets as biomarkers that can accurately predict the risk of age-related diseases. In addition to telomere shortening and genomic instability, epigenetics has been shown to capture the biological age with great accuracy. Indeed, alterations in DNA methylation patterns are associated with age. This so-called "epigenetic clock," developed based on generating a regression model that was trained using the chronological age of the sample and a set of CpG dinucleotides, has been developed to estimate age in various tissues [92, 93]. This concept allowed detection of an accelerated biological aging process that deviates from the individual's actual chronological age. Accelerated DNA methylation age has been suggested in patients with hypertension, dementia, and cancer [94–96]; however, the role is less clear in rheumatic diseases. In osteoarthritis (OA) patients, accelerated DNA methylation aging of 3.7 years in articular cartilage was observed compared with controls [97]. However there were no differences in epigenetic age in bone and blood between OA patients and controls. The epigenetic clock in autoimmune diseases including SSc has not been examined and should be of interest in future studies. Since DNA methylation changes associated with age in naïve CD4+ T cells from healthy controls are associated with pathways related to the immune system and defective in autoimmune diseases such as lupus [98], it is possible that an accelerated DNA methylation age is observed in autoimmune diseases including SSc.

The advancement of next-generation sequencing-based approaches has demonstrated how the development and progression of SSc is affected by multiple alterations at the cellular and molecular levels. These high-throughput techniques are indeed powerful tools for identifying putative targets and highlighting the key processes involved in SSc. Indeed, genome-wide epigenetic profiling studies in SSc have gained momentum in recent years. The concept of utilizing "system biology perspective in drug discovery" by integrating multinomic, multi-platform, and multi-dimensional data sets will no doubt aid in uncovering the functional consequences of the epigenetic changes observed in SSc. This approach will also accelerate the identification of potential biomarkers and therapeutic targets for this disease, as well as advancement of "personalized epigenetic medicine."

Conclusions

In conclusion, this is an exciting time for epigenetic research in SSc. Undoubtedly, intensifying research efforts in this area will lead to the introduction of new drugs targeting novel epigenetic marks, identification of epigenetic biomarkers, as well as initiation of clinical trials for SSc patients in the near future.

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Compliance with Ethical Standards

Conflict of Interest The author declares that she has no potential conflicts of interest relevant to this article.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by the author.

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- • Of importance
- •• Of major importance

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