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Unfolding the Pathogenesis of Scleroderma through Genomics and Epigenomics

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Abstract

With unknown etiology, scleroderma (SSc) is a multifaceted disease that comprises of immune activation, vascular complications, and excessive fibrosis in internal organs. Genetic studies, including candidate gene association studies, genome-wide association studies, and whole-exome sequencing have supported the notion that while modest, SSc patients are genetically predisposed to this disease. The strongest genetic association for SSc lies within the MHC region, with loci in *HLA-DRB1*, *HLA-DQB1*, *HLA-DPB1*, and *HLA-DOA1* being the most replicated. The non-HLA genes associated with SSc are involved in various functions, with the most robust associations including genes for B and T cell activation and innate immunity. Other pathways include genes involved in extracellular matrix deposition, cytokines, and autophagy. Among these genes, *IRF5*, *STAT4*, and *CD247* were replicated most frequently while SNPs rs35677470 in *DNASE1L3*, rs5029939 in *TNFAIP3*, and rs7574685 in *STAT4* have the strongest associations with SSc. In addition to genetic predisposition, it became clear that environmental factors and epigenetic influences also contribute to the development of SSc. Epigenetics, which refers to studies that focus on heritable phenotypes resulting from changes in chromatin structure without affecting the DNA sequence, is one of the most rapidly expanding fields in biomedical research. Indeed extensive epigenetic changes have been described in SSc. Alteration in enzymes and mediators involved in DNA methylation and histone modification, as well as dysregulated miRNA levels all contribute to fibrosis, immune dysregulation, and impaired angiogenesis in this disease. Genes that were affected by epigenetic dysregulation include ones involved in autoimmunity, T cell function and regulation, TGFβ pathway, Wnt pathway, extracellular matrix, and transcription factors governing fibrosis and angiogenesis. In this review, we provide a comprehensive overview of the current findings of SSc genetic susceptibility, followed by an extensive description and a systematic review of epigenetic research that has been carried out to date in SSc. We also

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summarize the therapeutic potential of drugs that affect epigenetic mechanisms, and outline the future prospective of genomincs and epigenomics research in SSc.

Keywords

scleroderma; genetics; epigenetics; DNA methylation; histone modification; miRNAs; therapeutics

1. Introduction

Systemic sclerosis (scleroderma, SSc) is a multisystem disorder with unknown etiology. It is characterized by early vascular injury, immune activation, and consequently fibrosis in the skin and internal organs. Tissue fibrosis often leads to organ dysfunction and is regarded as the major cause of disease-associated morbidity and mortality [1]. Although the overall survival in SSc has improved significantly over the years [2], mostly due to earlier diagnosis and routine evaluation of organ involvement, the therapeutic options for SSc are mainly symptom- and organ-based [3]. The lack of an efficacious treatment for SSc is partly due to the complex nature of the disease and the insufficient knowledge of the cause of this disorder.

Although there is clearly a genetic component in SSc as having a family history of SSc' is one of the highest risk factors for the development of this disease [4], there is evidence that subjects with genetic predisposition to SSc also need additional environmental triggers for the disease (Figure 1). A twin study conducted by Feghali-Bostwick et al. showed that the concordance rate for SSc twins was low compared to other autoimmune diseases and no significant differences were observed between monozygotic and dizygotic twins [5]. Environmental factors, including exposure to silica, organic solvents, welding fumes, viruses, and drugs, have been implicated in the development of SSc [6]. The molecular mechanism of how these external factors trigger an autoimmune response is still not known. However it has been postulated that these factors induce cellular and tissue damage leading to loss of immune tolerance and affecting both innate and adaptive immunity [7]. In addition, these factors can potentially affect gene expression profiles and therefore the behavior of different cell types through epigenetic mechanisms, including DNA methylation, histone modifications, as well as microRNA (miRNA) regulation.

In this review, we will provide a comprehensive overview of recent developments in the involvement of genetics and epigenetics in SSc pathogenesis. The therapeutic implications as well as the recent advances in the tools for epigenomics research will also be discussed.

2. Genetic involvement

SSc is a complex disease in terms of genetic susceptibility. This is supported by the higher familial relative risk of the disease itself or autoimmune diseases [4, 8], low disease concordance but high concordance of autoantibodies produced in monozygotic twins [5], and multi-case SSc families that are concordant for SSc-specific autoantibodies as well as human leukocyte antigen (HLA) haplotypes [9]. Twin studies have been performed in several autoimmune diseases. These studies are useful to dissect whether a complex disease

is determined by genetic or environmental factors, especially for monozygotic twins who share identical genetic background. In autoimmune diseases, the concordance rate in monozygotic twins is far from 100% [10]; the concordance rates of monozygotic twin pairs in systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis, primary biliary cirrhosis, and SSc are 11.1% [11], 12.3% [12], 16.7% [13], 77% [14], and 4.2% [5], respectively. Among other autoimmune diseases, SSc has a lesser role for genetic susceptibility compared to primary biliary cirrhosis for example, which is highly genetically determined. However, monozygotic discordant twins were shown to be concordant for gene expression profiles in skin fibroblasts in SSc [15]. Nonetheless, during the last two decades, studies focusing on identifying genetic markers for SSc have expanded tremendously (Table 1). Many Major Histocompatibility Complex (MHC) and non-MHC genetic variations have been found to be associated with SSc susceptibility. In this section we will discuss the novel genetic associations in SSc identified by candidate gene studies, genome-wide association studies (GWAS), Immunochip, and most recently exosome sequencing approaches.

2.1. HLA genes

As seen in other autoimmune diseases, the strongest genetic association for SSc lies within the MHC region. This region, located on chromosome 6, is high in gene density and the HLA alleles are highly polymorphic. Several studies have associated HLA polymorphism with SSc susceptibility, which was confirmed by multiples studies including GWAS [16–20], pan-meta GWAS [21], and Immunochip studies [22]. This includes genes that belong to both HLA-Class I and HLA-Class II, with Class II genes associated with SSc more frequently than Class I (Table 1). Among these genes, *HLA-DRB1*, *HLA-DQB1*, *HLA-DPB1*, and *HLA-DOA1* were replicated in various studies [23].

The HLA genes or haplotypes identified in SSc genetic studies have been shown to be associated with autoantibody profiles, general susceptibility to SSc, disease subtypes, or certain SSc clinical features. Moreover, these HLA alleles not only determine SSc susceptibility, several studies also showed that some of them have a protective effect. It should also be noted that these associations can be common in different ethnicities, while others are specific to certain populations [24, 25]. The first GWAS in SSc reported that a SNP located in *HLA-DQB1* had the strongest association [18]. Later studies confirmed its role in SSc. In a recent study by Furukawa et al., *DQB1*03:01* was protectively associated with SSc in Japanese patients while *DQB1*05:01* and *DQB1*06:01* were associated with anti-centromere antibodies (ACA+) and anti-topoisomerase I antibodies (ATA+) positive SSc, respectively [26]. Similarly, in Mexican SSc patients *DQB1*03:01* was associated with protection from SSc while *DRB1*11:04* was shown to be associated with SSc susceptibility [27]. In contrast, *DQB1*03:01* was found to be associated with SSc risk in European-Americans,, Hispanics, and African-Americans [24]. In European-Americans, *DQB1*05:01* was associated with ACA+ and ATA+ SSc patients, while in African-Americans, *DQB1*03:01* was associated with ATA+ SSc patients and *DQB1*0501* was associated with ACA.

The Immunochip study published in 2014 provided a more comprehensive analysis of the HLA region and further dissected the association between the HLA region and SSc

serological subgroups (ACA+ or ATA+) [22]. The authors generated a model that included 6 polymorphic amino acid positions and 7 SNPs; all of which explained all observed associations in the HLA region, either with SSc or autoantibody profile. In addition, the models included all the known SSc-related HLA alleles. Looking closer, the ACA model included *HLA-DRB1* amino acid 13, *HLA-DQA1* amino acid 69, and SNPs rs12528892 and rs6933319. The ATA model is built with *HLA-DRB1* amino acids 67 and 86 and *HLA-DPB1* amino acids 76 and 96. The SSc model included both the ACA and ATA models as well as SNPs rs17500468, rs9277052, rs2442719, and rs4713605. Further examination of the identified amino acid residues revealed that some of them could affect the 3D structure of MHC or located in the binding pocket of MHC thus potentially affecting epitope binding, suggesting a functional relevance of the amino acid-associated positions. In addition, most of the SNPs were associated with *cis*-expression Quantitative Trait Loci (eQTLs) that are responsible for controlling various HLA genes.

2.2. Non-HLA genes

In addition to HLA genes, many variants in non-HLA loci are also associated with SSc (Table 1). Some of these genes were reported in only one study, while others have been replicated in multiple studies. These reported non-HLA genes associated with SSc are located on different chromosomes and are involved in various functions, with the most robust associations including genes for B and T cell activation and innate immunity. Other pathways include genes involved in extracellular matrix deposition, cytokines, and cell signaling.

2.2.1. Genes located in the MHC region—Two non-HLA associated genes were found in the MHC region but do not code for HLA proteins: *PSORS1C1* [16] and *NOTCH4* [17]. Little is known about *PSORS1C1*, however it has been associated with other autoimmune diseases, such as rheumatoid arthritis [28], lupus [29], and Behçet's disease [30]. NOTCH proteins are transmembrane receptors and are involved in controlling fibrosis and vascular function [31, 32], and may play functional roles in SSc pathogenesis. NOTCH4 polymorphism was associated with ACA+ or ATA+ in SSc [17]. A recent study by Cardinale et al. showed that a rare missense variant in the *NOTCH4* gene was identified in a family with 3 generation history of SSc using whole exome sequencing (WES) [33].

2.2.2. Genes located outside the MHC region—The non-HLA genes identified that are associated with SSc include *IRF* genes, *IL* genes, genes that encode the cluster of differentiation (CD) molecules, TNF related genes, B-cell related genes, genes encoding enzymes, and genes involved in apoptosis, autophagy, and fibrosis (Table 1). Among these genes, *IRF5*, *STAT4*, and *CD247* were replicated most frequently while SNPs rs35677470 in *DNASE1L3*, rs5029939 in *TNFAIP3*, and rs7574685 in *STAT4* have the strongest associations with SSc [17]. Some SNPs were shown to be associated with SSc but others were associated with disease subtypes or autoantibodies. In addition, different SNP association exists in different populations. For example, SNP rs2056626 for *CD247* was identified to be associated with SSc in European populations [16, 18, 34], but not in Han Chinese [35].

SSc is a complex highly heterogeneous disease, and the genes that have been identified so far that are associated with SSc encompass a wide variety of functions. The *IRF* genes are involved in innate immunity. Type I interferons (IFNs) are essential for the innate immune system and increased IFN signature has been observed in SSc [36]. Several IFN regulatory factor (*IRF*) genes were identified in genetic studies in SSc (Table 1); *IRF5* being the first identified [37], and *IRF4* being the newest member [38]. Several genes are involved in adaptive immune responses including B and T cell function and cytokine production. *BANK1* and *BLK* are B cell-related genes. *BANK1* encodes a signaling molecule involved in B cell mobilization, and *BLK* encodes a tyrosine kinase crucial for B cell development and signaling. The association of *BANK1* in SSc susceptibility was revealed by two independent candidate gene association studies, and later confirmed by whole-exom sequencing [39–41]. The association of *BLK* was established in both European and Japanese populations [21, 42–44].

Interestingly an additive effect for *BANK1* and *BLK* was reported [44]. Several T cell-associated genes have also been reported. *CD247* encodes the zeta chain of the T cell receptor, and plays critical roles for signaling events in T cells after antigen activation. *CSK* and *PTPN22* encode the C-Src tyrosine kinase and lymphoid tyrosine phosphatase (LYP), and both are involved in negatively controlling T cell activation. It has been reported that LYP directly interacts with CSK [45] and the *PTPN22* SNP disrupts the interaction between the two [46], suggesting a functional relevance of these genes in SSc pathogenesis. A number of tumor necrosis factor (TNF)-α-related genes were also reported to have genetic association with SSc. *TNFAIP3*, *TNIP1*, and *TNFSF4* encode for TNFα-induced protein (A20), TNFAIP3-interacting protein, and the T cell co-stimulatory molecule OX40 ligand (OX40L), respectively. Both A20 and TNIP1 are involved in negatively regulating the TNF-induced NF-κB signaling pathway, while OX40L controls B- and T-cell proliferation and survival. Genetic association studies also highlighted the involvement of several IL-12-related loci, including *IL12A* (IL-12 subunit), *IL12RB1* and *IL12RB2* (IL-12 receptor chains), *TYK2* (tyrosine kinase in the JAK-STAT family for IL-12 signaling), and *STAT4* (transcription factor for IL-12 signaling), suggesting the relevance of this signaling pathway in SSc pathogenesis. In addition to the IL-12 pathway, STAT4 is also involved in IFN-regulated signaling [47]. The association of *STAT4* in SSc susceptibility was reported and confirmed in multiple studies [16–18, 21, 34, 48–53]. In addition, an additive effect was reported for *STAT4* and *IRF5* [48]. Its functional role in SSc pathogenesis was suggested in *Stat4* knockout mice; *Stat4* deficient mice showed decreased production of cytokines and were protected from bleomycin-induced fibrosis [54].

In addition to genes involved in immunity and inflammation, several SSc-associated SNPs were found in genetic loci related to apoptosis, autophagy, and fibrosis. *DNASE1L3* encodes for a protein in the deoxyribonuclease I family that is responsible for DNA breakdown during apoptosis. Identified and replicated by two Immunochip studies, this gene is one of the genes that show the strongest non-HLA association for SSc [22, 52]. *ATG* is a gene involved in autophagy, an intracellular degradation system that maintains cellular homeostatic state by breaking down their own components or recycle nutrients [55]. The *ATG* SNP rs9373839 was reported in two pan-meta-GWAS studies and an Immunochip study [21, 22, 38]. *PPARG* encodes the peroxisome proliferator-activated receptor gamma

that is involved in adipogenesis and anti-fibrotic processes that showed functional impact in SSc [56]. Its role in SSc susceptibility was first described in a GWAS follow-up study and later reported in a candidate gene study [57, 58].

Both GWAS and Immunochip studies have advanced our understanding of SSc genetic susceptibility. However, these studies can only capture common variants. The contribution of rare variants in SSc susceptibility remains largely unknown. Whole-exome sequencing (WES) is a strategy to identify rare coding variants that may be relevant to disease risk and pathogenesis. The first WES study in SSc was performed by Gao et al. and reported a novel gene, *ATP8B4*, as a risk factor for the disease [59], however this association was not replicated in a follow up study [60]. In a recent study, Mak et al. identified 68 novel genes (both rare and common variants) that may contribute to diffuse SSc with or without interstitial lung disease using WES [41]. Pathway analysis revealed that the genes identified are enriched in the extracellular matrix pathway and the DNA repair pathway.

As we are starting to understand the genetic landscape of SSc, the functional relevance of these genetic variants and the impact of the environment are still lacking. One recent study investigated the interactions between genetic and environmental factors (silica particles) in SSc using a fibroblast model [61]. The authors reported that the SNP rs58905141 in *TNFAIP3* was strongly and persistently associated with silica particles-stimulated *MMP1* and *MMP3* expression in fibroblasts, suggesting a potential interaction of the SSc susceptibility locus in *TNFAIP3* and silica particles, which might be associated with the development of SSc through inflammatory mechanisms.

3. Epigenetic mechanisms

Epigenetics, which was first envisioned by Conrad Waddington in 1942 [62], refers to heritable changes that affect gene expression independent of altering the DNA sequence. These critical events regulate DNA packaging and chromatin structure that coordinate gene transcription during various physiological processes such as cell differentiation, survival, as well as in response to external stimuli or biological changes, and explains how cells function with a limited number of genes but capable of doing so much more. The epigenome is in a metastable state; it can be stable under normal cellular events, however when there are environmental or pathological challenges it can dynamically alter its landscape to respond. Because of this, epigenetic concepts have been applied to not only genetic research but also, to name a few, physiology, autoimmunity, psychology, and cancer. Indeed, epigenetic research has gained its momentum in the past decade and in 2016 alone there were more than 7800 articles in PubMed using “epigenetic” as the search key word. Epigenetic mechanisms include methylation of DNA, modification of histones, as well as regulation by non-coding RNAs. In this section we will briefly discuss the basics of these mechanisms.

3.1. DNA methylation

DNA methylation is accomplished by addition of a methyl group from S-adenosylmethionine (SAM) to the C5 position of cytosine nucleotides, with majority of it occurring at cytosine-phosphate-guanine dinucleotides (CpG). CpG-rich sequences, called CpG islands, are mainly located within the promoter regions of many genes. When

methylation occurs, it generates a more condensed DNA configuration thereby blocking the accessibility of transcription factors while attracting the methyl-CpG-binding domain proteins (MBDs), resulting in repression of gene transcription. In contrast, lower methylation at the promoter region leads to increase in transcription activity. Methylation is catalyzed by a family of DNA methyltransferases (DNMTs) that consists of 3 members: DNMT1, DNMT3A, and DNMT3B. These members have their unique sequences, expression patterns, as well as regulatory mechanisms [63]. DNMT1 is considered a maintenance methyltransferase as it is involved in sustaining the existing methylation pattern during cell division and DNA replication. DNMT3A and 3B are responsible for *de novo* methylation, mainly during embryonic development.

As all epigenetic mechanisms are dynamically controlled, DNA methylation can be reversible. Passive demethylation occurs during cell division, where methyl groups are lost during the DNA replication process [64]. Active demethylation is mediated through enzyme catalysis. Methyl cytosines are modified sequentially through hydroxylation, deamination, and/or oxidation, followed by DNA repair mechanisms [65]. The enzymes capable of active demethylation include the ten-eleven translocation (TET), the activation-induced cytodine deaminase/apolipoprotein B mRNA editing enzyme component 1 (AID/APOBEC), and the base excision repair (BER) [64]. The oxidized form of 5-methylcytosine (5-mC), the 5-hydroxymethylcytosine (5-hmC), is often viewed as an indicator for active demethylation.

As described above, MBDs will bind to methylated CpG sites to act as transcriptional repressors. They play crucial roles in coordinating the interaction between DNA methylation, histone modifications, and chromatin structure. The MBD family includes eleven members: the methyl-CpG-binding protein (MeCP2) was the first that was discovered, in addition to MBD1-6, and SETDB1, SETDB2, BAZ2A, and BAZ2B [66–68]. Among these, only MeCP2 and MBD1-4 can bind to 5-mC, and therefore are considered core proteins in the MBD family. Although traditionally viewed as transcriptional repressors, growing evidence is supporting the notion that these proteins can also act as transcriptional activators [69].

3.2. Histone modification

While DNA is primarily methylated, histone proteins can undergo a wide array of post-translational modifications, and this process represents another major epigenetic regulatory mechanism. Histones are highly conserved proteins that form the nucleosome cores, and together with 146 base pairs of DNA that wraps around it form the chromatin. Each nucleosome constitutes two copies of the core histones (H2A, H2B, H3, and H4) as well as linker histones (H2 and H5). The nucleosomes rearrange their structures frequently through DNA unwrapping and rewrapping and histone core disassembly and assembly. Gene expression depends largely on the interaction between the histones and their surrounding DNA: an open chromatin configuration is associated with transcriptional activation, while a closed chromatin conformation is connected with transcriptional repression. Histone modifications, which occur at specific amino acid residues in the side chains of histones, affect such interaction, thereby changing the chromatin structure and influencing its accessibility. These post-translational modifications include acetylation, methylation,

phosphorylation, ubiquitination, sumoylation, and ADP ribosylation. Among these “histone codes”, histone acetylation and methylation are best characterized.

Histone acetylation typically occurs at lysine residues on H3 or H4, and can present as mono-, di-, or tri-acetylation. This modification eliminates the positive charges on the histone side chains thus reducing the interaction between the histones and the negatively charged DNA, resulting in an open chromatin conformation with gene promoter regions more accessible to transcription factors, and ultimately increased gene transcription. Histone acetylation is supported by histone acetyltransferases (HATs), including P300/CBP, PCAF, and MYST, by transferring an acetyl group from acetyl coenzyme A to the NH3+ groups on lysine. On the other hand, histone deacetylation is catalyzed by a series of histone deacetylases (HDACs), including class 1, 2, and 4 HDACs, as well as sirtuins (SIRT1-7, also known as class 3 HDACs). As mentioned above, histone acetylation, which is promoted by HATs, leads to increase in gene expression through transcriptional activation. Therefore specific acetylated histone lysine residues can encode specific transcriptional functions. For example, acetylation of lysine 27 of H3 (H3K27ac) is considered an active enhancer mark and associated with transcriptional activation.

Histone methylation is another form of histone post-translational modification that occurs frequently. It can either increase or decrease gene transcription depending on the number of methyl groups that are added and the location of the amino acid being methylated. For instance, trimethylation of histone H3 at lysine 27 (H3K27me3) is repressive while H3k4me3 is an active mark for transcription. Histone methyltransferases (HMTs) and histone demethylases (HDMs) control the methylation status of histones [70]. There are 3 classes of HMTs: SET domain lysine methyltransferase, non-SET domain lysine methyltransferase, and arginine methyltransferase [71]. As for HDMs, they are classified based on their demethylase domains: LSD1 domain and JmjC domain [70]. Each lysine or arginine can be modified by more than one HMT or HDM, and each HMT or HDM can work on different lysines or argninies. For instance, H3K27 can be methylated by EZH2 and demethylated by UTX or JMJD3, while EZH2 can also methylate H1K26, and JMJD3 can demethylate H3K4.

As mentioned above, histone modification patterns are regulated by enzymes that add and remove groups on different amino acid residues. Enzymes that add groups on histones (e.g. HATs and HMTs) are referred to as “writers” of the histone code, and ones that remove groups (e.g. HDACs and HDMs) are called “erasers”. In addition to directly affecting the histone-DNA or histone-histone interactions by these writers and erasers, histone modifications can also be targeted by “readers” or protein effectors to control gene transcription [72]. Although these histone readers have diverse structural differences, they contain one or more conserved domains that are capable of binding to specific histone modifications [73]. Several protein modular domains have been recognized: bromodomains bind to acetyl-lysines, PHD binds methyl-lysines, and “Royal Family” of reader modules including Tudor, chromo, PWWP, and MBT domains recognize methylated lysine or methylated arginine residues [74]. The interaction between readers and histone modifications are dynamic. Several readers can recognize one histone modification (e.g. H3K4me3 has 8 readers), while a single domain from a reader can recognize multiple

histone modifications (e.g. EED can read H3K9me3, H3K27me3, and H1K26me3 [74]). In addition, histone marks that possess opposite reactions can share the same binding motif [73], adding to the complexity of these proteins.

3.3. Non-coding RNAs

Non-coding RNAs (ncRNAs) lack the potential to encode proteins, but they are able to affect gene expression through a variety of mechanisms. This family of RNA is generally classified into 3 groups based on size: small ncRNAs (<50 nucleotides [nt]) that include microRNA (miRNA) and PIWI-interacting RNA (piRNA), medium-sized ncRNAs (<200 nt) such as small nucleolar RNA (snoRNA) and promoter-associated small RNAs (PASRs), and long ncRNAs (>200 nt) (lncRNAs) [75]. Among these non-coding RNAs, miRNAs and lncRNAs are the most studied in SSc pathogenesis.

miRNAs are approximately 22 nt in length and are synthesized in a step-wise fashion. Primary miRNAs which are longer transcripts are first transcribed from the non-coding regions of the genome [76]. They are processed by RNase III enzymes including Drosha and Dicer into mature double-stranded miRNAs. Each strand of the complimentary miRNA is subsequently incorporated in the RNA-induced silencing complex (RISC) to promote degradation of mRNA. This tightly regulated process allows the miRNAs to repress gene transcription through mRNA degradation or repression of mRNA translation.

lncRNAs are a group of heterogeneous non-coding transcripts that are more than 200 nt long, and believed to make up the largest portion of the non-coding transcriptome [77]. In addition to RNA silencing, lncRNAs are also involved in chromatin remodeling, transcriptional activation, post-transcriptional regulation, as well as DNA methylation [78].

It should be noted that the three epigenetic mechanisms mentioned above, DNA methylation, RNA-mediated silencing, or histone modifications, can act in concert to control chromatin state and gene transcription, and are therefore crucial for governing genomic functions.

4. Epigenetic impact in SSc pathogenesis

Scleroderma is considered an autoimmune disease that is characterized by three major self-amplifying events: microvascular alterations, immune activation, and fibrosis in multiple organs (Figure 2). The prominent cell types that are involved in this complex process include microvascular endothelial cells (ECs), immune cells including lymphocytes and macrophages, as well as fibroblasts. Microvascular injury triggering endothelial activation marks the initiation of the disease process, resulting in reduction of capillaries and increased smooth muscle cell proliferation. Endothelial damage allows increased infiltration of inflammatory cells, which release pro-inflammatory and pro-fibrotic mediators such as transforming growth factor β (TGFβ) and interleukin 6 (IL-6). In addition to blood vessel extravasation, circulating immune cells are found to be altered in number and function, such as preferred Th2 and M2 polarization, as well as distinct serum autoantibodies present in patients. On the other hand, immersed in the pro-fibrotic milieu, resident fibroblasts are activated and are subsequently transformed into α-smooth muscle actin (αSMA) positive

myofibroblasts, which produce excessive extracellular matrix (ECM) proteins such as collagen.

As mentioned above, the pathogenesis of SSc cannot be explained by genetics alone. Growing evidence is now suggesting that epigenetics contributes to a variety of disease-associated phenotypes, such as age of onset, disease severity, and response to medication. In this section, the recent advances regarding epigenetic research in SSc (as summarized in Figure 2) will be discussed in detail in the context of the various cell types involved in this disease.

4.1. DNA methylation

DNA methylation has been implicated to play a role in SSc. The DNA methylation status in various cell types involved in the triad of SSc pathogenesis, as well as the role of enzymes and MBDs, were documented (Table 2). It appears that global hypomethylation is common among various cell types (except for ECs, which have not been examined). In addition, a fair amount of differentially methylated genes has been reported (Table 3).

4.1.1 Immune system—Regulation of immune cell-specific transcription factors by DNA methylation has been implicated in SSc pathogenesis. One of the early studies showed a significant reduction in DNMT1, MBD3, and MBD4, leading to a global reduction of DNA methylation in CD4+ T cells from patients with SSc [79]. A later study revealed that the overexpression of CD40L in CD4+ T cells isolated from female SSc patients was due to DNA hypomethylation in the promoter and enhancer regions of CD40L [80]. Similarly, the highly expressed CD70 and CD11a in SSc CD4+ T cells was also due to demethylation of its promoter region [81, 82]. In the case of CD11a, the authors added mechanistic studies and reported that SSc CD4+ T cells and DNMT inhibitor-treated CD4+ T cells, both with CD11a overexpression, showed increased proliferation, influenced B cells to produce increased levels of IgG and normal fibroblasts to synthesize more collagen. Therefore, the functional consequence of CD11a overexpression may contribute to immune dysfunction and fibrotic processes in SSc patients. In contrast to the genes mentioned above, DNA hypermethylation was observed in the promoter region of forkhead fox protein 3 (FOXP3) in CD4+ T cells in patients with SSc, leading to a reduction in FOXP3 expression in these cells, and consequently lower numbers of Tregs [83]. Treating SSc CD4+ T cells with a DNMT inhibitor not only reduced DNA methylation levels, but also increased FOXP3 expression and number of Tregs. These results contradicted the recent study reported by Almanzar et al, where they showed increased FOXP3 expression and the number of Tregs in SSc patients, with no significant change in DNA methylation in FOXP3 regulatory regions in peripheral blood mononuclear cells (PBMCs) [84]. They reported that Th17-specific transcription factors, RORC1 and 2, were hypermethylated in PBMCs. The DNA methylation results obtained from whole blood [85] was similar to what was published in CD4+ T cells: FOXP3 and CD70 were hypomethylated, however CD11a was hypermethylated instead, pointing to the importance of examining epigenetic changes in individual cell types. These studies suggest an epigenetic impact on immune dysregulation in SSc.

In addition to immune regulation, DNA methylation is also involved in perturbation of X chromosome inactivation therefore the predominance of females among SSc patients. Selmi et al. immunoprecipitated methylated DNA from PBMCs from monozygotic twins discordant for SSc and hybridized them to a DNA methylation array that included promoters of all X chromosome genes [86]. They found 25 X chromosome sites that were consistently hypermethylated and 18 that were hypomethylated in affected twins. Genes that were affected include transcription factors, surface antigens, and genes related to cell proliferation, apoptosis, inflammation, as well as oxidative stress. These results, together with the study by Lian et al. that showed demethylation of the CD40L regulatory regions on the inactive X chromosome in CD4+ T cells from female SSc patients [80], support the notion that epigenetic mechanisms are indeed contributing to the gender bias in SSc.

4.1.2 Vascular dysfunction—The one report in DNA methylation in SSc ECs involves the bone morphogenetic protein type II receptor (BMPRII) [87]. Bone morphogenetic proteins belong to the TGFβ superfamily and signal through both canonical and non-canonical pathways to mediate their effects in cell proliferation and development [88]. BMPRII is among the receptors that are involved in the canonical signaling cascade for its substrate. In SSc ECs, BMPRII expression was significantly lower than healthy ECs due to hypermethylation at its promoter region [87]. Reduced BMPRII levels might contribute to the apoptotic nature of SSc ECs, as knocking down BMPRII in healthy ECs rendered these cells more apoptotic.

4.1.3 Fibrosis—In one of the pioneer studies of epigenetics in SSc fibroblasts, Wang et al. showed that a number of epigenetic mediators were upregulated in SSc fibroblasts compared to normal fibroblasts, which was confirmed by subsequent studies (Table 2) [89–91]. This observation prompted them to examine whether epigenetics plays a role in SSc fibrosis [89]. They showed that addition of the DNMT inhibitor 2-deoxy-5-azacytidine (5-aza) and HDAC inhibitor trichostatin A (TSA) decreased collagen content in SSc fibroblasts, possibly through normalizing the hypermethylated and hypoacetylated promoter region of the collagen suppressor gene Friend leukemia integration 1 (FLI1), hence increasing Fli-1 expression. The impact of DNA methylation on FLI1 expression was further confirmed by Bujor et al. [92]. They showed that the antifibrotic effect of ciprofloxacin, a broad spectrum antibiotic, in SSc dermal fibroblasts and SSc-interstitial lung disease lung fibroblasts stemmed from downregulation of DNMT1 and upregulation of FLI1. It was later shown that Kruppel-like factor 5 (KLF5), similar to FLI1, was epigenetically suppressed in SSc fibroblasts by hypermethylation at its promoter region and H3/H4 hypoacetylation [93]. The deficiency of the two transcription factors in animals led to enhanced fibrosis, vascular complications in the skin and lung, as well as immune dysfunction, suggesting crucial roles for FLI1 and KLF5 in SSc pathogenesis.

The canonical Wnt signaling pathway is involved in fibrosis and requires a tight control for Wnt proteins and their endogenous antagonists, so that β-catenin can ultimately accumulate in the cytoplasm and translocate into the nucleus to increase transcription. In SSc aberrant signaling and activation of the Wnt pathway has been reported [94, 95]. Dees et al. further investigated the impact of DNA methylation of two Wnt antagonists, DKK1 and SERP1 in

SSc fibrosis [96]. Hypermethylation at the promoter regions of DKK1 and SERP1 led to decreased expression of the two in SSc fibroblasts, and treating the cells with a DNMT inhibitor upregulated their expression. In the bleomycin mouse model, the DNMT inhibitor reactivated DKK1 and SERP1 that lead to inhibition of the Wnt pathway, thereby alleviated skin fibrosis.

Recent advance in technology allows examination of DNA methylation at a genome-wide level. Altorok et al. performed a genome-wide DNA methylation analysis in SSc patients with diffuse or limited disease, as well as age-, sex-, and ethnicity- matched healthy controls using the Illumina Infinium Human Methylation 450 BeadChip array, which covers over 485,000 CpG sites in the entire genome [97]. A total of 2710 and 1021 differentially methylated CpG sites were identified in diffuse SSc and limited SSc, respectively, with a large proportion of them being hypomethylated (61% in diffuse and 90% in limited), agreeing with previous studies that showed global hypomethylation in various cell types, including fibroblasts, in SSc [79, 85, 98]. This unbiased approach identified genes that were affected by DNA methylation in SSc including ones that encode collagen, ECM, transcription factors, as well as genes that are involved in the TGFβ pathway and Wnt pathway (listed in Table 3), echoing and expanding the results from hypothesis-driven studies mentioned earlier. There were drastic differences in DNA methylation patterns in the two subsets of patients, with only 6% of differentially methylated CpG sites common between diffuse and limited SSc. This study not only identified novel genes that are implicated in SSc pathogenesis, but it also revealed how the two different disease subsets are epigenetically distinct.

In addition to dermal fibroblasts, one study also examined the role of DNA methylation using DNA methylation arrays in lung fibroblasts from SSc patients. Evans et al. observed that the expression of anti-fibrotic cyclooxygenease-2 (COX2) and its product prostaglandin E2 (PGE2) were lower in SSc lungs [99]. The ability of the DNMT inhibitor in normalizing the fibroblast phenotype suggested that DNA methylation was involved. Since differential methylation of COX2 was not observed in SSc fibroblasts compared with controls, an Illumina Infinium Human Methylation 450 array was used to identify genes with altered methylation status in patient fibroblasts compared to healthy controls. Chromosome 8 open reading frame 4 (c8or4) was identified as a hypermethylated and downregulated locus/gene in SSc fibroblasts. Further functional studies confirmed that c8or4 regulated COX2 expression, serving as an indirect epigenetic regulator for the COX2 pathway.

As active demethylation had been recently discovered, the expression of TET and 5-hmC was characterized in SSc fibroblasts [98]. Upregulation of TET1 was observed, along with increased 5-hmc staining in SSc skin. However in another study, TET1 expression remained unchanged in SSc fibroblasts, suggesting more work needs to be done to elucidate the mediators for DNA demethylation [91].

4.2. Histone modification

Various studies have focused on the effect of histone modification in SSc pathogenesis. The aberrant expression of histone writers and erasers, along with histone codes in difference cell types were summarized in Table 2. While documentation of the changes in the histone codes

(mainly histone acetylation and methylation) and histone modifying enzymes were analyzed in immune cells, ECs, and fibroblasts, mechanistic studies were solely performed in fibrosis, with two recent reports in ECs. The detailed description of these studies and their implications will be discussed in this section, and the genes that are affected by histone modifications in SSc are summarized in Table 4.

4.2.1. Immune system—A comprehensive analysis examining the global histone acetylation/methylation status as well as enzymes involved was carried out in B cells isolated from controls and SSc patients [100]. Global H4 hyperacetylation and H3K9 hypomethylation in SSc B cells were reported, associated with expression changes in JHDM2A, HDAC2, HDAC7, and SUV39H2. In addition, global H4 acetylation was positively correlated with disease activity while HDAC2 expression was negatively correlated with skin thickness, suggesting that these histone alterations might be pathogenic in SSc.

The HDM JMJD3 was overexpressed in CD4+ T cells from SSc patients, contributing to lower levels of H3K27me3 in these cells, since the HMTs EZH1 and EZH2, as well as HDM UTX that also affects H3K27me3 were not altered [101]. As mentioned above, these cells showed increased levels of CD40L, CD70, and CD11a due to demethylation at the promoter regions of these genes. The overexpressed JMJD3 hence lower H3K27me3 levels might work in concert with DNA methylation to control these genes.

4.2.2. Vascular dysfunction—HDACs control the acetylation state of histones and are also involved in angiogenesis [102]. We reported an increase in HDAC4 and 5 expression, and a reduction in HDAC6 expression in SSc ECs compared to normal ECs [103]. We then focused on the anti-angiogenic HDAC5, and showed that when HDAC5 was knocked down in SSc ECs, the ability of these cells to form tube-like structures on Matirgel was restored. To determine the mechanism, an assay for transposase-accessible chromatin using sequencing (ATAC-seq) was performed to assess how HDAC5 knockdown altered chromatin accessibility at a genome-wide level in SSc ECs. A total of 75 genes were located in regions with increase accessibility following HDAC5 knockdown. Using bioinformatics analysis, we narrowed the genes down to 25 genes that are involved in angiogenesis and/or fibrosis. Among the 8 genes that were also significantly upregulated after HDAC5 knockdown in SSc ECs, three genes, FSTL1, CYR61, and PVRL2, appeared to play functional roles in SSc EC angiogenesis. Overexpression of these genes individually led to increase in tube formation in SSc ECs, and knockdown of these genes individually together with HDAC5 resulted in decrease in tube-like structures.

The HMT EZH2 is the catalytic component of a multiprotein complex, polycomb repressive complex 2 (PRC2) which is involved in chromatin compaction and gene repression. It catalyzes H3K27me3. Transcriptomic studies suggest a relatively high expression of the EZH2 in ECs [104], which regulates the expression of multiple angiogenic pathways [105, 106]. We showed that EZH2 was upregulated at the protein level in SSc ECs compared to normal ECs, accompanied with increased levels of H3K27me3 [107]. The effect of EZH2 in ECs isolated from normal subjects was examined and we showed that overexpression of EZH2 (18-fold increase in EZH2 mRNA) inhibited tube formation, while knockdown of

EZH2 (88% decrease in EZH2 mRNA) resulted in increase in tubes. Since EZH2 was overexpressed in SSc ECs, knocking down EZH2 in these cells led to increased tube formation. These data suggest that EZH2, possibly by repressing pro-angiogenic genes or activating anti-angiogenic genes, inhibits angiogenesis in SSc ECs.

4.2.3. Fibrosis—The studies that examined the impact of histone modifications in SSc fibrosis focused on (1) transcription factors (FLI1, KLF5, and Fos-related antigen 2 [FRA2]); (2) the TGFβ pathway; (3) the Wnt pathway.

In addition to DNA methylation as mentioned earlier, H3 and H4 hypoacetylation at the promoter region of the collagen suppressor FLI1 also led to increased collagen synthesis in SSc fibroblasts, and treating the cells with both DNMT inhibitor 5-aza and HDAC inhibitor TSA led to decrease in collagen levels [89]. A later study confirmed the beneficial effect of TSA in SSc dermal fibroblasts and animal model of bleomycin-induced skin fibrosis [108, 109]. In addition, the authors further showed that silencing HDAC7 in SSc fibroblasts was as effective as TSA in normalizing collagen content in these cells, however, silencing HDAC7 was more specific as TSA altered the expression of connective tissue growth factor (CTGF), and intercellular adhesion molecule 1 (ICAM-1) while HDAC7 knockdown did not. Similar to FLI1, KLF5 was also epigenetically controlled by both DNA methylation and H3/4 hypoacetylation in SSc fibroblasts [93]. Both downregulated in SSc, KLF5 and FLI1 appeared to be crucial in triggering the three major manifestations in SSc (fibrosis, vascular/EC dysfunction, and autoimmunity). In contrast to the aforementioned anti-fibrotic transcription factors, FRA2, a profibrotic transcription factor, was epigenetically controlled by EZH2 [110]. Inhibition of EZH2 by 3-deazaneplanocin (DZNep) resulted in increase in collagen production in SSc dermal fibroblasts as well as exacerbation of fibrosis in animal models of SSc [110]. This is believed to be mediated through induction of Fra2. However, we took a similar approach and found that DZNep instead possesses anti-fibrotic properties [107]. In our hands, overexpression of EZH2 was accompanied with elevated H3K27me3 levels, and SSc dermal fibroblasts treated with DZNep showed significant reduction in EZH2, collagen (COL), FRA2, and TGFβ. In addition, DZNep affected the DNA methylome in these cells, likely through its inhibitor effect on DNMT1, 3A, and MeCP2 [107]. To examine whether EZH2 played a role in cell migration, a scratch wound assay was performed. SSc fibroblasts treated with DZNep showed a delay in wound closure at 48 hrs compared to the PBS treated cells. This suggests that EZH2 inhibition might reduce the invasiveness of SSc fibroblasts. These inhibition studies were supported by EZH2 overexpression in normal dermal fibroblasts: overexpression of EZH2 in normal fibroblasts resulted in a pro-fibrotic phenotype, mimicking what was seen in SSc fibroblasts, and overexpression of EZH2 in normal fibroblasts led to increase in wound healing. The reasons for this apparent discrepancies between the two studies is not known. The DZNep doses used were similar in both *in vitro* studies, however Kramer et al. did not measure the expression of EZH2 therefore whether DZNep was effective in their study is not known [110].

Studies have shown that HAT p300 regulates fibrosis through myofibroblast transformation and ECM homeostasis acting as a transcriptional activator [111]. In SSc, p300 was upregulated in skin and fibroblasts, and is involved in TGFβ-mediated fibrosis [112, 113].

TGFβ induced p300 expression through early growth response 1 (egr-1) in dermal fibroblasts. Increased p300 in these TGFβ induced cells was associated with p300 recruitment and histone H4 acetylation at the COL1A2 locus, resulting in increased collagen expression. These results make p300 a desirable treatment target for TGFβ-mediated fibrosis.

In addition to HATs, HDACs have also been implicated in the TGFβ pathway. The role of class III HDAC sirtuin 1 (SIRT1) in SSc fibrosis is controversial, as two studies reported opposite results [114, 115]. Both studies showed downregulation of SIRT1 in SSc skin. However, Wei et al. suggested that SIRT1 mediated its potent anti-fibrotic effect through blockade of TGFβ-Smad-dependent transcription, in part by suppressing p300 [114]. The pro-fibrotic effect of TGFβ was ameliorated by the SIRT1 activator resveratrol, while knockdown or inhibition of SIRT1 showed pro-fibrotic effects. In the bleomycin skin fibrosis model, SIRT1 was also downregulated, and the extent of fibrosis and SIRT1 expression could be normalized with resveratrol. In contrast, Zerr et al. suggested that TGFβ downregulated SIRT1 and activation of SIRT1 with resveratrol enhanced the profibrotic effect of TGFβ while knockdown of SIRT1 inhibited TGFβ/Smad signaling and collagen synthesis in fibroblasts [115]. Consistent with the *in vitro* study, mouse models with fibroblast-specific depletion of SIRT1 showed less fibrosis compared to control mice. The reason for the discrepancies between the two studies is not known. Although resveratrol has been used as SIRT1 activator, several studies have questioned its selectivity towards SIRT1 and its effect could be dose-dependent [116, 117]. Indeed, the doses of resveratrol were different in the two studies, this might at least in part, explain the disagreement of the results.

The anti-fibrotic nuclear receptor NR4A1 was shown to be elevated in SSc skin [118]. Under normal wound healing conditions, TGFβ induced NR4A1 expression to halt fibroblast activation. In contrast, fibrotic conditions with persistent TGFβ activation inhibited the negative feedback mechanism by AKT- and HDAC-mediated NR4A1 repression and inactivation. This warranted the use of NR4A1 agonist cytosporone-B (Csn-B) in fibrosis animal models; this reagent alleviated tissue fibrosis through rebalancing TGFβ signaling.

As mentioned earlier, the Wnt/β-catenin pathway has been implicated in SSc fibrosis [94, 95]. Wnt inhibitor factor 1 (WIF1), which was repressed in SSc fibroblasts, disrupts Wnt signaling by binding directly to Wnt ligands [119]. The reduction of WIF1 expression was due to oxidative DNA damage-mediated histone deacetylation, resulting in Wnt pathway activation, and ultimately enhanced fibrosis in SSc. The authors showed that this complex process involved the DNA damage checkpoint kinase ataxia telangiectasia mutated (ATM) and activation of transcription factors as well as signaling molecules.

4.3. Non-coding RNA

Since the first study in miRNA revealed that the downregulation of anti-fibrotic miR-29a in SSc fibroblasts contributes to SSc pathogenesis [120], numerous studies have focused on non-coding RNAs in different cell types and tissues affected by this disease (summarized in Table 5). The aberrant expression of pro-fibrotic and anti-fibrotic non-coding RNAs in SSc likely plays critical roles in the disease. This section encompasses the current understanding

of the cellular and molecular mechanisms of miRNAs reported in SSc, with specific focus on the target genes of these non-coding RNAs, as well as the biological processes that are associated with them.

4.3.1. Immune activation—Surprisingly, none of the studies published as of March 2017 focused on non-coding RNAs in immune cells in SSc. Instead there are 3 studies that focused on how miRNAs were involved in immune mediators' effect on dermal fibroblasts [91, 121, 122]. Nakashima et al. showed that IL-17A exert its' anti-fibrotic effect through upregulation of miR-129-5p that downregulated COL1A1 [122]. However, this inhibitory pathway was suppressed by TGFβ in SSc dermal fibroblasts, amplifying the pro-fibrotic signals in these cells. In contrast to the anti-fibrotic effect of IL-17A, IL-13 promoted collagen deposition in SSc dermal fibroblasts via STAT6 [91]. miR-135b, which was downregulated in SSc dermal fibroblasts, modulated STAT6 expression. Therefore, the pro-fibrotic effect of IL-13 in SSc was enhanced by downregulation of miR-135b and upregulation of STAT6. Interestingly miR-135b was repressed by methylation in SSc fibroblasts as inhibition of DNMT1 significantly induced miR-135b expression. This is a great example of how epigenetic mechanisms work together. In another study, the effect of miR-30a-3p on BAFF (B cell-activating factor) in SSc dermal fibroblasts was examined [121]. Stimulation with IFNγ or Poly(I:C) led to concomitant BAFF upregulation and miR-30a-3p downregulation in fibroblasts. After confirming that miR-30a-3p directly modulated IFNγ or Poly(I:C)-mediated BAFF expression, the authors showed that miR-30a-3p decreased BAFF secretion from dermal fibroblasts that resulted in lower B cells survival, indicating that fibroblasts can modulate autoimmune responses through miRNAs in SSc.

4.3.2. Vascular dysfunction—The only study that focused on the effect of miRNAs on vasculopathy in SSc was published by Iwamoto et al. [123]. The authors showed that miR-193b was significantly downregulated in SSc fibroblasts and skin, and that manipulation of miR-193b in SSc dermal fibroblasts altered urokinase-type plasminogen activator (uPA) expression, confirming that uPA is indeed targeted by miR-193b. The impaired uPA and uPA receptor (uPAR) pathway has been shown to be involved in EC dysregulated angiogenesis in SSc [124, 125], and mice null of uPAR showed enhanced fibrosis and increased EC apoptosis [126], supporting a role of this pathway in SSc pathogenesis. The authors showed that uPA not only induced vascular smooth muscle cell proliferation but also inhibited cell apoptosis. Downregulation of miR-193b led to increased uPA production, which could contribute to proliferative vasculopathy in SSc.

4.3.3. Fibrosis—As the TGFβ pathway is considered the master regulator for fibrosis, the majority of the non-coding RNA studies in SSc fibrosis evolved around it, either focusing on activation of this pathway, downstream signaling molecules involved such as Smads, or TGFβ-targeted genes.

Integrins, which are regulated by miRNAs, can activate the latent form of TGFβ. Honda et al. performed a miRNA PCR array to measure miRNAs in normal and SSc fibroblasts. Among the miRNAs that were significantly altered, the authors chose to focus on miR-150 since its putative target was integrin β3, implicating its role in TGFβ activation [127]. In SSc

skin and dermal fibroblasts, miR-150 was downregulated. However, overexpression of miR-150 in SSc fibroblasts resulted in reduction of the upregulated intergrin β3, phosphorylated Smad3, as well as type I collagen. DNA methylation appears to be involved in the downregulation of miR-150 in SSc, as treating the fibroblasts with a DNMT inhibitor increased miR-150 expression.

In terms of TGFβ signaling, miR-21 appeared to exert its pro-fibrotic effect via Smad7 in SSc dermal fibroblasts [128, 129]. MiR-21 could be induced by TGFβ and in turn downregulates Smad7, which inhibits the pro-fibrotic signal of TGFβ. Therefore, miR-21 can be viewed as an enhancer that amplifies the effect of TGFβ in SSc fibrosis. In contrast, downregulation of miR-145 was observed in SSc fibroblasts, while its predicted target Smad3 was upregulated [128]. It is possible that miR-145 substantiates TGFβ signaling through Smad3, however more mechanistic studies need to be performed to confirm this link. Similarly, miR-206, let-7g, miR-125b, miR-140-5p, and miR-23b were found to be differentially expressed in SSc dermal fibroblasts and their predicted targets include TGFβ, TGFβ receptor and SMAD5, however whether these miRNAs play functional roles in promoting fibrosis needs to be further validated [130].

Several non-coding RNAs were found to mediate their anti-fibrotic or pro-fibrotic effect through TGFβ-targeted genes. MiR-29a, which is a classical anti-fibrotic miRNA that has been extensively studied, was found to be downregulated in SSc skin and fibroblasts when compared to healthy controls [120]. Overexpression of this miRNA led to decreased type I and type III collagen in SSc dermal fibroblasts, and its expression can be modulated by TGFβ, platelet-derived growth factor (PDGF), or IL-4. Blockade of the TGFβ and PDGF pathways by imatinib significantly restored miR-29a expression both *in vitro* and *in vivo*. In a separate study, miR-29a appeared to prevent fibrosis via direct repression of TGFβ activated kinase 1 binding protein 1 (TAB1), which led to decreased tissue inhibitor of metalloproteinase 1 (TIMP1) and increased functional metalloproteinase 1 (MMP1) secretion to break down collagen [131]. In addition, miR-29a promoted apoptosis in SSc fibroblast via altering the expression of Bcl-2 family proteins which governs a cell fate decision whether to enter apoptosis or not [123]. All of these studies support miR-29a's anti-fibrotic role in SSc. In addition to miR-29a, TGFβ also negatively modulated miR-196a and let-7a expression in fibroblasts and affects the expression of their target type I collagen [132, 133]. In contrast, TGFβ-mediated overexpression of lncRNA TSIX and miR-202-3p stabilized collagen mRNA and downregulated MMP1 expression, contributing to their pro-fibrotic properties in SSc dermal fibroblasts [134, 135]. Discoidin domain receptors (DDRs) regulate ECM remodeling and DDR2 negatively regulates miR-196a to control collagen synthesis [136]. In SSc fibroblasts, TGFβ lowered both DDR2 and mir-196a expression leading to excessive collagen accumulation.

In addition to TGFβ, a few studies explored the involvement of miRNAs in other pathways. MiR-155 enhanced the signaling of Wnt/β-catenin and Akt pathways in SSc fibroblasts by directly targeting casein kinase 1α (CK1α) and src homology 2-containinginositol phosphatase-1 (SHIP1) [137]. Mice null of miR-155 or ones treated with miR-155 antagonist showed reduced skin fibrosis accompanied with inhibition of Wnt/β-catenin and Akt pathways. MiR-130b appeared to exert its pro-fibrotic role in SSc by negatively

regulating peroxisome proliferator-activated receptor γ (PPARγ) thereby enhancing TGFβ signaling [138]. Activated Toll-like receptor 4 (TLR4) signaling downregulates miR-29 while upregulating Smad2/3, ultimately contributing to SSc fibrosis [139]. Thrombospondin-2 (TSP-2) is known to regulate ECM synthesis, and downregulation of TSP-2 in SSc fibroblasts resulted in upregulation of miR-7 ultimately leading to decreased collagen production [140]. However this negative feedback loop is insufficient to control dysregulated fibrosis in SSc. It should be noted that skin miR-7 expression was downregulated in patients with localized scleroderma (LSc) but not diffuse SSc, suggesting that mechanistic involvement of miR-7 in the two disease subsets might be distinct [141].

4.3.4. Circulating non-coding RNAs—While most studies focused on intracellular miRNAs, there is growing evidence that miRNAs exist in extracellular environments, often times associated with proteins, lipids, or enclosed in vesicles [142]. Because of this, miRNAs are stable and can be detected in biological fluids including serum, plasma, saliva, and urine [143]. In addition, circulating miRNAs are easily accessible and can be quickly assayed by many methods, making them desirable candidates as biomarkers.

In SSc, several studies have focused on circulating miRNAs to examine whether they are involved in SSc pathogenesis (Table 5). The first report was published by Kawashita et al. showing that miR-29a was detectable in serum [144]. Although miR-29a targets COL1A1, the authors did not detect any significant differences in serum miR-29a levels between controls and SSc patients. However, lower miR-29a levels were associated with higher right ventricular systolic pressure in SSc patients, suggesting the involvement of this miRNA in the pathogenesis of pulmonary hypertension. Interestingly patients with scleroderma spectrum disorder (SSD), patients that did not fulfill ACR criteria for SSc but considered still at risk of developing SSc [145, 146], showed significantly lower amount of miR-29a compared to healthy controls or SSc patients. Despite the small number of SSD patients recruited, these data suggests that miR-29a might be useful in identifying patients with SSD.

MicroRNAs including miR-142-3p, miR-92a, and miR-135, were analyzed in serum [147, 148], as their putative target was integrin αV which is involved in TGFβ activation therefore likely involved in SSc pathogenesis. Both miR-142-3p and miR-92a were elevated significantly in SSc patients (both diffuse and limited SSc) compared to healthy controls, while no changes were observed in patients with systemic lupus erythematosus (SLE), dermatomyositis (DM), or SSD. These results contradict a later study where plasma miR-142-3p was reduced significantly in SSc patients compared to controls [149]. The inconsistency might have arisen from using serum vs. plasma, in the former vs. latter study. No significant correlation between clinical or serological features and miR-142-3p was noted, although patient with elevated miR-142-3p levels appeared to have a shorter sublingual frenulum. As for miR-92a, it appears that patients with elevated levels of miR-92a had lower frequency of telangiectasia. Additional functional studies were carried out for miR-92a in dermal fibroblasts. The elevated expression of miR-92a can be reduced by knocking down TGFβ, and overexpression of miR-92a resulted in MMP-1 downregulation [148].

Later studies focused on examining a variety of miRNAs at the same time instead of one miRNA at a time. Tanaka et al. performed a comprehensive analysis on a panel of miRNAs in SSc serum that were predicted to target SSc-related genes *in silico* [150]. Nineteen of the 95 miRNAs were significantly lower in SSc patients compared to healthy controls, with miR-30b being the most downregulated. This miRNA not only was inversely correlated with the modified Rodnan skin scores, but additional analysis also showed that it targets PDGFRβ. Its lower expression in both SSc skin and bleomycin-induced skin fibrosis model might indicate its role in promoting fibrosis in SSc through upregulation of the PDGFRβ pathway.

Honda et al. also performed a comprehensive analysis of dermal miRNA expression in fibroblasts isolated from both healthy controls or SSc patients using a miRNA PCR array that included 88 miRNAs [127]. They focused on miR-150 as its putative target was integrin β3, which is implicated in SSc pathogenesis. Significantly lower miR-150 levels were observed in SSc patients compared to healthy controls, and lower serum levels appear to be associated with more severe clinical manifestations. Follow up studies further showed that down-regulation of miR-150 resulted in up-regulation of both integrin β3 and type 1 collagen expression in dermal fibroblasts, suggesting its involvement in promoting fibrosis in SSc.

A similar study was conducted using miRNA PCR array to examine the miRNA profiles in skin samples from SSc (including both diffuse and limited SSc), LSc, and keloid patients [133]. Ten out of the 88 miRNA showed significant changes in SSc/LSc skin compared to normal skin or keloid samples. As 5 of the 10 miRNAs belonged to the let-7 family, the authors focused on let-7a, which was the most down-regulated let-7 member in both SSc and LSc samples. Serum let-7a was significantly lower in SSc and LSc patients compared to controls, and let-7a levels were inversely correlated with the severity of the skin disease. Two additional studies using similar approaches further identified miR-7 and miR-196a to be down-regulated in LSc patients compared to healthy control [141, 151]. These studies indicate that these miRNAs may have the potential to serve as useful diagnostic markers for disease differentiation.

In addition to miRNAs, lncRNAs are also measurable in extracellular fluids. There is only one report that studied lncRNA in SSc so far. Wang et al. examined the serum levels of lncRNA TSIX in healthy controls, SLE, SSD, and SSc patients and found that this lncRNA was upregulated in patients with SSD and SSc (both diffuse and limited) [134]. Clinical correlation analysis suggested that higher TSIX levels are associated with higher ratio of dcSSc:lcSSc and higher modified Rodnan skin scores.

Although studies that focused on individual circulating non-coding RNAs were informative, they could not be always replicated and seemed to vary with different clinical or laboratory features, making it hard to draw an absolute conclusion as to whether or not they are suitable biomarkers for SSc. More recent studies started to examine miRNA “profiles” or “network” in serum and their relevance in clinical assessment. Koba et al. observed different miRNA expression patterns in SSc compared to healthy controls, and that combining miR-206 and miR-21 may be more informative in distinguishing SSc patients from healthy subjects than

either miRNA alone [152]. Wuttge et al. examined circulating miRNA expression profiles and their correlation with disease phenotype and autoantibody status [153]. They identified 4 miRNAs (miR-223, -181b, -342-3p, -184) that were differentially expressed between diffuse SSc and limited SSc, and that 5 miRNAs (miR-409-3p, -184, -92a, -29a, -101) were differentially expressed in one or more autoantibody groups; both of which further confirmed by logistic regression and receiver operating characteristic curve analysis. Steen et al. were able to build a statistical model from 26 circulating miRNAs extracted from 120 SSc patients to distinguish healthy controls, SSc, and SLE populations based on their miRNA profiles [149]. They found that a combination of the miR-17~92 cluster (including -17, -20a, -92a, -106a) that was significantly reduced in SSc and SLE, and miR-142-3p and -223 that were decreased in SSc but elevated in SLE, could discriminate between the control and diseased groups, and between the SSc and SLE groups. All these studies point to the merit of analyzing a cluster of circulating miRNA for better disease classification and prediction.

In addition to being a source of biomarkers, it is possible that circulating miRNAs transmit their signal to other cell types via exosomes [154]. It has been shown that SSc patients had lower amount of exosomes in sera [155]. In addition, the frequencies of vascular complications were significantly increased in patients with lower levels of serum exosomes [155]. In a recent study, the miRNA profiles in serum exosomes from SSc patients were examined [156]. These SSc exosomes appeared to be pro-fibrotic, as 6 pro-fibrotic miRNAs were upregulated while 10 anti-fibrotic miRNAs were downregulated compared to exosomes isolated from healthy serum. There were also significant differences in exosomal miRNA expression between diffuse and limited SSc. In addition, these SSc exosomes were able to induce pro-fibrotic genes in normal dermal fibroblasts, suggesting a role in maintaining and propagating the fibrotic potential of the disease to distal or unaffected sites.

Although circulating non-coding RNAs have their potential to be biomarkers for SSc, there are challenges that needs to be overcome. The choice of the biological fluid can significantly impact the expression profiles since each could be enriched for a distinct set of miRNAs. One example is the drastic difference of miR-142-3p levels assayed in SSc plasma and serum mentioned earlier [147, 149]. The difference of miRNA profiles between serum and plasma might be due to the use of heparin-coated tubes to isolate plasma, which would interfere with downstream steps that involve PCR protocols [157]. Contamination of intracellular RNA from plates or erythrocytes could introduce bias and should be minimized [158]. In addition, changes in non-coding RNAs in the skin and may not reflect similar changes in the circulation, adding to the difficulties in data interpretation. One other challenge for non-coding RNA profiling is the selection of the best analytical technique used (e.g. RT-qPCR and miRNA arrays), as well as the correct way, if any, to properly normalize non-coding RNA data to minimize technical variation (e.g. small nuclear RNA, specific mRNAs, and external spike-in synthetic oligonucleotide) [159, 160].

4.3.5. miRNA gene polymorphism—A miR-146a polymorphism tagged by the SNP rs2910164 (C/G) has been reported in skin samples that were collected from 52 SSc patients and 107 healthy controls in Japan [161]. Patients with the CC genotype had a significantly

higher prevalence of telangiectasia than in those with CG or GG genotype. This is the only study published to date regarding miRNA gene polymorphism in SSc.

5. Therapeutic implication

As most of the known epigenetic mechanisms are in metastable states, therapeutic interventions to reprogram the epigenomic landscape to improve human health seems promising. Indeed reversibility of epigenetic marks mediated by writers or erasers (DNMTs, HDACs, or HMTs etc) makes these enzymes attractive drug targets. As small molecule inhibitors are becoming more available, one should take into consideration their target specificity and cellular toxicity, along with their lack of cellular specificity.

The epigenetic modifiers that have been employed in SSc studies are summarized in Table 6. These modifiers include DNMT inhibitors 5-azacytidine (5-azaC, Vidaza) and 5-aza (Dacogen), pan-HDAC inhibitors TSA and Divalproex sodium, SIRT1 activator resveratrol, EZH2 inhibitor DZNep, and miRNAs or miRNA-containing exosomes. Other non-epigenetic modifiers, such as the antibiotic ciprofloxacin, NR4A1 agonist Csn-B, tyrosine inhibitor imatinib, and proteasome inhibitor bortezomib, that originally were not anticipated to have epigenetic effects, turned out to modify various epigenetic mediators in SSc and alleviated tissue fibrosis.

The DNMT inhibitors irreversibly inactivate DNMTs and trigger their degradation [162]. The prototypical drugs include 5-azaC and 5-aza; both have been approved by the FDA to treat myelodysplastic syndrome and acute myeloid leukemia. Usage of these DNMT inhibitors in SSc appeared to be beneficial for stopping fibrosis, as all studies showed potent antifibrotic effect of these drugs targeting transcription factors, the Wnt pathway, the COX2 pathway, and miRNAs [91, 93, 96, 99, 127]. They also modify the functions of immune cells, as shown in CD4+ T cells, where 5-azaC enhanced FOXP3 expression and regenerated Tregs [83]. However overall effects of these drugs can be mixed, since 5-azaC increased CD11a on CD4+ T cells that resulted in activation of B cells and induced collagen synthesis in fibroblasts [82].

Similar to DMNT inhibitors, HDAC inhibitor TSA showed promising antifibrotic effect both *in vivo* and *in vitro* in SSc [89, 108, 109, 119]. TSA, which is a pan-HDAC inhibitor, was the prototypic drug that was widely used in experiments. Suberoylanilide hydroxamic acid (SAHA; vorinostat) and depsipeptide (romidepsin), which are also pan-HDAC inhibitors, are currently approved by the FDA for cutaneous T cell lymphomas. Global inactivation of HDACs by these nonspecific inhibitors may not be ideal for a multifaceted disease such as SSc, since they may be beneficial for fibrosis treatment but may affect angiogenesis by inactivating pro-angiogenic HDACs [102]. Therefore specific HDAC inhibitors will be ideal for SSc, and this seems to be the trend for drug development, as recent development towards individual HDACs have been reported [163],

The role of other classes of epigenetic modifiers in treating SSc is less certain. As contradictory results were reported for SIRT1 [114, 115], the use of resveratrol as a possible anti-fibrotic is questionable. In addition to resveratrol, which is known have specificity

issues, new activators for SIRT1 have been identified [164]. Early phase clinical trials showed safe and well tolerated results for SRT2104 [165], and this new generation of SIRT1 activators might show more conclusive results in SSc studies. Similar to SIRT1, the role of EZH2 in SSc fibrosis is not clear [107, 110]. Several drugs targeting EZH2 are now in clinical trials, and perhaps usage of these drugs that have better pharmacokinetic profiles and specificities in SSc studies would provide more insight for the involvement of KMTs such as EZH2 in SSc. Apparent involvement of miRNAs in SSc pathogenesis has been well documented (Table 5), and their therapeutic potential has been examined in animal studies [129, 133, 137, 155] and in clinical trials for fibrotic conditions (MRG-201, ClinicalTrials.gov Identifier: NCT02603224). Surprisingly, no studies examined histone readers such as the bromodomain and extra-terminal (BET) family in SSc. Small-molecule inhibitors for BET bromodomains have been identified [166] and several are in clinical trials for cancer [167]. It will be intriguing to examine whether these histone readers are dysregulated in SSc.

The recognition of epigenetics as an important contributor to SSc pathogenesis has advanced new avenues for drug development and therapeutic options for this devastating disease. Combinatorial therapy should be considered, either with epigenetic modifiers or conventional immunotherapy, to limit the side effects of treatment, and maximize the therapeutic effect of the combined drugs.

6. Conclusions and future prospective

We highlighted the rapid development of genetic and epigenetic research in the field of SSc pathogenesis in this review. Thanks to these studies, we are starting to understand the impact of genetic predisposition, epigenetic dysregulation, and environmental factors in this disease. Although the genetic architecture of SSc has been laid out, more work has to be done to identify specific disease-associated functional variants. There is a need for understanding the cause of ethnic differences in SSc susceptibility, as well as determining the role of rare variants in this disease. As more SSc susceptibility loci are being identified, studies should focus on interactions between genes as well as between genes and the environment in this complex disease. In addition, there is still a lot to be done to really understand what triggers the epigenetic changes in SSc. The relative contribution of each epigenetic mechanism, DNA methylation, histone modifications, and non-coding RNAs, has not been determined. There is a need to examine epigenetics in SSc ECs, and more mechanistic studies are required to determine the impact of histone changes in immune cells. In addition, whether epigenetic marks, such as circulating miRNAs, can be used as reliable biomarkers for SSc remains unclear. Finally, delineating the relationship between SNPs and epigenetic modifications may shed light on understanding the roles of genetic susceptibility variants that are located in noncoding regions of the genome.

The advancement of next-generation sequencing (NGS) technologies in the last decade have facilitated epigenomic profiling at high resolution, hence revolutionized the field of epigenetics. A great example is profiling histone marks at the genome level that allowed predictions for functional genetic elements. The histone patterns, such as H3K27ac and H3K4me1 as enhancers, have been included in the NIH Roadmap Epigenomics Consortium

to generate reference epigenomes and to establish the differences in epigenomic signature between normal and diseased cells [168]. Indeed, examining epigenetics at the genome-wide level has started to gain its popularity in SSc [97, 103]. In addition, as epigenetic mechanisms are often cell type specific and may differ within the same tissue (i.e. ECs vs. myofibroblasts in skin biopsy), it is important to perform epigenetic studies in isolated cells as opposed to whole tissue or a mixture of cells such as PBMCs. The recent breakthrough in technologies allowing epigenomics analysis at a single cell level will indeed pushed the field toward further understanding how a cell maintains its phenotype, its lineage commitment, and how it is perturbed in a disease setting [169]. Finally, NGS technologies in the form of miRNA-seq can be utilized to profile genome-wide expression of the miRNA transcriptome. This approach, unlike the qRT-PCR and miRNA array methods mentioned earlier, allows identification of novel miRNAs and pre-miRNAs [170]. However, caution must be drawn that sequencing bias that is inherent in different sequencing platforms might introduce reads that are not real miRNAs [171].

One major challenge in the epigenetic field is to discriminate between genes that are driving the disease epigenetically and genes that are altered in the disease process but not necessary pertinent for the disease to occur. Data generated by NGS such as RNA-seq, ChiP-seq, or ATAC-seq should be integrated through bioinformatics to allow a more comprehensive understanding of the epigenetic landscape that is crucial for the disease to occur, and to help decipher genes that are causative or correlative. One example is our study using ATAC-seq to determine the underlying mechanism for HDAC5 involvement in dysregulated angiogenesis in SSc (experiment schematic in Figure 3) [103]. Since knocking down HDAC5 increases acetylated histones and relaxes the chromatin, we assessed genome-wide chromatin accessibility in SSc ECs by ATAC-seq, and identified a list of genes that were altered under this condition. To find out genes that were pertinent in HDAC5-mediated angiogenesis, we narrowed down the list of genes through functional assays, eventually identifying 3 genes that were crucial in this process.

Because of the dynamic nature of the epigenome, it has become an attractive therapeutic target, especially in the cancer field. In SSc, various studies have indeed demonstrated the efficacy of DNMT inhibitors or HDAC inhibitors in alleviating fibrosis (Table 6). Given how epigenetic mechanisms control cellular development and maintain normal physiological behavior, it would be more beneficial to target specific epigenetic alterations instead of disturbing global modifications to lower toxicity and side effects while enhancing therapeutic efficacy.

The heterogeneity of SSc makes it a complex disease to understand and at the same time a difficult disease to treat. Recent focus on genetics and epigenetics research not only helped us apprehend this disease more, but also raised more questions. With the rapid development of epigenetic concepts, genomic and epigenomic methodologies, as well as drugs that target different epigenetic mechanisms, there is no doubt we are poised to have more progress in identifying novel therapeutic targets for SSc in the near future.

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Highlights

- Genetic susceptibility within and outside of the HLA have been replicated in scleroderma
- Scleroderma is a complex disease and understanding the functional effect of genetic susceptibility loci identified remains a challenge
- Epigenomic studies have provided novel insight into the immune, vascular, and fibrotic components of scleroderma
- Studies focused on genetic-epigenetic interaction will help understand the complexity of scleroderma
- Epigenomic marks and regulators might provide novel biomarkers and therapeutic targets in scleroderma

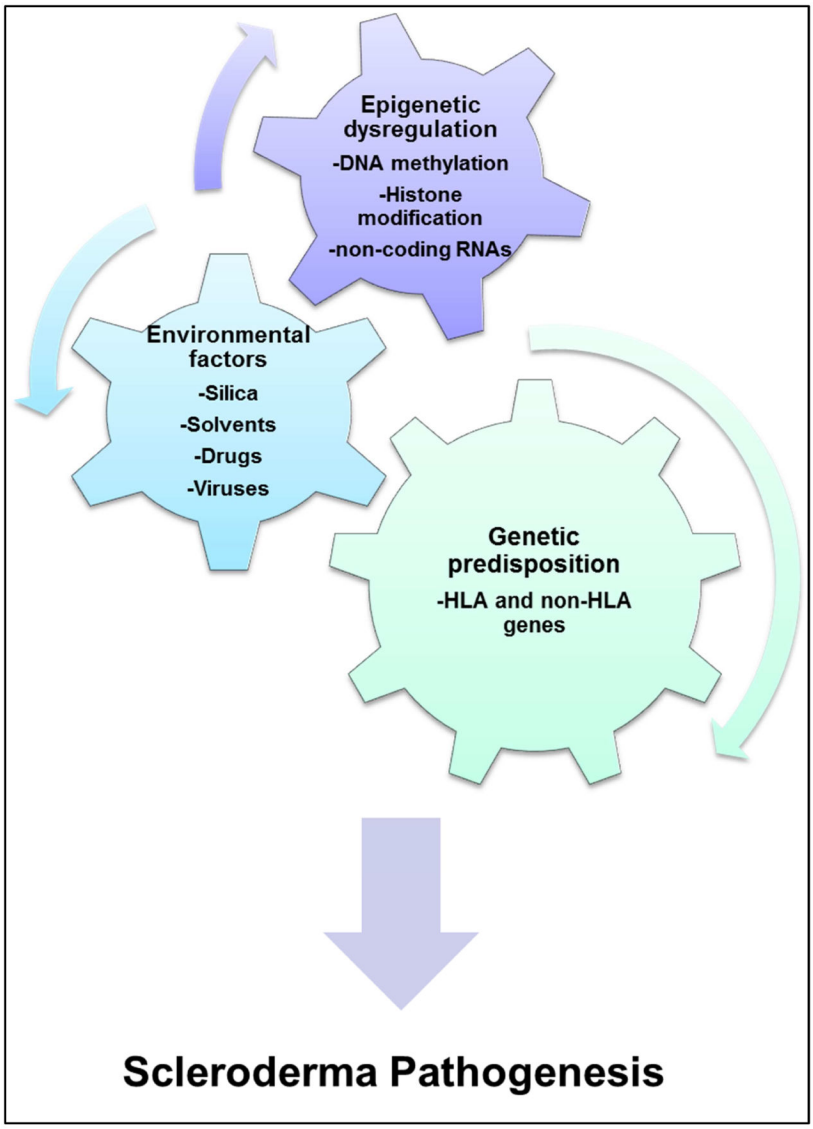

Figure 1. Key elements pertinent to the development of scleroderma (SSc)

In genetically predisposed individuals, environmental challenges, together with dysregulated epigenetic alterations, contribute to the development of SSc.

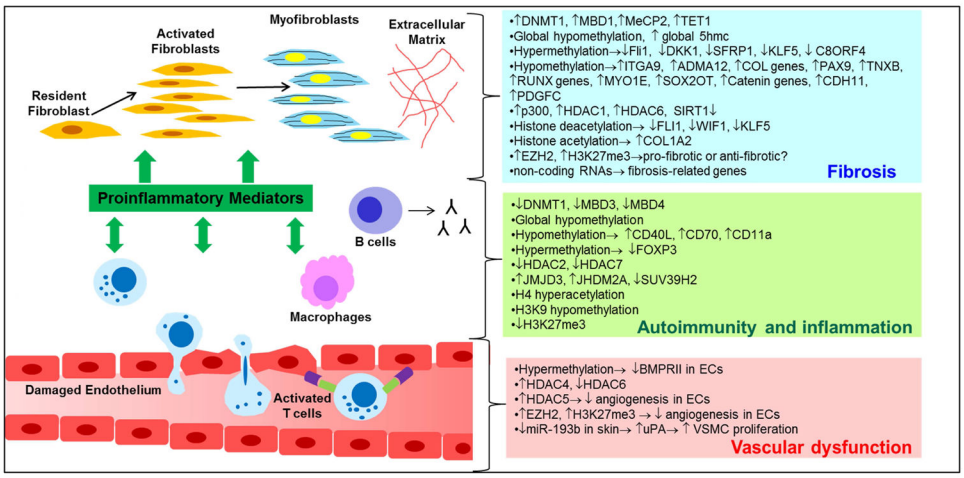

Figure 2. Summary of the pathogenesis of SSc, and the epigenetic mechanisms involved in this disease

There are three main components in SSc: vascular dysfunction, autoimmunity and inflammation, as well as fibrosis. These processes involve dysregulation of multiple cell types, including endothelial cells (ECs), immune cells such as lymphocytes and macrophages, and myofibroblasts, which ultimately result in extracellular matrix disposition and tissue fibrosis. Epigenetic changes, including alterations in DNA methylation, histone modifications, and non-coding RNAs, contribute to SSc pathogenesis by affecting the various cell types that are involved in the three major processes.

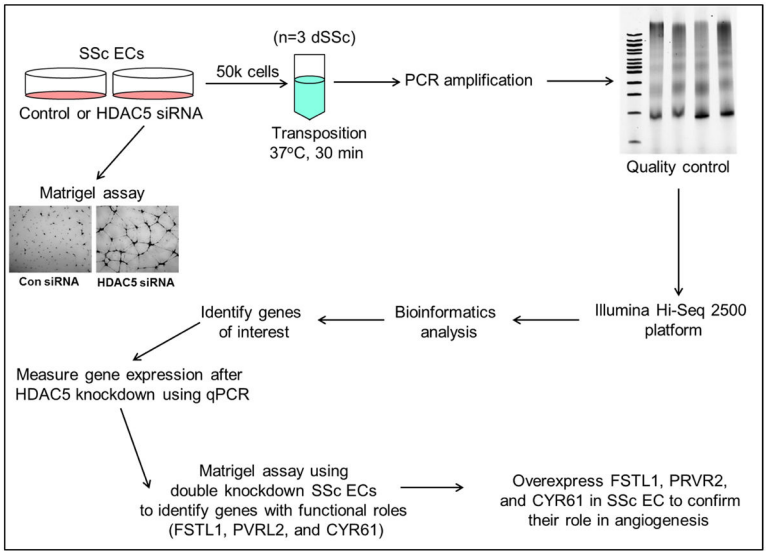

Figure 3. The application of ATAC-seq to identify novel genes that play functional roles in SSc angiogenesis

We hypothesized that the anti-angiogenic histone deacetylase 5 (HDAC5) contributes to impaired angiogenesis in SSc by repressing pro-angiogenic factors in endothelial cells (ECs). HDAC5 was indeed overexpressed in ECs from diffuse SSc compared to healthy controls, and silencing HDAC5 restored normal angiogenesis in SSc ECs. We then took an unbiased approach to examine genome-wide changes in chromatin accessibility after HDAC5 knockdown using ATAC-seq. HDAC5 knockdown showed increased chromatin accessibility compared to control cells. To identify genes related to angiogenesis or fibrosis, literature mining and bioinformatics analysis were performed. Using Matrigel tube formation assays, we identified 3 novel HDAC5-target genes, CYR61, PVRL2, and FSTL1 that are associated with impaired angiogenesis in SSc.

Table 1

Confirmed genes associated with SSc (established by associations reaching genome-wide significance or replicated in at least two studies).

Gene	Genetic variants	Method	Refs
HLA genes	*HLA-B* *HLA-C* *HLA-DMB* *HLA-DOA* *HLA-DPB1* *HLA-DQA1* *HLA-DQB1* *HLA-DR* *HLA-DRA* *HLA-DRB1* *HLA-DRB5*	Various	[23]
IRF4	rs9328192	GWAS	[38]
IRF5	rs2004640; rs10954213; rs2280714; rs10488631; rs12537284; rs4728142; rs3757385	Candidate Gene GWAS Immunochip	[16–18, 21, 22, 34, 36–38, 52, 53, 172–175]
IRF7	rs4963128; rs1131665; rs702966	Candidate Gene	[176]
IRF8	rs11642873; rs11117432; rs2280381; rs11117425; rs11644034; rs12711490; rs7202472	Candidate Gene GWAS	[17, 21, 34, 177, 178]
BANK1	rs10516487; rs3733197; rs17266594	Candidate Gene WES	[39–41]
BLK	rs2736340; rs13277113; rs13277113	Candidate Gene GWAS	[21, 42–44]
CD247	rs2056626	Candidate Gene GWAS	[16–18, 34, 179]
STAT4	rs7574865; rs11889341; rs8179673; rs10181656; rs6752770; rs3821236; rs10168266	Candidate Gene GWAS Immunochip	[16–18, 21, 34, 48–53]
TNFAIP3	rs5029939; rs2230926; rs6932056	Candidate Gene GWAS Immunochip	[21, 22, 178, 180–182]
TNFSF4	rs1234314; rs2205960; rs844644; rs12039904; rs844648; rs4916334; rs10798269; rs844665	Candidate Gene GWAS	[21, 34, 183–185]
TNIP1	rs2233287; rs4958881; rs3792783	GWAS GWAS follow up	[16, 21, 185]
PTPN22	rs2476601	Candidate Gene GWAS	[38, 186–189]
NOTCH4	rs443198; rs9296015 c.4245G>A:p.Met1415lle	GWAS WES	[17, 33]
TYK2	rs2304256; rs34536443; rs12720356; rs35018800	Immunochip follow up	[190]
IL12A	rs77583790	Immunochip	[22]
IL12RB1	rs2305743	Immunochip follow up	[191]
IL12RB2	rs3790567	GWAS follow up	[192]
ATG5	rs9373839	Immunochip GWAS	[21, 22, 38]
DNASE1L3	rs35677470	Immunochip	[22, 52]
PPARG	rs310746	Candidate Gene GWAS follow up	[57, 58]

Gene	Genetic variants	Method	Refs
CSK	rs1378942	GWAS GWAS follow up	[21, 181]
ATP8B4	rs55687265	WES	[59, 60]

GWAS: genome-wide association study; WES: whole-exon sequencing; ACA: anti-centromere antibodies; ATA: anti-topoisomerase I antibodies

Table 2

Summary of epigenetic modifiers and modifications in SSc

Epigenetic process	Modifiers/Modifications	Cell type	Findings in SSc	Refs
DNA methylation				
	DNMTs	CD4+ T cells	↓DNMT1 ↔DNMT3a ↔DNMT3b	[79]
	MBDs	CD4+ T cells	↔MBD-1 ↔MBD-2 ↓MBD-3 ↓MBD-4 ↔MeCP2	[79]
	DNMTs	¶Fibroblasts[d,l]	↑DNMT1 ↔DNMT3a ↔DNMT3b	[89, 90]
	DNA Demethylase	Fibroblasts	↑TET1 ↔TET2 ↔TET3	[98]
	DNA Demethylase	Fibroblasts[l]	↔TET1	[91]
	MBDs	Fibroblasts[d]	↑MBD-1 ↔MBD-2 ↑MeCP2	[89]
	MBDs	Fibroblasts[l]	↑MeCP2	[91]
	Global methylation	Whole blood[d,l]	↓	[85]
	Global methylation	CD4+ T cells	↓	[79]
	Global methylation	Fibroblasts	↓	[98]
	5-hydroxymethylcytosine	Fibroblasts	↑	[98]
	Genome-wide methylation	Fibroblasts[d,l]	↓	[97]
Histone acetylation				
	HATs	B cells[d,l]	↔p300 ↔CREBBP ↔PCAF	[100]
	HDACs	B cells[d,l]	↔HDAC1 ↓HDAC2 ↔HDAC3 ↔HDAC4 ↔HDAC5 ↔HDAC6 ↓HDAC7	[100]
	HDACs	¶ECs[d]	↑HDAC4 ↑HDAC5 ↓HDAC6 ↔HDAC7 ↔HDAC9 ↔HDAC10	[103]
	HATs	Fibroblasts[d]	↑p300	[112, 113]
	HDACs	Fibroblasts[d]	↑ HDAC1 ↑HDAC6	[89]
	HDACs	Fibroblasts	↔HDAC1-11	[108]
	HDACs	Fibroblasts[d,l]	↓SIRT1	[114, 115]
	Global H3 acetylation	B cells[d,l]	↔	[100]
	Global H4 acetylation	B cells[d,l]	↑	[100]
Histone methylation				

Epigenetic process	Modifiers/Modifications	Cell type	Findings in SSc	Refs
	HMTs	CD4+ T cells	↔EZH1 ↔EZH2	[101]
	HDMs	CD4+ T cells	↑JMJD3 ↔UTX	[101]
	HMTs	B cells[d,l]	↔SUV39H1 ↓SUV39H2 ↔EZH2	[100]
	HDMs	B cells[d,l]	↔JARID1A ↔JARID1B ↔JARID1C ↔JARID1D ↑JHDM2A ↔JMJD2A ↔JMJD2B ↔JMJD2C ↔JMJD2D	[100]
	HMTs	ECs[d]	↑EZH2	[107]
	HMTs	Fibroblasts[l]	↔ASH1 ↔EZH2	[91]
	HMTs	Fibroblasts[d]	↑EZH2	[107]
	H3K27me3	CD4+ T cells	↓	[101]
	H3K27me3	ECs[d]	↑	[107]
	H3K27me3	Fibroblasts[d]	↑	[107, 110]
	Global H3K4 methylation	B cells[d,l]	↔	[100]
	Global H3K9 methylation	B cells[d,l]	↓	[100]

d=diffuse SSc; l=limited SSc;

[¶]Dermal unless specified

Table 3

Summary of differentially methylated genes in SSc

Gene	Methylation changes	Cell type/tissue	Implications	Refs
PRF1	Hypermethylation	Whole blood[d,l]	May contribute to autoimmunity	[85]
CD11a	Hypermethylation	Whole blood[d,l]	May contribute to autoimmunity	[85]
FOXP3	Hypermethylation	Whole blood[d,l]	May contribute to autoimmunity	[85]
CDKN2A	Hypomethylation	Whole blood[d,l]	May be involved in cell apoptosis and senescence; role in SSc not know	[85]
CD70	Hypomethylation	Whole blood[d,l]	May contribute to autoimmunity	[85]
IL1RAPL2, ZBED1, EIF2S3, DDX3X, ZNF41, PGRMC1, UBE2A, ZNF280C, ENOX2, FAM122C, FAM127C, DDX26B, CXorf40A, SSR4, IDH3G	Hypomethylation	PBMCs[d,l]	X-chromosome genes	[86]
SMS, ARX, ARAF, CCDC22, SSX2, RGAG4, PGK1, IL1RAPL2, UTP14A, HSFX1, MTM1, LAGE3	Hypermethylation	PBMCs[d,l]	X-chromosome genes	[86]
RORC1, RORC2	Hypermethylation	PBMCs[d]	Th17-associated with transcription factors; correlated with inflammatory parameters, age, and Scl-70	[84]
CD40L	Hypomethylation	CD4+ T cells[d,l]	May contribute to female susceptibility in SSc	[80]
CD70	Hypomethylation	CD4+ T cells	May contribute to autoimmunity	[81]
CD11a	Hypomethylation	CD4+ T cells[d,l]	Increased CD4+ T cell proliferation, antibody overproduction by B cell, Increased collagen synthesis by fibroblasts	[82]
FOXP3	Hypermethylation	CD4+ T cells[d,l]	Leads to quantitative defects in Tregs	[83]
BMPRII	Hypermethylation	¶ECs/skin[d]	Leads to increased sensitivity to apoptosis	[87]
FLI1	Hypermethylation	¶Fibroblasts[d]	Results in collagen overexpression	[89]
DKK1, SFRP1	Hypermethylation	Fibroblasts[d,l]/PBMCs[d,l]/Animal model	Results in activation of the Wnt pathway	[96]
ITGA9	Hypomethylation	Fibroblasts[d,l]	Results in overexpression of *ITGA9*, possibly involved in myofibroblast transformation and TGFβ regulation	[97]
ADAM12	Hypomethylation	Fibroblasts[d,l]	Results in overexpression of *ADAM12*, possibly contributing to fibrosis through the TGFβ pathway	[97]
COL4A2, COL23A1	Hypomethylation	Fibroblasts[d,l]	Results in overexpression of *COL4A2, COL23A1*	[97]
PAX9	Hypomethylation	Fibroblasts[d,l]	Results in overexpression of *PAX9*, possibly contributing	[97]

Gene	Methylation changes	Cell type/tissue	Implications	Refs
			to overexpression of pro-α2 chain of type I collagen	
TNXB	Hypomethylation	Fibroblasts[d,l]	May lead to overexpression of ECM glycoproteins	[97]
RUNX1, RUNX2, RUNX3	Hypomethylation	Fibroblasts[d,l]	Possibly contribute to collagen synthesis	[97]
MYO1E	Hypomethylation	Fibroblasts[d,l]	Involved in actin assembly; role in SSc unclear	[97]
COL8A1, COL16A1, COL29A1	Hypomethylation	Fibroblasts[d]	May result in overexpression of collagen	[97]
CDH11	Hypomethylation	Fibroblasts[d]	Cadherin-11 may contribute to fibrosis through assisting myofibroblast differentiation	[97]
PDGFC	Hypomethylation	Fibroblasts[d]	Overexpressed PDGFC is a profibrotic factor	[97]
CTNNA2, CTNNB1	Hypomethylation	Fibroblasts[d]	Possible involvement in the Wnt/β-catenin pathway	[97]
SOX2OT	Hypomethylation	Fibroblasts[d]	Encodes lnc-RNAs; their roles in fibrosis is unknown	[97]
COL1A1, COL6A3, COL12A1	Hypomethylation	Fibroblasts[l]	May result in overexpression of collagen	[97]
CTNNA3, CTNND2	Hypomethylation	Fibroblasts[l]	Possible involvement in the Wnt/β-catenin pathway	[97]
KLF5	Hypermethylation	Fibroblasts[d]	Results in lower KLF5 expression; downregulation of KLF5 and Fli1 work synergistically to exacerbate fibrosis	[93]
C8ORF4	Hypermethylation	Lung fibroblasts	Lower expression of C8ORF4 contributes to lower levels of COX-2 and PGE2	[99]

d=diffuse SSc; l=limited SSc;

[¶]Dermal unless specified

Table 4

Summary of genes affected by histone modifications in SSc

Gene	Histone changes	Cell type/tissue	Implications	Refs
CYR61, PVRL2, FSTL1	HDAC5	¶ECs[d]	Confirmation of several genes that are crucial for dysregulated angiogenesis in SSc	[103]
FLI1	H3, H4 Hypoacetylation	¶Fibroblasts[d]	Results in collagen overexpression	[89]
KLF5	H3, H4 Hypoacetylation	Fibroblasts[d]	Results in lower KLF5 expression; downregulation of KLF5 and FLI1 work synergistically to exacerbate fibrosis	[93]
FRA2	H3K27me3	Fibroblasts[d]	Inhibition of H3K27me3 results in induction of Fra2	[110]
FRA2, TGFβ, VEGF, FGF2, DNMT1, DNMT3A, MECP2	H3K27me3	Fibroblasts[d]	Inhibition of H3K27me3 results in downregulation of the genes	[107]
WIF1	Histone hypoacetylation	FIbroblasts	The reduction of WIF1 in SSc was due to oxidative DNA damage-induced histone deaceylation, leading to activation of Wnt signaling and fibrosis	[119]
COL1A2	H4 hyperacetylation	Fibroblasts	TGFβ stimulated Egr-1 which increased transcription of both p300 and COL1A2. p300 acetylated COL1A2 promoter facilitating transcriptional activation by Smad2/3	[113]
NR4A1	H3, H4 hyperacetylation	Fibroblasts	Rapid acetylation occurs at NR4A1 promoter in the presence of TGFβ; The anti-fibrotic effect of NR4A1 was inhibited by AKT- and HDAC-dependent mechanisms under fibrotic conditions	[118]

d=diffuse SSc;

¶Dermal unless specified

Table 5

Non-coding RNAs involved in SSc

miRNAs	Target genes	Cell type/tissue	Function	Expression changes	Implications	Refs
let-7g-5p miR-17-5p miR-21-5p miR-23b-5p miR-29a-3p miR-150-5p miR-155-5p miR-215-5p miR-503-5p	[†] N.D.	Serum exosomes[d]	Profibrotic	↑	These exosomes stimulated profibrotic gene expression, collagen, and fibronectin in normal fibroblasts	[156]
let-7g-5p miR-17-5p miR-23b-5p miR-155-5p miR-215-5p miR-503-5p	N.D.	Serum exosomes[l]	Profibrotic	↑	These exosomes stimulated profibrotic gene expression, collagen, and fibronectin in normal fibroblasts	[156]
let-7a-5p miR-26b-5p miR-29b-3p miR-92a-3p miR-125b-5p miR-129-5p miR-133a-3p miR-140-5p miR-145-5p miR-146a-5p miR-196a-5p miR-200a-3p miR-200b-3p miR-223-3p	N.D.	Serum exosomes[d]	Antifibrotic	↓	These exosomes stimulated profibrotic gene expression, collagen, and fibronectin in normal fibroblasts	[156]
let-7a-5p miR-26b-5p miR-29b-3p miR-92a-3p miR-129-5p miR-133a-3p miR-140-5p miR-145-5p miR-146a-5p miR-196a-5p miR-200a-3p miR-223-3p	N.D.	Serum exosomes[l]	Antifibrotic	↓	These exosomes stimulated profibrotic gene expression, collagen, and fibronectin in normal fibroblasts	[156]
miR-29a	N.D.	Serum[d,l,L]	N.D.	↔	Significant decrease was found in patients with scleroderma	[141, 144]

miRNAs	Target genes	Cell type/tissue	Function	Expression changes	Implications	Refs
					spectrum disorder but not SSc or LSc	
miR-142-3p	Possibly *ITGAV*	Serum[d,l]	N.D.	↑	Elevated in specifically SSc patients; may be an useful biomarker	[147]
let-7g miR-21 miR-29b miR-125b miR-145 miR-206	N.D.	Serum[d,l]	N.D.	↔	Combination of circulating miRNAs may be more useful as biomarkers	[152]
let-7a, b, c, d miR-19a miR-26a miR-30b, miR-106b miR-181a, b miR-191 miR-203 miR-302c, d miR-376a miR-409-3p miR-410 miR-484 miR-549	N.D.	Serum[d,l]	N.D.	↓	Among these miRNAs, mir-30b appeared to be downregulated more strongly in dSSc then in lSSc, and that it correlated with disease severity	[150]
miR-181b miR-184	N.D.	Plasma[d,l]	N.D.	↑(dSSc vs. lSSc)	Circulating miRNA differed between disease phenotype as well as antibody profiles	[153]
miR-223 miR-342-3p	N.D.	Plasma[d,l]	N.D.	↓(dSSc vs. lSSc)	Circulating miRNA differed between disease phenotype as well as antibody profiles	[153]
miR-181b miR-638 miR-29b-3p miR-590-5p miR-150	N.D.	Plasma[d,l]	N.D.	↑	The circulating miRNAs in SSc showed distinct characteristics compared to controls and SLE samples	[149]

miRNAs	Target genes	Cell type/tissue	Function	Expression changes	Implications	Refs
miR-142-3p miR-342-3p miR-146a miR-20a miR-92a miR-145-5p miR-16 miR-223 miR-192-5p miR-221 miR-24 miR-146b miR-17 miR-106a	N.D.	Plasma[d,l]	N.D.	↓	The circulating miRNAs in SSc showed distinct characteristics compared to controls and SLE samples	[149]
miR-150 miR-20b miR-377 miR-23b let-7g miR-197 miR-28-5p miR-198 miR-26a	N.D.	Skin	N.D.	↑	Implication of miRNAs in SSc pathogenesis	[130]
miR-214 miR-125b miR-133a miR-486-5p miR-206 miR-685 miR-140-5p miR-125a-5p miR-145 miR-30a miR-801 miR-30d miR-92a miR-381 miR-27b	N.D.	Skin	N.D.	↓	Implication of miRNAs in SSc pathogenesis	[130]
miR-146a	N.D.	Skin[d,l]	N.D.	N.D.	Patients with the CC genotype showed higher prevalence of telangiectasia than in those with CG or GG genotype	[161]
miR-142-3p	N.D.	¶ Fibroblast exosomes	N.D.	↑	These exosomes stimulated type 1 collagen in normal fibroblasts	[155]

miRNAs	Target genes	Cell type/tissue	Function	Expression changes	Implications	Refs
miR-150 miR-196a	N.D.	Fibroblast exosomes	N.D.	↓	These exosomes stimulated type 1 collagen in normal fibroblasts	[155]
miR-7	*COL1A2*	Fibroblasts[d,l]/Skin[d,l]	Antifibrotic	↑	Downregulation of TSP-2 stimulates miR-7 to degrade collagen, however insufficient to stop fibrosis in SSc	[140]
miR-7	*COL1A2*	Fibroblasts[L]/Skin[L]/Serum[L]	Antifibrotic	↓	Implication in LSc pathogenesis	[141]
miR-21	*SMAD7*	Fibroblasts[d,l]/Skin[d,l]/Animal model	Profibrotic	↑	Can be stimulated by TGFβ; possible therapeutic target for SSc	[128, 129]
miR-29a	*COL1A1* *COL3A1* *TAB1*	Fibroblasts/Hair/Animal model	Antifibrotic	↓	Can be stimulated by TGFβ, PDGF-B, or IL-4, while imatinib restored its expression; downregulation of miR-29a resulted in increased TAB1 and hence TIMP1 upregulation that led to excessive collagen production in SSc	[120, 131, 193]
miR-29a	Possibly *BCL2, BCL2L1, TNFA*	Fibroblasts[d]	Proapoptotic	N.D.	Targeting this miRNA could benefit SSc	[194]
miR-29b	Possibly *COL1A1*	Fibroblasts[d,l]/Skin[d,l]	N.D.	↓	Can be regulated by TGFβ, possible involvement in SSc pathogenesis	[128]
miR-30a-3p	*BAFF*	Fibroblasts[d]	Immune activation	↓	Can be regulated by IFNγ and Poly(I:C); Downregulation of miR-30a-3p increased BAFF production and stimulated B cell survival	[121]
miR-30b	*PDGFRB*	Fibroblasts[d,l]/Animal model	Antifibrotic	↓	Can be stimulated by TGFβ; implied	[150]

miRNAs	Target genes	Cell type/tissue	Function	Expression changes	Implications	Refs
					its role in SSc pathogenesis	
miR-31	N.D.	Fibroblasts[d,l]/Skin[d,l]	N.D.	↑	Possible involvement in SSc pathogenesis	[128]
miR-92a	*MMP1*	Fibroblasts[d]/Serum[d,l]	Profibrotic	↑	Can be stimulated by TGFβ and influence collagen turnover via MMP1	[148]
miR-129-5p	*COL1A1*	Fibroblasts[d,l]	Antifibrotic	↓	The anti-fibrotic effect of IL-17 was mediated by miR-129-5p, however TGFβ blocked this pathway that led to SSc fibrosis	[122]
miR-130b	*PPARG*	Fibroblasts[d,l]/Skin[d,l]/Animal model	Profibrotic	↑	Can be stimulated by TGFβ; implication in SSc pathogenesis	[138]
miR-135b	*STAT6*	Fibroblasts[l]/Serum[d,l]/Monocytes/Animal model	Antifibrotic	↓	IL-13 stimulated collagen production through STAT6, which was controlled by miR-135b	[91]
miR-145	Possibly *SMAD3*	Fibroblasts[d,l]/Skin[d,l]	N.D.	↓	Can be stimulated by TGFβ; possible involvement in SSc pathogenesis	[128]
miR-146	N.D.	Fibroblasts[d,l]/Skin[d,l]	N.D.	↑	Possible involvement in SSc pathogenesis	[128]
miR-150	*ITGB3*	Fibroblasts/Skin/Serum[d,l]	Antifibrotic	↓	Patients with lower circulating miR-150 may show more severe disease; Controlled collagen expression through integrin β3 in fibroblasts	[127]
miR-155	*CSNK1A1* *SHIP1*	Fibroblasts[S,L]/Skin[S,L]/Animal model	Profibrotic	↑	It mediated its profibrotic effect through the Wnt/β-catenin and Akt pathways	[137]

miRNAs	Target genes	Cell type/tissue	Function	Expression changes	Implications	Refs
miR-193b	*PLAU*	Fibroblasts[d,l]/Skin[d,l]	Promotes proliferative vasculopathy	↓	Downregulation of miR-93b induced uPA in SSc, thereby increased smooth muscle cell proliferation	[123]
miR-196a	*COL1A1* *COL1A2*	Fibroblasts[d,l]/Skin[S,L]/Serum[L]/Hair shaft[d,l]/Animal model	Antifibrotic	↓	Can be regulated by TGFβ; DDR2 negatively impacts miR-196a and that TGFβ blocks both DDR2 and miR-196a in SSc leading to increased tissue fibrosis; serum levels were not significantly different between healthy subjects and SSc patients but do inversely correlate with disease severity	[132, 136, 151, 195]
miR-202-3p	*MMP1*	Fibroblasts[d,l]/Skin[d,l]	Profibrotic	↑	The overexpressed miR-202-3p mediated collagen levels in SSc fibroblasts through reduction of MMP1	[135]
miR-503	N.D.	Fibroblasts[d,l]/Skin[d,l]	N.D.	↑	Possible involvement in SSc pathogenesis	[128]
let-7a	COL1A1 COL1A2	Fibroblasts[d,l,L]/Skin[d,l,L]/Serum[d,l,L]Animal model	Antifibrotic	↓	TGFβ downregulated let-7a led to overexpression of collagen	[133]
TSIX	COL1A1 COL1A2	Fibroblasts[d]/Skin[d]/Serum[d,l,SSD]/Fibroblast culture media[d]	Profibrotic	↑	Upregulation of TSIX in SSc might be due to TGFβ stimulation; stabilizes type 1 collagen in SSc	[134]

d=diffuse SSc; l=limited SSc; S=systemic scleroderma; L=localized scleroderma; SSD=scleroderma spectrum disease; LSc=localized scleroderma; N.D.=not determined;

†Not validated by experiments;

¥versus healthy controls unless specified;

¶Dermal unless specified;

Table 6

Epigenetic modifiers used in SSc studies

Compound	Specificity	Cell type	Biological effect	Refs
5-azaC	Pan-DNMT inhibitor	CD4+ T cells[d,l]	Enhanced FOXP3 expression and Treg generation	[83]
5-azaC	Pan-DNMT inhibitor	CD4+ T cells[d,l]	Increased CD11a expression in control CD4+ T cells leading to increased proliferation of T cells, increased production of IgG by co-cultured B cells, and induced expression of collagen by co-cultured fibroblasts	[82]
5-aza + TSA	Pan-DNMT Pan-HDAC inhibitor	¶ECs[d]	Normalized BMPRII expression	[87]
5-aza + TSA	Pan-DNMT Pan-HDAC inhibitor	Fibroblasts[d]	Normalized collagen expression	[89]
5-aza + TSA	Pan-DNMT Pan-HDAC inhibitor	Fibroblasts[d]	Increased KLF5 levels while decreased COL1A2	[93]
5-aza	Pan DNMT inhibitor	Fibroblasts[d]	Increased KLF5 expression	[93]
5-aza	Pan DNMT inhibitor	¶Fibroblasts[d,l]/Animal model	Its antifibrotic effect stemmed from increasing DKK1 and SFRP1 transcription thereby inhibiting canonical Wnt signaling	[96]
5-aza	Pan DNMT inhibitor	Fibroblasts[l]	Reduced collagen levels, possibly through increasing miR-135b	[91]
5-aza	Pan DNMT inhibitor	Fibroblasts[d]	Significantly increased antifibrotic miR-150	[127]
5-aza	Pan DNMT inhibitor	Lung fibroblasts	Normalized COX-2, PGE2, and COL1A1 expression, and sensitivity to FasL-induced apoptosis	[99]
Ciprofloxacin	fluoroquinolone antibiotic	Fibroblasts[d]/Lung fibroblasts	Its antifibrotic properties was partly due to downregulation of DNMT1 and upregulation of Fli1	[92]
TSA	Pan-HDAC inhibitor	Fibroblasts/Animal model	Its antifibrotic effect was in part due to inhibition of Smad and cell proliferation	[109]
TSA	Pan-HDAC inhibitor	Fibroblasts	Increased HDAC3, CTGF, and ICAM-1 but decreased HDAC7, COL1A1, and COL3A1 expression	[108]
TSA	Pan-HDAC inhibitor	Fibroblasts/Animal model	TSA resulted in normalization of WIF1 and COL1A1 expression on SSc fibroblasts. In bleomycin model it prevented WIF1 loss, β-catenin accumulation, and collagen levels	[119]
Divalproex Sodium	Pan-HDAC inhibitor	Digital ulcers	This case study reported beneficial effect of this drug in a limited SSc patient	[196]
Cytosporone-B	NR4A1 agonist	Animal model	Lack of active NR4A1 resulted in persistent TGFβ activation. NR4A1 agonist inhibited TGFβ and alleviated fibrosis in animal model	[118]
Resveratrol	SIRT activator	Fibroblasts[d]/Animal model	Antifibrotic property possibly through suppressing the TGFβ-p300 pathway	[114]
DZNep	EZH2 inhibitor	Fibroblasts[d]/Animal model	Profibrotic properties through induction of Fra-2	[110]
DZNep	EZH2 inhibitor	Fibroblasts[d]	Antifibrotic properties through reduction of Fra-2, TGFβ, and affecting the DNA methylome	[107]
let-7a	miRNA	Animal model	IP injection of let-7a improved skin fibrosis induced by bleomycin in mice	[133]
Imatinib	Tyrosine kinase inhibitor	Fibroblasts/Animal model	Restored miR-29a expression	[120]
Bortezomib	Proteosome inhibitor	Animal model	Anti-fibrotic effect through restoring miR-21 levels and Smad7 in bleomycin model	[129]

Compound	Specificity	Cell type	Biological effect	Refs
Serum exosomes	exosomes	Animal model	Accelerated wound healing in mice, possibly through miRNA-mediated collagen synthesis	[155]
antagomiR-155	miR-155 inhibitor	Animal model	Topical application effectively treated bleomycin-induced skin fibrosis possibly through inhibition of the Wnt/β-catenin and Akt pathway	[137]

d=diffuse SSc; l=limited SSc;

¶Dermal unless specified