Epigenetics and systemic sclerosis: An answer to disease onset and evolution?

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Abstract

There is growing evidence that implicates epigenetic modification in the pathogenesis of systemic sclerosis (SSc). The complexity of epigenetic regulation and its dynamic nature complicate the investigation of its role in the disease. We will review the current literature for factors that link epigenetics to SSc by discussing DNA methylation, histone acetylation and methylation, and non-coding RNAs (ncRNAs), particularly microRNA changes in endothelial cells, fibroblasts (FBs), and lymphocytes. These three cell types are significantly involved in the early stages and throughout the course of the disease and are particularly vulnerable to epigenetic regulation. The pathogenesis of SSc is likely related to modifications of the epigenome by environmental signals in individuals with a specific genetic makeup. The epigenome is an attractive therapeutic target; however, successful epigenetics-based treatments require a better understanding of the molecular mechanisms controlling the epigenome and its alteration in the disease.

Keywords: Epigenetics, systemic sclerosis, angiogenesis, autoimmune disease, MicroRNAs DNA methylation

Introduction

Systemic sclerosis (SSc) is a chronic, heterogeneous multisystem autoimmune connective tissue disease. It is characterized by three pathological processes: vascular injury and endothelial dysfunction, resulting in vascular intimal proliferation and remodeling, vasoconstriction, and defective angiogenesis; immune dysregulation, resulting in cell-mediated immunity and autoantibodies production; and fibroblast (FB) activation, resulting in excessive collagen and extracellular matrix (ECM) production and accumulation in the skin and other organs (1-4). Of note, vascular manifestations can precede other disease manifestations by several years. The pathogenesis of SSc is still unclear. The disease occurs in a multi-step process involving interaction between genetic and environmental factors in a genetically susceptible individual. This process starts with microvascular endothelial cell dysfunction and overexpression of adhesion molecules and chemokines, attracting diverse types of immune cells, including T cells and activated B cells. These cells release their cytokines when they accumulate in the tissue, stimulating FBs to produce excessive amounts of collagen and other ECM components (5).

SSc is classified as limited cutaneous SSc (lcSSc) when it affects limited portions of skin and has minimal systemic involvement and as diffuse cutaneous SSc (dcSSc) when it affects large portions of skin and involves multiple internal organs. SSc is more prevalent in women with an overall female-to-male ratio of 3:1 or greater and marked ethnic differences (1, 6). There is no clear causative factor for SSc. Genetics plays an important role in the pathogenesis of SSc; however, despite the identification of multiple genetic risk loci such as the major histocompatibility complex (MHC) II (7), which are associated with increased susceptibility to the disease, genetic factors alone do not explain the occurrence of the disease (1-4, 8). Of note, vascular manifestations can precede other disease manifestations by several years. The pathogenesis of SSc is still unclear. The disease occurs in a multi-step process involving interaction between genetic and environmental factors in a genetically susceptible individual. This process starts with microvascular endothelial cell dysfunction and overexpression of adhesion molecules and chemokines, attracting diverse types of immune cells, including T cells and activated B cells. These cells release their cytokines when they accumulate in the tissue, stimulating FBs to produce excessive amounts of collagen and other ECM components (5).

Epigenetics is defined as heritable variations in gene expression patterns without alteration in the DNA sequence. These modifications are accomplished through various mechanisms, including DNA methyl-
DNA methylation
DNA methylation is the process by which a -CH₃ (methyl) group, derived from S-adenosyl-L-methionine, covalently binds to position 5 in the cytosine ring within the CpG dinucleotides in the DNA. This process is catalyzed by DNA methyltransferases (DNMTs), namely DNMT1, DNMT3a, and DNMT3b. Even DNMT3L belongs to this group; however, it has no catalytic activity. Nonetheless, it stimulates de novo methylation of cytosine by DNMT3a and DNMT3b (15). DNMT3a and DNMT3b play important roles in the de novo methylation and generation of specific DNA methylation patterns. DNMT3a has a significant role in genomic imprinting during gametogenesis (17-19), whereas DNMT3b has an important role in embryonic development. DNMT1 is responsible for the maintenance of DNA methylation patterns during replication, ensuring faithful inheritance of epigenetic changes during replication and mitotic divisions (20). Therefore, any abnormalities or changes in the inherited methylation patterns can be attributed to DNMT1 dysfunction (21). DNA methylation is the proposed mechanism for genomic imprinting and X chromosome inactivation (21, 22).

DNA methylation of the cytosine in the CpG sites marks the heterochromatin closed structure, resulting in transcriptional silencing by direct physical interference with the binding of transcriptional factors or by binding to methyl-CpG-binding proteins such as MeCP2 and methylated DNA-binding domain (MBD)-containing proteins (23). DNA methylation can be reversed by a demethylation process that can be active or passive. Active demethylation is mediated by 10-11 translocation oxidases and passive demethylation occurs through replication in the absence of DNMT1 activity (2). The demethylation of CpG islands in the gene promoter region results in increased transcriptional activity.

DNA methylation plays a significant role in SSC pathogenesis as described above in the three key cells associated with disease pathogenesis—FBs, endothelial cells, and lymphocytes. These cells mediate the major SSC manifestations of fibrosis, vasculopathy, and immune dysregulation.

Main Points
- Systemic sclerosis is the result of complex interaction between genetic susceptibility and environmental epigenetic factors.
- There is evidence for epigenetic changes in key pathways in the pathogenesis of the disease.
- Oxidation injury and hypoxia might be the trigger for epigenetic alterations in SSC.

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Table 1. Summary of epigenetic modifications in SSc (Continue).

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III-miRNAs anomaly

| miR-29                  | Downregulation | FB | Antifibrotic factor, putative target is type I collagen (80, 89) |
| miR-let-7a              | Downregulation | FB, serum | Putative target is type I collagen (83) |
| miR-196a                | Downregulation | FB, serum, hair shafts | Putative target is type I collagen (84, 85) |
| miR-150                 | Downregulation | FB, serum | Induction of integrin β3 (86) |
| miR-129-5p              | Downregulation | FB | Putative target is type I collagen (87) |
| miR-21                  | Overexpression | FB | Profibrotic factor, targets SMAD7 (89) |
| miR-31                  | Overexpression | Skin, FB | Putative target is type I collagen (89) |
| miR-145                 | Downregulation | Skin, FB | Putative target is SMAD3 (89) |
| miR-146                 | Overexpression | Skin, FB | Putative target is SMAD4 (89) |
| miR-503                 | Overexpression | Skin, FB | Putative target is SMAD7 (89) |
| miR-7                   | Overexpressed in SSc, down regulated in lcSSc | FB, skin, sera | Target type I collagen (91, 92) |
| miR-142                 | Overexpression | Serum | Regulation of integrin α-V expression (95) |
| miR-92-a                | Overexpression | FB, serum | Inhibits MMP-1 (96) |
| miR-483-5p              | Overexpression | FB, serum, MVECs | Promotes fibrosis, increases transcription of COL4A1 and COL4A2 in FBs and endothelial cells (97) |
| miR-152                 | Downregulation | MVECs | Overexpression of DNMT1 (98) |

All cell types are human in origin, unless otherwise specified. adiffuse SSc: blimitted SSc. ACTA: actin, alpha 2, smooth muscle, aorta; BMPRII: bone morphogenetic protein type II receptor; CDX-2: prostaglandin-endoperoxide synthase 2; DNA: deoxyribonucleic acid; DNMT1: DNA (cytosine-5-)methyltransferase 1; ECM: extracellular matrix; FLI1: Friend leukemia integration 1; FB: fibroblast; H3K27me3: trimethylation of histone H3 on lysine 27; HDACs: histone deacetylases; IgG: immunoglobulin G; MBT1: methyl-CpG-binding domain protein 1; MeCP2: methyl-CpG-binding protein 2; MMP1: matrix metalloprotease 1; MVEC: microvascular endothelial cell; NOS: nitric oxide synthase; PGE2: prostaglandin E2; PBMC: peripheral blood mononuclear cell; RNA: ribonucleic acid; RORC: RAR-related orphan receptor C; SMAD: intracellular proteins that transduce extracellular signals from TGF-β ligands; SSc: systemic sclerosis; TGF-β: transforming growth factor beta; Tregs: regulatory T cells.
genes. The hypermethylated genes identified in SSc MVECs were NO51, DNMT3A, DNMT3B, HDAC4, and ANGPT2. Gene ontology analysis demonstrated enrichment of genes involved in angiogenesis (30).

**Lymphocytes:** Both CD4+ and CD8+ cells participate in the pathogenesis of SSc. CD8+ cells infiltrate the tissue at an early phase of the inflammatory response, followed by CD4+ predominance when fibrosis is evident (31). CD4+ T cells in SSc are characterized by global hypermethylation with decreased expression and activity of DNMT1 (32). The global hypermethylation of DNA in CD4+ T cells may lead to the reactivation of endoparasitic sequences, such as LINE-1 retrotransposable elements that contribute to autoimmune responses (33). In animal models of autoimmunity, defects in the extracellular signal-regulated kinase signaling pathway in CD4+ T cells were reported (34, 35).

A significant reduction in the number of regulatory T cells (Tregs) was observed in SSc (36). Tregs are CD4+ cells with immunosuppressive activity aimed at maintaining self-tolerance, regulating immune responses, and averting autoimmunity (37, 38). The reduced number of Tregs in SSc is linked to the gene methylation status of forkhead box P3 (FOXP3) transcription factor (39). FOXP3 is a lineage-specifying factor with an important role in the differentiation and regulation of Tregs. The all-trans retinoic acid (ATRA), which is an active metabolite of vitamin A, was found to improve skin manifestations of SSc and decrease DNA methylation at the promoter region of FOXP3 gene, leading to increased levels of mRNA and protein of FOXP3 and the percentage and number of SSc Tregs. The addition of 5-azacytidine to SSc CD4+ T cells resulted in a similar effect (40). This study suggested a potential role for ATRA as a therapeutic agent in SSc and explained the epigenetic mechanism for its effect.

The maturation of B cells involves the interaction between CD40 on B cells and CD40 ligand (CD40L, also known as CD154) on CD4+ T cells. The CD40L gene is located on the inactivated X chromosome. Hypomethylation of CD40L promoter leads to the overexpression of the ligand, resulting in extensive interaction with CD40 on B cells and additional B cell activation (41, 42).

Another co-stimulatory molecule that is expressed on both B and T cells is CD70. The promoter region of CD70 in SSc CD4+ T cells was found to be hypomethylated, leading to its overexpression (41, 43). Similarly, CD11a was found to be overexpressed in SSc CD4+ T cells and its gene promoter region was found to be hypomethylated (44).

Other examples of hypomethylated genes include ACTA (45), CTKNA3, CTNND2, COL1A1, COL6A3, COL12A1, PDGF-C, TNXB, PAX9, ADAM12, and ITGA9 (46). Examples of hypermethylated genes are C8ORF4 (47), KLF5 (48), SOX20T (46), DKK1, SFRP1 (49), and RORC1 and RORC2 (50). Table 1 summarizes the biological consequences of these modifications.

It is interesting to note hyper- and hypo-methylation patterns in different cells that are likely to contribute to SSc pathogenesis. The epigenetic modification differs depending on the cell type (hypermethylation in FBs and MVECs and hyper- and hypo-methylation in CD4+ T cells). Mapping all the patterns of epigenetic modifications in cells is essential to completely understand the role of DNA methylation in the pathogenesis of SSc.

**Histone modification**

Histones are an essential part of the eukaryotic nucleosomes and are the key building blocks of chromatin (16). There are five different types of histones, which are divided into two main groups: core histones (H2A, H2B, H3, and H4) and linker histones (H1 and H5) (9). Post-translational modifications of histones occur on their N-terminal domains. These modifications include methylation, acetylation, phosphorylation, citrullination, ubiquitination, and sumoylation (2). The most studied modifications are histone acetylation and methylation. Histone acetylation results from the transfer of an acetyl group from acetyl coenzyme A to the N-terminal domain of lysines on histone H3 or H4 (4, 51, 52). This process is regulated by histone acetyltransferases (HATs) such as P300/ CBP, PCAF, and MYST; histone deacetylases (HDACs) such as class 1, 2, and 4 HDACs; and sirtuins such as SIRT1-7, which is also known as class 3 HDAC (4). Histone acetylation relaxes the chromatin structure by reducing the interaction between the positively charged histones and the negatively charged DNA, as acetylation removes the positive charge on histones. Thus, it allows transcription factors to gain access to the promoter region and initiate transcription activation (22). On the contrary, histone deacetylation represses transcription. The general acetylation state of histones is based on the balance between HATs and HDACs (19). Histone methylation can activate or inhibit gene contingent on the site of methylation and regulate the number of methyl groups that are added (2, 53). For example, methylation of histone H3 lysine 4 (H3K4) induces gene expression, whereas that of H3K9 and H3K27 induces gene repression (3, 4, 19). It is important to recognize that DNA methylation and histone modification are linked (24). Accordingly, when MBD proteins bind methylated cytosines, they recruit HDACs, resulting in heterochromatin conformation that inhibits the transcription machinery (1, 3, 54).

The role of histone methyltransferase, enhancer of zeste homolog 2 (EZH2), was recently studied in SSc FBs and endothelial cells (55). EZH2 catalyzes the trimethylation of histone H3 lysine 27 (H3K27me3) to repress transcription. This enzyme has a role in T cell differentiation, endothelial cell angiogenesis (56), and myofibroblast transformation. The levels of expression of EZH2 and H3K27me3 are elevated in SSc FBs when compared with control cells. Inhibition of EZH2 by DZNep decreases fibrosis both in vitro and in vivo. DZNep decreases the expression of EZH2, H3K27me3, COL1A1, TGFβ, FRA2, and LRRRC16A in a dose-dependent manner. Similarly, DZNep decreases the expression of DNMT1, DNMT3A, and MECP2, resulting in reduced DNA methylation. In addition, DZNep and GSK126 (another EZH2 inhibitor) prevent bleomycin-induced skin fibrosis. Exposure of SSc FBs to GSK126 results in decreased matrix gel contraction, indicating decreased myofibroblast contractility. The effect of overexpression of EZH2 in normal FBs was analyzed using a wound closure model. The results showed that the overexpression of EZH2 resulted in increased wound closure, supporting a role of EZH2 in myofibroblast contraction. Migration of FBs was negatively affected when EZH2 was overexpressed in LRRRC16A (a gene encoding cell membrane cytoskeleton-associated protein) knockdown FBs, indicating a significant role of LRRRC16A in EZH2-mediated FB migration.

Similarly, the collagen suppressor gene FLI1 was found to have more deacetylated H3 and H4 and more methylated regions in its promoter region as compared with controls. The addition of a HDAC inhibitor (trichostatin A, TSA) and a DNA methyltransferase inhibitor normalized the expression of type I collagen in SSc FBs (24). Moreover, TSA can reduce TGFB-β-induced FB activation by decreasing the nuclear translocation of SMAD3/SMAD4 and DNA binding of SMAD transcription factors (57). As TSA is a broad deacetylase inhibitor, its clinical use is limited by its safety profile; therefore, more specific HDAC inhibitors are required for clinical use. Specifically inhibiting HDAC7 using small interfering RNA resulted in decreased TGFB-β-induced accumulation of type I and type III collagen (58). Another HDAC inhibitor is suberoylanilide hydroxamic acid (SAHA), which
was found to prevent TGF-β-induced collagen deposition and FB activation (59).

Another important profibrotic factor is the HAT p300 that is regulated by SIRT1 (60). P300 modifies transcription factors affecting the regulatory region of the collagen gene. Levels of SIRT1 are significantly decreased in SSC dermal FBs compared with controls. A SIRT1 activator resulted in decreased response of FBs to TGF-β stimulation and reduced collagen production (61). However, another study revealed opposite effects of the SIRT1 activator on FB response (62); more studies are required to clarify the effects of SIRT1.

The overexpression of EZH2 in SSC endothelial cells affected cell adhesion and migration (63-65). The knockdown of EZH2 in SSC endothelial cells significantly increased angiogenesis, which is similar to the effect of the addition of DZNep to cell cultures. The treatment with DZNep upregulated the expression of notch ligands JAG1, JAG2, DLL4, notch receptor NOTCH2, and notch target gene HES1, whereas it downregulated the expression of notch signaling inhibitors NOTCH1, NOTCH3, NUMB, and FBXW7. These results suggest that EZH2 activates certain genes and inhibits others. When compared with normal endothelial cells, SSC endothelial cells showed increased levels of JAG2 and NUMB, and decreased levels of DLL4, HES1 and HEY2. Moreover, it was found that EZH2 inhibited SSC endothelial cells tube formation by repressing the notch ligand DLL4 through increased binding of EZH2 and H3K23me3 at the promoter region of DLL4 (55). The effect on tube formation was reversed when endothelial cells were treated with an EZH2 inhibitor.

Histone modifications such as increased H4 acetylation and decreased H3K methylation were associated with activating B cell genes that are responsible for the production of antibodies (66).

Histone deacetylases regulate the proliferation and migration of endothelial cells. The expression of HDAC5, an antiangiogenic factor, is significantly increased in SSC dermal MVECs, and it may play a significant role in SSC vasculopathy. Vascular damage in SSC is an early event that occurs before the onset of tissue fibrosis (67). The proposed mechanism for HDAC5 in inhibiting angiogenesis is that HDAC5 represses pro-angiogenic genes. The pro-angiogenic genes identified after HDAC5 was knockdown were FGF2, SLIT2, EPHB4, PVR2L (cell adhesion molecule that improves angiogenic ability of MVECs), FSTL1 (plays a role in fibrosis and MVEC proliferation and tube formation), and CYR61 (a member of the CCN protein family that supports angiogenesis). Moreover, knockdown of HDACS increased the levels of bFGF, which is encoded by FGF2 and increased the expression of FSTL1. Although these observations are interesting, the results infer limited potential clinical utility of HDAC inhibitors as antiangiogenic therapy because of their detrimental effects on MVECs that could potentially contribute to SSC vasculopathy. The ideal HDAC inhibitors as a potential therapeutic agent must have a specific target profile with no effects on multiple genes and multiple processes in different cell types.

Other histone modifications such as histone hypoacetylation in WIF1 (68), H3 and H4 hyper-acetylation in NRRA1 (69), and H4 hyperacetylation in COLIAZ (60) were observed. Table 1 summarizes the biological consequences.

Non-coding RNA mechanisms

Non-coding RNAs (ncRNAs) are functional RNA molecules that are transcribed from DNA but not translated into proteins. These RNA molecules are biologically active and can affect gene expression, epigenetic modulation, and post-translational modification throughout the body (4, 70). The ncRNA molecules are divided into different groups based on the number of nucleotides: long ncRNAs (lncRNAs) that have more than 200 nucleotides and can be present in both the nucleus and the cytoplasm; medium-sized ncRNAs (<200 nucleotides) that include small nucleolar RNAs (snoRNAs) and promoter-associated small RNAs (PASRs); and small ncRNAs (<50 nucleotides) that include miRNAs and PIWI-interacting RNAs (piRNAs) (4).

MicroRNAs are a group of small non-coding RNAs, ranging from 18 to 22 nucleotides that are synthesized initially as a longer precursor, which is degraded to miRNAs (71). miRNAs are involved in post-transcriptional silencing and regulation of gene expression (23, 72, 73) by binding to the complementary sequence in the 3’ prime region of the mRNA, resulting in translational repression or mRNA degradation (74-76). Therefore, the upregulation of miRNAs results in gene repression, whereas their downregulation results in gene activation. The expression of miRNAs is regulated through epigenetic mechanisms; for example, miRNAs can be silenced by DNA methylation. miRNAs differ from siRNAs in that siRNAs target a single gene, whereas miRNAs can target multiple genes (77, 78). The involvement of miRNAs in tissue fibrosis was initially reported in cardiac fibrosis after myocardial infarction. It was found that the expression of the miR-29 family decreased in the cardiac cells adjacent to the infarct area (79).

As miR-29 regulates fibrosis-related genes, its downregulation resulted in tissue fibrosis (9).

In 2010, the first study focusing on miRNA levels in SSC dermal FBs found that miR-29a was downregulated. The same finding was observed in bleomycin-induced skin fibrosis (80). Interestingly, downregulating miR-29a in normal dermal FBs increased the formation of collagen types I and III, and overexpressing it in SSC FBs decreased the expression of collagen. Furthermore, miR-29a plays an important role in liver (81) and kidney fibrosis (82). Profibrotic cytokines, such as TGF-β1 and IL-4, decrease the levels of miR-29a (9). Further analysis of miRNAs showed that in SSC skin, 9 miRNAs were upregulated and 15 miRNAs were downregulated. Of these, the expression of miR-206, miR-125b, and let-7g was confirmed by real-time polymerase chain reaction (PCR). As miR-125b functions as a regulator of multiple molecules involved in SSC pathology, including SMAD5, interleukin (IL)-1F10, IL-6R, and IL-13, its downregulation results in increased levels of these molecules. Moreover, the expression of multiple collagen-related miRNAs is decreased in SSC FBs and TGF-β-stimulated normal dermal FBs (83). Alpha 1 and 2 type I collagen are regulated by miR-196a (84) and let-7a, the expression of both is reduced in SSC FBs, both in vivo and in vitro (9). Transfection by miR-196a and let-7a inhibitors resulted in increased expression of α 1 and α 2 type I collagens, whereas transfection with their mimics resulted in decreased expression. It is suggested that the activation of TGF-β in SSC dermal FBs results in miR-196a and let-7a downregulation, which in turn upregulates the expression of collagen. Interestingly, levels of miR-196a decreased in shafts of hairs obtained from patients with SSC (85).

Another miRNA with an important role in SSC is miR-150 that is underepressed in SSC (86). MiR-150 is a regulator of intergrin-B3, a known inducer of TGF-β. Interestingly, the overexpression of miR-150 in SSC dermal FBs resulted in decreased integrin-B3, phosphorylated SMAD3, and type I collagen deposition. Knocking down miR-150 resulted in opposite changes.

IL-17A is known to have antifibrogenic effects; it stimulates the overexpression of miR-129-5p, which in turn downregulates the production of α 1 type I collagen. IL-17 receptor is downregulated in SSC FBs in association with decreased expression of miR-129-5p and overproduction of α 1 type I collagen (87).

Circulating levels of miRNAs are proposed as sensitive biomarkers of disease activity, as changes in miRNAs appear earlier than those in
levels were associated with higher mRSS scores. Of note, miR-152 (98), Table 1 summarizes their biological consequences.

The future of miRNAs as a therapeutic option for SSc is promising. However, this approach needs to overcome several obstacles. The most troubling is the potential for miRNAs to alter the function of several genes that may result in undesirable outcomes. Moreover, appropriate dosing and the method of delivery are other obstacles in this emerging field.

Figure 1. An overview of the effect of epigenetics on immune cells (T and B cells), fibroblasts, and endothelial cells that contribute to the pathogenesis of SSC. Histone acetylation switches chromatin configuration from condensed to relaxed, permitting the transcription machinery to access the DNA to initiate transcription. This process is catalyzed by histone acetyltransferases and reversed by histone deacetylases. DNA methylation results in transcription repression, as the addition of methyl groups to DNA prevents transcription factors from accessing the DNA. This process is catalyzed by DNA methyltransferases and reversed by DNA demethylases. Inhibition of gene expression by miRNAs through translational repression and degradation of mRNA. MiRNAs can upregulate profibrotic molecules or downregulate antifibrotic molecules. The results of epigenetic alterations in SSc are the activation of the immune system leading to autoimmunity; increased collagen and ECM production resulting in tissue/organ fibrosis; and endothelial cell injury and vascular dysfunction. The figure was created using BioRender.com.

Conclusion

In this review, we provided evidence for a key role of epigenetic regulation in the pathogenesis of SSc involving disparities in DNA methylation, anomalies in the histone code, and altered expression of miRNAs in different tissues and cell types (Figure 1). Although it is likely that environmental cues trigger epigenetic regulatory mechanisms, this needs to be confirmed in detail, possibly in a longitudinal cohort study starting with epigenetic profiling of individuals at risk of developing SSc and repeating the epigenetic profile for those who develop the disease. This would provide a better understanding of how environmental stimuli interact and trigger the epigenetic regulatory mechanisms. Furthermore, this approach will provide us a better understanding of whether these epigenetic variations among individuals are a cause or a result of the disease process. In addition, we should develop an experimental model of SSc that we can use for further analysis to obtain an epigenetic map for each cell type involved in the disease process, including endothelial cells, T cells, and FBs. A huge collaborative effort, similar to genome-wide association studies, is required to reveal the epigenetic map. With the ever-expanding discoveries of epigenetic targets, understanding the epigenetic basis of SSc is important for finding potential therapeutics. It is possible that in the near future, epigenetic research may lead to the development of epigenomic tools that can both uncover the risk and offer effective therapeutic options.
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