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Review

Epigenetics in autoimmune connective tissue diseases

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Summary. *Background.* Autoimmune connective tissue diseases (ACTDs) encompass a heterogeneous group of chronic immune-mediated inflammatory disorders, primarily affecting connective tissues and clinically characterized by variable multisystem manifestations, frequently overlapping. Environmental factors are thought to promote ACTD development in genetic predisposing/endocrine permissive background through the induction of epigenetic modifications, consisting of stable, heritable, but potentially reversible changes in gene expression, occurring without alterations of the DNA sequence. Actually, epigenetic mechanisms (such as histone modifications, DNA methylation, nucleosome positioning, and RNA interference) link genotype upstream and phenotype downstream, and, if persistently aberrant, may cause a variety of human diseases, including ACTDs. We aimed to review the recent advances in the knowledge of the ACTD epigenetic alterations. *Methods:* A detailed search of the available literature was performed in the PubMed (U.S. National Library of Medicine) database. *Results:* Growing evidence underlines the relevant role of epigenetic defects in the ACTD pathogenesis, and specific epigenetic patterns can represent disease biomarkers. In patients with rheumatoid arthritis (RA), epigenetic variations interact determining the typical "aggressive" phenotype displayed by RA synovial fibroblasts. Epigenetic modifications are involved in the profibrotic process that characterizes systemic sclerosis. In systemic lupus erythematosus and Sjögren's syndrome, complex epigenetic changes altering gene expression have been demonstrated. *Conclusions:* Comprehensive studies will contribute to further define the aberrant epigenetic mechanisms involved in the ACTDs etiopathogenesis. Moreover, being epigenetic changes potentially reversible, the identification of ACTDs epigenetic biomarkers will allow the development of therapeutic strategies addressed to target dysregulated genes and correct aberrant epigenomic alterations.

Key words: Epigenetics, epigenotype, epigenome, epigenetic changes, histone acetylation, DNA methylation, RNA interference, microRNA, autoimmunity, autoimmune connective tissue disease, inflammatory rheumatic diseases

Introduction

Autoimmune connective tissue diseases (ACTDs) encompass a heterogeneous group of chronic immune-mediated inflammatory disorders, primarily affecting connective tissues and clinically characterized by variable multisystem manifestations, frequently overlapping (1). A complex interactions among genetic factors, endocrine status, and environmental triggering agents is likely involved in their etiopathogenesis: environmental factors are thought to promote the ACTD development in genetic predisposing/endocrine permissive background through the induction of epigenetic modifications (or "epimutations"), that consist of stable, heritable, but potentially reversible changes in gene expression, occurring without alterations of DNA sequence (and in that differentiating from genetic mutations) (2,3).

Epigenetic mechanisms constitute the molecular processes that convert genetic information into observable traits through the modulation of gene activation/silencing, and collectively define the "epigeno-

type", which interfaces genotype upstream and phenotype downstream, conditioning both physiological and pathological processes (2,4) (Figure 1).

Whereas genetic information (genome) is homogeneously and permanently shared by all the cells in an organism (except for eventually occurring somatic mutations), epigenetic pattern (epigenome) is lineage-specific, determining the cell-type identity and development, and may vary over time according to the environmental context, so conferring metabolic plasticity to cells (4-6). Actually, under the pressure of environmental factors (including lifestyle, dietary factors, drugs, pollutants, heavy metals, radiations, pathogens, etc), epigenetic variations progressively accumulate during lifetime, biochemically reflecting the individual biological experience. The age-related epimutations have been studied in a large cohort of monozygotic (MZ) twins: while genetically identical twins are epigenetically indistinguishable in the early years of life, older MZ twins exhibit remarkable differences (6). These inter-individual epigenetic diversities can also explain the discordant disease susceptibility observed in identical twins (6,7).

The main mechanisms that define the cell epigenotype are represented by histone modifications, deoxyribonucleic acid (DNA) methylation, ribonucleic acid (RNA) interference, and nucleosome positioning (8-12).

Histones, categorized as core histones (H2A, H2B, H3, and H4) and linker histones (H1 and H5), are conserved proteins that package and organize DNA. A core histone octamer, composed of a central H3-H4 heterotetramer, flanked by two H2A and H2B heterodimers, is wrapped by two turns of DNA specific sequences (each composed of 147 base pairs) to form the nucleosomes, the basic chromatine subunits (8) (Figure 2 and 3). Nucleosomes are interconnected by linker DNA chains, constituting "beads-on-a-string" structures of 10-11 nanometers (nm) in diameter (Figure 2 and 3). These chromatin fibers are then folded into progressively more condensed arrangements (from 30 nm to 100-700 nm thick) by H1 and H5 linker histones, ultimately shaping the highly

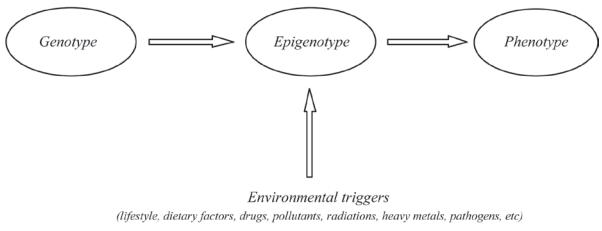

Figure 1. Epigenotype interfaces genotype upstream and phenotype downstream

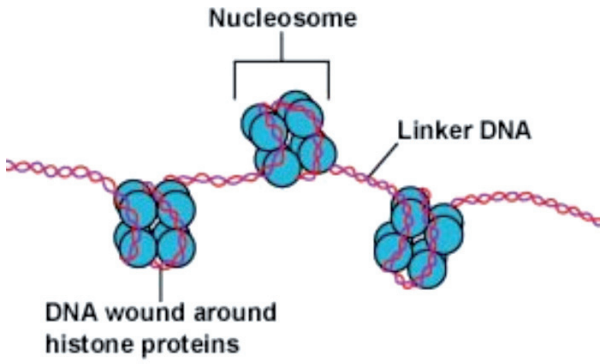

Figure 2. The nucleosome structure

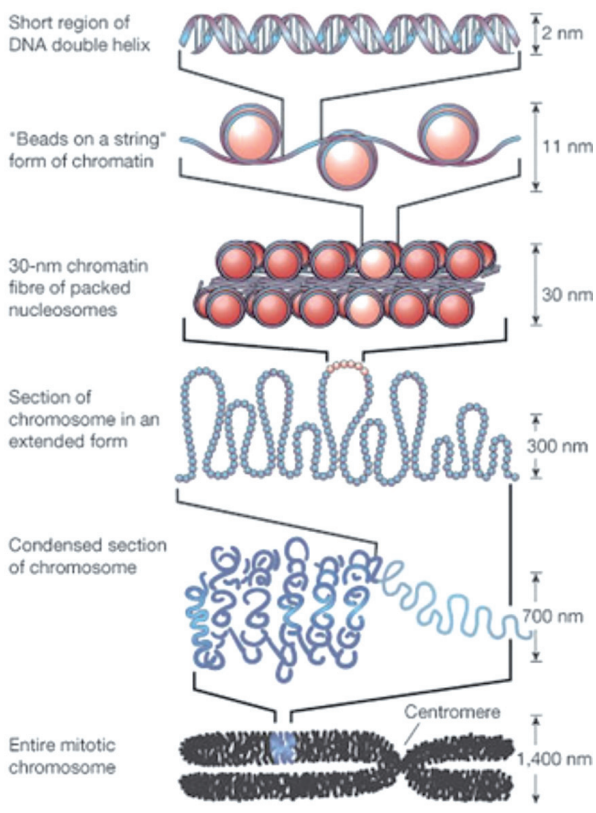

Figure 3. The chromosome structural organization

aggregated building blocks of the chromosome (that during cell division has a diameter of 1,400 nm) (8-10) (Figure 3). In its basic conformational state, chromatin displays a strongly packed and transcriptionally silenced structure that sterically hinders the access of polymerases (11).

Changes in chromatin organization primarily depend on histone reversible site-specific post-translational modifications, among which acetylation is one of the most studied. Histone acetylation (operated by acetyltransferases at the ε amino groups of lysine residues in the N-termini) neutralizes lysine's positive charge, consequently weakening the electrostatic interaction with the negatively charged DNA, and so leads to a more "open" chromatin conformation that allows transcriptase accessibility (8,11). Conversely, the targeted deacetylation by histone deacetylases (HDACs) [including the ubiquitously expressed class I HDACs (HDACs 1-3 and 8), the tissue-restricted class II HDACs (HDACs 4-7, 9, 10), the class III (sirtuins 1-7) and the class IV (HDAC-11)] compacts the chromatin structure and induces gene silencing (8,11,13,14). Ultimately, histone acetylation is generally associated with activation of gene expression, and deacetylation with repression; these processes are normally in dynamic equilibrium, tightly regulating DNA transcription, replication, and repair.

Other than acetylation/deacetylation, histones may undergo methylation, phosphorylation, ubiquitylation, sumoylation, and ribosylation (8,9). While phosphorylation, ubiquitylation, sumoylation, and ribosylation are usually related to DNA transcription and repair, the histone methylation consequences vary depending on the number and location of the methyl groups (15).

DNA methylation is an epigenetic covalent modification that induces heritable gene silencing (9,16). In prokaryotes, DNA methylation occurs on cytosine and adenosine residues, whereas in eukaryotes it is restricted at position 5 of cytosines followed by guanines (the CpG dinucleotides). DNA methylation is mediated by the DNA methyltransferase (DNMT) family, being DNMT1 the predominant enzyme, while demethylation depends on the activity of cytosine deaminases (16). DNMTs use S-adenosylmethionine (SAM) as a donor of methyls, whose generation is mainly modulated by nutritional folic acid. Briefly, folate is metabolized to dihydrofolate and tetrahydrofolate (THF); vitamin B_{12} facilitates the generation of methyl-THF, which, in turn, catalyzes the conversion of homocysteine to methionine. Methionine is further metabolized by the methionine adenosyltransferase to SAM, the substrate for DNMTs (16).

CpG dinucleotides, the sites of the eukaryotic methylation, are underrepresented in human DNA, but clusters of CpGs (called CpG islands) are electively located in promoter and first exon of about half of all genes (17). Their methylation triggers gene repression directly interfering with transcription factor binding to recognition sites on DNA; in addition, methyl-CpG binding domain proteins can reinforce silencing by recruiting co-repressor complexes that harbor HDACs or histone methyltransferases. In physiological situations, most promoter CpG islands are demethylated (allowing transcription to proceed), whereas CpG sites located in repetitive sequences are methylated (contributing to genomic stability) (9,17).

DNA methylation tightly controls cell differentiation/development (9) and responses to environmental stimuli, such as hypoxia (18), viral latency and reactivation (19), or hormonal signaling (20). Hypermethylation plays a pivotal role in genomic imprinting (namely, the modulation of one of the two maternally or paternally inherited alleles, that results in monoallelic gene expression) (9,21), as well as in female X-chromosome inactivation (22), possibly contributing to the observed autoimmunity predisposition of female gender (23). Conversely, DNA hypomethylation is a major contributor to both genomic instability and generation of disease-causing germline mutations or somatic mutations that cause oncogenesis (15,17).

RNA interference is an evolutionarily conserved mechanism involved in the post-transcriptional regulation of gene expression. It is carried out by "non-coding" RNAs (ncRNAs), categorized as small (<200 nucleotides) and large (>200 nucleotides) ncRNAs. While large ncRNAs show poor sequence conservation across species and less clearly defined functions, small ncRNAs are highly evolutionarily conserved and display well characterized activity, generally functioning as negative regulators of gene expression (15). The small ncRNA family includes piwi-interacting RNAs

(piRNAs), short interfering RNAs (siRNAs), and microRNAs (miRNAs).

piRNAs derive from single stranded precursors and exert their functions primarily in the germline, while siRNAs (21-23 nucleotides) are generated from longer double-stranded (ds) RNAs by the ribonuclease (RNase) III cleavage, and induce sequence-specific mRNA degradation (15,24).

miRNA are small single-stranded ncRNA molecules (21-25 nucleotides) representing the main actors of RNA interference (25). After transcription in the nucleus, long primary miRNAs are processed by the Drosha RNase into approximately 70-nucleotide precursor miRNAs (pre-miRNAs). Then, the pre-miRNA is transferred into the cytoplasm by Exportin, where the Dicer RNase cleaves it into approximately 21-nucleotide miRNA duplex (miRNA/miRNA*), structurally similar to siRNA. This duplex is unwound by an helicase, and, while a strand (miRNA*) is usually released and degraded, the other is loaded into the RNA-induced silencing complex, where it binds the 3'-untranslated region (UTR) of its target messenger RNA (mRNA) by base-paring, negatively influencing both mRNA stability and translation (26). Each miRNA may suppress multiple mRNA targets (average 200), and each mRNA may be targeted by many miRNAs. miRNAs can also bind to the 5'-UTR region of mRNA and to protein coding sequences, albeit causing relatively weak translational repression (27).

Owing to about 30% of human genes are their putative targets, miRNAs can influence a variety of biological processes, including cell differentiation, proliferation, and apoptosis (25). They play a crucial role in hematopoiesis, inflammation, and oncogenesis, as well as in the immune cell differentiation, development, survival, and functions (9,25,28-32) (Table 1).

Nucleosome positioning in regions critical for gene regulation or other specific chromosome functions is precisely encoded in eukaryotic genomes. The occupation of transcription start sites (TSSs) by nucleosomes induces gene repression, whereas their displacement from TSSs correlates with gene activation. Moreover, the 5' and 3' ends of genes possess nucleosome-free regions, needed to provide space for transcriptional machinery assembly/disassembly (10). Nucleosome positioning is influenced by histone affinity for particular DNA sequences, by histone post-translational modifications, and by chromatin remodeling complexes. These chromatin remodelers are large macromolecular ATP-dependent molecules that may over-ride preferences in sequence, and eject, slide, restructure nucleosomes, or move them to new locations whenever needed (10,33).

Due to the pivotal role carried out in physiologic processes by the above mentioned epigenetic mechanisms, it is not surprising that alterations of the epigenetic machinery may cover a wide human disease continuum, including malignancies, neurodevelopmental and neurodegenerative diseases, and autoimmune disorders (12,23,34-40).

Oncogenesis (entailing autarky in growth signals, insensitivity to growth-restrictive signals, evasion of apoptosis, limitless potential to replicate, sustained angiogenesis, and tissue invasion) is strictly linked to abnormal epigenetic regulation of gene expression, and epigenetic defects have been demonstrated in all types of cancer, such as aberrant histone acetylation, DNA hypermethylation (leading to silencing of tumor-suppressor genes), DNA hypomethylation (inducing overexpression of proto-oncogenes), or oncogenic miRNA interference (that can promote cancer progression by targeting tumor suppressor, apoptotic, or differentiation genes) (12,35-37). Notably, miRNA profiles are valuable in accurately distinguishing tumor tissue from benign tissue and premalignant lesions from malignant ones (36); moreover, circulating miRNAs are considered as stable blood-based markers for cancer detection (41).

Well-recognized epigenetic defects characterize neurodevelopmental disorders, such as Prader-Willi syndrome (PWS) and Angelman syndrome (AS), that are caused by genetic and epigenetic mechanisms involving the proximal long arm of chromosome 15: the lack of a functional paternal copy of a gene within 15q11-q13 induces PWS, while AS is due to the absence of a functional maternal copy (38).

Epigenetic changes have been also described in various autoimmune disorders, such as multiple sclerosis (23) and ACTDs (23,40,42-46). In this paper, we aimed to review the recent acquisitions as regards the epigenetic alterations involved in the ACTDs etiopathogenesis, focusing on rheumatoid arthritis (RA),

systemic sclerosis (SSc), systemic lupus erythematosus (SLE), and primary Sjögren's syndrome (pSS) epigenomic backgrounds.

Methods

A detailed search of the available literature was performed in the PubMed (United States National Library of Medicine) database, using the following key words: epigenetics, epigenetic changes, histone modifications, histone acetylation, DNA methylation, RNA interference, microRNA, nucleosome positioning, autoimmune connective tissue disease (and relative syndromal names), inflammatory rheumatic diseases.

Results

Rheumatoid arthritis

RA is a chronic polyarticular disorder, whose hallmarks are represented by progressive inflammation and

Table 1. miRNAs involved in immune functions

miRNAs	Functions
miR-155	Regulates macrophages/monocyte response to bacterial and viral infection Involved in B and T cell differentiation Regulates germinal centre B cell response Regulates immunoglobulin production Regulates acute inflammatory response (IL-8 and RANTES/CCL5 release)
miR-150	Involved in B and T cell development
miR-181	Involved in B and T cell development
miR-146	Involved in immune cell development; drives Th1-effector cell specificity; regulates acute inflammatory response (IL-8 and RANTES/CCL5 release); miR-146a suppresses nuclear factor-kappa B (NF-kB) activity and the lipopolysaccharide (LPS)-induced inflammatory response
miR-196b	Modulates haematopoietic stem cell (HSC) homeostasis and lineage commitment
miR-223	Involved in maturation of promyelocytic precursors into granulocytes; negatively regulates the proliferation/activation of neutrophils
miR-221/miR-222	Regulates haematopoietic cell proliferation and engraftment
miR-126	Enhances colony formation in vitro and may promote the production of downstream progenitors by HSCs
miR-17-92 cluster	Enhances T cell survival during development
miR-326	Promotes Th17 cell development
miR-142-3p	Increases suppressor function of T regulatory cells
miR-424	Promotes monocyte differentiation
miR-17-5p/-20a/-106°	Promotes monocyte differentiation
miR-9	Regulates NF-κB expression during TLR4-mediated activation
miR-21	Suppresses inflammatory pathway activation in myeloid cells
miR-16	Restriction of inflammatory mediators production; regulates immunity through the cooperation with other miRNAs
miR-34	Perturbs B cell development
miR-122	Required for hepatitis C virus replication
miR-196, miR-296, miR-351, miR-431, miR-448	Antiviral defense; the over-expression of these miRNAs in infected cells attenuates the viral replication

hyperplasia of the synovium, with consequent erosion of articular cartilage and adjacent subchondral bone, resulting in severe pain, functional impairment, and, ultimately, disability. The genetic basis of RA is supported by the identification of more than 30 susceptibility genetic variants, that, however, individually make only a slight contribution to the risk of disease (47), suggesting the intervention of epigenetic mechanisms (40,42).

Changes of the three main processes of the epigenetic control (histone modifications, DNA methylation and miRNA interference) have been found to interact in the development of the "intrinsically" activated and aggressive phenotype displayed by RA synovial fibroblasts (RASFs), the effector cells of cartilage and bone destruction. The RASF activated state, characterized by the increased production of matrix-degrading enzymes and adhesion molecules, is conserved over long-term passage *in vitro* due to epigenetic imprinting (48).

Histone modifications

Some epigenetic studies reported the decreased expression of HDACs in RA synovial tissue, resulting in histone hyperacetylation (49,50). However, a recent study demonstrated that nuclear HDAC-1 is specifically up-regulated in RA synovial tissue, and positively related to the amount of cytoplasmic tumor necrosis factor (TNF)-α, suggesting that the HDAC-1 over-expression might be associated with enhanced inflammatory activity (51).

Peripheral blood mononuclear cells (PBMCs) of RA patients exhibit significantly enhanced HDAC activity, compared to PBMCs from healthy individuals, and the increase remains unaltered after 12 weeks of etanercept therapy (52).

As revealed by genome-wide microarray analyses, HDACs play a critical role in the expression of host defense genes, such as pattern-recognition receptors, adaptor molecules, kinases, transcription regulators, complement factors, cytokines, chemokines, and growth factors (53). Therefore, the potential therapeutic application of HDAC inhibitors (HDACis) in RA has been suggested (54-57): HDACis have been found to suppress synovial macrophage inflammatory activation, and production of interleukin (IL)-6 and TNF-α by RA synovial macrophages and synovial tissue explants, as well as to uniformly ameliorate inflammation and prevent joint destruction in animal models of arthritis (56,57). These compounds can interfere with the inflammatory cytokine synthesis also via regulation of mRNA stability: recently, it has been observed that, in RASFs cultures, the HDACi treatment enhances IL-6 mRNA degradation (57).

DNA methylation

Notably, DNA demethylation of normal SFs was found to induce a cellular phenotype resembling that of activated RASFs; in addition, a global genomic hypomethylation was demonstrated in RA synovial tissue (58,59), and was conserved *in vitro* even after >5 passages (58).

The genomic hypomethylation was proven to depend on the reduced expression of DNMT1 in RASFs, either in unstimulated cells (58,59) or after exposure to proinflammatory cytokines (58). One hundred eighty-six genes resulted to be up-regulated >2-fold by hypomethylation in RASFs, consistent with the increased expression of multiple receptors, adhesion molecules, and matrix-degrading enzymes, that play a pivotal role in RA (58).

As compared with osteoarthritis (OA) controls, the CpG islands of the death receptor 3 (DR3) gene (*DR3*) promoter are highly methylated in RA synovial cells and in synovial tissue lymphocyte fraction, with consequent down-modulated *DR3* activation and significantly reduced expression of DR-3 protein (60). DR-3 protein is a member of the apoptosis-inducing TNF receptor superfamily, that includes the apoptosis-inducing death receptor Fas, known to play an important role in peripheral deletion of potentially autoreactive T cells and B cells (61). Thus, decreased expression of DR-3 protein could be responsible for the defective apoptosis in rheumatoid synovium (60).

Comparing DNA methylation of the *IL-6* gene promoter in PBMCs from RA patients and healthy controls, a specific CpG site (-1099C) was found to show a lower level of methylation in RA cells, with consequently higher IL-6 mRNA levels, suggesting that hypomethylation at this site might contribute to the risk of developing the disease (62). Moreover, in PBMCs from RA patients compared with healthy controls, the proximal CpG at -145 of the *IL-10* gene was noted to be significantly demethylated, correlating with higher mRNA and serum level of IL-10 (63).

RNA interference

The up-regulation of specific miRNAs (miR-16, miR-132, miR-146a, and miR-155) was demonstrated in PBMCs (64,65) and in synovial tissue (65,66) of RA patients. In patients with RA, compared to OA patients and healthy controls, miR-146a was strongly up-regulated not only in synovial tissue, SFs, macrophages, B cells, and $CD3^+$ T cells along the superficial and sublining layers, but also in $CD4^+$ T cells from the synovial fluid (65,66). In RA synovial tissue, the enhanced expression of miR-155 was shown to correlate with decreased levels of matrix metalloproteinase (MMP)-3, and to block the induction of MMP-1 and MMP-3 after stimulation with proinflammatory cytokines and Toll-like receptor (TLR) ligands, suggesting that miR-155 might play a role in the modulation of the RASF destructive behavior (66). Not surprisingly, among the miRNAs over-expressed in RA patients, both miR-146a and miR-155 are involved in the development and functions of innate and adaptive immune cells (67,68) (Table 1).

The synovial fluid concentrations of miR-16, miR-146a, miR-155 and miR-223 resulted significantly higher in RA than in OA patients (69). Moreover, miR-223 was over-expressed in RASFs and $CD4^+$ naive T-lymphocytes from RA patients, compared with healthy donors (70).

The high levels of miR-146a observed in PBMCs of RA patients were found to positively correlate with the disease activity (64,71), and to be strongly associated with IL-17 expression in both PMBCs and synovium, especially at the early stage of the disease (71). The enhanced expression of miR-146a might reflect a defense mechanism, owing to the miR-146 family (including miR-146a, whose gene resides on human chromosome 5, and miR-146b, whose gene is on human chromosome 10) plays a critical role as negative regulator of inflammatory and innate/adaptive immune responses (28,68,72,73). As a matter of fact, miR-146a controls TLR and cytokine signaling and suppresses both NF-κB activity and the LPS-induced inflammatory response (72,74) (Table 1).

The abnormal expression of other miRNAs in RA has been further documented, resulting mostly up-regulated (71,75,76). miR-150 was found to be intensely expressed by PBMCs of RA patients with severe joint destruction (71). Recently, the basal levels of miR-203 were reported to be increased in RASFs compared with OASFs, and were shown to correlate with enhanced production of MMP-1 and IL-6 (76). Conversely, miR-124a, involved in the control of synoviocyte proliferation and production of monocyte chemoattractant protein 1, is down-regulated in RASFs (77).

In RASFs, a methylation-dependent specific down-regulation of the proapoptotic miR-34a* was demonstrated, conditioning the resistance to apoptosis of these cells (78). Actually, the promoter of miR-34a/34a* was hypermethylated, and neither TNF-α, IL-1β, TLR ligands, nor hypoxia altered its expression, whereas the gene transcription was induced upon treatment with demethylating agents levels (78).

Ultimately, a complex picture of altered expression of miRNAs emerges in RA, having functional relevance in the development of the disease phenotype.

Systemic sclerosis

SSc, also known as scleroderma, is a complex multisystem disorder, characterized by severe fibrosis of the skin and internal organs (79). SSc fibroblasts exhibit altered phenotype defined by excessive deposition of extracellular matrix and reduced expression of matrix-degrading enzymes. This profibrotic phenotype is stable in multiple generations of SSc fibroblasts, suggesting the intervention of epigenetic imprinting (79).

Histone modifications

Changes in the expression of HDACs were found to play a crucial role in SSc (80-83). The nonselective HDACi trichostatin A (TSA) displays potent antifibrogenic effects on SSc skin fibroblasts *in vitro*, and abrogates the stimulating effects of profibrotic cytokines [such as transforming growth factor β (TGF-β), platelet-derived growth factor (PDGF), and IL-4] on the extracellular matrix production; in addition, *in vivo*, in a mouse model of bleomycin-induced fibrosis, TSA prevents the development of skin fibrosis, as quantified by changes to the dermal thickness within the bleomycin injection sites (80). The treatment with TSA almost completely inhibited the transcription of the class II HDAC-7 in SSc fibroblasts *in vitro*, while the class I HDAC-3 was significantly up-regulated; further, the silencing of HDAC-7

decreased the constitutive and cytokine-induced production of type I and type III collagen (81).

Friend leukemia integration factor 1 (Fli-1), a negative regulator of collagen synthesis, is consistently absent from fibroblasts and significantly reduced in endothelial cells of clinically involved scleroderma skin (82,83). In SSc fibroblasts, compared with controls, the *FLI1* gene was proven to be epigenetically repressed, due to significant deacetylation of H3 and H4 histones in its promoter region, as a consequence of significantly higher levels of HDAC-1 and HDAC-6 (82,83).

The transcriptional coactivator p300 is a ubiquitous nuclear phosphoprotein, which acts as transcriptional cofactor with intrinsic acetyltransferase activity, controls the expression of numerous genes, and regulates cellular proliferation, apoptosis and embryogenesis; its expression is significantly elevated in SSc skin fibroblasts, and seems to be implicated in the TGF-β-induced fibrotic responses (84).

DNA methylation

In SSc fibroblast cultures, compared with control cells, an increased methylation of CpG islands in the promoter region of *FLI1* was observed and conserved for multiple generations, whereas no detectable methylation was noted in control fibroblasts (82). Similar results were demonstrated in freshly obtained SSc skin biopsy specimens, compared with controls, as well as in the cloned DNA from these cell-lines (82).

A global DNA hypomethylation was proven in $CD4^+$ T cells of patients with SSc, in comparison with controls (85).

The interaction between CD40 (TNF receptor superfamily member 5) and CD40 ligand (CD40L, CD154) is likely involved in the development of SSc, and the CD40L expression is significantly elevated in $CD4^+$ T cells of female SSc patients, relative to controls, due to hypomethylation of the related DNA regulatory sequences (86). In contrast, no significant difference was observed in the expression of CD40L between male patients with SSc and male control subjects. Of note, the DNA demethylation in healthy women reactivates the silent X chromosome, resulting in CD40L over-expression. The CD40L up-regulation by DNA demethylation and the subsequent reactivation of the silent X chromosome in female patients with SSc may explain the well-known susceptibility of women to SSc (86).

CD70, a membrane bound B cell co-stimulatory molecule encoded by *TNFSF7* gene and typically expressed on activated $CD4^+$ and $CD8^+$ T cells (also known as CD27 ligand, due to its interaction with CD27 during B/T cell contact), was found to be over-expressed in $CD4^+$ T cells from patients with SSc, as consequence of *TNFSF7* promoter regulatory elements demethylation, possibly contributing to the autoimmune response (87).

RNA interference

Recently, a miR-29a dysregulation was reported in SSc fibroblasts, leading to abnormal collagen expression (88). In fact, the miR-29 family members (namely, miR-29a, miR-29b and miR-29c), and, in particular, miR-29a are consistently down-regulated in skin fibroblasts and skin tissue sections from SSc patients, compared with healthy controls. Intrinsic epigenetic mechanisms are likely involved in this down-regulation, owing to the low expression of miR-29 family members is maintained from early to late cell cultures, without additional profibrotic stimuli by TGF-β, PDGF-B, and IL-4 (88).

miR-196a expression was found to be decreased in SSc fibroblasts both *in vivo* and *in vitro,* and patients with lower serum levels showed significantly higher ratio of diffuse/limited cutaneous SSc, higher modified Rodnan total skin thickness score, and higher prevalence of pitting scars than those without (89).

The serum level of the Homo sapiens miR-142 stem-loop (hsa-miR-142-3p), one of the miRNAs regulating the expression of α5 integrin, has been recently suggested as a specific disease marker for SSc, being significantly higher in SSc patients than in those with SLE and dermatomyositis, or in healthy controls (90).

The miR-92a expression was demonstrated to be significantly increased in serum and dermal fibroblasts of SSc patients, compared with normal subjects (91). Furthermore, the forced over-expression of miR-92a in normal dermal fibroblasts resulted in the down-regulation of MMP-1, suggesting that MMP-1 might be its target, and that the enhanced miR-92a expression might play a role in excessive collagen accumulation in SSc, via the down-regulation of MMP-1 (91).

Systemic lupus erythematosus

SLE is a prototypic systemic autoimmune disease with polymorphic clinical manifestations, immunologically characterized by T and B cell hyperactivation, auto-antibody production, and immune complex deposition. The role of genetic predisposition has been extensively investigated, and genome-wide association studies have identified more than 30 associated loci, being the SLE heritability rate nearly 66%, and ranging the concordance rates from 24% to 56% in MZ twins and from 2% to 4% in dizygotic twins (92). The inability of genetic association studies to comprehensively account for disease heritability has suggested the participation of epigenetic mechanisms, further underlined by data derived by drug-induced lupus, a syndrome related to more than 40 medications, among which hydralazine and procainamide have been proven to cause DNA hypomethylation in T and B cells, with consequent gene activation and molecule over-expression, contributing to the generation of pathogenic autoreactivity (93-95).

Histone modifications

In comparison with controls, $CD4^+$ T cells of SLE patients with active disease showed global H3 and H4 histone hypoacetylation, which negatively correlated with disease severity (96), while H3 acetylation levels within the *TNFSF7* gene promoter were significantly elevated, positively correlating with disease activity (97). By increasing CD70 expression in $CD4^+$ T cells, the *TNFSF7* H3 hyperacetylation is thought to provide a significant pathogenic contribute to the disease development (97).

The H3 lysine 4 trimethylation, an important epigenetic modification associated with active transcription, has been observed in PBMCs of SLE patients (98).

A global H4 hyperacetylation was found in monocytes from patients with SLE, and 63% of the H4 hyperacetylated genes had the potential for regulation by interferon (IFN) regulatory factor 1, which was overexpressed in SLE monocytes (99). Moreover, enhanced H4 acetylation occurred at the TNF-α locus, consistently with the overproduction of this proinflammatory cytokine in SLE patients (99).

DNA methylation

Notably, a cohort of MZ twins discordant for SLE, RA, and dermatomyositis, compared with matched controls, underwent methylation profiling analyses on DNA extracted from white blood cells, being the direct comparison of identical twins an excellent approach to investigate epigenomic differences and to identify epigenetic targets (100). Only MZ twins discordant for SLE featured widespread DNA hypomethylation of 49 genes, and reduction of DNMT1 and DNMT3B mRNA levels. Gene ontology analysis revealed enrichment in categories associated with immune function, some of which had been previously referenced as markers in the context of SLE pathogenesis (100). Moreover, significantly decreased methylation of the 18S and 28S ribosomal genes was observed in all SLE samples, relative to their respective healthy siblings, and correlated with higher transcript levels, suggesting that hypomethylation and over-expression of ribosomal RNA genes might be associated with an increased assembly of ribosomal particles stimulating the generation of auto-antibodies (100).

A global DNA hypomethylation was demonstrated in SLE PBMCs, causing over-expression of autoimmune-related genes (101). The DNA methylation in SLE PBMCs resulted to be further decreased upon *in vitro* exposure to both moderate and high doses of ultraviolet (UV) B radiation, especially in patients with malar rashes and leucopenia, suggesting that the SLE photosensitivity may be due, in part, to epigenetic mechanisms (102).

T cells from SLE patients were found to display a significant decrease in DNMT activity (103), and global DNA demethylation (103,104).

Notably, widespread DNA hypomethylation is considered hallmark of SLE $CD4^+$ T cells (85,105-108): demethylated autoreactive T cells could interact with macrophages *in vivo* causing apoptosis, release of antigenic nucleosomes, and induction of anti-DNA antibodies, as well as over-stimulate B cells, increasing autoantibody production (109).

Various agents known to inhibit DNA methylation in T cells, including procainamide, hydralazine, 5-azacytidine (5-azaC, the prototypic DNA methylation inhibitor), and UV light, were shown to induce the over-expression of Leukocyte Function-associated Antigen 1 (LFA-1, CD11a/CD18 dimer, αLβ2 integrin), a

heterodimeric integrin consisting of αL (whose gene is named *ITGAL*) and β2 (whose gene is named *ITGB2*) subunits and expressed in all leukocytes, which contributes to make T cells auto-reactive; the LFA-1 up-regulation has been proven to depend on demethylation of the encoding *ITGAL* regulatory sequences (110).

$CD4^+$ T cells from SLE patients abnormally over-express the "Growth Arrest and DNA-Damage-induced 45 alpha" (GADD45α) protein, and GADD45α mRNA production correlates with disease activity (106). UV irradiation was found to induce *GADD45A* gene expression and to reduce DNA methylation in both normal and SLE $CD4^+$ T cells, suggesting that UV light may lead to autoimmune-related gene over-expression through aberrant T cell DNA demethylation (106).

Other than global, also a gene-specific DNA demethylation has been demonstrated in SLE, occurring in genes known to be associated with the development of SLE, namely *ITGAL* (whose hypomethylation is proportional to disease flare severity) (110), *PRF1* (perforin) (111), *TNFSF7* (CD70) (97,112,113), and *CD40LG* (CD40L) (114). Of note, $CD4^+$ T cells from women but not men with SLE were proven to over-express the B cell costimulatory molecule CD40L, due to the demethylation of *CD40LG* on the normally inactive X chromosome, that can contribute to the striking female predisposition to the disease (114). The simultaneous and tightly associated over-expression of *ITGAL*, *PRF1*, *TNFSF7* in SLE $CD4^+$ T cells has been suggested to play relevant role in the pathogenesis of the disease (115).

The extracellular signal-regulated kinase (ERK) signaling pathway, that regulates the methyltransferase levels, can contribute to the DNA demethylation in SLE T cells (103). Signaling via the ERK pathway is decreased in $CD4^+$ T cells from patients with active disease, causing decreased DNMT1 expression and CD70 over-expression (94). Notably, transgenic animals generated with defective ERK signaling in T cells similarly showed reduced expression of DNMT1, over-expression of the methylation-sensitive *ITGAL*, *TNFSF7* and IFN-regulated genes, and also developed anti-dsDNA autoantibodies (116).

The hypomethylation of the CpG islands in the *IL-4* and *IL-6* gene promoters was demonstrated in T cells from SLE patients, and correlated to the significantly increased IL-4 and IL-6 mRNA expression (117).

The DNA methylation within *IL-10* and *IL-13* gene regulatory domains is reduced in SLE $CD4^+$ T cells, relative to healthy controls, and correlates with enhanced IL-10 and IL-13 levels (118). Moreover, the *IL-10* gene hypomethylation was found to be associated with greater disease activity (119).

The DNA methylation levels in SLE activated B cells are reduced, compared with B cells of healthy controls, and, as a result of this demethylation, B lymphocytes display reduced CD5 expression on their membrane (120). The CD5 surface levels are determined by the relative rates of two alternative CD5 isoforms: a membrane-expressed variant [which uses the exon 1A (*E1A*) of the *CD5* gene, and is named CD5-E1A], and a cytoplasmic isoform (which uses the *CD5-E1B*, and is termed CD5-E1B). High IL-6 levels abrogate the B cells ability to induce DNMT1 and then to methylate DNA, altering the CD5-E1A/CD5-E1B balance in favor to the CD5-E1B cytoplasmic variant. As CD5 modulates B cell receptor signal strength, changes in its cell surface expression may contribute to the activation and expansion of autoreactive B cells in SLE patients (120).

RNA interference

In PBMCs of SLE patients, a 27 miRNA signature was identified; 8 miRNAs were found to be specifically deregulated in T cells, 4 miRNAs in B cells, and 19 was proven to correlate with disease activity (121). Of note, the levels of miR-21, miR-25, miR-106b, and miR-148b positively correlated with SLE activity, while the miR-196a and miR-379 expression was inversely correlated, suggesting a potential role for miRNA profiling as disease biomarker (121).

Moreover, 3 miRNAs (miR-21, miR-126 and miR-148a), over-expressed in $CD4^+$ T cells from both SLE patients and lupus-prone MRL/lpr mice, were noted to promote cell hypomethylation by repressing DNMT1 expression, which leads to the over-expression of autoimmune-associated methylation-sensitive genes (122,123). In particular, miR-21 indirectly down-regulates DNMT1 expression by targeting an important autoimmune gene, *RASGRP1*, which mediates the Ras/ mitogen-activated protein kinase (MAPK) pathway upstream of DNMT1, whereas miR-126 and

miR-148a directly down-regulate DNMT1 expression by targeting the protein coding region. Additionally, the inhibition of these miRNA expression in $CD4^+$ T cells from SLE patients increases DNMT1 and attenuates DNA hypomethylation (122,123).

Conversely, miR-146a levels were found to be intrinsically under-expressed in PBMCs of SLE patients, negatively correlating with disease activity and leading to the over-activation of the type I IFN pathway (124). Assessing whether genetic variants modulate miR-146a expression and contribute to the risk of developing SLE, the promoter and key regulatory regions of the miR-146a precursor were sequenced, and the rs57095329 single-nucleotide polymorphism in the promoter was identified as a functional variant affecting miR-146a levels in PBMCs and conferring SLE susceptibility in Asians (125). In Europeans, the rs2431697 genetic variant in an intergenic region between the pituitary tumor-transforming 1 and miR-146a genes is associated with SLE susceptibility, and the risk allele correlates with a down-regulation of miR-146a (126).

Whereas in SLE patients decreased levels of miR-146 have been demonstrated, RA patients showed increased levels, as compared with healthy controls (127). The finding that the miR-146 values are contradictory in these ACTDs simply reflects a difference in their overall cytokine profiles, with type I IFN playing a dominant role in SLE, and being TNF-α, IL-1, and IL-6 pivotal cytokines in RA (127).

Abnormalities in the IL-2-related signaling pathways in SLE patients were found to be linked to the miR-31 marked under-expression in T cells (128). The under-expression of miR-125a was also reported in SLE $CD4^+$ T cells, leading to increased levels of the "Regulated on Activation, Normal T Cell Expressed and Secreted" (RANTES)/CCL5 proinflammatory chemokine, and, in turn, the normalization of the miR-125a expression resulted in reduced RANTES/CCL5 levels (129).

miR-142-3p and miR-142-5p are significantly down-regulated in SLE $CD4^+$ T cells, compared with healthy controls, being miR-142-3p/5p levels inversely correlated with the SLE-related putative targets, such as the "signaling lymphocytic activation molecule-associated protein" (SAP), CD84 and IL-10: miR-142-3p and miR-142-5p directly inhibit SAP, CD84, and IL-10 translation, and the reduced miR-142-3p/5p expression in $CD4^+$ T cells significantly increases their levels (130). The inhibition of miR-142-3p/5p in healthy donor $CD4^+$ T cells induces T cell over-activation and B cell hyperstimulation, whereas the over-expression of miR-142-3p/5p in SLE $CD4^+$ T cells has the opposite effect (130).

In PMBCs from SLE patients with nephritis, compared with unaffected controls, 5 miRNAs (such as hsa-miR-371-5P, hsa-miR-423-5P, hsa-miR-638, hsa-miR-1224-3P and hsa-miR-663) resulted to be differentially expressed across diverse ethnicities, and were suggested to represent specific biomarkers for the diagnosis of lupus nephritis (131). Intra-renal miR-638, miR-198 and miR-146a were differentially expressed between SLE patients with nephritis and normal controls (131). Moreover, the degree of change in glomerular miR-146a and tubule-interstitial miR-638 expression correlated with the clinical severity, suggesting that these miRNAs may play a role in the pathogenesis of lupus nephritis (132).

The profile of circulating miRNAs was determined in SLE patients, in comparison with RA patients and healthy controls (133). Among the 8 identified miRNAs (miR-126, miR-21, miR-451, miR-223, miR-16, miR-125a-3p, miR-155, and miR-146a), miR-126 was specifically enriched only in the SLE patients blood, and 4 miRNAs (miR-21, miR-451, miR-223, and miR-16) were proven to be significantly increased in patients with both SLE and RA. In contrast, miR-125a-3p, miR-155, and miR-146a showed a trend toward significantly reduced levels in SLE patients. Most of the dysregulated circulating miRNAs are involved in various signal transduction pathways and cell interactions, particularly in the MAPK signaling pathway (133).

Sjögren's syndrome

SS, classified as primary (pSS) when isolated, or secondary if associated with another autoimmune disease, is a chronic autoimmune disease mainly affecting exocrine glands (autoimmune exocrinopathy), but the pSS clinical spectrum may space from an organ-specific disorder to a multisystemic process with heterogeneous extraglandular manifestations. Aberrant B-cell hyperactivation, reflected by hypergammaglobulinemia and autoantibody production, is a pSS pivotal immunologic abnormality, while the disease histological hallmark is the exocrine gland infiltration by mononuclear cells (134).

Histone modifications

In pSS, TNF-α is thought to play an important role in the destruction of acinar structures, and the TNF-α inhibition of the membrane water channel aquaporin-5 expression in human salivary gland acinar cells was proven to be due to epigenetic mechanisms by suppression of H4 acetylation (135).

DNA methylation

In $CD4^+$ T cells of pSS patients compared with controls, the CD70 expression was found to be significantly elevated, and correlated with a decrease in *TNFSF7* promoter methylation, contributing to autoreactivity (136).
In salivary glands of patients with pSS, the cell/basal lamina interactions and tight junctions are damaged, being adhesion physiologically mediated by α 6β1 and α 6β4 integrins and proteins BP180 and BP230 [the entire adhesion complex constitutes the type I hemidesmosomes (I HD)]. Abnormal I HDs impede extracellular matrix/acinar cell communication and the loss of anchorage contributes to abnormal cell death in pSS salivary glands (137). Changes in the expression of BP230 and BP180 may occur as a consequence of epigenetic modifications: in labial salivary glands of pSS patients, the *BP230* gene was hypermethylated and the mRNA levels were decreased, with consequent alteration of BP230 protein levels, leading to modified localization and distribution of integrins and abnormal I HD assembly (137).

RNA interference

Expression of miR-146a, a negative regulator of immune and inflammatory responses (28,68,73,73), was examined in PBMCs of pSS patients and healthy donors, as well as in PBMCs, salivary and lacrimal glands of SS-prone mice (46). Expression of miR-146a was significantly increased in pSS patients compared with healthy controls, and was up-regulated in the salivary glands and PBMCs of the SS-prone mouse at both 8 weeks (prior to disease onset) and 20 weeks (full-blown disease) of age. More importantly, functional analysis revealed roles for miR-146a in increasing phagocytic activity and suppressing inflammatory cytokine production. Taken together, these data suggest that abnormal expression/regulation of miRNAs in innate immunity may contribute to, or be indicative of the initiation and progression of pSS (46).
The expression of miR-146a/b and their target genes [IL-1 receptor-associated kinase (*IRAK*) 1 and 4, and TNF receptor-associated factor 6 (*TRAF6*) genes] was measured in PBMCs of patients with pSS, compared to healthy controls (138). Both miR-146a and miR-146b, as well as *TRAF6* were significantly over-expressed, while the expression of *IRAK1* was significantly decreased in pSS patients. The expression of *IRAK4* did not differ significantly. Whereas *IRAK1* has been regarded a crucial gene in the pathogenesis of SLE, the *TRAF6* over-expression may be a pSS specific biomarker, confirming and partly explaining the existence of different pathogenic pathways in these two diseases (138).
miRNA profiles from the minor salivary glands of healthy controls and patients with pSS (with low-grade or high-grade inflammation, and impaired or normal saliva production) were studied to identify patterns specific to salivary gland inflammation or dysfunction (139). Among an identified set of 27 putative housekeeper miRNAs, 2 miRNAs were found to change in opposite directions: hsa-miR-768-3p increased, while hsa-miR-574 decreased with increasing focus score. A good correlation between their differential expression and focus scores was observed, so that these two miRNAs have been suggested as specific markers of inflammation in pSS (139).

Conclusions

During lifetime, according to the environmental context, several epigenetic modifications progressively are added to the genomic message, conferring adaptive and dynamic variability, so that everyone records his own biological experience in a physiologically different way that establishes the personal biodiversity. Epigenerator signals, including environmental cues or niches (such as viral and bacterial pathogens, self antigens, etc.), may activate or decrease epigenetic initiators (transcription factors, ncRNAs, etc.). Once induced, an epigenetic maintainer (histone acetylases, HDACs, DNMTs) coordinates the overall cell response. Thus, stable chromatin landscape changes occur as a result of expression of modifying enzymes, and their persistence requires the cooperation between both initiator and maintainer signals.

If persistently aberrant, stable epigenetic modifications may biologically condition disease development. Over lifetime, age-related epigenetic defects may condition the late onset and strong age dependency of some diseases or the progressive nature of many common diseases.

In explaining the complex ACTDs pathogenesis, either genetic studies or analyses of the potential role of environmental factors have been only partly successful, suggesting the intervention of epigenetic mechanisms. Presently, some generalizations about the ACTDs development can be made: first, the genetic components of these diseases are significant, but do not comprehensively describe their onset and heritability; second, epigenetic variations correlate with variable expression of genes in pathways involved in disease pathogenesis; third, the path forward in the ACTD knowledge will be the understanding of the emergent phenotypes that result from genome/environment/epigenome interactions over time; fourth, due to the complexity and heterogeneity of ACTDs, the efforts addressed to identify specific epigenetic alterations will likely result in the identification of mosaics of epigenetic changes, defining peculiar epigenomic signatures. In this regard, specialized miRNA networks have been identified in a variety of diseases, including rheumatic diseases and cancers (27,41,140,141).

Moreover, identifying the cell-specific targets of the epigenetic deregulation in ACTDs not only will provide clinical markers for diagnosis and disease progression, but also, being epimutations reversible, will offer the opportunity to use pharmacologic approaches to reverse them, and ameliorate their effects on a specific phenotype.

Several compounds that modulate the epigenetic reactions in rheumatic diseases have already been tested *in vitro* and in animal models. In particular, HDACis and demethylating agents are thought to have therapeutic value, but targeted agents that modify the epigenetic patterns could be designed, increasing the efficacy and minimizing toxic effects.

HDACis, originally developed as anti-cancer drugs, exhibit anti-proliferative activity through multiple mechanisms, such as induction of cell cycle arrest, apoptosis and promotion of differentiation, modulating gene expression. HDACis can also reduce the expression of mediators involved in the pathogenesis of inflammatory diseases, such as TNF-α , IL-1β, IL-6, IL-8, TGF-β and nitric oxide (142). HDACis efficacy in animal and cell culture disease models has been reported, and their clinical use in the treatment of human autoimmune and inflammatory diseases seems to provide interesting promises (54,55,143). Recently, the safety and efficacy of ITF2357 (givinostat), a orally administered hydroxamic acid with HDACi activity, have been evaluated in a phase II, open-label, international, multicenter clinical trial, enrolling a small cohort of patients with systemic-onset juvenile idiopathic arthritis, according to the International League Against Rheumatism criteria (144). Givinostat has been reported to exhibit significant therapeutic benefit in treated patients, with an excellent safety profile (144). However, long-term studies on large cohorts of patients are needed to infer valid conclusions.

Demethylating agents, such as 5-azaC, have been used in animal models of diseases, but their clinical use in humans is limited by their lack of specificity.

Moreover, recent technological advances have proposed the use of promoter-directed small interfering RNA techniques to alter the methylation status of individual CpG motifs. RNA interference by ncRNA is a relatively new field, but holds great promises. The introduction of miRNAs via cell-specific transfection or transduction could directly affect transcription of important disease-associated genes, both by modulating expression of single genes, or more globally by targeting DNA methylation machinery via upregulating DNMT1 (122).

Ultimately, new scenarios are opening in which disease-related epigenetic variants can be specifically manipulated, thus providing novel therapeutic strategies in the treatment of ACTDs.

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