Published in final edited form as:

Expert Rev Clin Immunol. 2015 January ; 11(1): 45–58. doi:10.1586/1744666X.2015.994507.

Autoimmune disease in the epigenetic era: how has epigenetics changed our understanding of disease and how can we expect the field to evolve?

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Abstract

Autoimmune diseases are complex and enigmatic, and have presented particular challenges to researchers seeking to define their etiology and explain progression. Previous studies have implicated epigenetic influences in the development of autoimmunity. Epigenetics describes changes in gene expression related to environmental influences without alterations in the underlying genomic sequence, generally classified into three main groups: cytosine genomic DNA methylation, modification of various sidechain positions of histone proteins, and noncoding RNAs. The purpose of this article is to review the most relevant literature describing alterations of epigenetic marks in the development and progression of four common autoimmune diseases: systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), systemic sclerosis (SSc), and Sjögren's syndrome (SS). The contribution of DNA methylation, histone modification, and noncoding RNA for each of these disorders is discussed, including examples both of candidate studies and larger epigenomics surveys, and in various tissue types important for the pathogenesis of each. The future of the field is speculated briefly, as is the possibility of therapeutic interventions targeting the epigenome.

Keywords

epigenetics; methylation; histone modification; microRNA; autoimmune disease; systemic lupus erythematosus; rheumatoid arthritis; systemic sclerosis; Sjögren's syndrome

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Financial and competing interests disclosure

This work was supported by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health under award number R01AI097134. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

Introduction

Epigenetic modifications function as an integrator of environmental input and the underlying genetic code. Originally defined as heritable changes in gene expression or function that were not caused by alterations in the underlying genomic code, an updated definition that encompasses a more modern interpretation was proposed by Bird et al. in 2007, who dropped the ‘heritability’ argument in favor of “the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states”; thus, epigenetic mechanisms are key to integrating the cellular response to environmental cues [1]. As it pertains to autoimmune disease, this definition becomes particularly relevant. The human immune system is constituted by a wondrously diverse set of cell types, each adapted to serve a particular role in the defense of the human organism as a whole against influences that seek to disrupt homeostasis. As such, immune cell transcription in particular must be remarkably plastic to adapt to these ever-changing conditions. It comes as no surprise, then, that differential epigenetic patterns play a pivotal role in the development and proper maintenance of the immune system and prevention of autoimmunity. Indeed, even nuanced cellular subsets can be robustly identified by one type of epigenetic control, differential DNA methylation patterns [2]. Drawing from this research, it then follows that derangements in epigenetic marks would be strongly associated with the development autoimmune disease.

The focus of this article is to review what we feel to be the most relevant contributions of epigenetics as they relate to the most prevalent autoimmune disorders; namely, systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), systemic sclerosis (SSc), and Sjogren’s syndrome (SS), and briefly describe where the field is headed in the future, both in the near and long-term.

Epigenetic mechanisms

First, we shall review the structure and function of the epigenome (Figure 1). There are three fundamental mechanisms of epigenetic control as we understand it today: methylation of genomic DNA, post-translational modification of various histone sidechains, and noncoding RNA modulation of gene expression. We will explore each of these individually.

DNA Methylation

Methylation of genomic DNA occurs on the 5’ carbon position of the pyrimidine ring of cytosine residues. Although classically described in the context of CpG dinucleotides, it is now understood that other motifs (e.g. CHG or CHH) are methylation substrates in embryonic tissue and induced pluripotent stem cells [3]. Interestingly, CG dinucleotides represent approximately 4% of the genome, significantly less than the statistical expectation of 6%, due to CG drift caused by the instability of methylated cytosines[4]. CpG pairs are found throughout the genome; however, their transcriptionally relevant activity is generally ascribed to regions within the 5’ upstream promoter; particularly, as our group and others have shown, between 1000bp and 800bp upstream of the transcription start site [5]. Furthermore, the importance of methylation at distantly upstream enhancer regions has been

recently demonstrated; in cancer, the methylation state at these enhancers is actually better correlated with gene transcription than are traditional proximal promoter elements [6].

Methylation of cytosine residues is mediated by an evolutionarily divergent set of DNA methyltransferases (DNMTs); DNMT1 recognizes hemimethylated cytosines and functions as the maintenance methyltransferase enzyme, copying epigenetic information during DNA replication in S phase of the cell cycle to new DNA strands, whereas DNMT3a and DNMT3b set the methylation code *de novo* [7]. DNMT2 mediates RNA methylation, an area of study that is still in its infancy. Demethylation of DNA is a long debated topic; it now appears that both active and passive demethylation occurs (review, [8]). Passive demethylation occurs when the maintenance methylation machinery (e.g. DNMT1) is inactivated during DNA replication, thereby precluding the copying of methyl marks to nascent DNA. The most convincing to-date description of an active DNA demethylation-associated protein comes in the form of TET family members, which participate in active DNA demethylation by creating a 5-hydroxymethylcytosine intermediate with subsequent development of a T:G mismatch, which is excised through double strand breaks utilizing base excision repair (BER) complexes to add an unmethylated cytosine [9].

Histone modification

Histones are highly conserved proteins that function principally to stabilize and organize DNA within the nucleus. Composed of repeating octamers, they contain dimers of each of the four core histone proteins (H2A, H2B, H3, and H4) and wrap genomic DNA around their outer surface. Owing to their packaging role, they function in concert with DNA methylation and transcription machinery; particularly, methylcytosine binding proteins (e.g. MeCP-2) to alter the local chromatin environment and either permit transcription or repress it. These various functions occur via post-transcriptional modifications of the amino acid sidechains attached to various histone proteins themselves, including acetylation, methylation, ubiquitination, sumoylation, deimination, and poly(ADP)-ribosylation, among others [10]. The best studied histone modifications are acetylation and methylation. Acetylation of terminal lysine residues is associated with an increase in transcription, and deacetylation with repression; thus, various histone deacetylase inhibitors have been widely studied as a means to increase transcription of a wide variety of genes, including in autoimmunity, as will be discussed later. Methylation of histones results in different transcriptional outcomes depending upon the particular residue involved; the addition of three methyl groups to lysine 27 of histone 3 is repressive, whereas methylation of lysine 4 in histone 3 is activating [11].

Noncoding RNA

The field of noncoding RNA research, particularly in autoimmune disease, is burgeoning. The first and most widely-studied of these RNA subtypes are micro RNAs. Composed of small, noncoding nuclear-DNA-encoded RNAs of approximately 22 nucleotides in length, miRNAs effect expression by one of three principal mechanisms. First, they may bind to complementary sites on the 3' tails of messenger RNA transcripts and target them for degradation by the RNA-induced silencing complex (RISC), composed of microRNAs bound to target mRNA and the various cleavage proteins, notably including members of the

Argonaut family [12]. A second mechanism involves the binding and destabilization of mRNAs without cleavage, a third reduces the efficiency of ribosomal translation (review, [12]). Several novel functions of miRNAs have been proposed, and several new classes of larger noncoding RNA with important transcriptional significance have been recently described, and will no doubt lead to much future research in this area.

Methods, design of epigenetic studies, utility of publically available data

A variety of methods and study designs have been utilized to perform epigenome-wide association studies (EWAS), and each carries with it strengths and weaknesses. Monozygotic twin studies have been quite informative and have been performed both in SLE and RA, detailed below. These are particularly adept at isolating environmental contributions to disease, as they negate the effects of genetic variation disease:control comparisons. The most obvious negative aspect of this design is the exceptional difficulty of locating and studying monozygotic twins in rare disease, frequently prompting reductions in sample size and concomitant reduction in study power. With advancing technology, we are now seeing ever more comprehensive genetic evaluations of risk for particular diseases, data from which should eventually allow non-twin studies to computationally correct for genetic variation in EWAS studies and make twin studies less relevant. Cross-sectional and case-control studies are perhaps the most frequently used design, as they lend themselves to rapid and relatively easy collection of biospecimens, with a wide population base from which to select. Downsides include the need to carefully select the appropriate timepoint for analysis, and the need to scrutinize genetic variation to reduce false associations. Unfortunately, it is not always obvious when the appropriate time is to gather epigenetic data for disease association. For example, would collecting EWAS data from lupus patients' T cells one week after the onset of a flare reveal the same data as collecting those same cells one day after the onset of symptoms? Another difficulty in these studies that is often overlooked is the appropriate selection of control patients. Many EWAS reports in rheumatoid arthritis, for example, compare joint tissue DNA methylation data to "control" tissue from osteoarthritis patients. Recent reports have clearly shown, however, that OA cartilage is abnormal, leading to some questioning of reported disease-specific associations.

To overcome this, longitudinal studies could be conducted, wherein samples are obtained repeatedly over a predefined disease-specific timecourse and compared to one another using internal controls that are more likely to reveal true disease-associated epigenetic changes. These can be quite burdensome, particularly if the tissue being studied requires a surgical extraction, and require a significant amount of time for both patients and researchers. Nevertheless, autoimmune diseases that require only minimally invasive procedures to procure the specimen of interest, e.g. differential DNA methylation patterns in circulating immune cells from lupus patients or animal models of disease could be technically feasible and would be quite useful in their own right as well as informing appropriate design for larger case-control studies. So far, there have not been any examples of large studies performed using this design in autoimmunity.

A final study design that has yet to be implemented widely in autoimmunity research involves the EWAS analysis of pre-symptom biobank collections. These will no doubt be of

particular importance in the decades to come as our EWAS technologies continue to advance. A significant hurdle remains sample selection; for example, most biobanks include DNA obtained from mixed peripheral blood cell populations that are difficult if not impossible to draw conclusions regarding epigenetic associations. Thankfully, the rapid growth of large epigenetic databases have made bioinformatic adjustment of EWAS data mixed cellular populations cautiously possible, and we have seen a few examples of this technology in the literature [13].

These large databases have afforded us other novel insights as well. The publically available Encyclopedia of DNA Elements (ENCODE) database is a particularly relevant example, and was used to great effect in a 2013 study by Trynka et al., who used colocalization of known genomic variants and histone 3 triacetylation marks (H3K4me3) to identify cell types associated with particular disease; of relevance to this review, 31 SNPs associated with RA colocalized with H3K4me3 marks in CD4+ T cells [14]. Bioinformatic analysis of this nature could help guide future epigenetic studies in a variety of diseases to tissue types likely to exhibit epigenetic dysregulation in a particular disease state. Studies of this type also highlight an important and as-yet incompletely understood reality in the field of epigenetics. For many diseases, it remains unclear how and to what extent genetic and epigenetic variation interact to produce a given phenotype. For example, do genetic variations associated with a disease occur because of a history of epigenetic aberration rendering the DNA sequence unstable and prone to mutation, or do genetic variants drive epigenetic changes by altering the local chromatin environment? To put it another way, are altered epigenetic signals simply “capturing” the underlying genetic effects? We do not yet have a clear answer to these concepts and, subsequently, one must be cautious in the interpretation of EWAS studies where segregation of epigenetic and genetic effects in data analysis is not clearly defined.

How, then, might we imagine the “optimal” approach to an epigenetic analysis of a particular disease? Perhaps it starts not in the laboratory, but rather by mining the myriad publically accessible databases, narrowing down the potential tissue types of interest with an analysis similar to the one described above, then moving towards small longitudinal epigenetic studies to identify appropriate timepoints for sample collection, and culminating in large EWAS case-control studies with these optimized parameters. Although this approach is certainly idealistic and likely to be more fiction than fact, we can nonetheless construct such an overarching theoretical experiment and analyze data published in the field, filling in these various components to draw more robust conclusions about epigenetic associations in a given disease.

Epigenetics in autoimmune disorders

We will now turn our attention to a review of the relevant literature of epigenetic mechanisms influencing the development and progression of four of the most prevalent autoimmune diseases: SLE, RA, SSc, and SS. Contributions of the three major classes of epigenetic control will be discussed in each; namely, differential DNA methylation, alterations of histone sidechains, and expression of various micro RNA species.

Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is perhaps the best studied autoimmune disease with regard to epigenetic modification. A chronic autoimmune condition that can affect virtually any organ system, it is characterized by the production of autoantibodies targeted to a variety of nuclear antigens. Discordance noted in early monozygotic twin studies, as well as racial and geographic variations in prevalence, suggest a strong interaction between the environment and underlying genomic code in the development of SLE. Early studies in SLE CD4⁺ T cells treated with DNA methylation inhibitors demonstrated a conversion to autoreactivity, and the introduction of synthetically demethylated CD4⁺ T cells into mouse models resulted in a lupus-like syndrome [15–17]. Two medications known to both induce the formation of anti-nuclear antibodies and cause drug-induced lupus were found to inhibit methylation and produce murine lupus, either by blocking the activity of the maintenance DNA methyltransferase DNMT1 or the ERK signaling pathway upstream of it [18–20].

Several abnormal biological features of SLE contribute to DNA hypomethylation and disease pathogenesis. There is strong evidence that apoptotic DNA is important. Lupus patients are less able to clear apoptotic cells than disease-free controls due to a defect in phagocytosis [21,22]. The introduction of DNA extracted from *ex vivo* stimulated lymphocytes into BALB/c mice is capable of inducing an autoimmune disease closely resembling human SLE, but the development of autoimmunity is entirely dependent upon the methylation level of this DNA. Apoptotic DNA, which is demethylated, generates a robust autoimmune response, whereas remethylation of this same DNA by treatment with a DNA methylase does not produce such autoimmunity. Conversely, demethylation of necrotic or normal DNA via treatment with the drug 5-azacytidine, when injected, causes florid autoantibody production and proteinuria, nearly as strong as seen with apoptotic DNA [23]. This reaction is mediated by signaling through Toll-like receptor 9 (TLR9), an innate immunological receptor thought to have originally evolved to detect generally unmethylated bacterial DNA [24]. Similarly, DNA demethylation has been noted in PBMCs of SLE patients upon exposure to moderate and high levels of ultraviolet radiation, particularly UV-B, independent of the transcriptional activity of DNMT1, suggesting an epigenetic component to the ubiquitous lupus photosensitive rash [25].

The methylation status of many specific genes have been linked with SLE pathogenesis and progression. In CD4⁺ T cells, *ITGAL* (*CD11a*) is an integrin involved in both costimulation and cellular adhesion. Upon dimerization with CD18, it forms leucocyte function-associated antigen 1 (LFA-1). CD11a is overexpressed in SLE patients' CD4⁺ T cells and correlates with disease activity, and the upstream promoter of *ITGAL* is demethylated in active lupus patients compared to controls, the degree of this demethylation correlating with disease activity [26]. Some years ago, our group generated a mouse with an inducible ERK signaling block, a pathway known to be dysregulated in SLE and upstream of the maintenance DNA methyltransferase DNMT1. Upon induction, this mouse model demonstrated demethylation and upregulation of CD11a, as well as autoantibody production and the induction of a number of interferon-regulated genes [27].

Another SLE associated T-cell gene is CD40L, a type II transmembrane protein coded on the X chromosome by *CD40LG* and functioning as a critical costimulatory molecule. Investigations of genes encoded on the X chromosome are of particular relevance to SLE given its female predominance and apparent gene-dose effect noted regarding incidence of the disease in patients with duplications of this chromosome in outstanding work conducted by Scofield et al several years ago [28]. Elevations of soluble CD40L are found in human SLE patients and several lupus mouse models [29–31]. This overexpression of CD40L is at least partly driven by demethylation of the *CD40LG* promoter. Treatment of human T cells with DNA methylation inhibitors (5-azacytidine, procainamide) or ERK pathway inhibitors (hydralazine, PD98059) cause demethylation of *CD40LG* regulatory sequences, lead to overexpression of CD40L, and are sufficient to induce T-cell autoreactivity *in vitro* [32]. Female lupus patients have roughly double the expression of CD40L compared to their male counterparts. They exhibit demethylation and reactivation of *CD40LG* sequences on the inactivated X chromosome, a likely explanation for this apparent gene-dose effect [33].

Another costimulatory molecule of particular interest is CD70, encoded by *TNFSF7*, a ligand of CD27. Abrogation of CD70-CD27 interaction via a blocking antibody is sufficient to fully mitigate lectin-stimulated B cell IgG production *in vitro* [34]. The epigenetic state of the *TNFSF7* promoter drives CD70 expression, and overexpression in mouse models and *in vitro* can be induced not only by traditional demethylating agents (e.g. 5-azacytidine) but also by medications associated with drug-induced lupus, including hydralazine and procainamide. Lupus patients' CD4+ T cells exhibit demethylation of these same promoter regions *in vivo* and overexpress CD70 [35]. Furthermore, our group has shown that CD4+ T cells from the commonly-used lupus prone mouse model MRL lpr demonstrate demethylation of *Tnfsf7* and overexpression of CD70 both as they develop autoimmunity with age and when compared to age-matched disease free MRL $+/+$ mice [36]. Following these and other strong associations with lupus, Zhang and colleagues in 2010 developed a novel high-throughput approach to the measurement of *CD70* and *ITGAL* (CD11a) DNA methylation. In it, they combine traditional bisulfite DNA treatment with an oligonucleotide microarray-based method with specific probes for each CpG of interest, in a manner similar to modern high-density genome-wide methylation arrays. This allows for highly sensitive, rapid quantitation of methylation values for these genes and provides hope for a future clinical assay to measure specific SLE-associated epigenetic alterations [37].

In cytotoxic CD8+ T and NK cells, Perforin (*PRF1*) is a key gene producing a cytolytic protein which, in concert with granzyme B, allows for the disruption and lysis of membranes of target cells [38]. Perforin is overexpressed in CD4+ T cells of lupus patients, where it exhibits activity analogous to that in its native cytotoxic T cell milieu. Perforin activity in both CD4+ and CD8+ cells have been linked to lupus, driven by the methylation status of its promoter region. Similar to *CD11a*, the degree of demethylation of the *PRF1* promoter is associated with lupus disease activity in both of these cell populations, and this differential methylation and overexpression has additionally been linked to a distinct subset of patients afflicted by the uncommon subacute cutaneous lupus (SCLE) form of the disease [39–42]. IL10 is a cytokine pivotal to the development of both human and murine lupus. It is a well-described lupus-susceptibility gene overexpressed in lupus CD4+ T cells and functions to

regulate many important other via several signaling pathways [43]; review, [44]. It is hypomethylated in SLE patients compared to disease-free controls. Another interleukin with an important role in Th2 cellular differentiation, *IL13*, was also hypomethylated and overexpressed in the same experiment [45].

Several genome-wide DNA methylation surveys have been completed with samples from lupus patients. The first was done by Javierre et al in 2010, with an intriguing study design: they examined global DNA methylation levels of 1505 CpG pairs in peripheral blood leukocytes from 17 pairs of monozygotic twins discordant for SLE. First, they confirmed that the SLE-afflicted twin did indeed have lower overall DNA methylation content, and that this was associated with significant reduction in the expression of both *DNMT1* and the *de novo* DNA methyltransferases *DNMT3b*. Furthermore, they showed significantly decreased methylation of various ribosomal genes. Among non-ribosomal DNA, 49 genes were differentially methylated in the SLE twin, corresponding to a variety of functional ontologies, including ‘defense response’, ‘cell activation’, ‘immune response’, ‘cell proliferation’, and ‘cytokine production’ [46].

In 2011, our group completed the first genome-wide DNA methylation study in CD4+ T cells from 12 female lupus patients compared to 12 age- and sex-matched healthy controls among ~27,000 CpG sites. Of the 337 CpG sites which met criteria for differential methylation, we again saw the majority (~70%) were hypomethylated, and identified several novel methylation associations with SLE. Matrix metalloproteinase 9 (*MMP9*), previously overexpressed in Sjogren’s syndrome and RA, was hypomethylated [47,48]. *PDGFRA*, also found to be hypomethylated, has been previously identified as a target of autoantibody in active lupus patients [49]. The T cell coactivator *CD9* was hypomethylated, a finding which corroborated a previous report of hypomethylation of this gene in the Javierre twin study [46]. Gene pathway analysis identified pathways and ontologies which were overrepresented among differentially methylated genes, the most prominent of which included ‘folate biosynthesis’, the pathway whereby S-adenyl methionine, the methyl donor for DNA methylation, is generated and recycled. A number of transcription factors were also overrepresented, including *RUNX3*, which interacts with the promoter region of *ITGAL* (CD11a), mentioned previously. A final and important observation was the correlation of the DNA methylation pattern of several genes to clinical disease activity as measured by SLEDAI score. We identified five CpG sites with r^2 values > 0.60, three positively and two negatively correlated to disease severity [50].

Hypothesizing that alterations in DNA methylation were a cause of human lupus rather than a response to the presence of disease, we next examined the effects of differential methylation of naïve CD4+ lupus T cells in 2013 using a newer, more comprehensive platform consisting of >485,000 CpG sites among a discovery cohort of 18 SLE and 18 age-, race-, and sex-matched controls, and a validation cohort of the same number of patients and controls. 86 differentially methylated CpG sites were identified, again the majority (75%) being hypomethylated. Several genes, including the retroviral cellular membrane anchor tetherin (*BST2*) and the interferon-induced *IFI44L* were similarly differentially methylated as in our previous study in total CD4+ T cells. Furthermore, we found hypomethylation without evidence of overexpression of interferon-regulated genes, suggesting strongly that

naïve CD4⁺ T cells in lupus are “poised” for a rapid type-I interferon regulated gene expression upon stimulation. Indeed, our data demonstrated that the majority of hypomethylated genes in naïve CD4⁺ T cells in lupus are interferon-regulated. These data provided the first evidence that DNA methylation changes in interferon-regulated genes can provide a mechanistic explanation for type-I interferon hyper-responsiveness in lupus [51], and were subsequently replicated and extended to B cells and monocytes [52]. Intriguingly, we also identified hypermethylation of two genes known to be SLE genetic susceptibility loci (*CD247* and *IL21R*) [51], suggesting epigenetics as an alternate mechanism for disruption of these genes in human lupus.

Further linking epigenetic modification and genetic risk for SLE are recent data characterizing the lupus associated genetic locus in *MECP2/IRAK1*. Methyl cytosine binding protein 2, or MeCp-2, is a master regulator of gene expression and intimately involved in regulating the expression of methylation-sensitive genes. It is known to recruit histone deacetylase 1 and 2, leading to chromatin condensation, as well as the recruitment of DNMT1 during DNA replication [53,54]. A comparative DNA methylation analysis was undertaken between SLE patients with the risk haplotype compared to those with the protective haplotype. Although no global changes in DNA methylation levels were seen, differential methylation was noted among several genes, including hypomethylation of the aforementioned *BST2* and the interferon-related genes *IFI6* and *IRF6*, offering further evidence for an interferon methylation phenotype signature SLE. Furthermore, we showed that the lupus risk *MECP2* haplotype is a gain of function haplotype, associated with increased expression of at least one *MECP2* mRNA isoform, and that human *MECP2* transgenic mice develop an interferon gene expression signature and antinuclear antibodies [55]. Taken together, these findings strongly reinforce the ability of epigenetic mechanisms to modulate genetic risk, similar to that discussed later in rheumatoid arthritis.

How this global hypomethylation phenotype develops initially is not clear, but several recent articles have identified novel mechanisms that may contribute. A recent work by Sunahori and Tsokos, et al., demonstrated that knockdown of protein phosphatase 2A (PP2Ac), which is overexpressed in T-cells from SLE patients, increased phosphorylation and activity of components of the aforementioned ERK pathway and increased DNMT1 expression, which subsequently suppressed expression of *CD70* [56]. Further supporting this finding, the development of a PP2Ac subunit-overexpressing transgenic mouse was more susceptible to immune-mediated glomerulonephritis (a hallmark of human SLE), likely by promoting aberrant T cell activation through IL-17 production [57]. Zhao, Lu, and colleagues in 2010 reported that binding of the transcription factor RFX1 in CD4⁺ T cells from human lupus patients could mediate epigenetic control of both *CD70* and *CD11a* by recruiting both the maintenance DNA methyltransferase DNMT1 and the histone deacetylase HDAC1 [58]. Conversely, levels of the growth arrest and DNA damage-induced 45α (*GADD45α*) are increased and inversely proportional to levels of DNA methylation in human lupus CD4⁺ T cells. Furthermore, after exposure to ultraviolet-B irradiation, a known trigger of SLE, *gadd45A* levels increased and were accompanied by expected increases in levels of *CD11a* and *CD70*. Interestingly, the transfection of *gadd45A* *in vitro* was also sufficient to increase expression of these methylation-sensitive genes, as well as induce autoreactivity [59].

Recent data on oxidative stress in SLE also provide important insights. Increased oxidative stress leads to nitration of PKC δ , preventing its activation and decreasing signaling through the ERK pathway, previously identified as key to DNMT1-mediated DNA methylation [60]. Furthermore, increases in oxidative stress lead to activation of the mTOR pathway, which can directly inhibit DNMT1 [61].

Histone modification patterns in lupus have been less extensively studied than has DNA methylation. MRL/lpr lupus prone mouse splenocytes exhibit hypermethylation and hypoacetylation of histone H3 and H4 compared to control mice, and treatment with histone deacetylase inhibitors (HDACi) reversed these changes and reduced disease activity [62]. Contrasting with these data, Dieker et al have demonstrated that a lupus-associated monoclonal antihistone antibody KM-2, isolated from both lupus prone mice and SLE patients, reacts more strongly with triacetylated H4 and hyperacetylated and apoptotic histones than with normal or nonacetylated H4. Furthermore, injection of triacetylated H4 to young lupus-prone mice accelerated their disease phenotype and increased mortality [63]. Administration of hyperacetylated histones to dendritic cells *in vitro* is sufficient to cause syngeneic T cell activation [64]. Following on these data, Zhang et al performed a chromatin immunoprecipitation study in 2010 where they determined H4 acetylation levels and concordance to gene expression data. Unsurprisingly, 179 genes were found to have significant increases in H4 acetylation in SLE monocytes compared with controls, and network analysis revealed an association with IFN- α , NF- κ B, and MAP kinase pathways. Administration of IFN- α to control monocytes increased H4 acetylation and gene transcriptional upregulation in a similar pattern to that observed in lupus monocytes [65].

Several recent studies have begun to unravel the contributions of noncoding RNAs to lupus pathogenesis. One screened 585 miRNAs in lupus-prone MRL/lpr mice and identified miR-21 and miR-148a as significantly overexpressed in CD4⁺ T cells. In an interesting link to another epigenetic mechanism, they subsequently transfected these two miRNAs into an immortalized human T cell line and found they directly altered global DNA methylation. miR-148a reduced the catalytic function of DNMT1, whereas miR-21, suppression of the MAPK pathway, previously linked by several studies to SLE, with subsequent downregulation of DNMT1 expression [66]. miR-148a transfection induced the expression of the CD70 and CD11a, similar to lupus patients [67]. A study by Dai and colleagues identified a miRNA “signature” consisting of 29 miRNAs downregulated in lupus patients’ PBMCs [68]. There have been subsequent studies linking no less than fourteen miRNAs with SLE (reviewed in [69]), a number that will no doubt only increase as more attention is paid to this particular effector of epigenetic regulation.

Rheumatoid arthritis

Early RA methylation studies revealed that patients’ T cells display a remarkable phenotype, similar to SLE, characterized by global hypomethylation [70,71]. Later studies confirmed this, but did not find evidence for association between methylation levels or the DNA methyltransferases and disease activity; furthermore, our most powerful therapeutic agents (TNF α inhibitors) did not alter the RA PBMC methylome [72]. The recent advent of

large DNA methylation assays have allowed for more unbiased evaluations of DNA methylation levels in a genome-wide fashion. Unfortunately, these studies have to date looked only at mixed cell populations (peripheral blood mononuclear cells, PBMCs). Owing to the specificity of methylation marks to particular cell types, it comes as no surprise that these data have not revealed significant differences between patients and controls. Candidate gene studies have identified a pattern similar to that found in SLE, wherein female RA patients have demethylation of the immune costimulator CD40L, which is not found in male RA patients, perhaps offering insight into the female predominance of the disease [73]. More study is certainly warranted in this area, with a focus on particular immune cell subtypes using more modern arrays with truly genome-wide coverage.

A landmark recent study did, however, provide remarkable insights into the interaction of the RA PBMC epigenome and the underlying genomic code. This study compared a large number of patients (354 RA patients positive for anti-citrullinated peptide antibody), analyzing genome-wide DNA methylation data from >450,000 CpG sites alongside SNP genotyping analysis. Importantly, they developed and implemented algorithms to correct for discordant cellular populations in their mixed-cell samples. They identified ten DNA methylation phenotypes that appeared to mediate the underlying genetic risk for RA, with nine being within the major histone compatibility (MHC) complex, previously strongly implicated in RA in extensive previous genetic polymorphism studies [74].

More robust differential methylation association has been found among the RA synovium and synoviocytes. Early candidate gene studies identified hypomethylation of a transposable element, the L1 retrotransposon, known to drive the expression of MAP kinase, creating an aggressive cartilage breakdown phenotype in RA fibroblast-like synoviocytes (FLS) [75,76]. Other candidate gene studies have identified hypomethylation of the matrix metalloproteinase *CXCL12* and hypermethylation of the apoptosis receptor *DR3* [77,78]. DNMT1, the maintenance DNA methyltransferase, is downregulated in RA synovial fibroblasts [79]. This same group subsequently published data pointing to the increased expression of S-adenosyl methionine decarboxylase (AMD), spermidine/spermine N1 acetyltransferase (SSAT1), and polyamine-modulated factor 1-binding protein 1 (PMFBP1), which work in concert to enhance the catabolism and recycling of polyamines and reduce the level of S-adenosyl methionine, the principal methyl donor required for DNA methylation, offering a potential mechanism for the observed differential methylation in RA fibroblasts [80].

The advent of large genome-wide methylation arrays have provided more robust data in RA fibroblast-like synoviocytes. In 2012, Nakano and colleagues reported the first epigenome-wide study comparing RA and osteoarthritis (OA) FLS cell lines. Of the 1859 differentially methylated loci, around 60% were hypermethylated, in stark contrast to the global hypomethylation previously described in circulating immune cells in RA patients. Gene ontology analysis demonstrated overrepresentation of differentially methylated genes in pathways related to immune cell trafficking, cell adhesion, and extracellular matrix interactions [81]. Interestingly, other studies indicated chronic immune stimulation by IL-1, TNF, or toll-like receptor ligands in various combinations in vitro resulted in significant suppression of DNA methyltransferases; in fact, IL-1 supplementation had an effect similar

to the addition of a pharmacologic DNMT inhibitor, leading them to postulate that chronic inflammation may be related to the differential methylation phenotype noted in RA FLS [82].

A second recent genome-wide study by de la Rica et al, published in 2013, compared RASF DNA methylation with miRNA expression and RASF transcriptome data from the Gene Expression Omnibus (GEO) in an integrated analysis. Novel target genes were identified as being differentially methylated, including *IL6R*, *CAPN8*, and *DPP4*. More than 200 of the 714 genes they identified as being differentially methylated had inverse gene expression. Several known-associated and novel miRNAs were identified. This group then performed a rigorous analysis attempting to integrate the omics data from these three potentially interacting spheres. They identified several CpG sites found to be hypermethylated with concomitantly reduced miRNA expression, but only four hypomethylated CpGs associated with increased miRNA expression. Furthermore, they identified an interesting pattern of epigenetic-transcriptional-miRNA interaction including groups of genes in which DNA methylation and miRNA post-translational targeting work in concert to reduce gene expression and several in which these control mechanisms are at odds. This work provides important insights into the depth of epigenetic control and interaction with other transcriptional mechanisms that has yet to be fully explored in detail by the general research community, but is certainly at play in autoimmune disease [83].

Histone modifications in RA have been less well studied, although alterations in the enzymes responsible for these modifications have been cursorily evaluated. A study by Kawabata et al. reported in 2010 that histone deacetylase 1 (HDAC1) expression was increased in response to TNF-alpha supplementation in RA synovial fibroblasts [84]. Targeting HDACs therapeutically does have robust support in the literature: these drugs demonstrate anti-inflammatory effects in FLS, synovial macrophages, and alleviate disease in animal models of RA [85–88]. Indeed, a small open-label trial of the HDAC inhibitor Givinostat in a similar disorder, juvenile idiopathic arthritis, described improvement in arthritis with an impressive safety profile [89].

Studies examining the effects of noncoding RNAs on RA pathogenesis are gaining momentum. Candidate studies have implicated reduced expression of miR-146a in increasing activation of the immune modulator STAT1 and altering the phenotype of regulatory T cells in RA [90], whereas modulation of the MAPK pathway by miR-451 causes an increase in neutrophil chemotaxis [91]. A recent miRNA survey examined the expression of 270 human miRNAs in T cells isolated from RA patients and identified miR-223 and both possessing the ability to reduce IGF1-mediated IL-10 production in activated RA T cells and correlated with the important autoantibody rheumatoid factor (RF) titer [92]. A larger genome-wide analysis correlated downregulation of miR-363 and miR-498 and upregulation of miR-146a positively with TNF-alpha in CD4+ T cells from RA patients [93]. Of direct practical relevance, scrutiny of studies such as these may soon identify biomarkers of RA activity, which remains difficult to fully assess clinically.

Systemic sclerosis

Like SLE, the development of systemic sclerosis is intimately linked to T cell dysfunction, particularly in early disease, where autoreactive T cells transfer signals to surrounding fibroblasts leading to the deposition of collagen and initiation of fibrosis [94]. Also like SLE, it appears that hypomethylation of CD4⁺ T cells, driven at least in part by downregulation of DNA methyltransferases, contributes significantly to the overexpression of various genes important to disease progression [95]. Demethylation of the ordinarily quiescent copy of *CD40L* on the female Barr body has been described, giving a hint about the female predominance of the disease [96]. In addition to its role as an adaptive immunity costimulatory molecule, it also appears that CD40L plays an integral role in the aberrant fibrosis of SSc [97]. CD40 is overexpressed in plasma and skin fibroblasts of SSc patients [98,99], and blockade of the CD40/CD40L interaction can reduce fibrosis in a mouse model of SSc [100]. Also similar to other autoimmune diseases, *CD70* is hypomethylated and its transcript overexpressed in CD4⁺ T cells from SSc patients [101].

Moreover, there are several other pathways unique to systemic sclerosis that are epigenetically dysregulated. One such pathway, Wnt, contributes to skin fibrosis in SSc patients [102]. Hypermethylation and reduced expression of several Wnt pathway antagonists is a feature of systemic sclerosis; particularly, *Dickkopf-1* and *SFRP-1* are both hypermethylated in dermal fibroblasts and PBMCs of the bleomycin-induced skin fibrosis SSc mouse model. Furthermore, reactivation of expression of these genes via treatment with the demethylating agent 5-azacytidine reduces canonical Wnt signaling and eliminates the fibrosis phenotype [103]. Similarly, the collagen suppressor gene *FLII* is epigenetically inactivated via hypermethylation in fibroblasts from SSc patients' skin biopsies and transient transfection of *FLII* into these cells reduced the expression of type I collagen. In contrast to immune cells, the expression of DNMT1, MeCP-2, several histone acetylases and several methyl binding domain proteins are significantly increased in dermal fibroblasts of SSc patients [104].

Fibroblasts play a key role in SSc pathogenesis, particularly in skin and organ sclerosis where they overproduce collagen and other extracellular matrix components. Furthermore, they produce a variety of cytokines that may serve to further dysregulated immune cells. Altorok et al., recently published the first broad genome-wide methylation assay examining large-scale differential methylation patterns in dermal fibroblasts from systemic sclerosis patients [105]. They compared 6 patients with diffuse SSc (dSSC), 6 patients with limited SSc (lSSC), and 12 healthy controls, all being age-, sex-, and ethnicity-matched. In contrast to what would be expected from the increase in DNMT1 seen in previous studies, they found that the majority of differentially methylated CpG sites were hypomethylated in SSc compared to controls. In dSSC, 2710 CpG sites were differentially methylated compared to controls, and in lSSC, 1021 CpG sites were differentially methylated. Interestingly, only 203 of these CpG sites were concordant among the two disease subtypes. Among shared sites, several involved collagen genes (*COL4A2*, *COL23A1*) or collagen breakdown enzymes (*ADAM12*). Among the subsets, there was further hypomethylation of *COL8A1*, *COL16A1*, and *COL29A1* in dSSC, and *COL1A1*, *COL6A3*, and *COL12A1* in lSSC. The transcription factors *RUNX1* and *RUNX2*, also associated by our group with eroded cartilage in

osteoarthritis [106], and the T-cell associated transcription factor *RUNX3* were hypomethylated among both disease subtypes. Pathway analysis identified the expected overrepresentation of the Wnt pathway. Several hypomethylated genes were also found to have overexpression of their gene products in RT-PCR analysis, as were genes associated with TGF β , which contributes to fibrosis in SSc. This study further reiterates the importance of these two pathways in the pathogenesis of SSc.

Several histone modification patterns similar to those in SLE have been described in association with SSc. Both H3 and H4 acetylation is reduced in SSc fibroblasts [104]. Global hyperacetylation of H4 has been noted in B cells from SSc patients [107]. Chemical inhibition of H3K27me3 in murine models of SSc exacerbates bleomycin-induced fibrosis, through the induction of the transcription factor Fra-2; furthermore, knockdown via siRNA of Fra-2 completely abrogated this effect [108]. Future drug therapy of targeting histone modification has proven quite fruitful in models of SSc: the general HDAC inhibitor trichostatin A or direct HDAC inhibitors reduces fibrosis in animal models [109], and reduces expression of collagen in cultured SSc fibroblasts [104,110].

MicroRNAs have been implicated in SSc as well, particularly as it pertains to dysregulated fibrosis. miR-29a is decreased in SSc fibroblasts, and transfection of miR-29a into diseased fibroblasts leads to a reduction in Col 1 and 3 expression [111]. miR-29a transfection into an animal model of SSc can prevent pulmonary fibrosis, and knock-down of miR-29 increases levels of pro-fibrotic TGF β and PDGF-B [112]. miR-21 is regulated by TGF β and targets Smad-7, a key profibrotic gene in SSc [113,114]. miR-155 is elevated in animal models of SSc, and appears to mediate fibrosis by targeting of keratinocyte growth factor [115]. miR-196a serum levels correlate with the modified Rodnan skin score, and has been proposed as a biomarker of dSSc [116]. This group also proposes that miR-196a is a direct regulator of collagen 1 *in vitro*, as transient overexpression of miR-196a reduces collagen levels.

Sjogren's syndrome

Epigenetic mechanisms have a long history in the Sjogren's syndrome (SS) literature, dating back to the demonstration of a link between administration of hydralazine and the development of SS and autoantibodies. Similar to other autoimmune diseases, DNA methylation in SS circulating immune cells is generally reduced. The T-cell costimulatory gene *CD70* has been demonstrated as hypomethylated and overexpressed in SS CD4+ T cells [117]. Furthermore, as SS is a prototypical autoimmune disease, one would expect that the population of regulatory T cells would be reduced with disease. This is indeed the case, as CD4+FOXP3+ cells are reduced, owing to hypermethylation and reduced expression of the *FOXP3* gene [118].

Our group reported earlier this year in a genome-wide DNA methylation study in naïve CD4+CD45RA+ cells, comparing 11 patients with primary SS to age-, sex-, and race-matched controls 553 differentially methylated CpG sites. The majority of these (64%) were hypomethylated. Several genes of interest were differentially methylated. *LTA*, encoding lymphotoxin α , is part of a receptor complex that is involved in the activation of follicular dendritic cells and release of interferon-alpha. This is a particularly interesting finding, as

LTA is overexpressed in both salivary gland tissue and sera of SS patients (23). In addition, it is upregulated in the salivary glands of SS mouse models, and deletion of *LTA* in these same mouse models prevent the development of SS [119]. Indeed, blockade of the LT receptor by a LT β R fusion protein, a subunit of the heterodimeric receptor complex, is ongoing as a clinical trial for SS (ClinicalTrials.gov ID# NCT01552681).

Several genes involved in type I interferon pathway were also noted to be differentially methylated, including hypomethylated *STAT1*, hypomethylated *IFI44L* (also differentially methylated in SSc and SLE), *IFITM1*, and *USP18*. This is a finding similar to, though less expansive, than in SLE. The *RUNX1* gene, a transcription factor particularly important in T cell development, was hypermethylated among SS patients. The T-cell receptor ζ chain gene *CD247* was hypomethylated, highlighting the importance of T cell signaling in SS.

Although less well defined than circulating immune cells, epithelial cells from salivary gland (SGEC) in SS patients also exhibits global hypomethylation, owing to reduced expression of DNMT1 and an increase in GADD45alpha. Interestingly, this may in part be driven by infiltrating B lymphocytes. Following treatment with the anti-CD20 monoclonal antibody rituxumab, salivary gland biopsies from SS patients demonstrated significant increases in global DNA methylation levels. This finding was corroborated with *in vitro* co-culture experiments [120].

To date, no large-scale studies of histone modification patterns have been conducted in SS, and only a few miRNA experiments have been reported. The best demonstrated association is with miR-146a, also seen in RA patients. In primary SS, miR-146a expression is increased in PBMCs, prior to the onset of clinical symptoms, and persists during disease in salivary gland tissues [121].

Expert Commentary: The future of epigenetics in autoimmunity

Throughout this review, we have sought to highlight the most important discoveries in epigenetic regulation, and dysregulation, of pathways involved in the development of prevalent autoimmune diseases. With this in mind, where will further work in the field of autoimmune epigenetics take us? Perhaps the first step should be a concerted effort to investigate the functional consequences of the epigenetic associations previously described. Unfortunately, we do not yet have technology to efficiently and accurately manipulate the epigenome in a site-specific way to allow us to do this in primary cells *in vivo*. The recent description of endogenous bacterial DNA targeting systems (e.g. TALE DNA binding domains) fused with various epigenetic effectors, a masterful example of bioengineering, is starting to move the field in this direction [122]. In the future, one can envision site-specific modification of most any epigenetic mark in a similar way; this will not doubt provide remarkable insights into the contributions of particular epigenetic aberrations to disease phenotypes, but perhaps also offer targeted therapeutic options for use in the clinic.

Drugs broadly targeting the epigenome are already in widespread use, particularly in the field of oncology, where the DNMT1 inhibitor decitabine is approved for the treatment of several liquid malignancies, and is under investigation as an adjunctive treatment for the “sensitization” of tumors pre-chemotherapy [123–125]. Histone deacetylase inhibitors are

enjoying a similar flurry of interest as potential oncotherapeutic agents and have been described in laboratory studies of autoimmunity, as discussed previously. Unfortunately, the epigenotype of most autoimmune disease involves global hypomethylation, which is quite difficult to target with our current therapeutic arsenal.

Five-year view

In the next five years, further studies examining the epigenome of as yet undescribed, particular tissue types in autoimmunity will be forthcoming, aided by the development of ever more comprehensive and more accessible technologies. At some point in the near future, the cost per sample of performing whole-genome bisulfite sequencing will fall to parity with that of microarrays, allowing the development of large epigenome biorepositories, which may be mined for associations among the various autoimmune diseases' clinical presentations and biological manifestations that have not yet been described. Of particular interest in this area are the contributions of the endogenous microbiome to immunological epigenetics and the development of autoimmune disease, a field of study that will no doubt only increase in importance in the coming years and may offer novel therapeutic opportunities [126–128]. Along these same lines, we anticipate the development of epigenetic biomarkers of both disease presence and activity, particularly in lupus, where the prediction of flares might lead to prevention by early interventions, sparing the administration of high doses of often expensive, damaging immunosuppressives, reducing both cost and patient morbidity.

Taken together, the future of epigenetics in autoimmunity looks bright indeed, as contributions in this field have moved us ever closer to understanding the development and progression of these complex diseases.

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Key Issues

- Studies describing epigenetic modifications (changes in DNA and chromatin protein secondary structure and noncoding RNA feedback affecting expression of gene products without alterations in the underlying DNA sequence) are becoming commonplace in the autoimmunity literature, but must be scrutinized carefully, as the interplay between epigenetic and genetic variation as it relates to disease phenotype is complex and not completely understood.
- The overwhelming majority of studies performed so far in the field of autoimmune epigenetics have been case-control designs, and most have described differential DNA methylation in an epigenome-wide association study (EWAS) manner.
- Systemic lupus erythematosus is characterized by a global hypomethylation phenotype accompanied by a reduction in the maintenance DNA methyltransferase DNMT1, particularly in CD4⁺ T cells, where most epigenetic research has been focused.
- A variety of hypomethylated genes appear to be key to SLE pathogenesis and have been described in several studies, including CD11a, CD70, Perforin, and CD40L in CD4⁺ T cells.
- In SLE naïve CD4⁺ T cells a permissive epigenetic alteration in interferon-regulated genes is present before active gene expression, and explains the hypersensitivity of lupus PBMCs to type-I interferon.
- Genetic / epigenetic interactions have highlighted the complexity of SLE, particularly as it relates to *MECP2/IRAK1* associations.
- Rheumatoid arthritis patients' T cells also exhibit a hypomethylated phenotype and a reduction in DNMT1, with strong evidence for genetic/epigenetic interaction in the MHC complex. By contrast, fibroblast-like synoviocytes present a hypermethylated phenotype.
- Systemic sclerosis is characterized by hypomethylation within CD4⁺ T cell genes, including CD40L, like SLE.
- In SSc, hypomethylation of collagen genes appears to play a key role in the fibrotic phenotype of this disease.
- Sjögren's syndrome has been less well studied than other autoimmune diseases; in naïve T cells, a hypomethylated phenotype is seen and several type I interferon genes are particularly differentially methylated.
- Histone modifications have been less well studied in all autoimmune diseases than DNA methylation patterns.
- In lupus, generalized hypoacetylation of H3 and H4 have been observed in mouse models, and reversing this with HDAC inhibitors reduces disease. Human studies have shown significant increases in H4 acetylation in SLE

monocytes; furthermore, patients and mouse models have antibodies that react more strongly to hyperacetylated H4 than “normally” acetylated protein.

- In RA, histone deacetylase 1 expression is dysregulated, and targeting of HDACs does have therapeutic benefit in animal models.
- SSc fibroblasts have a histone pattern similar to SLE; both H3 and H4 hypoacetylation is seen. Treatment of animal models with HDACi improves some aspects of disease.
- miRNA studies are in their infancy in each of these autoimmune diseases. In lupus, large miRNA screens have been performed. Both miR-148a and miR-21 seem to have dual epigenetic effects, as they reduce the expression or function of DNA methyltransferase machinery. A miRNA “signature” consisting of 29 downregulated miRNAs has been described in SLE. Similarly, miR-363, miR-498, and miR-146a are differentially expressed in RA CD4+ T cells. miR-21 is also implicated in SSc.
- No large histone or miRNA studies have been conducted in Sjögren’s syndrome.
- In the next five years, we look forward to further epigenotyping of other, perhaps less obvious cell types in these diseases, guided by our growing databases of genetic and epigenetic disease associations. We anticipate the development of biomarker assays for several of these diseases based upon epigenetic alterations, and perhaps the utilization of epigenetically-active therapeutics to complement traditional drugs.

Figure 1.
A depiction of the three main epigenetic mechanisms in regulating gene expression. A) Histone tail modifications, B) DNA methylation, and C) microRNA regulation.

Table 1

General aspects of the three commonly described epigenetic mechanisms in systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), systemic sclerosis (SSc), and Sjogren's syndrome (SS)

	DNA methylation	Histone modification	Noncoding RNA
SLE	T cells: global ↓, IFN signature	↑ H3, H4 methylation ↓ H3, H4 acetylation	↑ miR-21, ↑ miR-148a
	<i>CD70↓, CD11a↓, CD40L↓, Perforin↓</i>		
RA	T cells: global ↓ Synoviocytes: global ↑	No large-scale analysis. <i>HDAC inhibitors effective in animal models.</i>	↑ miR-146a, strong link between many miRNAs and DNA methylation
SSc	T cells: global ↓ Fibroblasts: global ↓	Fibroblasts: ↓ H3, H4 acetylation. <i>HDAC inhibitors effective in animal models.</i>	↑ miR-29a, ↑ miR-196a
	Wnt pathway genes ↑		
SS	T cells: global ↓, including naïve	No large-scale analysis.	↑ miR-146a prior to symptom onset
	Type-I IFN pathway genes ↓		