Annals of the Rheumatic Diseases collection on epigenetics: from three dimensional chromatin organisation to microRNA

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INTRODUCTION

The term 'epigenetics' describes various mechanisms that in their entirety control the gene expression of a cell. These include chromatin folding, DNA packaging around histones and histone modifications, direct modifications on the DNA (figure 1) and non-coding RNA such as microRNA or long non-coding RNA. These epigenetic modifications determine cellular differentiation, maintain the specific phenotype of a cell and ensure the maintenance of genomic integrity, for example, by silencing retroviral sequences.

In a strict sense, epigenetics has been defined as mitotically and meiotically heritable.[1] This is of considerable interest in rheumatic and musculoskeletal diseases (RMD) to understand the development of chronicity and heritability, which cannot be explained by genetic factors alone. However, research in recent years has shown that epigenetic control of gene expression may well be transient and susceptible to modulation. Furthermore, technical advances and simultaneous genome-wide analysis of different epigenetic mechanisms in combination with genetic analysis revealed how interconnected genetic and different epigenetic mechanisms really are.

Several studies published in the *Annals of the Rheumatic Diseases* (*ARD*) have significantly contributed to the understanding of how epigenetic mechanisms might steer pathogenic mechanisms in RMD. In this article, published work in *ARD* from 2021 to 2023 is summarised and discussed. These manuscripts gave insights in how chromatin folding, histone modifications, DNA methylation and microRNA can influence disease development and drive pathogenic cellular activation in RMD.

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THREE-DIMENSIONAL CHROMATIN ORGANISATION AND HISTONE MODIFICATIONS

Nuclear organisation and three-dimensional chromatin structure are important higher order epigenetic regulators of gene expression. Within the nuclear territories covered by the different chromosomes (figure 1A), DNA strands bound to histones form loops that divide into active A compartments and inactive B compartments (figure 1B). Within these large compartments, insulators separate topologically associating domains (TAD) from each other, so that gene regulatory interactions between enhancers and promoters predominantly occur within one TAD (figure 1C). Enhancers are marked by methylation of lysine 4 of the histone tails in histone 3 (H3K4me1), promoters by H3K4me3 modifications (figure 1D). Their activity is regulated by the presence of H3K27 acetylation of the histone tails. Inactive promoters/enhancers are tri-methylated at H3K27 (H3K27me3).

Most recently, Zhao *et al*[2] analysed whether changes of this chromatin organisation occur in CD4+T cells from patients with systemic lupus erythematodes (SLE). Principal component analysis suggested differences in TAD and loop formation between healthy controls and patients with SLE with more pronounced changes in patients with higher SLE Disease Activity Index (SLEDAI). The authors could identify specific chromatin loops that correlated with high SLEDAI. The size of these 'SLE-loops' were on average longer than the average loop size and more often spanned different TADs (inter-TAD loops). In over 90%, these loops connected enhancers and promoters and the differentially expressed genes within these loops were enriched in inflammatory and immune-related pathways. In conclusion, this study showed profound higher order chromatin changes underlying or at least accompanying gene expression changes in SLE CD4+T cells.

Similar to SLE T cells, in which changes in DNA methylation were described over 30 years ago,[3] it has been known for a long time that rheumatoid arthritis (RA) synovial fibroblasts are affected by epigenetic changes.[4] Now, Tsuchiya *et al*[5] used an extensive set of epigenetic, transcriptomic and genetic measurements in resting and stimulated synovial fibroblasts to delineate the effect of epigenetics on RA synovial fibroblast gene expression. By measuring enhancer promoter interactions in basal and stimulated conditions, they could show that stimulation specific responses of synovial fibroblasts were more often connected to so-called superenhancers, which are defined by high density of enhancer specific histone marks (H3K4me1), activation marks (H3K27ac) and transcription factor binding, than traditional enhancers. RA genetic risk loci were enriched at superenhancers that formed after stimulation of synovial fibroblasts with a mix of 8 cytokines (IFN-α, IFN-γ, TNF-α, IL-1β, IL-6/sIL-6R, IL-17, TGF-β1, IL-18). The results suggested that the transcription factor MTF1 was strongly associated with the formation of superenhancers in stimulated synovial fibroblasts and inhibition of MTF1 showed beneficial effects in collagen-induced arthritis in mice. Thus, therapeutic targeting of MTF1 might be able to influence epigenetic remodelling of synovial fibroblasts after an inflammatory stimulus.

Therapeutic modulation of the epigenetic landscape is a major scientific interest in RMD, as it may be the key to 'reprogramming' chronically active pathogenic cell types. Based on their previous studies showing a lack of H3K79me2 in osteoarthritis (OA) chondrocytes,[6 7] Assi *et al*[8] explored the expression of histone demethylases in human articular cartilage. They could show that the expression of the JmjC demethylases KDM6B and KDM7 was increased in healthy chondrocytes after IL-1β stimulation and that levels of KDMA3A and KDM7 were strongly upregulated in affected areas of OA cartilage compared with unaffected areas. Silencing of KDM7A or KDM7B in primary human OA chondrocytes increased H3K79me2 levels as well as transcription of COL2A1 and decreased expression of MMP13 and ASAMTS5. Pharmacological blockade of KDM7 by using the KDM2/7 inhibitor daminozide restored H3K79me2, reduced cartilage damage and COLX protein levels and prevented aggrecan degradation in mice after destabilisation of the medial meniscus. Thus, daminozide might be an

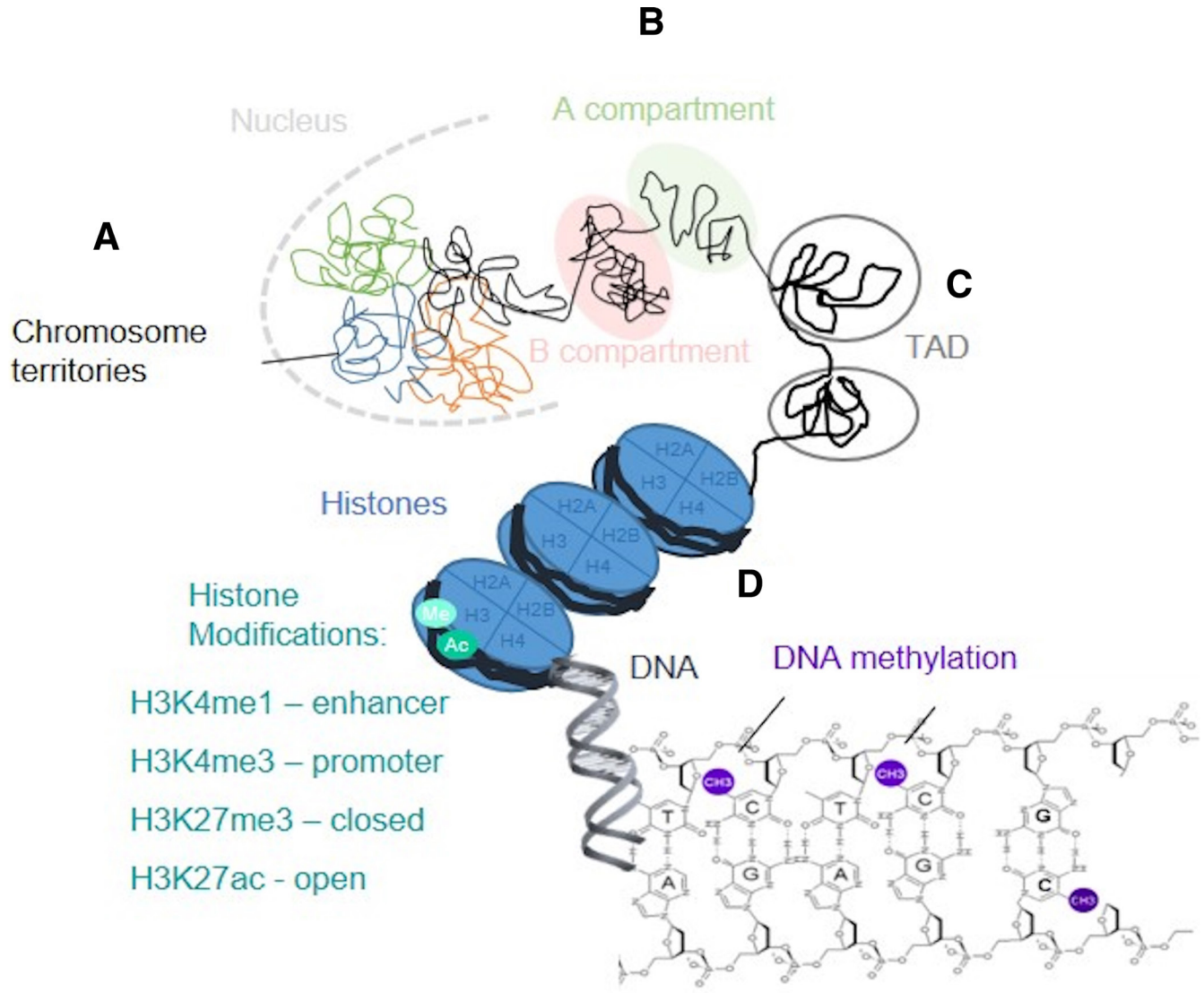

Figure 1 Mechanisms of epigenetic gene regulation. In the nucleus, the different chromosomes occupy distinct territories (A). Chromosomal chromatin is found as active A compartments or inactive B compartments (B). Within the compartments, the chromatin is organised in topologically associating domains (TAD), in which internal chromatin interactions preferentially take place (C). The chromatin comprises the DNA strand and the histones around which the DNA strand is wrapped (D). Transcriptional activity and regulatory function of a DNA locus is regulated by chemical modifications of the histone tails, for example, acetylation, methylation and of the cytosine base, for example, DNA methylation.

effective regulator of pathogenic loss of H3K79me2 in human OA.

The effect of a gain of H3K4me3 histone marks in the RANK promoter was deciphered by Ansalone *et al*.[9] They first showed that, in addition to classical HLA−DR+CD14+CD16− monocytes, surprisingly circulating CD14−CD16−CD11c+myeloid cells differentiated into mature osteoclasts in vitro. This population showed increased H3K4me3 histone marks in the RANK promoter with higher transcription of RANK, when compared with classical monocytes. The latter only increased H3K4me3 histone marks at the RANK promoter during the course of osteoclastogenesis, after stimulation with RANK-L. At an early time point of osteoclast differentiation, addition of TNF to classical monocytes inhibited this gain of H3K4me3 at the RANK promoter and consequently inhibited osteoclast formation via TNFR1 mediated signalling. Osteoclast formation from CD14−CD16−CD11c+ myeloid cells was not affected by early addition of TNF, potentially because these cells already had high H3K4me3 marks in the RANK promoter at baseline and lower levels of TNFR1 compared with classical monocytes. Circulating CD14−CD16−CD11c+ cells were reduced in RA patient's blood compared with healthy controls, which could be a sign of their depletion and differentiation in the joint. Most interestingly, classical monocytes of patients with RA showed increased H3K4me3 marks in the RANK promoter with increased RANK transcription compared with healthy monocytes. Subsequently, TNF addition to differentiating classical RA monocytes at early time points did not show the previously mentioned inhibitory, but a promoting effect on osteoclastogenesis in 56% of patients with RA. This study nicely illustrates how histone marks steer cell differentiation and how alterations of these marks in disease can modulate cellular response.

DNA METHYLATION

DNA methylation is the most studied epigenetic modification in RMD. DNA methylation occurs mainly in CG rich regions of the DNA, so-called CpG islands. DNA methylation of CpG islands

in promoter regions is associated with transcriptional repression, while promoter hypomethylation correlates with increased gene expression. Many aspects of how DNA methylation regulates gene expression outside of promoters are not yet well understood.

In RMD, changes of DNA methylation have been described in various immune and stromal cells mainly in RA, OA and SLE. After a publication in 2016 in *ARD*, showing changes in DNA methylation in temporal artery tissues,[10] Estupiñán-Moreno *et al*[11] analysed for the first time changes in the DNA methylome in circulating CD14+ monocytes in people with giant cell arteritis (GCA). As in GCA temporal arteries, the measured regions were mainly hypermethylated in GCA compared with healthy CD14+ monocytes. These changes mostly did not occur in gene promoters, but in gene bodies and intergenic regions and were not associated with CpG islands, suggesting epigenetic changes in distal regulatory regions to be involved in gene regulation in GCA. Subgroup analysis of the samples showed substantial differences between DNA methylation in CD14+ monocytes of active untreated GCA patients compared with patients in drug free remission. Of note, in contrast to changes in gene expression, only very few differences in DNA methylation (27 CpG sites) were detected when cells from patients in remission with and without glucocorticoid treatment were compared. No significant changes in DNA methylation were detected between healthy controls and patients in remission with treatment. Together, this suggests that glucocorticoid treatment successfully reverted GCA-associated DNA methylation changes in monocytes.

A study from Cooles *et al*[12] analysed changes in DNA methylation in acohort of patients with early RA naïve for disease modifying antirheumatic drugs (DMARDs) and glucocorticoids to elucidate epigenetic mechanisms underlying the interferon gene signature (IGS) that has been connected to a worse disease outcome in patients with RA.[13] They first confirmed that patients with the IGS had a smaller probability of achieving a good response 6 months after initiation of treatment and showed that the IGS score, which is based on gene expression of five interferon response genes in blood cells, strongly correlates with serum interferon (IFN) α levels. By comparing DNA methylation levels in peripheral B and T cells of patients with a high IGS score to patients with a low IGS score, they found 330 CpGs differentially methylated in CD4+T cells and 287 differential CpGs in CD19+B cells. Intriguingly, several interferon response genes were affected from these changes in DNA methylation such as *IFI44L, RSAD2, MX1* as well as *STAT1* in CD4+T cells and *PARP9* and *EPSTI1* in CD19+B cells. STAT1 is a key transcription factor in driving the IFN response, PARP9 is known to regulate type I IFN production and EPSTI1 is an interferon response gene. All three of them were higher expressed in CD4+T cells or peripheral blood mononuclear cells (PBMCs) isolated from patients with a high ISG score and remained high 6 months after treatment and even though IFNα levels went down with treatment. This pointed to a stable epigenetic imprinting of the IFN response, which might be a driver of the poor outcome in this subgroup of patients.

Ha *et al*[14] used analysis of DNA methylation in RA T cells to understand how RA genetic risk variants exert functional effects. Integration of genetic, DNA methylation and gene expression data revealed a substantial impact of RA genetic risk genes located in differentially methylated regions and associated with changes in gene expression on RA heritability. However, only 83 differentially expressed genes between healthy and RA T cells were identified whose changed expression pattern could be explained by meQTL, that is, a change in DNA methylation that is associated with a genetic variant. This small number is most likely due to the fact that the study was not sufficiently powered for this analysis with 82 patients. However, the study proved that at least some of the effects of genetic risk variants on gene expression in RA are mediated via changes in DNA methylation.

A similar approach was used by Coit *et al*[15] who analysed the connection between genetic factors and DNA methylation in CD4+T cells of patients with SLE. First of all the data confirmed their previous reports on DNA hypomethylation in interferon-regulated genes in SLE T cells.[10 16] Additionally, the authors found that several loci encoding for microRNA were hypomethylated in diseased cells and provided evidence that in particular increased expression of miR-18a might be epigenetically regulated in SLE. A genome wide comparison between genotype and DNA methylation profile in SLE and healthy control T cells showed that less than 1% of differentially methylated sites were meQTL. Therefore, the authors concluded that changes in DNA methylation in SLE CD4+T cells are not driven by genetic background. However, also this conclusion has to be taken with caution since usually meQTL studies include thousands of individuals and this study used only 63. Accordingly, an earlier study analysing over 1000 samples found evidence of genetic regulation of changes in DNA methylation in CD4+T cells.[17] Nevertheless, Coit *et al* identified three SLE genetic risk variants that were associated with changes in DNA methylation namely, rs1131665 within the *IRF7* gene and rs56154925 between *TMEM86B-PTPRH,* both associated with hypomethylation and rs170942 in the CFB gene associated with DNA hypermethylation.

The influence of genetics on epigenetic traits as shown in the above-mentioned studies, while of interest, interferes with measuring the influence of environmentally induced epigenetic changes. Studies in monozygotic twins permit analysis of the effects of epigenetic changes without the influence of genetics. An interesting study by Malaab *et al*[18] used a cohort of twins discordant for systemic sclerosis (SSc) (seven monozygotic and two dizygotic) to study DNA methylation differences between healthy and SSc cultured dermal fibroblasts. They found 174 significantly differentially methylated CpG sites that mapped to gene bodies of 83 distinct genes. Several genes with the strongest difference in DNA methylation between healthy and diseased dermal fibroblasts were T-box and homeobox transcription factors such as TBX5, MEIS1 and genes from the HOX cluster with a known role in embryonic limb formation. Changes in DNA methylation often affect these 'embryonic' gene loci, because their expression is strongly controlled by epigenetic mechanisms in a time and location specific manner during embryogenesis and later in life. Accordingly, microRNA encoded in the HOX locus, miR-10a and miR-10b were downregulated, in particular in fibroblasts from patients with limited cutaneous SSc, and regulated the expression of HOXD10 and TBX5 in healthy human dermal fibroblasts. The study further showed that silencing of TBX5 increased the levels of the anti-fibrotic transcription factor KLF4. Thus, the authors concluded that changes in DNA methylation and microRNA expression lead to aberrant expression of TBX5, which via KLF4 lead to profound changes in the anti-fibrotic programme of dermal fibroblasts in SSc.

MICRORNA

MicroRNA are crucial post-transcriptional regulators of gene expression. They are strongly interlinked with DNA

methylation and histone modifications and are regarded as a major mechanism of intergenerational inheritance of environmentally induced epigenetic changes.[19] MicroRNAs are found in the cell, but also circulate in body fluids such as serum, urine or saliva. This makes them ideal candidates for biomarker studies.

Emmi *et al*[20] analysed the microRNA profile in the plasma of 16 patients with Behcet's disease compared with 18 healthy controls using microarray technology and validated their findings in a cohort of 30 patients and healthy controls, respectively. They found 29 differentially secreted microRNA. The predicted targets of these microRNA were enriched in cell–matrix interaction, oxidative stress and blood coagulation pathways which pointed towards the regulation of thromboinflammatory pathways by these altered microRNA. Six of the most changed microRNA were measured in the validation cohort, from which miR-224–5 p, miR-206 and miR-653-5 p could be confirmed to be upregulated in plasma of patients with Behcet's disease. The expression of these microRNA correlated with the levels of reactive oxidant species in immune cell populations and plasma oxidation, supporting their role in thromboinflammation. Furthermore, these microRNA were also higher in Behcet's disease compared with plasma from patients with SLE or GCA and could discriminate Behcet's disease from SLE with a specificity of 0.80 and a sensitivity of 0.77 and from GCA with a specificity of 0.81 and a sensitivity of 0.70. The discriminating power of these three microRNA between active and inactive Behcet's disease was with 0.94 very specific, but only had a sensitivity of 0.42.

Circulating microRNA mostly are packed in exosomes, which is considered an important cell–cell communication pathway between tissues. Tavasolian *et al*[21] isolated exosomes from plasma of 22 patients with ankylosing spondylitis (AS) and 18 healthy controls to analyse their content and function. Small RNA sequencing of the RNA contained in the exosomes showed that 22 microRNA were present in higher amounts in AS exosomes compared with healthy and only let-7b-5p and let7c-5p were less abundant in AS exosomes. Interestingly, exosomes isolated from AS patients contained significantly less IL-8 and IL-10 and induced the expression of IFNα and IL-33 in healthy CD4+T cells. Furthermore, coculture with AS exosomes but not control exosomes inhibited proliferation of healthy CD4+T cells and FOXP3+T regulatory (Treg) cells. One of the most strongly enriched microRNA in AS exosomes was miR-30c-5p, which targets IRF4, a critical factor in Treg differentiation. AS exosome treatment increased the levels of miR-30c-5p in CD4+T cells and reduced the presence of FOXP3+IRF4+ Treg as did direct transfection of CD4+T cells with miR-30c-5p mimic. In summary, this study proposed that altered microRNA content in AS exosomes modulates T cell differentiation, shifting the balance to activated T cells in AS.

CONCLUSIONS

Overall, the studies discussed impressively illustrate the broad spectrum of functions that epigenetic mechanisms exert in health and disease and the breadth of research in the field of epigenetics in RMD. These studies have shown us that changes in gene expression can be related to profound remodulation of histone modifications and chromatin organisation. They highlighted the interplay between genetics and epigenetics, identified epigenetic changes that mediate pathogenic pathways in RMD and gave insights in the functional role of microRNA in pathogenic mechanisms and cell activation.

Do these studies suggest a role for epigenetics as a biomarker in clinical practice? MicroRNAs are considered to hold the most potential to be used as biomarkers. However, due to a lack of specificity and reliability, microRNA measurements have not yet been used as predictive or prognostic biomarkers for any disease. Although the study on microRNA profiles in Behcet's disease discussed here has potential, it still needs to be shown that these measurements are reliable, robust and practicable. In contrast, DNA methylation profiles already fulfil these requirements in other fields. Measurements of DNA methylation with Illumina arrays have become key in the diagnosis, classification and prognosis of central nervous system tumours.[22] Given the abundance of DNA methylation data in RMDs and the decreasing cost of DNA methylation measurements, this is a promising area for the RMD field to move towards.

Currently, large meQTL studies and studies analysing the influence of RMD environmental risk factors on the occurrence of the detected epigenetic changes are lacking. The functional role of microRNA in RMD is still poorly understood, and the role of epigenetic inheritance in RMD has not been investigated. Given the RMD research community's great expertise and interest in epigenetics, we can be confident that future studies will elucidate these fundamental questions of RMD epigenetics.

Handling editor Josef S Smolen

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Contributors CO wrote the manuscript.

Funding The authors have not declared a specific grant for this research from any funding agency in the public, commercial or not-for-profit sectors.

Competing interests None declared.

Patient consent for publication Not applicable.

Provenance and peer review Commissioned; externally peer reviewed.

To cite Ospelt C. *Ann Rheum Dis* Epub ahead of print: [*please include* Day Month Year]. doi:10.1136/ard-2023-224857

Received 20 October 2023
Accepted 23 November 2023

Ann Rheum Dis 2023;**0**:1–5.
doi:10.1136/ard-2023-224857

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