

## Epigenetics in atherosclerosis and inflammation

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### Abstract

Atherosclerosis is a multifactorial disease with a severe burden on western society. Recent insights into the pathogenesis of atherosclerosis underscore the importance of chronic inflammation in both the initiation and progression of vascular remodelling. Expression of immunoregulatory molecules by vascular wall components within the atherosclerotic lesions is accordingly thought to contribute to the ongoing inflammatory process. Besides gene regulatory proteins (transcription factors), epigenetic mechanisms also play an essential and fundamental role in the transcriptional control of gene expression. These epigenetic mechanisms change the accessibility of chromatin by DNA methylation and histone modifications. Epigenetic modulators are thus critically involved in the regulation of vascular, immune and tissue-specific gene expression within the atherosclerotic lesion. Importantly, epigenetic processes are reversible and may provide an excellent therapeutic target. The concept of epigenetic regulation is gradually being recognized as an important factor in the pathogenesis of atherosclerosis. Recent research provides an essential link between inflammation and reprogramming of the epigenome. In this review we therefore discuss the basis of epigenetic regulation – and the contribution thereof in the regulation of inflammatory processes in general and during atherosclerosis in particular. Moreover we highlight potential therapeutic interventions based on epigenetic mechanisms.

**Keywords:** atherosclerosis • epigenetics • inflammation • chromatin • histone modifications • CAD • KDAC inhibitors

### Introduction

Cardiovascular diseases (CVD) are the number one cause of death in western societies [1, 2]. Atherosclerosis is the primary cause of coronary artery disease (CAD) and stroke and is regarded as a (chronic) inflammatory disease [3, 4]. Atherosclerosis is characterized by asymmetrical focal thickenings of the arterial intima that are

often referred to as atherosclerotic lesions or atheromas [5]. These thickenings are caused by the accumulation of lipids and inflammatory cells in the vessel wall [5]. Especially T cells and macrophages play an important role in pathogenesis of atherosclerosis [6]. Besides lipids and inflammatory cells, the lesion – depending on

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the stage of the disease – further consists of vascular endothelial cells (vECs) and vascular smooth muscle cells (vSMCs), and extracellular matrix (ECM) [7]. Extreme thickening of the intima or even plaque rupture with subsequent thrombus formation may seriously hamper coronary blood flow, eventually leading to myocardial infarction [8]. Thus, atherosclerosis is a potential life-threatening condition and understanding the cause of atherosclerosis may contribute to the treatment and possibly prevention of this disease.

Atherosclerotic plaque formation is a dynamic multi-cellular process in which the activity of the different cell types involved is essentially determined by the regulation of different genes [3–8]. Nowadays we are fully aware of the important involvement of epigenetic processes in the regulation of gene expression. Understanding these processes is critical for our understanding of inflammatory responses and disease. For instance, not only is cytokine expression under epigenetic control, cytokines themselves induce (indirect) changes to the chromatin, providing an essential link between inflammation and epigenetic programming [9]. Here, we review the role of epigenetic regulation in vascular remodelling and in the activity of immune cells involved in atherosclerotic plaque formation.

## Epigenetics explained

Although all cells in our body contain the same genetic material, each cell acts in a cell-type specific manner, as determined by its gene expression profile. Epigenetic mechanisms control gene expression without modifying the actual genetic code, while the altered gene expression patterns can be passed on to the daughter cells upon cell division or even transgenerationally [10–12].

In its natural state DNA is packaged into chromatin, a highly organized and dynamic protein-DNA complex which consists of DNA, histones and non-histone proteins [13]. Epigenetic mechanisms alter the accessibility of chromatin by modifying DNA and by modification or rearrangement of nucleosomes, which include post-translational modifications of histones [14–16]. Accessible chromatin allows gene-regulatory proteins (transcription factors) to interact with their cognate binding sites within the regulatory regions of genes, such as proximal promoters and enhancers/silencers. In this way, global gene activation and local control of gene-specific transcription is exerted by components of the epigenetic machinery. Moreover, environmental factors have an important role in the establishment of the epigenome [17–21]. Since epigenetic alterations can accumulate in time, environmental factors can have profound effects on the cellular repertoire of expressed genes [10].

Already in 1975 DNA methylation was proposed to play a role in regulating gene transcription [22, 23]. Generally speaking, DNA methylation is associated with low gene activity. Actively transcribed genes are usually maintained in an unmethylated state in their promoter regions [24–26]. DNA methylation involves methylation at the C5 position of cytosine residues in a CpG dinucleotide

(*i.e.* cytosine followed by a guanine) context, exerted by DNA methyltransferases (DNMTs). DNMTs are capable of both methylation and demethylation – as has been postulated in two recent reports – thus making the modification reversible [27, 28]. Notably, CpG dinucleotides are underrepresented in the genome of eukaryotes, but can be found in clusters – so-called ‘CpG islands’ – which in turn are mainly found in promoter regions. The term ‘CpG island’ is defined as a region of at least 500 base pairs with a CG content greater than 55% [29].

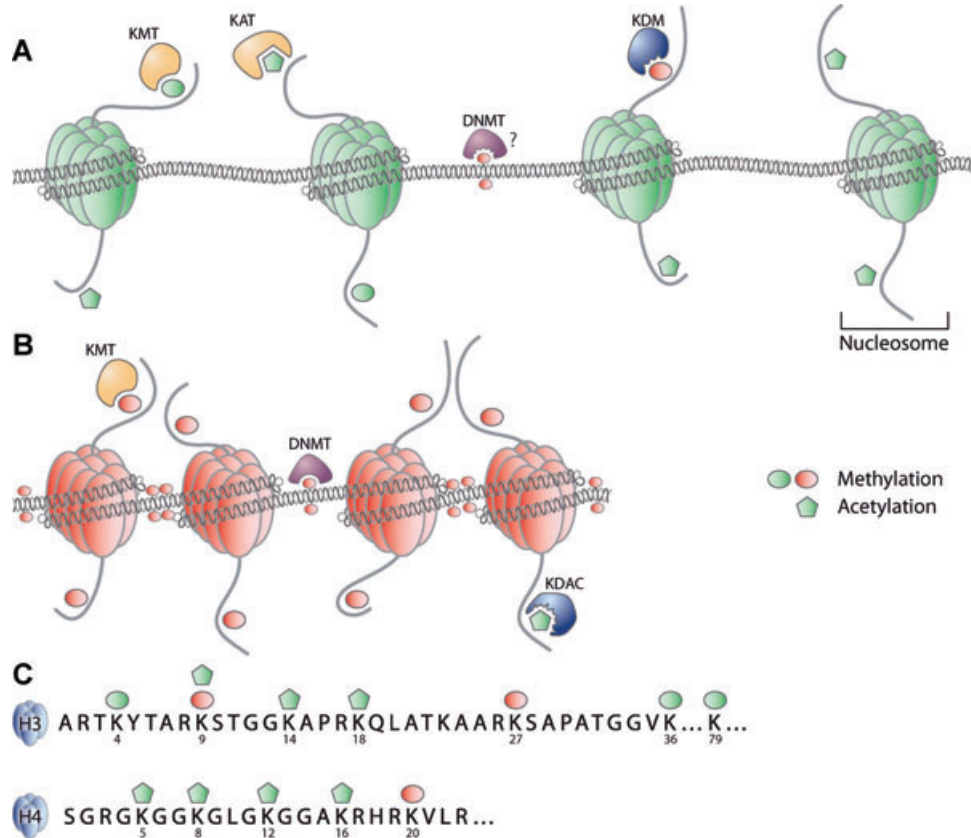
For many years it was thought that functionally relevant DNA methylation occurs at the CpG islands within the promoter region. However, in a recent study by Irizarry *et al.* it was shown that most tissue-specific DNA methylation occurs in regions up to 2 kb distant of the promoter region [30], which were dubbed ‘CpG island shores’ by the authors. Furthermore they showed that cancer-specific methylation also occurs at conserved tissue-specific CpG island shores.

Besides methylation of DNA, post-translational modifications of N-terminal tails of histone proteins are key components in the epigenetic regulation of genes. Over 60 distinct modifications are currently known – mostly in the histone tails – although some have been observed in the globular domain [31, 32]. Modifications of histone tails include (amongst others) acetylation and methylation of lysine residues. Whereas acetylation of histone tails is correlated with gene activation [33–35], the influence of histone methylation depends on the exact residue methylated and the number of added methyl groups [36–38]. Histone modifications and DNA methylation provide a close interplay with respect to gene regulation as both activities are functionally linked [39].

Whereas lysine methylation and acetylation are the most studied modifications, there are many more histone modifications known. Arginine residues can also be methylated and acetylated, just as lysine residues. As is the case with lysine methylation, whether arginine methylation results in repression or activation of transcription depends on which arginine residue is methylated [40]. In addition, SUMOylation and ubiquitination of histones has also been observed [32]. SUMOylation appears to be associated with transcriptional repression [41], whereas ubiquitination has been suggested to play a role in transcriptional activation and elongation [42]. However, the exact function of these modifications remains to be elucidated.

## Epigenetic alterations are reversible

Interestingly, epigenetic modifications are reversible, which is illustrated by the counterbalancing actions of the various enzymes that are responsible for maintaining the epigenome. Lysine acetyltransferases (KATs, formerly known as histone acetyltransferases) are counteracted by lysine deacetylases (KDACs, formerly known as histone deacetylases) and sirtuins in establishing histone acetylation modifications at lysine residues in the N-terminal tails. Lysine methyltransferases KMTs, (formerly known as histone methyltransferases), finally, are counteracted by the recently discovered lysine demethylases (KDMs, formerly known as histone



**Fig. 1** Schematic representation of chromatin. DNA is wrapped around the octamer of core histones to form a structure called the nucleosome. Transcriptionally active chromatin (euchromatin) is hallmarked by low frequency of DNA methylation. High levels of histone acetylation and histone methylation correlated with activation (MeK4H3, MeK36H3, MeK79H3) are detected in euchromatin. KATs are responsible for acetylation of histone tails, KMTs for methylation, whereas KDMs remove methylation marks of histones. DNMTs probably remove methylation marks from DNA, although this has not yet been proven definitively (A). Transcriptionally silent chromatin (heterochromatin), is hallmarked by high frequencies of repressive methylation markers (*i.e.* DNA methylation and MeK9H3, MeK27H3 and MeK20H4). Methylation of histone tails is catalysed by KMTs whereas DNA methylation is catalysed by DNMT. Acetylation markers, associated with activation, are removed by KDACs (B). Post-translational modifications of histone tails include (but are not limited to) acetylation (Ac) and methylation (Me), which can be associated with transcriptionally active (green) or silent (red) chromatin (C).

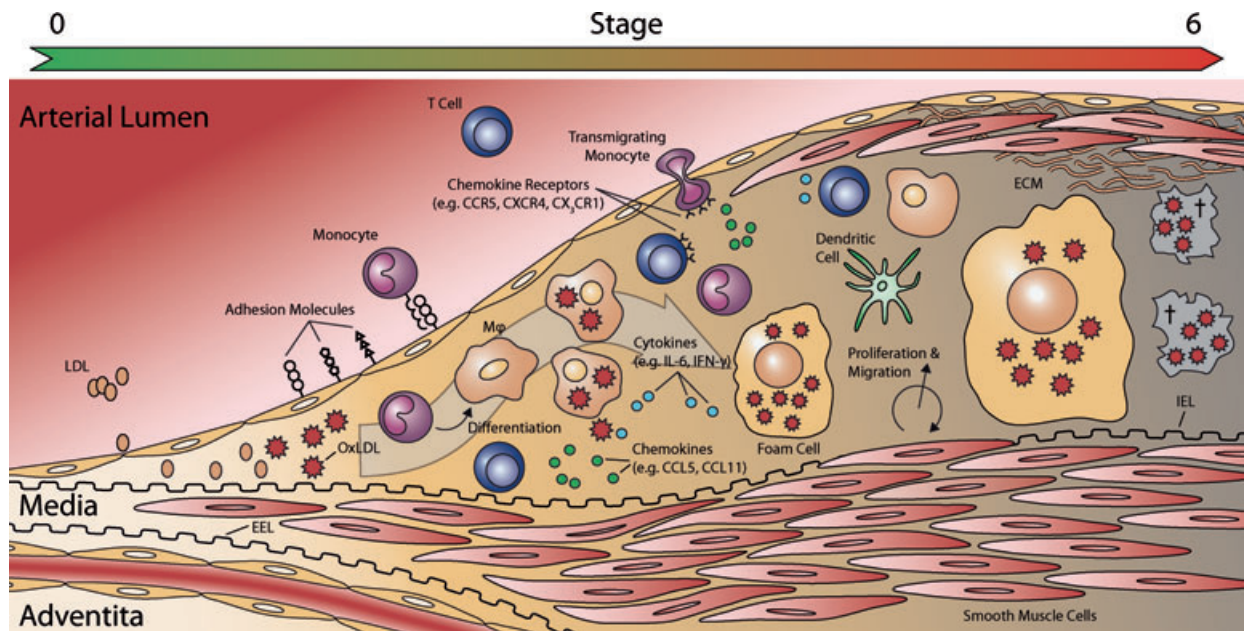
demethylases) in establishing histone methylation modifications (Fig. 1). In this way these enzymes promote a return to, respectively, repressive or active chromatin structure [43–45].

Importantly, the reversible nature of these epigenetic modifications makes the chromatin-modifying enzymes interesting therapeutic targets [46–48] and a myriad of small molecule inhibitors that can influence the enzymatic activity of these chromatin-modifying enzymes are currently being tested for their efficacy [49 and references therein]. These inhibitors are mostly evaluated in the field of cancer research, in cell lines, in animal models as well as in clinical trials [49]. Notably suberoylanilide hydroxamic acid (SAHA, or Vorinostat) and valproic acid (KDAC inhibitors [KDACi]) are already FDA approved for cancer treatment and epilepsy, respectively [50–52]. With respect to atherosclerosis, administration of curcumin (a KAT inhibitor) resulted in significantly lowered low-density lipoprotein levels and raised high-density lipoprotein

levels in healthy volunteers [53–55]. It has also been reported that curcumin has an anti-proliferative effect on peripheral blood mononuclear cells (PBMCs) and vSMCs [56].

## Atherosclerosis

The first step in atherosclerosis is thought to be endothelial dysfunction [57], possibly triggered by oxidized low-density lipoprotein (OxLDL) [5]. Endothelial activation results in the expression of cytokines and chemokines, enhancement in the permeability of the endothelial cell (EC) layer and an increment in the expression of adhesion molecules. Immune and inflammatory cells, such as monocytes and T lymphocytes, are then attracted by chemokines



**Fig. 2** Schematic representation of plaque development. OxLDL is thought to trigger endothelial dysfunction. OxLDL is often associated with proteoglycans in the subendothelium. Activated ECs start to express various adhesion molecules. Immune cells, mainly monocytes and T cells, are attracted by chemokines and migrate across the endothelium, using adhesion molecules. Monocytes then differentiate into macrophages (M $\phi$ ) and DCs. These macrophages take up OxLDL and transform into 'foam cells'. This process of foam cell formation is indicated with the large grey arrow. T cells recruited to the plaque express several cytokines and chemokines. In response to the stimuli, vSMCs start to proliferate and migrate, and start to produce ECM. Abbreviations: ECM: extra cellular matrix; EEL: external elastic lamina; IEL: internal elastic lamina; LDL: low-density lipoprotein; OxLDL: oxidized low-density lipoprotein. Illustration inspired on [192].

(chemotaxis), followed by firm adhesion and transendothelial migration. These cells subsequently infiltrate the subendothelium of the vascular wall and form the main players in the formation of the 'fatty streak' – the first identifiable lesion – (Fig. 2) [57].

Foam cells – cholesterol-engorged monocyte-derived macrophages – are the dominant type of immune cells found within the lesions. Macrophages take up the OxLDL present in the subendothelium, but are unable to digest it sufficiently, resulting in the formation of foam cells [5]. Although fatty streaks are not clinically significant, they are thought to be the precursors to more advanced lesions, although there is some dispute whether fatty streaks are truly precursors of advanced lesions [7, 58]. If the causative agents for this endothelial dysfunction are not effectively removed, the inflammatory response can continue indefinitely. The atherosclerosis-associated immune response is driven by monocyte-derived macrophages and specific subsets of T cells [6, 59]. Monocytes and T lymphocytes migrate from the blood and proliferate within the lesion, resulting in the accumulation of inflammatory cells. If these cells become activated they release cytokines, chemokines and growth factors [57], contributing to the ongoing inflammatory process. Moreover, immune activation eventually induces focal necrosis leading to the necrotic core found in advanced lesions.

In later stages of the disease other cell types (*e.g.* vSMCs) become involved. vSMCs start to proliferate as a response to the various signalling molecules present in the lesion, thereby thickening the arterial wall. Eventually the lesion will evolve into a 'fibrolipid plaque', consisting of a lipid-rich necrotic core covered by a fibrous cap of vSMCs and a collagen-rich matrix [3, 60]. The resulting thickening of the arterial wall caused by the plaque formation will partially be compensated by gradual dilation (remodelling). If the plaque volume becomes too large, this process cannot compensate enough, leading to decreased lumen size and hampered blood flow [61].

As stated above, the development of the atherosclerotic lesion is for the larger part determined by the release of various cytokines and chemokines. Pro-inflammatory cytokines such as tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interferon- $\gamma$  (IFN- $\gamma$ ) released by activated T cells – recruited to the vascular cell wall – play an essential role in disease development. This critical role for pro-inflammatory cytokines in atherosclerosis development is illustrated in atherosclerotic mouse models. It has been shown that deletion of the genes coding for pro-inflammatory cytokines (*e.g.* IL-12, IFN- $\gamma$ , IFN- $\gamma$ -receptor and TNF- $\alpha$ ) resulted in reduced atherosclerosis development in these knockout mice [62–67]. Conversely, deletion of anti-inflammatory cytokine genes (*e.g.* IL-10

and TGF- $\beta$ ) resulted in increased atherosclerosis [68, 69]. Different genetic variants of the *TNF- $\alpha$*  gene also showed to be important for the outcome of percutaneous coronary intervention (PCI, one of the treatments used for opening of occluded vessels) with respect to restenosis – one of the major limitations of the PCI technique [66]. Genetic variants of the *IL-10* gene have also proven to be important for the development of restenosis [70].

The cross-talk between immune system and vascular wall results in up-regulation of major histocompatibility complex (MHC) class II (MHC-II) molecules and of the MHC class Ib molecule, HLA-E [3, 71]. Furthermore, it results in up-regulation of chemokines such as CX<sub>3</sub>CL1 (fractalkine), CCL2 (MCP-1), CCL5 (RANTES) and their receptors [3, 72–75]. All of these factors influence the gene expression profiles of cells present within the lesion. These observations not only feed the current theory that atherosclerosis is an inflammatory disease – and not just a disease of lipid metabolism. For many of these inflammatory and immune modulating factors epigenetic components have been identified that are responsible for the transcriptional regulation of the genes encoding these factors. Thus epigenetic processes might prove to be important contributing factors to disease pathogenesis that should be taken into consideration.

## Epigenetics and association with atherosclerosis

Epigenetics provides an attractive explanation how diet, environment and lifestyle may contribute to disease. In principle, epigenetics explains how such external factors can impose aberrant gene expression patterns in an individual lifetime and even transgenerationally. One of the earliest studies linking DNA methylation to atherosclerosis showed that the extracellular superoxide dismutase (*ec-SOD*) gene was hypomethylated in atherosclerotic lesions in rabbits [76]. The importance of DNA methylation as a contributing factor to the pathogenesis of atherosclerosis is underscored by a study linking global DNA hypermethylation with predisposition to, and natural history of atherosclerosis [77]. In this study the correlation was made by comparing methylation sensitive restriction of peripheral blood leucocyte DNA. However, this approach evaluates only the inflammatory component of the disease process in PBMCs and not other components such as vECs and vSMCs. In particular, DNA hypermethylation was found to be significantly associated with both all-cause and cardiovascular mortality, even following the adjustment for age, CVD and diabetes mellitus. Hypermethylation was found in patients suffering from inflammation (high C-reactive protein levels) and also in 13 patients who died from CVD. This paper suggests that global DNA hypermethylation is associated with inflammation and increased mortality in chronic kidney disease. Importantly, hypermethylation was found to be the strongest independent risk factor for CVD mortality.

In the case of CAD an epigenetic component associated with disease was also identified [78]. Sharma *et al.*, found that angiographically confirmed CAD patients showed a higher level of genomic DNA methylation – determined in peripheral blood lymphocytes – when compared to healthy controls [78]. Furthermore, a positive correlation was found between global DNA methylation and homocysteine levels. Homocysteine is known to be an independent risk factor for CAD [79, 80]. Sharma and coworkers also tested the specific methylation status of the *ApoE* gene by bisulphite-sequencing. The *ApoE* promoter was previously shown to have a degree of methylation that varies with homocysteine levels [81]. However, no significant difference could be detected between patients and controls [78]. Both these studies underline the potential effect DNA methylation can have with respect to disease development and outcome. However, the precise genes targeted by DNA methylation and thus the precise mechanisms that induce DNMT activity remain to be elucidated.

Although DNA methylation is one of the most studied epigenetic phenomena, more and more research is performed on the specific contribution of histone modifications. Recently, a study was published directly linking epigenetic histone modifications and atherosclerosis. Hastings *et al.* showed that atherosclerosis-prone shear-stress profiles globally decreased histone H4 acetylation and increased H4 acetylation at the *c-fos* promoter in smooth muscle cells (SMCs) [82]. Perhaps more importantly, they showed that atheroprone haemodynamics resulted in differentially regulated phenotypes for ECs and SMCs. Shear stress (flow) was applied to ECs that were layered on top of SMCs in forces that correlate to atheroprone or atheroprotective conditions. Atheroprone flow reduced the expression of endothelial nitric oxide synthetase (eNOS), Tie-2 and kruppel-like factor in ECs, and smooth muscle actin and myocardin in SMCs, whereas vascular cell adhesion molecule 1 (VCAM-1), IL-8 and MCP-1 were increased in both cell types. This correlated to a decrease in total H4 acetylation and serum response factor (SRF) binding to the SMC actin CArG-box in the promoter region. Furthermore atheroprotective conditions induced a polygonal shape in EC whereas atheroprone conditions induced an elongated EC phenotype. In vSMCs atheroprotective conditions induced an elongated shape whereas atheroprone conditions showed a random SMC organization and cells aligned toward a perpendicular orientation relative to the direction of flow [82].

Another linkage between atherosclerosis and histone modifications was found when the administration of the KDACi trichostatin A (TSA) in *Idr*<sup>-/-</sup> mice led to exacerbated neointimal lesions [83]. TSA increased CD36 mRNA, protein and cell surface expression levels, which were related to increased acetylated histone binding at the promoter region. CD36 recognizes OxLDL, thus the obtained results might be due to increased OxLDL uptake by macrophages. Also levels of TNF- $\alpha$  and VCAM-1 were increased in aortic plaques. It has also been noted that application of TSA resulted in inhibition of SMC migration [84]. Unfortunately, the exact mechanisms are not yet fully understood, but these studies clearly indicate a role for histone acetylation in disease pathogenesis.

Interestingly, some components relevant for the development of atherosclerosis, such as OxLDL, are able to modulate the activity of lysine deacetylases [85]. In cultured ECs it was shown that OxLDL reduced expression of KDAC1 and KDAC2. Global KDAC activities were partly restored by statins: pretreatment of ECs for 24 hrs with simvastatin or fluvastatin blocked the OxLDL-related modifications in H3 and H4. Addition of mevalonate could revert the effects observed after statin treatment. The researchers postulate that this, at least in part, may be due to inhibition of small GTP-binding Rho proteins. Furthermore decreased expression of KDAC2 in ECs was shown in atherosclerotic plaques of human coronary arteries [85]. Taken together, these data show the importance of histone modifications in atherosclerosis.

A study performed by Barker *et al.* in 1989 sheds light on how long-term cellular memory can have an effect on the development of atherosclerosis. Nowadays it is generally accepted that long-term cellular memory is mediated *via* epigenetic phenomena. Barker *et al.* demonstrated that low birth weight is associated with a higher risk of cardiovascular events in adult life [86]. This led to the hypothesis that the environment the foetus is exposed to during pregnancy might partially determine cardiovascular risk. This so-called 'foetal origins hypothesis' states that adaptation to an unfavourable maternal environment is beneficial to the developing embryo *in utero* [86]. However, when the adult environment differs from the *in utero* environment, this may lead to an increased risk for CVD. Studies in pregnant rats show that a protein-restricted diet results in reduced expression of Dnmt1, leading to hypomethylation of specific promoters [87]. These observations provide a strong indication that the *in utero* environment leads to epigenetic (re)programming, which later in life might contribute to disease development.

## Epigenetic regulation of cell activity

Cell differentiation (*e.g.* monocyte differentiation into macrophages) and cell-specific gene expression in most cases is controlled by epigenetic mechanisms. Below we will discuss the most important cellular factors that contribute to atherosclerotic plaque formation and how their phenotype is regulated by epigenetic processes.

### T cells

Several lines of investigation illustrate that T cells are important in the formation of (early) lesions [88–91]. Within the plaque, several subsets of T cells can be found. Mainly CD4<sup>+</sup> T cells are dominantly found in plaques, but there are some CD8<sup>+</sup> T cells found as well [5]. The T cells present in human plaques are predominantly of the Th1 phenotype [91]. Th1 cells produce IFN- $\gamma$  – a macrophage activating cytokine – and are generally considered pro-atherogenic. Th2 cells are rarely detected in lesions [92]. They are generally considered anti-atherogenic, but may also contribute to aneurysm formation [93]. Regulatory T cells (T<sub>reg</sub> cells) control

the balance between Th1 and Th2. T<sub>regs</sub> are considered to be atheroprotective [94].

All T cells derive from the same precursor: the naïve T-cell. Not surprisingly, differentiation of *e.g.* CD4<sup>+</sup> T cells into a Th1, Th2 or T<sub>reg</sub> phenotype is regulated by epigenetic processes [95, 96]. The differentiation of naïve T cells into Th1 or Th2 is determined by IL-12 and IL-4 cytokines, respectively. In response to these signals, transcription is initiated of lineage specific cytokine genes including IFN- $\gamma$  and IL-4 [97]. The IFN- $\gamma$  and IL-4 loci are maintained in a poised state in naïve T cells, meaning they show both repressive and activating epigenetic marks. In 1998 it was first proposed that epigenetic, chromatin-based structural changes, underlie the Th1/Th2 differentiation [98]. These epigenetic mechanisms – that are necessary to stably maintain gene expression patterns and eliminate the need for feedback loops – will be discussed below.

In Th1 cells expression of IFN- $\gamma$  is preceded by remodelling of the IFN- $\gamma$  locus [99]. Upon initial stimulation of naïve T cells, the lineage determining factors GATA3 and T-bet mediate many of the structural changes to the chromatin [95]. These factors will, respectively, render the IFN- $\gamma$  or IL-4 genes accessible to regulatory enzymes and other transcription factors [100–102]. On the level of DNA methylation, there are numerous findings supporting the epigenetic regulation of IFN- $\gamma$  expression. Increased expression of IFN- $\gamma$  was found in T cells from *Dnmt* knockout mice [103] and in T cells treated with DNMT inhibitors (DNMTi) [104, 105]. In addition, expression of IFN- $\gamma$  by NK and NKT cells is also controlled by epigenetic processes [99]. The ability of NK and NKT cells to rapidly produce substantial amounts of IFN- $\gamma$  implies that the IFN- $\gamma$  locus is accessible to transcription factors, meaning that the chromatin at the IFN- $\gamma$  locus is maintained in an 'open' state. Indeed the IFN- $\gamma$  locus in NK cells was found to be hyperacetylated and in a poised state [106, 107].

In the differentiation to Th2 cells, IL-4 expression is preceded by remodelling of the IL-4 locus, similarly to the IFN- $\gamma$  locus remodelling in Th1 cells. In naïve T cells, the IL-4 locus is 'poised', allowing rapid, early transcription. The IL-4 promoter region exhibits a low basal level of H3 acetylation and DNA hypomethylation, but also shows 3MeK27H3 (*i.e.* triple methylation of lysine 27 on histone H3) modifications. When naïve T cells are stimulated under Th1 conditions, transcription activating chromatin marks at the IL-4 locus are replaced with repressive marks, whereas the contrary happens under Th2 stimulating conditions (*e.g.* at the IFN- $\gamma$  locus). These processes have been excellently reviewed by Ansel *et al.* [108 and references therein].

The Foxp3 transcription factor is considered the master switch for T<sub>reg</sub>. The promoter of this transcription factor showed a difference in methylation levels between T<sub>regs</sub> and non-T<sub>reg</sub> CD4<sup>+</sup> cells [109]. Furthermore, this study also showed a difference in activating histone marks (AcH3, AcH4 and 3MeK4H3) in Foxp3 promoter chromatin. Epigenetic regulation of T cell subtypes has also been shown *in vivo*. Mice which were treated with the KDACi TSA showed an increase in Foxp3<sup>+</sup> CD4<sup>+</sup> T<sub>reg</sub> cells – both proportionally as well as in absolute numbers – in the lymphoid tissues [96]. Besides influencing T cell populations, the differentiation of monocytes to dendritic cells (DCs) can also be modulated by KDACi [110].

## Monocytes

Monocytes are critical players in the formation of atherosclerotic plaques. Monocytes migrate from the vessel lumen into the arterial wall, where they differentiate into either DCs or macrophages (Fig. 2). These macrophages later transform into foam cells, one of the major components of the atherosclerotic plaque. It has been shown by two independent researches that interfering with KDAC function during differentiation resulted in the formation of function-impaired DCs. Notably, treatment with the KDACi's butyrate and MS-275 resulted in lowered expression of CD1. CD1 expression is a marker for mature DCs, but notably it is also important in the presentation of lipid antigens and thus may play an important role in the development of the atherosclerotic plaque [110, 111].

## Endothelial cells

Epigenetic mechanisms are also directly involved in the transcriptional regulation of immune genes in the vascular wall. For instance, in cultured human ECs it was found that administration of the KDACi TSA inhibited TNF- $\alpha$  induced monocyte adhesion. This was the result of down-regulation of the *VCAM-1* gene; however, ICAM-1 and E-selectin – two other cytokines inducible genes – were not affected [112]. This suggests that the KDACi modulated TNF- $\alpha$ -mediated signalling specifically targets the VCAM-1 promoter, instead of general inhibition of the NF- $\kappa$ B pathway. This notion is interesting, since NF- $\kappa$ B is a target for chromatin modifying enzymes as well (see 'Non-histone targets' below). Similar results have been presented for the induction of tissue factor (TF) in human ECs. The human TF promoter contains a NF- $\kappa$ B related binding site: TF- $\kappa$ B. KDACi administration inhibited agonist induced (TNF- $\alpha$ , IL-1 $\beta$  or LPS) TF activity in ECs by blocking activation of TF- $\kappa$ B (TF activity was reduced 90% in HUVECs; 50% reduced in PBMCs, *in vitro*). TSA nearly abolishes TF- $\kappa$ B binding, without affecting NF- $\kappa$ B binding (as determined by electrophoretic mobility shift assays (EMSAs), chromatin immunoprecipitation (ChIP) and luciferase promoter reporter assays). These results were achieved using a variety of structurally distinct KDACi's [113].

Work performed in the group of Stefanie Dimmeler showed an important role for KDACs in regulating several endothelial-specific genes. Using KDACi's, it was shown that eNOS mRNA and protein levels decreased after KDACi administration [114]. Furthermore the 'master-switch' for some typical endothelial expressed genes (*HoxA9*, which regulates eNOS, VEGF receptor 2 and VE-cadherin expression) was also down-regulated in its expression by KDACi administration [115].

## Smooth muscle cells

The data reported in literature regarding epigenetic regulation in SMCs provides an explanation how epigenetics may control lineage specificity. Nearly all SMC-restricted protein genes contain the

CArG box DNA sequence within their promoter and this sequence is also required for SMC gene transcription *in vivo*. Genes important for SMC phenotypic switching (*e.g.* genes important for migration, proliferation and ECM production) also contain CArG boxes within their promoters [116]. CArG boxes serve as a binding site for the transcription factor SRF [117]. However, SRF expression is not limited to SMCs and serves many functions in different cell types including cell migration and cell-cell adhesion [118].

Multiple pieces of evidence suggest that SRF binding to CArG boxes in SMCs is regulated at the level of the chromatin structure. First of all, the x-ray crystal structure of SRF showed that SRF requires several contacts within the minor groove of DNA. The minor groove would be obscured if DNA was wrapped around the nucleosome structure. Also from thermodynamic considerations, binding of SRF to the CArG box would be greatly inhibited if the CArG box was bound to nucleosomes [119 and references therein].

Secondly, using EMSAs it was shown that SRF can bind to synthetic DNA with the same affinity as to DNA obtained from lysates of both SMC and non-SMC cell cultures. Conversely, by using ChIP assays, it was shown that SRF binds to promoter regions in SMCs much more effectively than to promoter regions in non-SMCs. This suggests that in SMCs the chromatin is in such a state that it is accessible to SRF [120–123]. This observation is supported by micrococcal nuclease digestion experiments, which showed that the CArG-box of gene promoters in SMCs is more accessible to digestion than in non-SMCs [124].

Finally, it was shown that histone H3 and H4 acetylation was increased in SMCs when compared to numerous different cell-types, including cultured endothelial stem cells. In addition, non-SMCs were enriched for the repressive chromatin marks 3MeK27H3 and 2MeK20H4 at SMC-specific promoter regions [119 and references therein].

Evidence from diabetic mouse models suggests an important role for epigenetic regulation in the inflammatory phenotype of vSMCs. The recently discovered KDM, lysine-specific demethylase (LSD-1 or KDM1, a 2MeH3K27 demethylase), was shown to play a role in the pro-inflammatory phenotype of vSMCs in diabetic mice [125]. In this mouse model levels of 2MeH3K4 (a histone modification correlated with expression) were increased at the MCP-1 and IL-6 promoters. Upon stimulation with TNF- $\alpha$  there was recruitment of RNA polymerase II to the promoter and increased expression of MCP-1 and IL-6. Expression of LSD-1 was shown to be decreased in vSMCs. Knockdown of LSD-1 with siRNA increased inflammatory gene expression whereas over expression decreased inflammatory gene expression in vSMCs of these mice.

It may very well be possible that similar mechanisms apply in the case of atherosclerosis. As discussed in a previous section, vSMCs isolated from rat thoracic aorta, which were treated with the KDACi TSA, displayed an inhibition of vSMC proliferation [84]. It was subsequently shown that expression of p21<sup>WAF1</sup> was induced by this treatment. However, KDACi treatment had no effect on SMCs isolated from p21<sup>WAF1</sup> knockout mice.

Together, these findings illustrate that epigenetic mechanisms play an important role in the processes that control differentiation

and activation of haematopoietic cells, but also of vECs and vSMCs [119, 126].

## Chemokines, their receptors and other genes involved in inflammation

Expression of immune response genes in vECs and vSMCs is a major determinant in atherosclerosis onset and development because it contributes to the vascular-immune cross talk. The vascular wall modulates inflammation by the expression of numerous cytokines and chemokines. For many of these genes epigenetic components, influencing expression, have been identified. Below, we will discuss some of the best-studied examples in detail.

### eNOS

As stated in the previous sections, many inflammatory processes are recognized by an epigenetic component. One of the best-studied cases of epigenetic regulation in the vascular wall involves the transcription of eNOS, an enzyme which is regarded as endothelial specific. Its catalytic product, nitric oxide, was first identified as a vasodilator [127], although recent evidence also suggest a role in inflammation. Nitric oxide has been attributed roles in leucocyte adhesion and vSMC proliferation [128]. Furthermore, dysfunctional eNOS is believed to be implicated in atherosclerosis development [129].

The human eNOS promoter does not contain a canonical TATA box, nor does it contain a proximal CpG island. Expression of eNOS is restricted to ECs *in vivo*. Various assays in both expressing and non-expressing cell types showed that a majority of the non-expressing cell types displayed transgene promoter activity [130–132]. In eNOS expressing ECs, eNOS promoter DNA was only slightly methylated or unmethylated, whereas non-expressing cells showed a high amount of DNA methylation. Treatment of non-expressing cell types with 5-azacytidine (a DNMTi) led to an increase in eNOS mRNA levels [132]. Later experiments showed the involvement of histone modifications in the expression of eNOS as well. ChIP showed enrichment of AcH3, AcH4 and (2/3)MeK4H3 in chromatin of the eNOS promoter in eNOS expressing cells. Treatment of non-expressing cells with the KDACi TSA – effectively increasing histone acetylation – also led to an increase in eNOS mRNA [128 and references therein].

### iNOS

Not only is eNOS regulated by epigenetic mechanisms, of interest is the observation that also inducible nitric oxide synthase (iNOS) is actively regulated by the epigenetic mechanisms [133]. This gene is expressed in macrophages, but also in ECs and vSMCs,

under inflammatory conditions. It has been suggested that nitric oxide – derived from macrophages expressing iNOS – can result in apoptosis of vSMCs, promoting plaque instability [134]. A study performed by Mellott *et al.* showed that cytokine induction of iNOS resulted in a change of chromatin structure at the iNOS promoter [9]. Later work showed that the *NOS2A* gene, encoding iNOS, had high levels of DNA methylation and of the 2MeK9H3 and 3MeK9H3 repressive marks in non-expressing cells. Cell lines capable of iNOS induction had lower levels of CpG-methylation and histone 3 lysine 9 methylation at the *NOS2A* promoter. Treatment with a DNMTi resulted in an increase of iNOS mRNA [133].

### CCL11 (eotaxin)

In human atherosclerotic plaques, the chemokine CCL11 (also known as eotaxin) was shown by immunohistochemistry to be expressed [135]. In healthy tissue only negligible amounts of CCL11 were found. Polymorphisms within the *CCL11* gene are also associated with the development of restenosis after PCI [136]. CCL11, an eosinophil chemoattractant, has been suggested to play a role in atherosclerosis development, although the precise relation remains to be elucidated [135, 137, 138]. At the same time CCL11 is expressed by SMCs, while macrophages in the human atherosclerotic plaque express the CCL11 receptor, CCR3 [135]. *CCL11* transcription is induced by inflammatory TNF- $\alpha$  signalling and is mediated through NF- $\kappa$ B. TNF- $\alpha$  induces acetylation of histone H4 in the *CCL11* promoter. This results in an open chromatin structure, promoting subsequent binding of the NF- $\kappa$ B subunit p65 to the *CCL11* promoter (as shown by ChIP) [139]. Notably, glucocorticoids repress *CCL11* transcription through selective inhibition of histone H4 acetylation [139]. Although only histone H4 acetylation and no other epigenetic markers were investigated, this research provides a very strong indication for epigenetic regulation of the *CCL11* gene [139].

### CCR5

CCR5 is important for plaque formation as it attracts T cells and mononuclear cells. Ccr5 knockout mice show less neointima formation and an increase in production of the anti-inflammatory IL-10 molecule by SMCs [140, 141]. One of the ligands for the CCR5 receptor, CCL5 or RANTES, has also been shown to be involved in unstable angina pectoris (UAP) [142]. UAP is generally the result of erosion or rupture of an atherosclerotic plaque. *CCR5* is epigenetically regulated in T cells. In naïve T cells, lacking CCR5 expression, DNA of the *CCR5* receptor is hypermethylated and the acetylation level of histone tails is strongly reduced in *CCR5* promoter chromatin. When T cells are activated and express CCR5, DNA-methylation is significantly lowered combined with an increase in histone acetylation modifications (our own unpublished observations).

The observations above indicate that epigenetic mechanisms play a key role also in regulation of (immune) genes in cells that



participate in atherosclerosis and vascular remodelling, and in the cross-talk of immune cells and vascular wall components.

## Epigenetics in (vascular) Inflammation

### KDM6B

De Santa *et al.* recently showed that inflammation and Polycomb-mediated gene silencing are linked [143]. Polycomb group (PcG) proteins are important for the maintenance of a repressive chromatin state that is stable over many cell divisions. As such, PcG proteins play a critical role in differentiation processes and maintenance of cellular identity. Gene silencing is mediated by the Polycomb repressive complex 2 (PRC2) through the 3MeK27H3 chromatin mark, which is subsequently read by the PRC1 maintenance complex [144 and references therein].

The JmjC-domain protein Jmjd3 (KDM6B, a H3K27 demethylase) is expressed in macrophages upon stimulation with bacterial products and pro-inflammatory cytokines [143]. Jmjd3 binds to PcG target genes, regulates 3MeK27H3 levels and therewith their transcriptional activity [145]. For the first time, an inducible enzyme was shown to erase epigenetic modifications. Later work showed that 70% of lipopolysaccharide-inducible genes in macrophages are Jmjd3 targets [146]. This provides an essential link between inflammation and reprogramming of the epigenome and might prove to be of vital importance in chronic inflammation and autoimmune diseases including atherosclerosis in the near future.

### Oestrogen receptor

Oestrogen receptors (ERs) are present in the coronary arterial wall on both SMCs and ECs [147–149]. ERs may play an important role in protection against atherosclerosis [148]. Deficiencies in ER- $\alpha$  lead to accelerated atherosclerosis in men, furthermore ER- $\alpha$  mediates the loss of expression of some cytokine induced cell-adhesion molecules [150 and references therein]. ER  $\alpha$  was shown to have a varying degree of methylation throughout the cardiovascular system [151]. In the same study it was shown that an age related increase of methylation occurred in the right atrium. Furthermore, higher degrees of methylation were found in coronary atheromas when compared to macroscopically normal tissues.

Similarly, the ER- $\beta$  also displays a correlation in methylation of its promoter and atherosclerosis [152]. Contrary to ER- $\alpha$  expression, expression of ER- $\beta$  correlates with atherosclerosis independent of age [153]. By comparing plaque *versus* non-plaque tissue from the same vessels, it was recently shown that the ER- $\beta$  promoter has higher methylation levels in atherosclerotic lesions [152]. In this study, increased expression of ER- $\beta$  was also observed – *in vitro* – in ECs and SMCs after administration of a

DNMTi (5-aza-2'-deoxycytidine) and a KDACi (TSA). These experiments provide supporting evidence for epigenetic regulation of the ER- $\beta$  receptor.

### COX2

Another example for the linkage between epigenetics and CVD-related inflammation is transcription of the *cyclooxygenase-2* (*Cox-2*) gene. TNF- $\alpha$  – as well as several other cytokines – can induce expression of Cox-2, a protein associated with atherosclerosis development [154]. Cytokines (such as TNF- $\alpha$  and IFN- $\gamma$ ) can also induce (indirect) changes to the chromatin, allowing effector genes to be expressed upon stimulation [9]. For Cox-2 it was demonstrated that mRNA expression and protein synthesis can be repressed *in vitro*, in cell lines by the KDACi's sodium butyrate and TSA revealing a significant role for epigenetic components in Cox-2 expression [155]. These experiments were performed in a colon cancer cell line, and thus need to be confirmed in cells relevant for vascular biology and atherosclerosis (*e.g.* vECs, vSMCs or monocytes) to extrapolate these results to atherosclerosis.

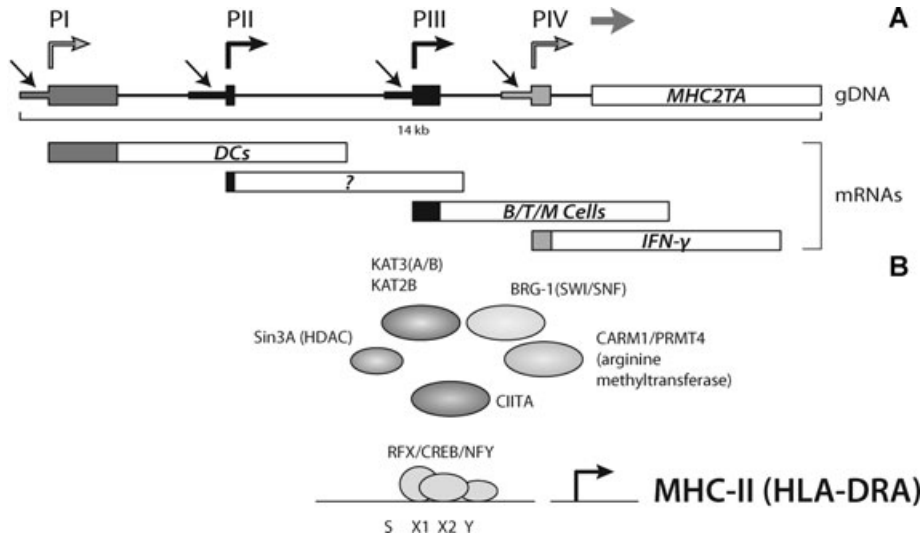
### Transcriptional regulation of MHC molecules – the role of CIITA

Constitutive expression of MHC-II surface molecules is restricted to professional antigen presenting cells (APC) of the immune system. On all other cell types their expressions can be induced in an environment rich in inflammatory cytokines – such as the atherosclerotic plaque – or upon activation such as in human T cells [156]. Therefore under inflammatory conditions MHC-II molecule expression can be induced on vECs and vSMCs, which normally are not expressing MHC-II [157, 158].

The 'master regulator' of MHC-II expression is the co-activator CIITA (class II transactivator) [159]. Within the context of atherosclerosis, CIITA is of importance, since it has been shown to be involved in transcriptional regulation of collagen type 1 [160, 161]. Collagen 1 is one of the main components of ECM and is essential in cap formation and plaque stabilization [57].

Transcriptional regulation of *MHC2TA*, the gene encoding CIITA, is mediated through the activity of four independent promoter units (CIITA-PI through CIITA-PIV, Fig. 3) [162]. These promoter units are employed in a cell type- and activation-specific manner. In aortic SMCs CIITA-PIII and -PIV are expressed after IFN- $\gamma$  stimulation and CIITA is needed for IFN- $\gamma$ -mediated repression of collagen type 1 genes (*COL1A1* and *COL1A2*) [160, 161]. Furthermore, collagen transcription is also repressed by the RFX family proteins. RFX1 only weakly interacts with the unmethylated collagen promoter, but binds with higher affinity when the promoter is in a methylated state [161].

In MHC-II transcription, CIITA exerts its transactivating function through protein–protein interactions with a multi-protein



**Fig. 3** Organization of the *MHC2TA* multipromoter region. Each promoter (indicated with black arrows) transcribes a unique first exon (coloured boxes). Transcription start sites are indicated with coloured, angles arrows. Promoter PI is mainly used for constitutive CIITA expression in DCs, whereas PIII is mainly used in B Cells, activated T cells and macrophages. Promoter PIV is mainly used for IFN- $\gamma$  induced CIITA expression in non-bone-marrow-derived cells. The function of PII is still poorly understood (A). Factor assembly on the MHC-II promoter. Shown is the regulatory SXY module, to which the MHC enhanceosome binds (B).

complex, which comprises RFX, CREB/ATF and NFY (Fig. 3B) [163–165]. Together with CIITA, this complex acts as an enhanceosome driving transactivation of these genes [163–165]. In the MHC-enhanceosome, CIITA acts as a platform for recruitment of chromatin-modifying activities which include KATs, KDACs and an arginine methyltransferase [166–168]. Interactions of these chromatin-modifying enzymes and CIITA result in higher-order structural changes within the chromatin, effectively activating or silencing MHC-II gene transcription [167, 169]. A number of studies now have shown that epigenetic processes not only control MHC-II transcription, but also contribute to the activating/silencing of *MHC2TA* transcription. This is illustrated by the observation that in DC maturation deacetylation occurs at the *MHC2TA* locus coinciding with transcriptional inactivation [170]. During DC differentiation histone acetylation of the type I promoter is increased. This increase was blocked by IL-10, inhibiting *MHC2TA* transcription [171].

## Non-histone targets

Acetyltransferases/deacetylases and methyltransferases/demethylases discussed in the previous sections also target non-histone proteins which could lead to alterations in function of the targeted proteins or the generation of novel epitopes which now can evoke an adaptive (auto-) immune response [172]. Especially since NF- $\kappa$ B – playing crucial roles in inflammation – is showing up as one of the non-histone substrates. Both NF- $\kappa$ B and p53 are acetylated by KAT3A (CBP) and KAT3B (p300) [173–176], whereas p53 can also be acetylated by KAT2B (PCAF) [177, 178].

As is the case with most genes, transcription of pro-inflammatory cytokine genes such as IFN- $\gamma$  and TNF- $\alpha$  is positively regulated by transcription factors. However, these cytokines can also induce changes to the epigenome. Thus not only are cytokines

**Table 1** Overview of genes regulated by epigenetic processes involved in atherosclerosis development. Genes for which evidence of epigenetic regulation is solely based on administration of inhibitors of histone modifying enzymes are not shown.

Gene	Type of regulation	References
ec-sod	DNA methylation	[76]
c-fos	Histone code	[82]
CD36	Histone code	[83]
IFN- $\gamma$	DNA methylation and histone code	[99–101]
IL-4	DNA methylation and histone code	[108]
Foxp3	DNA methylation and histone code	[109]
eNOS	DNA methylation and histone code	[128, 132]
iNOS	DNA methylation and histone code	[9, 133]
CCL11	Histone code	[139]
CCR5	DNA methylation and histone code	Own observations
ER- $\alpha$	DNA methylation	[151]
ER- $\beta$	DNA methylation and histone code	[152]
CIITA	DNA methylation and histone code	[166–171]

under epigenetic control, cytokines themselves induce (indirect) changes to the chromatin, allowing effector genes to be expressed upon stimulation. Activation of the IFN- $\gamma$  pathway results in activation of *e.g.* NF- $\kappa$ B. NF- $\kappa$ B is found in the cytoplasm of

unstimulated cells, together with its inhibitor, I $\kappa$ B, that prevents NF- $\kappa$ B from entering the nucleus [179]. Upon stimulation with inducers – e.g. reactive oxygen species (ROS) and lipid peroxidase products – I $\kappa$ B is rapidly degraded *via* the ubiquitin–proteasome pathway [180, 181]. Hereupon, NF- $\kappa$ B is transported to the nucleus, where it can activate the transcription of various targets with  $\kappa$ B-elements in their promoter [182, 183]. This process is known to be activated by KATs and repressed by KDACs [172 and references therein]. The p65 subunit of NF- $\kappa$ B is acetylated by KAT3A/B (P300/CBP) at lysines 218, 221 and 310 [184]. The acetylated form of p65 shows weak affinity for I $\kappa$ B. KDAC3 in turn deacetylates p65, promoting association with I $\kappa$ B $\alpha$  and thereby nuclear export of NF- $\kappa$ B [175]. In addition to KDAC3, KDAC1 has been shown *in vitro* and *in vivo* to deacetylate p53 [185].

Besides to acetylation, methylation of non-histone proteins has also been observed. For example, methylation occurs at lysine 831 of the vascular endothelial growth factor receptor 1, which is dimethylated by Smyd3 (a H3K4 methylase) [186]. The biological function of methylation at lysine 831 is yet to be discovered.

These examples perfectly illustrate how epigenetic and inflammatory processes are interwoven.

## MicroRNAs

In recent years it became apparent that microRNAs (miRNAs) are critically involved in the regulation of gene expression. Synthesized as a larger precursor in the nucleus, miRNAs are processed in the cytoplasm into mature miRNAs, where they target specific mRNAs for degradation or translational inhibition [187]. A large number of miRNAs have already been identified at the moment and, not surprisingly, miRNAs have also been found to be involved in inflammation and atherosclerosis [188]. Unfortunately for most miRNAs the target they act upon is yet to be identified.

For some miRNAs, which might play a role in atherosclerosis development or inflammation the miRNA target gene has been identified. For instance the EC specific miR-126 inhibits the (TNF- $\alpha$  mediated) expression of VCAM-1 [189, 190]. This miRNA might

thus be involved in transmigration of mononuclear cells into the subendothelium. Several other miRNAs have also been identified to be involved in inflammation [191 and references therein].

## Conclusions

Epigenetic control is one of the most fundamental regulatory systems within the cell. Not surprisingly it fulfils essential roles in the regulation of inflammatory and immune responses (summarized in Table 1). The link between inflammation and reprogramming of the epigenome, shown by the induction of *jmjd3* after LPS treatment, is an important discovery. Furthermore, the involvement of KDM1 in the inflammatory phenotype of vSMCs is of importance, as it does provide evidence for a link between inflammation and epigenetics, relevant for atherosclerosis. Correlations between global DNA methylation and atherosclerosis have provided valuable clues that epigenetics plays a role in atherosclerosis development; however, it remains to be further elucidated which genes are directly affected by DNA methylation.

Similarly, precise determination of histone modifications at specific genes might prove its value in understanding gene expression profiles in vascular disease. Especially since small molecules can inhibit the function of histone modifying enzymes, altering the expression of genes. Therefore intervention in epigenetic gene regulation might prove to be the therapeutic intervention of the future, especially in complex multi-factorial diseases such as atherosclerosis, multiple sclerosis and rheumatoid arthritis.

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