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On epigenetic regulation in atherosclerosis pathology

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On

Epigenetic Regulation

In

Atherosclerosis Pathology

Rutger J. Wierda

On
Epigenetic Regulation
In
Atherosclerosis Pathology

Proefschrift

ter verkrijging van

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1

General Introduction

Adapted from:

Epigenetics in atherosclerosis and inflammation

Rutger J. Wierda, Sacha B. Geutskens,J. Wouter Jukema,
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Introduction

Cardiovascular diseases (CVD) are the number one cause of death in the western society.[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 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] Understanding these processes is critical for our understanding of inflammatory responses and disease. Nowadays we are fully aware of the important involvement of epigenetic processes in the regulation of gene expression. 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] To understand the involvement of epigenetic process in atherosclerosis it is important to have a clear understanding of the disease process as well as an understanding of epigenetic phenomena. A brief introduction of both atherosclerosis as well as epigenetic gene regulation is provided below, before the potential involvement of epigenetic regulation in atherosclerosis is discussed.

Atherosclerosis

The first step in atherosclerosis is thought to be endothelial dysfunction,[10] possibly triggered by oxidized low-density lipoprotein (OxLDL).[5] Activation of the arterial endothelium results in the expression of cytokines and chemokines, enhancement in the permeability of the endothelial cell layer and an increased expression of adhesion molecules. Immune and inflammatory cells such as monocytes and

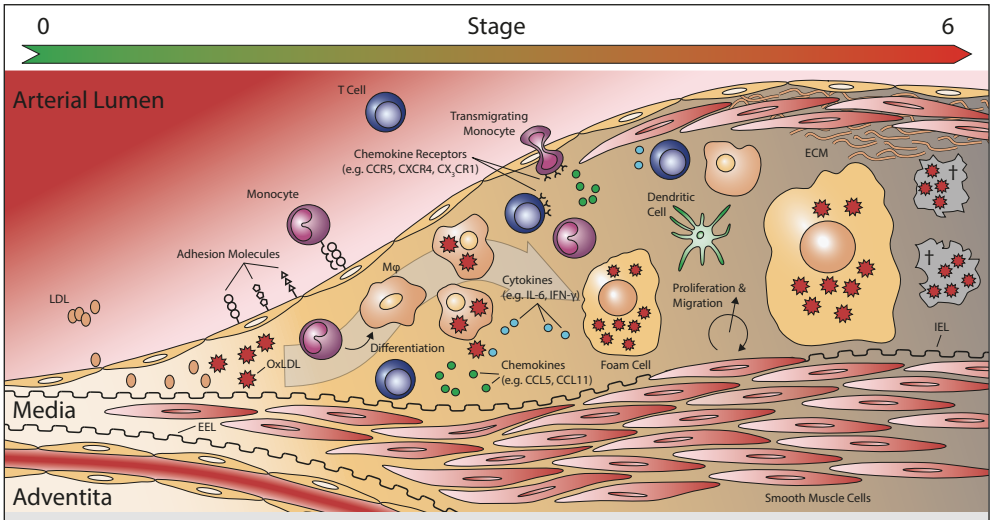

Figure 1–1. Schematic representation of plaque development. Oxidized low-density lipoprotein (OxLDL) is thought to trigger endothelial dysfunction. OxLDL is often associated with proteoglycans in the subendothelium. Activated endothelial cells start to express various adhesion molecules. Immune cells, mainly monocytes and T cells, are attracted by chemokines and migrate across the endothelium, using adhesion molecules. Monocytes then differentiate into macrophages (Mφ) and dendritic cells. 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; Mφ: macrophages; OxLDL: oxidized low-density lipoprotein. Illustration inspired on Sherer *et al.*[11]

T lymphocytes are then attracted by chemokines (a process know as 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.[10] The process of plaque formation is shown schematically in figure 1–1.

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.[7,12] 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.[6,13] 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,[10] contributing to the on-going 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. Vascular SMCs 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.[3,14] 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.[15]

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 α (*TNF-α*) and interferon γ (IFNγ) 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, IFNγ, IFNγ receptor and TNF-α) resulted in reduced atherosclerosis development in these knockout mice.[16–18] Conversely, deletion of anti-inflammatory cytokine genes (e.g. IL-10 and TGF-β) resulted in increased atherosclerosis.[19,20] Different genetic variants of the *TNF-α* 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.[21] Genetic variants of the *IL-10* gene have also proven to be important for the development of restenosis.[22]

The cross-talk between immune system and vascular wall results in upregulation of major histocompatibility complex (MHC) class II (MHC-II) molecules and of the MHC class Ib molecule, HLA-E.[3,23] Furthermore, it results in up regulation of chemokines such as CX3CL1 (fractalkine), CCL2 (MCP-1), CCL5 (RANTES) and their receptors.[3,24–27] 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 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, whilst the altered gene expression patterns can be passed to the daughter cells upon cell division or even transgenerationally.[28–30]

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.[31] Epigenetic mechanisms alter the accessibility of chromatin by modifying DNA and by modification or rearrangement of nucleosomes, which include post-translational modifications of histones.[32–34] 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 enhancer/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.[35–38] Since epigenetic alterations can accumulate in time, environmental factors can have profound effects on the cellular repertoire of expressed genes.[28]

Already in 1975 DNA methylation was proposed to play a role in regulating gene transcription.[39,40] Generally speaking, DNA methylation is associated with low gene activity. Actively transcribed genes are usually maintained in an unmethylated state in their promoter regions.[41–43] 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.[44,45] 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%.[46]

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 2kb distant of the promoter region,[47] 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.[48,49] Modifications of histone tails include (amongst others) acetylation and methylation of lysine residues. Whereas acetylation of histone tails is correlated with gene activation,[50–52] the influence of histone methylation depends on the exact residue methylated and the number of added methyl groups.[53–55] Histone modifications and DNA methylation provide a close interplay with respect to gene regulation as both activities are functionally linked.[56]

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.[57] In addition, SUMOylation and ubiquitination of histones has also been observed.[49] SUMOylation appears to be associated with transcriptional repression,[58] whereas ubiquitination has been suggested to play a role in transcriptional activation and elongation.[59] 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 or HATs) are counteracted by histone deacetylases (HDACs) and Sirtuins (SIRT) in establishing histone acetylation modifications at lysine residues in the N-terminal tails. Lysine methyltransferases (KMTs, formerly known

as histone methyltransferases or HMTs), finally, are counteracted by the recently discovered lysine demethylases (KDMs, formerly known as histone demethylases or HDMs) in establishing histone methylation modifications (Figure 1–2). In this way

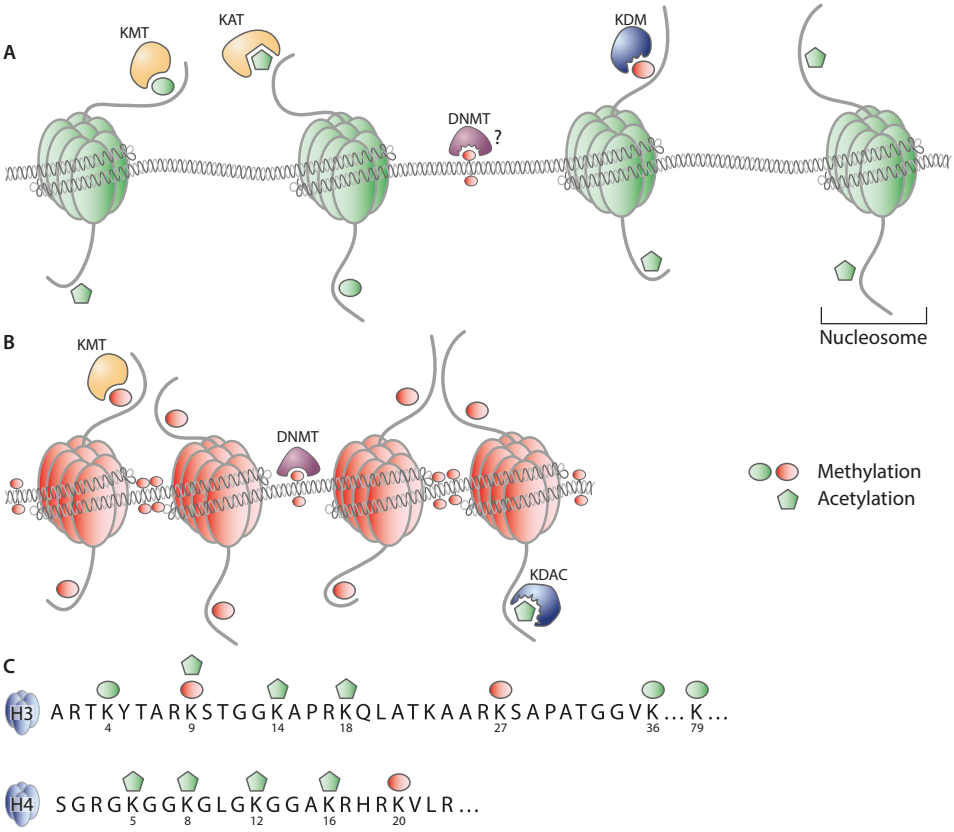

Figure 1–2. Schematic representation of chromatin. DNA is wrapped around the octamer core of histones to form a structure called the nucleosome. Transcriptionally active chromatin (euchromatin) is hallmarked by low frequency of DNA methylation. High levels of histone acetylation and histone methylation correlated with activation (MeK4H3, MeK36H3, MeK79H3) are detected in euchromatin. KATs are responsible for acetylation of histone tails, whereas KDMs remove methylation marks 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 HDACs (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).

these enzymes promote a return to respectively repressive or active chromatin structure.[60–62]

Importantly, the reversible nature of these epigenetic modifications makes the chromatin-modifying enzymes interesting therapeutic targets[63–65] and a myriad of small molecule inhibitors (SMI) that can influence the enzymatic activity of these chromatin-modifying enzymes are currently being tested for their efficacy (see Mai *et al.*[66] 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.[66] Notably suberoylanilide hydroxamic acid (SAHA) and valproic acid (VPA) (HDAC inhibitors (HDACi)) are already FDA approved for cancer treatment and epilepsy respectively.[67–69] With respect to atherosclerosis, administration of curcumin (a KAT inhibitor) resulted in significantly lowered low-density lipoprotein (LDL) levels and raised high-density lipoprotein (HDL) levels in healthy volunteers.[70–72] It has also been reported that curcumin has an anti-proliferative effect on peripheral blood mononuclear cells (PBMCs) and vSMCs.[73]

Epigenetics and association with atherosclerosis

Epigenetics provides an attractive explanation on 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 trans-generationally. 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.[74] 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.[75] In this study the correlation was found by comparing methylation sensitive restriction of peripheral blood leukocyte 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 (CRP) 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 coronary artery disease (CAD) an epigenetic component associated with disease was also identified.[76] 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.[76] Furthermore, a positive correlation was found between global DNA methylation and homocysteine levels. Homocysteine is known to be an independent risk factor for CAD.[77,78] Sharma and co-workers 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.[79] However, no significant difference could be detected between patients and controls.[76] 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).[80] Perhaps more importantly, they showed that atheroprone hemodynamics resulted in differentially regulated phenotypes for endothelial cells (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 eNOS, TIE2 and Krüppellike factor in ECs, and smooth muscle actin and myocardin in SMCs, whereas 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 binding to the smooth muscle cell actin CArG promoter. 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.[80]

Another linkage between atherosclerosis and histone modifications was found when the administration of the HDACi trichostatin A (TSA) in *LDR*-/- mice led to exacerbated neointimal lesions.[81] 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-α and VCAM-1 were increased in aortic plaques. It has also been noted that application of TSA resulted in inhibition of SMC migration.[82] 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.[83] In cultured ECs it was shown that OxLDL reduced expression of HDAC1 and HDAC2. Global HDAC activities were partly restored by statins: pre-treatment of ECs for 24h 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 HDAC2 in ECs was shown in atherosclerotic plaques of human coronary arteries.[83] 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 live.[84] 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*.[84] However, when the adult environment differs from the in utero environment, this may lead to an increased risk for cardiovascular disease. Studies in pregnant rats show that a protein-restricted diet results in reduced expression of Dnmt1, leading to hypomethylation of specific promoters.[85] 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, the most important cellular factors that contribute to atherosclerotic plaque formation and how their phenotype is regulated by epigenetic processes will be discussed.

T cells

Several lines of investigation illustrate that T cells are important in the formation of (early) lesions.[86–89] Within the plaque, several subsets of T cells can be found. Mainly $CD4^+$ T cells are dominantly found in plaques, but there are some $CD8^+$ T cells found as well.[5] The T cells present in human plaques are predominantly of the Th1 phenotype.[89] Th1 cells produce IFN-γ – a macrophage activating cytokine – and are generally considered pro-atherogenic. Th2 cells are rarely detected in lesions.[90] They are generally considered anti-atherogenic, but may also contribute to aneurysm formation.[91] Regulatory T cells (Treg cells) control the balance between Th1 and Th2. Tregs are considered to be atheroprotective.[92]

All T cells derive from the same precursor: the naïve T-cell. Not surprisingly, differentiation of e.g. $CD4^+$ T cells into a Th1, Th2 or Treg phenotype is regulated by epigenetic processes.[93,94] 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-γ* and *IL-4*.[95] The *IFN-γ* 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.[96] 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-γ is preceded by remodelling of the *IFN-γ* locus.[97] Upon initial stimulation of naïve T cells, the lineage determining factors GATA3 and T-bet mediate many of the structural changes to the chromatin.[93] These factors will respectively render the *IFN-γ* or *IL-4* genes accessible to regulatory enzymes and other transcription factors.[98–100] On the level of DNA methylation, there are numerous findings supporting the epigenetic regulation of IFN-γ expression. Increased expression of IFN-γ was found in T cells from *Dnmt* knockout mice[101] and in T cells

treated with DNMT inhibitors (DNMTi).[102,103] In addition, expression of IFN-γ by NK and NKT cells is also controlled by epigenetic processes.[97] The ability of NK and NKT cells to rapidly produce substantial amounts of IFN-γ implies that the *IFN-γ* locus is accessible to transcription factors, meaning that the chromatin at the *IFN-γ* locus is maintained in an 'open' state. Indeed the *IFN-γ* locus in NK cells was found to be hyperacetylated and in a poised state.[104,105]

In the differentiation to Th2 cells, IL-4 expression is preceded by remodelling of the *IL-4* locus, similarly to the *IFN-γ* 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 H3K27Me3 (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-γ* locus*)*. These processes have been excellently reviewed by Ansel *et al.*[106]

The Foxp3 transcription factor is considered the master switch for Treg. The promoter of this transcription factor showed difference in methylation levels between Tregs and non-Treg $CD4^+$ cells.[107] Furthermore, this study also showed difference in activating histone marks (H3Ac, H4Ac and H3K4Me3) in *FOXP3* promoter chromatin. Epigenetic regulation of T cell subtypes has also been shown *in vivo*. Mice, which were treated with the HDACi TSA showed an increase in $Foxp3^+$ $CD4^+$ Treg cells – both proportionally as well as in absolute numbers – in the lymphoid tissues.[94] Besides influencing T cell populations, the differentiation of monocytes to dendritic cells (DCs) can also be modulated by HDACi.[108]

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 (Figure 1–1). 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 HDAC function during differentiation resulted in the formation of function-impaired DCs. Notably, treatment with the HDACi's butyrate and MS275 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.[108,109]

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 HDACi TSA inhibited TNF-α induced monocyte adhesion. This was the result of downregulation of the *VCAM-1* gene, however *ICAM-1* and E-selectin – two other cytokines inducible genes – where not affected.[110] This suggests that the HDACi modulated TNF-α–mediated signalling specifically targets the *VCAM-1* promoter, instead of general inhibition of the NF-κB pathway. This notion is interesting, since NF-κB is a target for chromatin modifying enzymes as well (see "Non-histone Targets" on page 31). Similar results have been presented for the induction of tissue factor in human ECs. The human tissue factor (TF) promoter contains an NF-κB–related binding site: TF-κB. HDACi administration inhibited agonist induced (TNF-α, IL-1ß or LPS) TF activity in ECs by blocking activation of TF-κB (TF activity was reduced 90% in HUVEC; 50 % reduced in PBMC, *in vitro*). TSA nearly abolishes TF-κB binding, without affecting NF-κB 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 HDACi's.[111]

Work performed in the group of Stefanie Dimmeler showed an important role for HDACs in regulating several endothelial specific genes. Using HDACi's, it was shown that Endothelial Nitric Oxide Synthetase (eNOS) mRNA and protein levels decreased after HDACi administration.[112] Furthermore the 'master-switch' for some typical endothelial expressed genes (*HOXA9*, which regulates eNOS, VEGF receptor 2 and VE-cadherin expression) was also downregulated in its expression by HDACi administration.[113]

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.[114] CArG boxes serve as a binding site for Serum Response Factor (SRF), a transcription factor.[115] However SRF expression is not limited to SMCs and serves many functions in different cell types including cell migration and cell-cell adhesion.[116]

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 (see McDonald *et al.*[117] 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 then to promoter regions in non-SMCs. This suggests that in SMCs the chromatin is in such a state that it is accessible to SRF.[118–121] 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 then in non-SMCs.[122]

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 where enriched for the repressive chromatin marks H3K27Me3 and H4K20Me2 at SMC-specific promoter regions (see McDonald *et al.*[117] 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 H3K27Me2 demethylase), was shown to play a role in the pro-inflammatory phenotype of vSMCs in diabetic mice.[123] In this mouse model levels of H3K4Me2 (a histone modification correlated with expression) were increased at the MCP-1 and IL-6 promoter. Upon stimulation with TNF-α 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 HDACi TSA, displayed an inhibition of vSMC proliferation.[82] It was subsequently shown that expression of p21WAF1 was induced

by this treatment. However, HDACi treatment had no effect on SMCs isolated from p21WAF1 knockout mice.

Together, these findings illustrate how epigenetic mechanisms play an important role in the processes that control differentiation and activation of hematopoietic cells, but also of vECs and vSMCs.[117,124]

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 crosstalk. 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, some of the best-studied examples will be discussed 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 (NO), was first identified as a vasodilator,[125] although recent evidence also suggest a role in inflammation. NO has been attributed roles in leukocyte adhesion and vSMC proliferation.[126] Furthermore, dysfunctional eNOS is believed to be implicated in atherosclerosis development.[127]

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 demonstrated transgene promoter activity.[128–130] 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.[130] Later experiments showed the involvement of histone modifications in the expression of eNOS as well. ChIP showed enrichment of H3Ac, H4Ac and H3K4Me(2/3) in chromatin of the eNOS promoter in eNOS expressing cells. Treatment of non-expressing cells with the HDACi TSA

– effectively increasing histone acetylation – also led to an increase in eNOS mRNA (See Fish *et al.*[126] 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.[131] This gene is expressed in macrophages, but also in ECs and vSMCs, under inflammatory conditions. It has been suggested that NO – derived from macrophages expressing iNOS – can result in apoptosis of vSMCs, promoting plaque instability.[132] 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 H3K9Me2 and H3K9Me3 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.[131]

CCR5

CCR5 is important for plaque formation as it attracts T cells and mononuclear cells. CCR5 knock-out mice show less neointima formation and an increase in production of the anti-inflammatory IL-10 molecule by SMCs.[133,134] One of the ligands for the CCR5 receptor, CCL5 or RANTES, has also been shown to be involved in unstable angina pectoris (UAP).[135] 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. These results are extensively discussed in **chapter 3**.

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 crosstalk of immune cells and vascular wall components.

CCL11 (eotaxin)

In human atherosclerotic plaques, the chemokine CCL11 (also known as eotaxin) was shown by immunohistochemistry to be expressed.[136] 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.[137] CCL11, an eosinophil chemoattractant, has been suggested to play a role in atherosclerosis development, although the precise relation remains to be elucidated.[136,138,139] At the same time, CCL11 is expressed by SMCs, while macrophages in the human atherosclerotic plaque express the CCL11 receptor, CCR3.[136] CCL11 transcription is induced by inflammatory TNF-α signalling and is mediated through NF-κB. TNF-α induces acetylation of histone H4 in the CCL11 promoter. This results in an open chromatin structure, promoting subsequent binding of the NF-κB subunit p65 to the CCL11 promoter (as shown by ChIP).[140] Notably, glucocorticoids repress CCL11 transcription through selective inhibition of histone H4 acetylation.[140] 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.[140]

Epigenetics in (Vascular) Inflammation

KDM6B

De Santa *et al.* recently showed that inflammation and Polycomb-mediated gene silencing are linked.[141] Polycomb Group (PcG) proteins are important for the maintenance of a repressive chromatin 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 H3K27Me3 chromatin mark, which is subsequently read by the PRC1 maintenance complex (reviewed by Kohler *et al.*[142]).

The JmjC-domain protein Jmjd3 (KDM6B, a H3K27 demethylase) is expressed in macrophages upon stimulation with bacterial products and pro-inflammatory cytokines.[141] Jmjd3 binds to Polycomb Group (PcG) target genes, regulates H3K27Me3 levels and therewith their transcriptional activity.[143] 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.[144] 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.[145–147] ERs may play an important role in protection against atherosclerosis.[146] Deficiencies in oestrogen receptor alpha (ER-α) lead to accelerated atherosclerosis in men, furthermore ER-α mediates the loss of expression of some cytokine induced cell-adhesion molecules (see Miller *et al.*[148] and references therein). ER-α was shown to have a varying degree of methylation throughout the cardiovascular system.[149] 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 oestrogen receptor beta (ER-β) also displays a correlation in methylation of its promoter and atherosclerosis.[150] Contrary to ER-α expression, expression of ER-β correlates with atherosclerosis independent of age.[151] By comparing plaque vs. non-plaque tissue from the same vessels, it was recently shown that the ER-β promoter has higher methylation levels in atherosclerotic lesions.[150] In this study, increased expression of ER-β was also observed – *in vitro* – in ECs and SMCs after administration of a DNMTi (5-aza-2′-deoxycytidine) and a HDACi (TSA). These experiments provide supporting evidence for epigenetic regulation of the ER-β receptor.

COX-2

Another example for the linkage between epigenetics and cardiovascular disease related inflammation is transcription of the cyclooxygenase-2 *(COX-2)* gene. TNF-α – as well as several other cytokines – can induce expression of COX-2, a protein associated with atherosclerosis development.[152] Cytokines (such as TNF-α and IFN-γ) 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 HDACi's sodium butyrate (NaBu) and TSA revealing a significant role for epigenetic components in Cox-2 expression.[153] 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.[154] Therefore, under inflammatory, conditions MHC-II molecule expression can be induced on vECs and vSMCs, which normally are not expressing MHC-II.[155,156]

The 'master regulator' of MHC-II expression is the co-activator CIITA (Class II Transactivator)[157] Within the context of atherosclerosis, CIITA is of importance, since it has been shown to be involved in transcriptional regulation of collagen type 1.[158,159] Collagen 1 is one of the main components of extracellular matrix and is essential in cap formation and plaque stabilisation.[10]

Transcriptional regulation of *MHC2TA*, the gene encoding CIITA, is mediated through the activity of four independent promoter units (CIITA-PI through CIITA-PIV, Figure 1–3A).[160] These promoter units are employed in a cell type- and activation-specific manner. In aortic SMCs CIITA-PIII and –PIV are expressed after IFN-γ stimulation and CIITA is needed for IFN-γ–mediated repression of collagen type 1 genes (*COL1A1* and *COL1A2*).[158,159] 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.[159]

In MHC-II transcription, CIITA exerts its transactivating function through protein-protein interactions with a multi-protein complex, which is comprised of RFX, CREB/ATF and NFY (Figure 1–3B).[161–163] Together with CIITA, this complex acts as an enhanceosome driving transactivation of these genes.[161–163] In the MHC-enhanceosome, CIITA acts as a platform for recruitment of chromatin-modifying activities, which include KATs, HDACs and an arginine methyltransferase.[164–166] 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.[165,167] 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

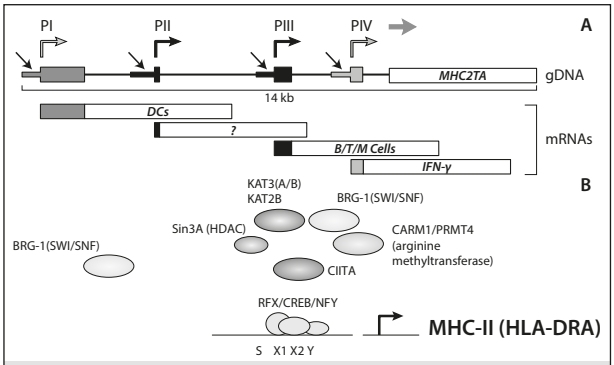

Figure 1–3. (A) Organization of the MHC2TA multipromoter region. Each promoter (indicated with black arrows) transcribes an unique first exon (shaded boxes). Transcription start sites are indicated with coloured, angled arrows. Promoter PI is mainly used for constitutive CIITA expression in dendritic cells, whereas PIII is mainly used in B cells, activated T cells and macrophages. Promoter PIV is mainly used for IFN-γ induced CIITA expression in non-bone marrow-derived cells. The function of PII is still poorly understood. (B) Factor assembly on the MHC-II promoter. Shown is the regulatory SXY module, to which the MHC enhanceosome binds.

maturation deacetylation occurs at the *MHC2TA* locus coinciding with transcriptional inactivation.[168] During DC differentiation histone acetylation of the type I promoter is increased. This increase was blocked by IL-10, inhibiting *MHC2TA* transcription.[169]

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.[170] Especially since NF-κB – playing crucial roles in inflammation – is showing up as one of the non-histone substrates. Both NF-κB and p53 are acetylated by KAT3A (CBP) and KAT3B (p300),[171–174] whereas p53 can also be acetylated by KAT2B (PCAF).[175,176]

As is the case with most genes, transcription of pro-inflammatory cytokine genes such as IFN-γ and TNF-α is positively regulated by transcription factors. However, these cytokines can also induce changes to the epigenome. Thus not only are cytokines under epigenetic control, cytokines themselves induce (indirect) changes to the chromatin, allowing effector-genes to be expressed upon stimulation. Activation of the IFN-γ pathway results in activation of e.g. NF-κB. NF-κB is found in the cytoplasm of unstimulated cells, together with its inhibitor, IκB, that prevents NF-κB from entering the nucleus.[177] Upon stimulation with inducers – e.g. reactive oxygen species (ROS) and lipid peroxidase products – IκB is rapidly degraded via the ubiquitin-proteasome pathway.[178,179] Hereupon, NF-κB is transported to the nucleus, where it can activate the transcription of various targets with

κB-elements in their promoter.[180,181] This process is known to be activated by KATs and repressed by HDACs (See Glozak *et al.*[170] and references therein). The p65 subunit of NF-κB is acetylated by KAT3A/B (P300/CBP) at lysines 218, 221 and 310.[182] The acetylated form of p65 shows weak affinity for IκB. HDAC3 in turn deacetylates p65, promoting association with IκBα and thereby nuclear export of NF-κB.[171] In addition to HDAC3, HDAC1 has been shown *in vitro* and *in vivo* to deacetylate p53.[183]

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 (VEGFR-1), which is di-methylated by Smyd3 (a H3K4 methylase).[184] The biological function of methylation at lysine 831 is yet to be discovered. These examples perfectly illustrate how epigenetic and inflammatory processes are interwoven.

Thesis Outline

As discussed above, 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 involved in atherosclerosis pathology (summarized in Table 1–1). 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, modulation of 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.

Table 1–1: 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	Reference
ec-sod	DNA Methylation	74
c-fos	Histone Code	80
CD36	Histone Code	81
IFN-γ	DNA Methylation & Histone Code	97–99
IL-4	DNA Methylation & Histone Code	106
Foxp3	DNA Methylation & Histone Code	94,107
eNOS	DNA Methylation & Histone Code	126,130
iNOS	DNA Methylation & Histone Code	9,131
CCL11	Histone code	140
CCR5	DNA Methylation & Histone Code	185
oestrogen receptor α	DNA Methylation	149
oestrogen receptor ß	DNA Methylation & Histone Code	150
CIITA	DNA methylation & Histone Code	164–169

In atherosclerosis much information has been gathered over the years regarding the individual components involved in plaque formation as discussed above. Systematic investigation however, of epigenetic phenomena in atherosclerosis has so far only been conducted in mouse models.[186,187] In **chapter 2** epigenetic modifications in human plaques are examined. The plaques investigated are cross-sectional for disease pathology. Subsequently, epigenetic regulation of monocytes is investigated in **chapter 3**. The transcriptional patterns of the genes encoding epigenetic modifying enzymes where studied during the differentiation of monocytes in macrophages and DCs. In **chapter 4** interventions by small molecule inhibitors is discussed by studying the transcriptional regulation of the CCR5 gene. **Chapter 5** illustrates the interplay between genetic and epigenetic code and discusses a SNP found in the upstream promoter region of the epigenetic modifying enzyme PCAF. Finally, in **chapter 6** the findings presented in this thesis will be summarized and discussed.

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2

Global histone H3 lysine 27 triple methylation levels are reduced in vessels with advanced atherosclerotic plaques

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Abstract

Aims:

Alterations in epigenetic processes are frequently noted in human disease. These epigenetic processes involve methylation of DNA and post-translational modifications of histones. It is well established that in particular histone methylation plays a key role in gene transcription. In this study, we have investigated the relationship between triple methylation of lysine 27 in histone H3 (H3K27Me3) modifications and atherosclerotic plaque stage.

Materials and Methods:

28 peri-renal aortic tissue patches covering the entire spectrum of atherosclerotic plaque development were evaluated by immunohistochemistry for the levels of H3K27Me3, EZH2, JMJD3 and BMI1.

Key Findings:

The results of our studies are in support of a reduction in global levels of the H3K27Me3 modification in vessels with advanced atherosclerotic plaques. This reduction in H3K27Me3 levels is not accompanied by alterations in global levels of the corresponding histone methyltransferase EZH2, the catalytic subunit of the polycomb repressive complex 2 (PRC2). Likewise no alterations in global levels of BMI1, a component of the PRC1 complex, which binds to H3K27Me3-modified histones or the global expression levels of the histone demethylase JMJD3, which removes the methyl marks on H3K27, were observed.

Significance:

Together, our data show that in atherosclerosis development alterations in global levels of H3K27Me3 occur. The reduction in the number of nuclei in the *tunica media* that display the repressive H3K27Me3 mark in vessels with advanced atherosclerosis plaques therefore could be a reflection of the dynamic pattern of smooth muscle cell differentiation and proliferation associated with atherosclerotic disease.

Introduction

Risk factors for atherosclerosis include many environmental factors.[1] Not surprisingly, research over the past few years has focused on epigenetic contributions to the disease. Nowadays, we are fully aware of the important involvement of epigenetic processes in the regulation of gene expression (see Geissmann *et al.*[2] and references therein). Understanding these epigenetic processes is critical for our understanding of inflammatory responses and disease.

Epigenetic mechanisms change the accessibility of chromatin.[3] The effect of these epigenetic processes on transcription depends on the presence of various post-translational histone modifications, commonly referred to as the histone code. Epigenetic gene regulation is a dynamic process, although the post-translational modifications themselves are chemically stable. Proteins that put the histone code in effect can be divided into three classes: 'writers', 'erasers', or 'readers'. One of the well-studied post-translational histone modifications is triple methylation of lysine 27 in histone H3 (H3K27Me3), which is linked with transcriptional repression.[4] Loss of H3K27Me3 is associated with cell proliferation (see Moore *et al.*[4] and references therein). Gene silencing via H3K27Me3 is orchestrated by two protein-complexes: the polycomb repressive complex 2 (PRC2), the silencing initiation complex, and the polycomb repressive complex 1 (PRC1); the maintenance complex.[4]

The PRC2 core consists of a number of proteins with enhancer of zeste homolog 2 (EZH2) as the catalytic subunit.[5] As such, EZH2 has been associated with X-inactivation, germline development, stem cell pluripotency, cancer metastasis and cell proliferation.[6,7] The PRC1 complex consists of (amongst others) chromobox homolog (CBX) 2, 4, 6, 7, 8 and B lymphoma Mo-MLV insertion region 1 homolog (BMI1; see Mestas *et al.*[5] and Jonasson *et al.*[8] and references therein). This complex recognizes the H3K27 trimethylation mark via the chromodomain of the CBX proteins, but does not possess methyltransferase activity itself.[8] Although the exact function of BMI1 is still unknown, it has been suggested that H2A ubiquitylation regulates BMI1-mediated gene silencing.[8] Furthermore, reduced expression of BMI1 is associated with cellular senescence.[9]

The histone demethylase jumonji domain containing 3 (JMJD3) removes methylation marks from H3K27.[10] JMJD3 has previously been found to be upregulated under nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) mediated inflammatory conditions, providing a link between epigenetic regulation and

inflammation.[11] Furthermore JMJD3 has been found to be upregulated in macrophages after lipopolysaccharide (LPS) exposure.[11,12] In general polycomb group (PcG) proteins are broadly expressed; their expression however, can be modulated by environmental stimuli, thus linking extracellular cues to reprogramming of the epigenome.[13,14]

By using immunohistochemistry (IHC), a recent study in ApoE knockout mouse identified histone lysine methylation marks as contributing factors to atherosclerosis development.[15] In particular, these studies revealed a significant decrease in vSMCs for global H3K27Me3 levels in ApoE+/– mice from ApoE-deficient mothers when fed with a high cholesterol diet. The effect of post-natal high cholesterol diet on global H3K27Me3 levels was also noted when offspring from wild-type mothers and ApoE-deficient mothers were compared.[15]

At the moment, not much is known on the role of epigenetic mechanisms to the pathology of atherosclerosis in humans. In the current study, we therefore have evaluated global H3K27Me3 levels in vessels representing different stages of atherosclerotic plaque development. Furthermore, we also investigated global levels of the writer, eraser and reader of this repressive histone mark in these atherosclerotic vessels. Using IHC with antibodies directed against H3K27Me3, EZH2, BMI1 and JMJD3, and an automated evaluation method we found that the numbers of nuclei that display the H3K27Me3 mark in vessels with more advanced atherosclerotic plaques were reduced. The global decrease in the number of H3K27Me3 immunopositive nuclei in the *tunica media* reveals the different epigenetic states of these cells during progression of atherosclerotic disease.

Materials and Methods

Donor and Tissue sampling

A total of 28 peri-renal aortic tissue patches were studied. The patches were obtained during clinical organ transplantation, as has been described previously.[16] The formalin fixed, paraffin embedded sections where cut in 4 µm thick consecutive slides. Donor age ranged from 12 to 71 years (Table 2–1). Sample collection and handling was performed in accordance with the guidelines of the Medical and Ethical Committee in Leiden, the Netherlands and the code of conduct of the Dutch Federation of Biomedical Scientific Societies (http://www.federa.org/code-goed-gebruik-van-lichaamsmateriaal-2011).

Table 2–1: Classification of aortic tissue according to the classification of the AHA proposed by Stary *et al.*[17]

Stage	Median age	*n*	
		male	female
0 (normal vessel)	42	1	2
1	14	1	2
2	50	3	2
3	55	3	1
4	48	1	2
5	57.5	2	2
6	58	5	1

Immunohistochemistry

Slides were deparaffinised in xylene (Merck, Darmstadt, Germany) and rehydrated in graded ethanol (Merck) into water. Antigen retrieval was performed in 0.01M citric acid (Sigma Aldrich, Zwijndrecht, the Netherlands) buffer, pH 6.0 at 98°C. Slides were stained with the following antibodies against: H3K27Me3 and H3K9Me3 (both repressive histone marks), H3Ac and H3K4Me3 (both active histone marks), EZH2, JMJD3, BMI1, or isotype control (Table 2–2). Depending on the antibody, slides were permeabilized, pre-blocked by human serum (HS) incubation, and/or avidin/biotin-block (Vector Laboratories, Burlingame, CA, USA; Table 2–2). Permeabilization was performed by 5 min. incubation in 0.5% (v/v) Triton® X-100 (Sigma Aldrich) in phosphate buffered saline (PBS), followed by 10 min. incubation in 0.1M glycine in PBS. Pre-blocking with HS (LUMC Pharmacy, Leiden, The Netherlands) was performed by incubating slides for 1h in 5% HS (v/v) in PBS with 0.05% (v/v) Tween® 20 (Sigma Aldrich; PBS-T). Avidin/biotin block (AB block) was performed according to manufacturer's instructions. Primary antibody incubations were all performed overnight in 1% (w/v) bovine serum albumin (Sigma Aldrich) in PBS-T unless noted otherwise. Either goat anti-rabbit (Vector Laboratories) or horse anti-mouse (Vector Laboratories) biotin conjugate was used as secondary antibody, depending on the species (Table 2–2). The secondary antibodies where diluted in PBS-T with goat or horse normal serum (Vector Laboratories; dilution 1:66). Secondary antibody incubation (1h) was followed by incubation with Vectastain ABC (Vector Laboratories). Visualization was performed using 3–3′ -diaminobenzidine tetrachloride (DAB; Sigma Aldrich) and Mayer's haematoxylin (Sigma Aldrich) was used for counterstaining.

Image Analysis

Complete slides where digitized with a digital (microscopic) Mirax slide Scanner system (3DHISTECH, Budapest, Hungary) equipped with a 20× objective with a numerical aperture of 0.75 and a Sony DFW-X710 Fire Wire 1/3″ type progressive SCAN IT CCD (pixel size 4.65 × 4.65 µm). After scanning the locations of the *tunica intima*, *tunica media* and *tunica adventitia* were annotated by hand using the Panoramic Viewer software (3DHISTECH) and exported in the TIFF image-format. The total number of nuclei and the number of positive cells for each slide where counted in an automated fashion using ImageJ (available at http://rsbweb.nih.gov/ij/). Briefly, the algorithm is as follows: slides where separated into haematoxylin and DAB components using the colour deconvolution plugin of Ruifrok and Johnston.[18] The MultiThresholder, with parameter settings "Triangle apply", was used to threshold both the DAB and the haematoxylin specific images. Potential nuclei where identified using particle analysis and subsequently mapped to the haematoxylin counterpart. Nuclei consisting of both DAB and haematoxylin components where considered as positively stained nuclei, thus eliminating possible background staining. The total number of nuclei was counted using particle analysis on the haematoxylin image.

Statistical analysis

The relative numbers of positively stained cells are represented as average ±SEM. The data were analysed using a linear model (ANOVA) with a sex effect, age effect, layer effect, AHA (American Heart Association) score effect and interaction effects. The analysis was performed in R (R, http://www.rproject.org). All tests were performed using t– or F-tests as appropriate. To correct each test for multiple testing a Bonferroni correction was used. Differences were considered to be significant if $p < 0.05$ after multiple testing correction. Correlations were tested using Pearson's correlation based on the total percentage of positive stained nuclei per slide and no discrimination was made between tunica media, tunica adventitia or tunica intima. Pearson's correlation was assessed using the SPSS (IBM) software version 20.0.0.2.

Table 2–2: Antibodies (with respective isotypes), dilutions and blocking steps used for immunohistochemistry.

Antibody reactivity	Manufacturer	Species	Isotype	Catalogue no.	Dilution	Triton permeabilization	Serum Block	AB Block
H3K27Me3	Abcam	mouse	IgG3	ab6002	1:200	no	no	no
H3K4Me3	Abcam	rabbit	IgG (polyclonal)	ab8580	1:30,000	no	no	no
H3K9Me3	Millipore	rabbit	IgG (polyclonal)	07-442	1:750	no	no	no
H3Ac	Millipore	rabbit	IgG (polyclonal)	06-599	1:3,000	no	no	no
EZH2	BD Bioscience	mouse	IgG1	612667	1:400*	yes	no	no
BMI1	Abcam	mouse	IgG1	ab14389	1:400	yes	no	no
JMJD3	Abcam	rabbit	IgG (polyclonal)	ab38113	1:700	no	yes	yes
Isotype control	Santa Cruz	rabbit	IgG (polyclonal)	sc2027	Similar to respective specific antibody			
	DAKO	mouse	IgG1	X0931				
	Abcam	mouse	IgG3	ab18394				

*Diluted in PBS + 1/66 Human serum.

Results

Distribution of H3K27Me3, EZH2, BMI1 and JMJD3 in the vessel wall

H3K27Me3, EZH2, BMI1 and JMJD3 staining was performed on serial cross-sections of 28 different aortic samples. The tissue samples were collected during clinical organ transplantation as has been described previously.[16] Donors varied in age from 12 to 71 years old and samples were specially chosen to cover all stadia (including 3 normal, stage 0, vessels) described by Stary *et al.* (Table 2–1).[17] Representative staining patterns of normal vessels (stage 0) and vessels with advanced atherosclerotic plaques (stage 6) are shown in figure 2–1. It can also be established that the staining appears not homogeneous across the entire section (Figure 2–2). Especially in the *tunica media*, positive staining cells are focally observed (Figure 2–2). Interestingly, this patchy staining does not show any correlation to plaque size or location of the plaque. The presence of these clusters of positive cells most likely reflects different chromatin states within the vessel wall.

The number of stained nuclei was determined following digitization of the slides. The *tunica intima*, *tunica media* and *tunica adventitia* were exported as separate images and by using the ImageJ software package the percentage of positive stained cells was subsequently counted, using an algorithm that only takes nuclear staining into account. The results of these analyses show that, when compared with the levels of intimal staining, the abundance of BMI1, EZH2, H3K27Me3, JMJD3 is significantly more in the adventitia (Figure 2–3). These varying staining patterns might be a reflection of the different cellular compositions of the various vessel wall components.

Relationship between H3K27Me3 levels and plaque stage

To investigate the relationship of global H3K27Me3 abundance and plaque stage, we correlated the percentage of positively stained cells in the complete vessel wall to plaque stage. For this purpose we used the lesion stage classification according to the AHA as proposed by Stary *et al.*[17] The results of these analyses revealed a significant decrease in the number of nuclei that stained positive for the H3K27Me3 mark in vessels with more advanced lesions (Figure 2–4). Interestingly, in these vessels with more advanced lesions there is also a reduction in the number of nuclei that stained positively for BMI1 and JMJD3, albeit that this reduction did not reach statistical significance (Figure 2–4). The EZH2 levels showed no significant difference between vessels with early and late stage plaques (Figure 2–4).

Since sex is considered a risk factor for atherosclerosis we also evaluated the sex effect in our statistical model and found for H3K27Me3 a significant effect ($p = 0.006$; data not shown).

To estimate the specificity of the H3K27Me3 antibody, IHC with antibodies directed against H3K9Me3, H3K4Me3 and H3Ac was also performed in a number of cases. Both H3K9Me3 and H3K27Me3 are associated with transcriptional repression whereas H3K4Me3 and H3Ac are associated with transcriptional activation.

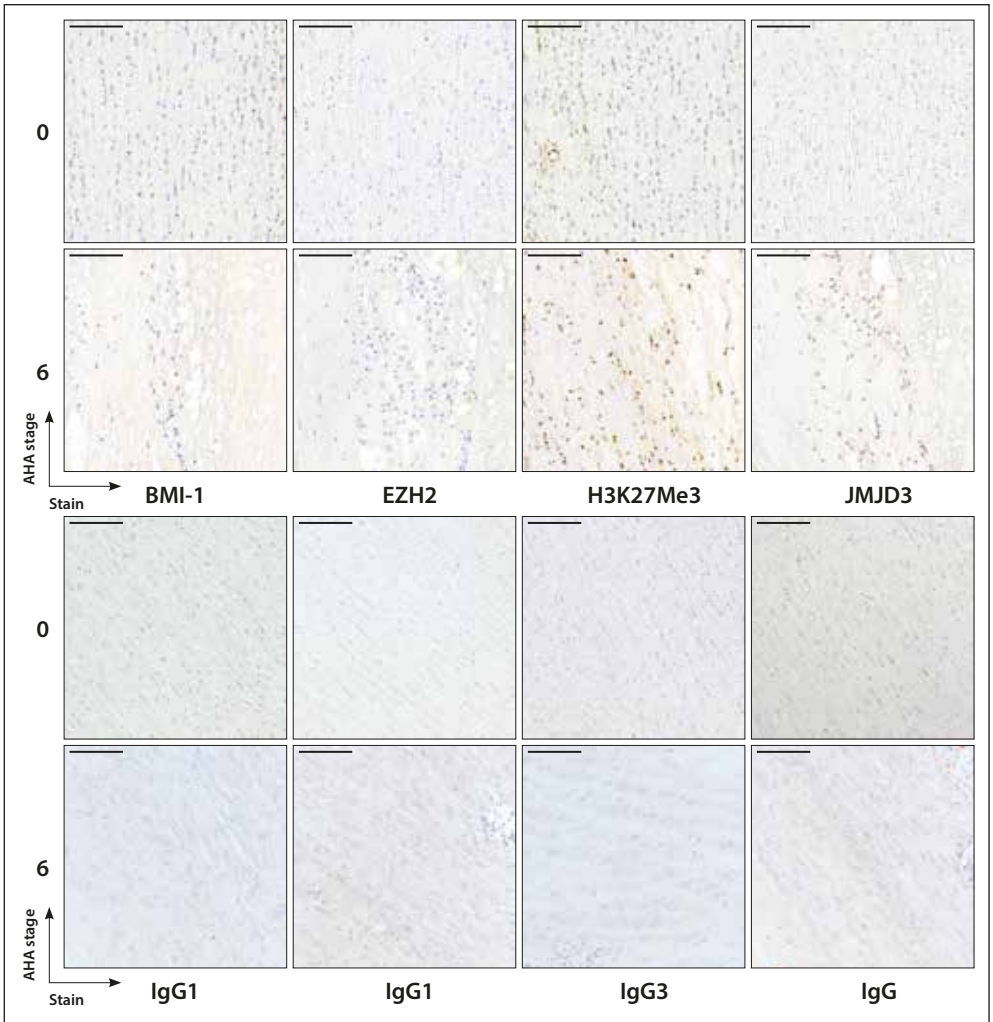

Figure 2–1. Representative staining for BMI1, EZH2, H3K27Me3, JMJD3 (upper panel) and their respective isotype controls (lower panel) staining in a normal vessel (stage 0) and an advanced plaque vessel (stage 6). Scale bars indicate 100 µm.

Shown in figure 2–5 (upper panel), is the staining pattern for H3K4Me3, H3Ac, H3K9Me3 and H3K27Me3 in vasculature-associated lymphoid tissue (VALT). Since VALT has a higher cell density, staining patterns are more easily discriminated. The staining patterns for H3K4Me3 and H3Ac marks are noticeably distinct from the H3K27Me3 and H3K9Me3 marks. Staining patterns for H3K9Me3 and H3K27Me3 show more similarities, but still yield a distinctively different pattern.

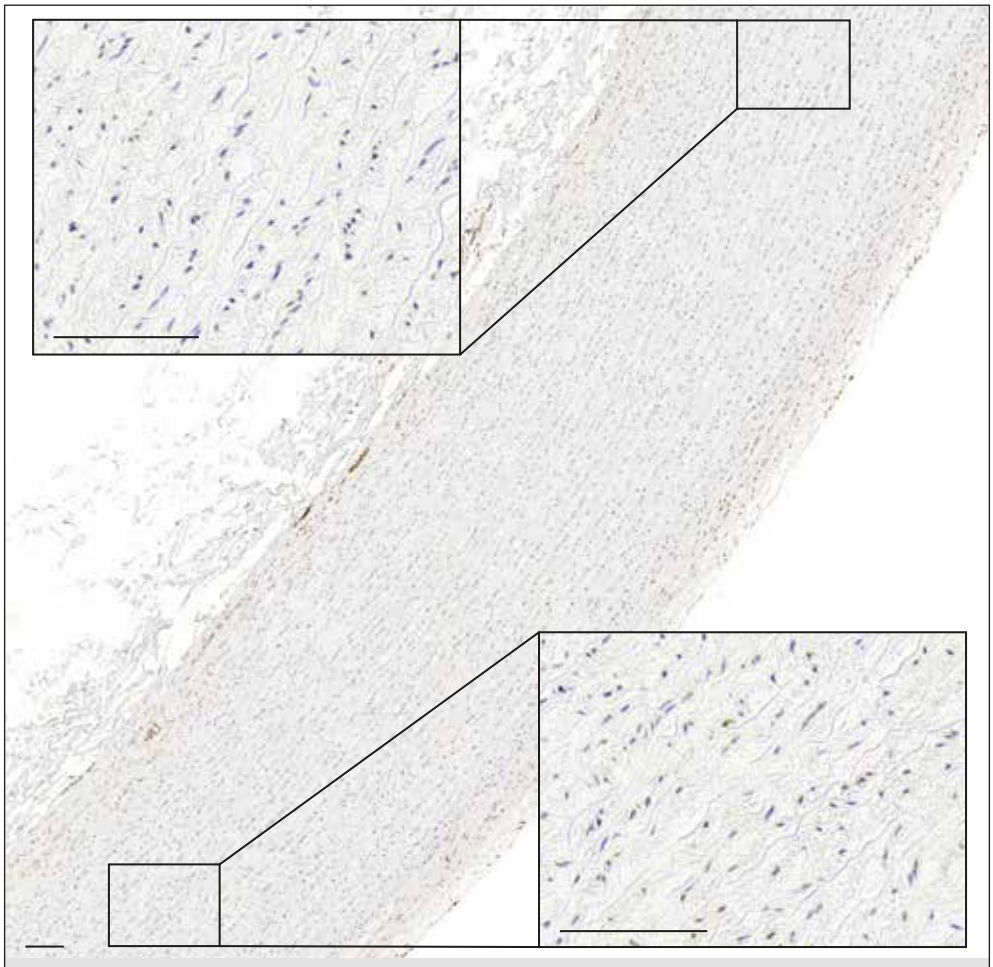

Figure 2–2. Representative slide showing nuclear H3K27Me3 staining in a normal vessel (stage 0) of a 16–year-old female. Staining is non-homogeneous throughout the vessel wall. Differential staining-patterns can clearly be observed between the *tunica intima*, media and adventitia, as well as focal staining within the *tunica media*. Scale bars indicate 100 µm.

Besides assessing VALT, the *tunica media* of vessels was also assessed to estimate the specificity of the H3K27Me3 antibody used in IHC. The staining for H3K4Me3, H3Ac, H3K27Me3 and H3K9Me3 yield patterns distinctive from each other (Figure 2–5, lower panel), although it is harder to visually discern the staining pattern in the tunica media compared to in VALT. Together, these distinct staining patterns reflect the specificity of the H3K27Me3 antibody for detecting the H3K27Me3 histone mark.

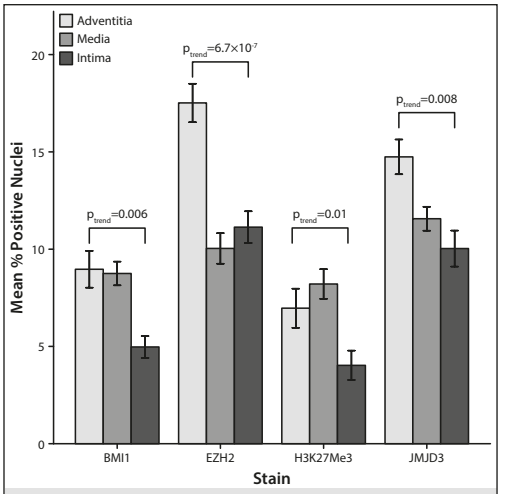

Figure 2–3. Percentage of positive nuclei found in *tunica intima*, *media* or *adventitia*. A significant increase of positively stained cells can be found in the *tunica adventitia* when compared with the intima, regardless of the used antibody. Error bars indicate ±SEM.

To further define the vessel wall components driving this change in global H3K27Me3 abundance, we investigated the distribution of this mark across the various vessel layers and plaque stage. As revealed in figure 2–6 all three layers showed lower levels in the number of nuclei displaying the H3K27Me3 mark. However, the reduction in the global levels of H3K27Me3 was more pronounced in the *tunica media* of vessels with late stage atherosclerotic plaques. The numbers of nuclei that reacted with the anti-EZH2, anti-JMJD3 and anti-BMI1 antibodies in the various vessel wall components remained similar in vessels with early and vessels with late stage atherosclerotic plaques (data not shown).

Correlation between H3K27Me3 levels and BMI1, EZH2 and JMJD3 levels

Given the functions of EZH2, JMJD3 and BMI1, a correlation between H3K27 Me3 levels and EZH2, JMJD3 and BMI1 levels can be expected. Correlations between the various staining levels were tested using Pearson's correlation. We found a weak correlation between levels of H3K27Me3 and BMI1 ($p = 0.014$; $r = 0.494$; Figure 2–7), but no correlation between levels of H3K27Me3 and JMJD3 or EZH2 (Figure 2–7).

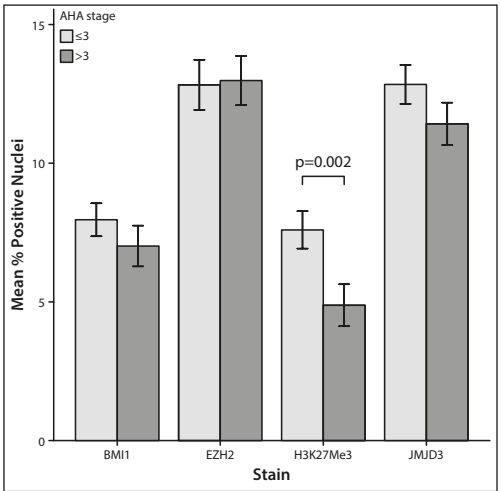

Figure 2–4. Quantitative analysis of staining patterns. Shown is the percentage of nuclei staining positive for proteins involved in H3K27Me3 methylation (i.e. BMI1, EZH2, JMJD3) and the H3K27Me3 mark itself, in early and late stage atherosclerotic plaques. A significant reduction of the H3K27Me3 mark was observed in more advanced lesions. Error bars indicate ±SEM.

Discussion

Currently it is widely appreciated that epigenetic processes contribute to disease pathogenesis including atherosclerosis. This is illustrated by recent research, which showed global alterations in DNA methylation in patients with CVD.[19–21] Some studies have also shown effects of small molecule inhibitors that interfere in the activities of histone modifying enzymes on disease parameters such as plasma cholesterol levels (see Wierda *et al.*[2] and references therein). In a previous study from our institute using the ApoE mouse model, an association between histone methylation and diet-induced hypercholesterolemia in vSMCs of ApoE+/– offspring from ApoE-deficient mothers was revealed.[15] In particular a significant decrease in vSMCs for global H3K27Me3 levels was noticeable following a high cholesterol diet postnatal in these ApoE+/– offspring.[15] A similar effect of this diet was also noted on global H3K27Me3 levels when offspring from wild-type mothers and ApoE-deficient mothers were compared.[15] To date, nothing is known with respect to alterations in global histone methylation patterns in human atherosclerotic processes. Therefore, in the current study, we have evaluated the association of global levels of H3K27Me3 with varying grades of atherosclerotic lesions. The results of this study revealed a significant decrease in the number of nuclei that stained positive for the H3K27Me3 repressive mark in vessels with late atherosclerotic lesions in comparison to vessels with early lesions. Particularly this difference was most pronounced in the *tunica media,* which comprises mostly vSMCs. Similar to the observations made in the ApoE-mouse model, high levels of cholesterol may contribute to the observed reduction in H3K27Me3 levels in the vessel wall of patients with advanced atherosclerotic plaques.

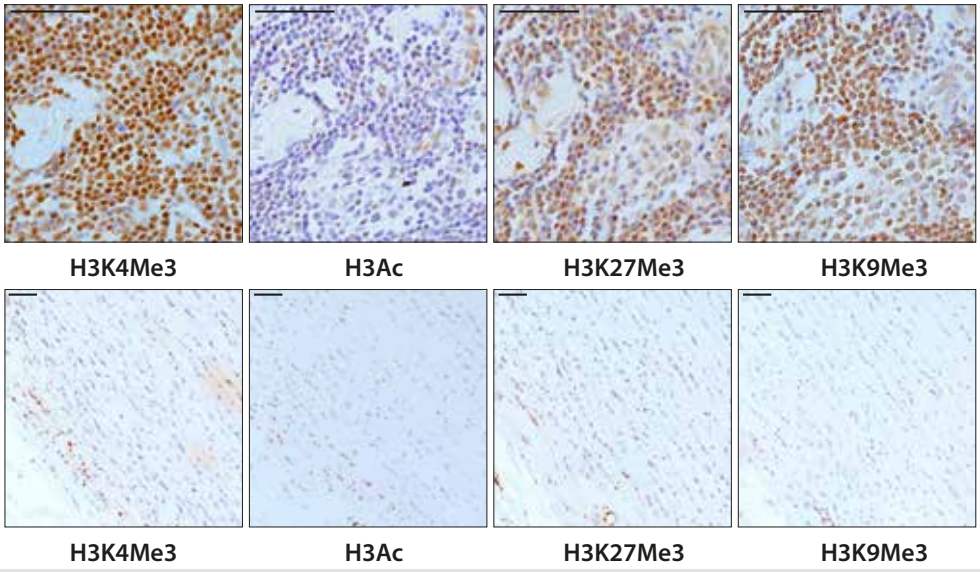

Figure 2–5. Specificity of the H3K27Me3 antibody was estimated by IHC staining with antibodies detecting other posttranslational modifications in histone H3 in serial sections of vasculature-associated lymphoid tissue (VALT) and of the tunica media. Shown in this figure are the staining patterns for H3K4Me3, H3Ac, H3K9Me3 and H3K27Me3. (Upper panel): Staining in VALT of a vessel with a ruptured plaque (stage 6) of a 57–year-old male. Since VALT has a higher cell density, staining patterns are more easily discriminated. The H3K4Me3 and H3Ac marks (associated with transcriptional activation) are notably distinct from the H3K27Me3 mark (associated with transcriptional repression). Staining patterns for H3K9Me3 and H3K27Me3, both marks being associated with transcriptional repression, show a higher degree of similarity. (Lower Panel): Staining pattern in the *tunica media* in a normal vessel (stage 0) of a 16–year-old female; vessel is identical to the vessel shown in figure 2–2. The H3K4Me3, H3K9Me3 and H3Ac marks all show a visually distinct pattern from the H3K27Me3 mark. Scale bars indicate 50μm.

We also studied the association of EZH2, BMI1 and JMJD3 in these atherosclerotic processes. No significant differences were observed in the number of nuclei that reacted with the immune reagents against EZH2, BMI1 and JMJD3 between vessels with early (including normal vessels) or with late atherosclerotic plaques. Therefore, no direct correlations could be made between levels of H3K27Me3 and levels of EZH2 (in the context of the PRC2 complex, the writer of the H3K27Me3 mark), of BMI1 (in the context of the PRC1 complex, the reader of the H3K27Me3 mark) or with JMJD3 (the eraser of the H3K27Me3 mark). The percentage of immunopositive H3K27Me3 and BMI1 nuclei however does show a significant, albeit weak correlation. This provides an indication that the observed change in H3K27Me3 results in a PRC-controlled cellular response.

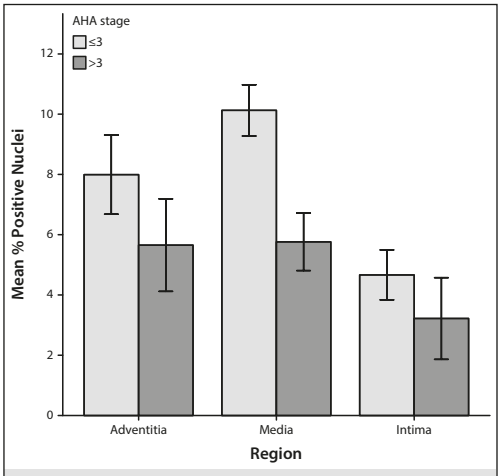

Figure 2–6. Location of H3K27Me3 within the vessel wall for early and late stage atherosclerotic plaques. A reduction of H3K27Me3 levels can be observed in all layers of the vessel wall. The most pronounced reduction of H3K27Me3 can be observed in the *tunica media*. Error bars indicate ±SEM.

The change in global histone modification levels without a change in the associated writer or eraser, observed in this study, has been noted before as well.[22] In a different study it was observed that, although EZH2 levels were significantly increased between patients and controls, there was no difference in global H3K27 methylation levels in PBMCs of patients with psoriasis vulgaris.[23] It must furthermore be realized that IHC does not address the functional activities of the proteins investigated or the catalytic capacity of the active complexes.

Of interest is the notion that regardless of the antibody used in the immunohistochemical staining, there is significantly more staining in the *tunica adventitia*. This is most likely a reflection of the cell types commonly found in the adventitia, which differ from those in the *tunica media* and *tunica intima*. A most likely explanation is that (activated) immune cells present in the *tunica adventitia* contribute to this skewing.

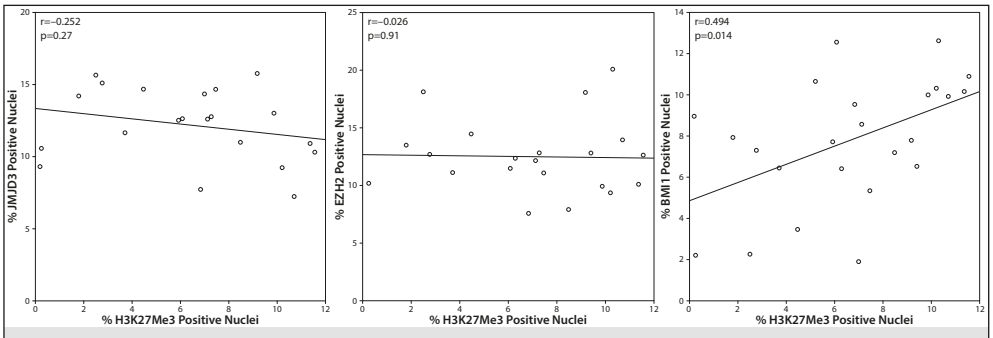

Figure 2–7. Correlations between H3K27Me3 levels and BMI1, EZH2 and JMJD3 levels. Correlations were tested, using Pearson's correlation, between the percentage of immunopositive H3K27Me3 nuclei and JMJD3, EZH2 or BMI1 immunopositive nuclei. No correlation was found between the percentage of H3K27Me3 immunopositive nuclei and JMJD3 or EZH2 immunopositive nuclei. A significant, albeit weak, correlation was found between the percentage of H3K27Me3 immunopositive nuclei and BMI1 immunopositive nuclei.

Interestingly, in vessels with advanced plaque stages, the observed decrease in H3K27Me3 positively stained cells occurs mostly in the *tunica media*. Given the role of the H3K27Me3 modification in chromatin condensation and gene silencing, the reduction in global H3K27Me3 levels in the *tunica media* infers that chromatin accessibility is increased, which could affect also the rate of transcription of genes involved in cell proliferation. This may reflect the proliferation of vSMCs commonly described in plaque formation. Whether this decrease in global H3K27Me3 levels proceeds or follows vSMC proliferation remains to be established.

In previous *in vitro* studies, a relation was found between cell activation and JMJD3 expression in macrophages.[11,12] In these studies cultured cells were subjected to LPS stimulation for several hours. Peak JMJD3 expression occurred after 2 hours whereafter JMJD3 levels diminished, showing that this is a temporary event. Both inflammation and macrophages form a central hub in the pathology of atherosclerosis. However, neither early, nor advanced atherosclerotic plaques showed significant alteration in JMJD3 levels in the various vessel wall components. The use of cross sectional material in this present study makes it therefore very unlikely to encounter macrophages that have been activated in the same time frame as in these previous *in vitro* studies.[11,12] Secondly, although macrophages are one of the major constituents of atherosclerotic plaques, they are present in relatively small numbers when the entire cross-section is taken into account, as in this study. Even if the effect observed by De Santa *et al.*[11,12] is present in human atherosclerotic plaques, it will probably not be reflected in the type of data we show here. Finally, the effect seen in the study by De Santa *et al.* may have been a LPS-specific effect, whereas LPS is not commonly associated with atherosclerosis.

Limitations of the current study

IHC results are notoriously hard to quantify, as staining intensity is not always correlated with protein concentration.[24] Furthermore DAB staining does not follow Lambert Beers Law complicating quantification. Also, DAB and heamatoxillin are hard to spectrally unmix by image processing.[25] In the data presented here, the heamatoxillin staining is often overpowered by strong DAB intensity. Using immunofluorescence the limitation of spectral unmixing could be overcome, but care must be taken in correlating intensity to protein expression with regards to quantitative analysis. The results presented here would therefore need to be confirmed by more quantitative methods, such as western blotting or ELISA. By using such methods distribution of protein expression across tissue however will be lost.

Trimethylation of H3K27 plays a definitive role in random X-chromosome inactivation26 and lineage determination.[27,28] It could therefore be expected that all, or at least a large number of cells of female-derived vessels stain positive for H3K27Me3 or for the commonly expressed modifying proteins EZH2, JMJD3 and BMI1. However, the seemingly low level of immunopositive nuclei that we found both in normal and in atherosclerotic vessels is observed quite frequently also in other studies. In several studies aimed to evaluate global levels of specific histone acetylation and methylation modifications it has been shown, both in humans and in mice, that these histone modifications, including H3K27Me3, are not detected in the nucleus of every cell by IHC.[15,29–31] This seemingly lack of the presence of a specific histone acetylation or methylation modification in the nucleus could be the due to the detection limitations of IHC. However, given the fact that this type of staining pattern has previously been observed in IHC,[15,29–31] it would be very unlikely that in the current study this relates to atherosclerosis pathology.

Conclusion

Our findings reveal the dynamics in the numbers of nuclei that display the H3K27Me3 mark during disease progression. The reduction in the number of nuclei that display the H3K27Me3 could reflect a phenotype switching of vSMCs. Due to sensitivity limitations of the current study, these results need to be confirmed by more quantitative methods.

Because epigenetic processes are involved in the transcriptional regulation of many, if not all genes, it remains to be elucidated which specific genes whose products contribute to atherosclerosis formation are actively controlled by H3K27Me3 in these different vSMCs (see Wierda *et al.*[2] and references therein). With many environmental risk factors contributing the atherosclerosis development, epigenetic phenomena may hold the key to understanding the onset and progression of this disease. Furthermore, these enzymes may also prove to be effective targets for clinical management of the disease, given the wide availability of Small Molecule Inhibitors that interfere in the activities of the enzymes that modify histones by acetylation or by methylation.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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3

A role for KMT1C in monocyte to dendritic cell differentiation

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3

Abstract

Monocytes play a key role in immune system function. Chromatin remodeling is crucial for various differentiation and gene regulation processes and is rather well studied in T cells. However, for monocytes not much is known regarding how the epigenetic machinery influences the differentiation into various effector cell types. In the work presented here, we explore the epigenetic underpinnings of monocyte differentiation. By transcriptional profiling we show that transcription of lysine methyltransferases (KMTs) and in particular KMT1C is markedly up regulated after differentiation of monocytes into immature dendritic cells (iDCs). Specifically inhibiting KMT1C function, using the small-molecule inhibitor BIX-01294, changes the transcription levels of the DC marker DC-SIGN, but does not affect surface protein expression. Blocking global KMT activity, using DZNep, does influence monocyte differentiation into iDCs, indicated by a loss of DC-SIGN surface expression. When BIX-01294 and DZNep treatment was combined DC-SIGN expression was almost lost completely. This work shows that the activities of KMTs are required for successful differentiation of monocyte-derived dendritic cells. Furthermore it shows the importance of KMT inhibitors in the field of epigenetic immune therapy, which is still much focused around HDAC inhibitors.

Introduction

Monocytes are key players in the functioning of our immune system. They are the precursor cells that upon differentiation form dendritic cells (DCs) and macrophages.[1,2] In diseases such as atherosclerosis and multiple sclerosis, monocyte dysfunction contributes to a great extend to disease initiation and progression.[3] In atherosclerosis particularly monocytes navigate to sites of endothelial damage, adhere to the vessel wall and transmigrate over the endothelial cell layer to infiltrate the underlying tissue.[4] When monocyte entry of the subendothelium is inhibited, by blocking chemokines or their receptors, atherogenesis is retarded or prevented in mouse models.[5]

Differentiation of monocytes into macrophages is a critical step in the pathogenesis of atherosclerosis. Upon transendothelial migration, monocytes differentiate into functionally defined subsets: the classically activated or M1 macrophages or the alternatively activated or M2 macrophages. Classically activated macrophages, a profile induced by Th1 cytokines, mainly display an inflammatory phenotype and produce pro-inflammatory cytokines such as IL-6, IL-12 and TNF-α. In contrast, alternatively activated macrophages are induced by Th2 cytokines and display a more atheroprotective phenotype, exemplified by the secretion of e.g. IL-10.[6] In addition, monocytes may also differentiate into DCs, depending on the stimulus.[7–9]

Dendritic cells, once activated, play an important role in the pathogenesis of atherosclerosis and are particularly harmful in destabilizing the atherosclerotic plaque.[6] Studies of DCs in patients indicate that aberrant DC activation or functions are associated with different autoimmune diseases including rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus, psoriasis, and inflammatory bowel disease (e.g. Crohn's disease and ulcerative colitis).[10]

How epigenetic regulation potentially influences various pathological conditions has been subject of several studies in recent years.[11,12] Epigenetic regulation of gene expression is thought to regulate lineage-specific gene expression in a manner that is stable across cell divisions. In humans, the chromatin remodelling involved in T cell differentiation has been rather well studied.[13–16] The epigenetic processes underlying differentiation of monocytes into dendritic cells and into macrophages, however, have not yet been studied in much detail. The work published on epigenetic regulation involving monocytes focuses on the epigenetic regulation of single genes, including following the response to interferon stimulation.[17–19] In addition to epigenetic regulation at a single gene level, some studies have been published

on genome-wide distribution of epigenetic markers, in particular H3K4me3 and H3K27me3.[20,21] That epigenetic processes indeed play an important role also in monocyte differentiation is also illustrated by the observation that inhibition of HDAC activity by the HDAC inhibitor (HDACi) butyrate results in inhibition of functional differentiation of human monocyte-derived dendritic cells.[22] While all previous work has mainly focused on the activity of epigenetic regulatory enzymes, in this study we present for the first time the expression characteristics of the genes encoding epigenetic regulatory enzymes. The transcription characteristics of these genes were measured during monocyte differentiation into various effector cells. By using specific inhibitors, an important functional role for lysine methyltransferases (KMTs) and in particular KMT1C in monocyte differentiation into dendritic cells is revealed.

Materials and Methods

Cell isolation and culturing

For the initial screening of transcript levels of epigenetic modifiers, peripheral blood was obtained by venipuncture from three individuals undergoing phlebotomy at Sanquin (Amsterdam, the Netherlands). For the validation of *KMT1C* transcript levels peripheral blood of institutional volunteers was drawn by venipuncture and collected into sodium citrate tubes (Greiner). For both screening and validation experiments, peripheral mononuclear cells (PBMCs) were then isolated by density gradient centrifugation using Ficoll Isopaque plus (GE Healthcare). Written informed consent was obtained from all participants. The study protocol conforms to the Declaration of Helsinki and was approved by the ethics committees of the institutions. Five parts of blood were diluted with one part of buffer consisting of phosphate buffered saline (PBS; LUMC Pharmacy) + 10% v/v GPO (Sanquin) + 10% v/v Sodium Citrate (LUMC Pharmacy). PBMC fraction was recovered and washed three times in buffer. Monocytes were isolated from the PBMC fraction by magnetic separation with anti-CD14 magnetic beads (MACS; Milteny Biotech).

Between 0.25 and 1.0×10^6 freshly isolated monocytes were seeded in 1 mL in a 24-wells plate. Cells were cultured in RPMI-1640 medium (Gibco, Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (FCS; PAA), 100 IU/mL streptomycin, 100 IU/mL penicillin (both Lonza) and 2 mM L-glutamine (Gibco). Cytokines were added to the medium to obtain DCs (1000 U/mL GM-CSF, 500 U/mL IL-4), M1 (50 U/mL GM-CSF) and M2 (50 ng/mL M-CSF) as previously described

by Verreck et al.[23] When indicated, cells were cultured in presence of lysine methyltransferase inhibitors (KMTi's): 5 μM 3-Deazaneplanocin A (DZNep, kind gift of V.E. Marquez), 5 μM BIX-01294 (Sigma Aldrich) or a combination of BIX-01294 and DZNep (both in final concentration of 5 μM). The used concentrations of inhibitors are based on the maximum tolerated concentration, allowing for at least 80% viable cells (assessed by eosin staining), when cells were harvested.

After 5 days of culture, fresh medium and cytokines (and inhibitors) were added to the cells and the cells were cultured for an additional 2 days. To obtain activated macrophages or maturated dendritic cells (mDCs), the fresh medium was supplemented with 100 ng/mL (final concentration) LPS at day 5 of culture and cells were cultured for an additional 2 days.

RNA isolation and cDNA synthesis

Either directly after isolation, after 24 hours or 7 days of culture, cells were scraped of the culture plate and centrifuged for 5 min at 500×g. Thereafter the pellet was lysed in RNAbee (Tel-Test) according to manufacturer's instructions. Lysates were stored at 80°C until further processing. Total RNA was extracted according to manufacturer's instructions.

From 1 μg total RNA, cDNA was synthesized using SuperScript III (Invitrogen), with random hexamers (Promega), at 50°C for 1 hour according to manufacturer's instructions.

qPCR

Quantitative PCR was performed on a Bio-Rad iCycler 5 with SYBR Green supermix (Bio-Rad) and the primers shown in supplementary table 3–S1. For each reaction, 125 ng of cDNA and 5 pmol of forward and reverse primer were used in a total volume of 25μL. The thermal profile for the PCR reactions was 3 min 95°C (enzyme activation) followed by [15s 95°C; 30s T_{ann}; 30s 72°C] for 50 cycles. All primers were designed for 60°C annealing temperature. For some primers however, optimal performance was achieved at a higher annealing temperature. The used annealing temperatures are shown in supplementary table 3–S1. Primers were designed to pick up all splice variants of a given gene, with the use of NCBI Primer design tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). All primers were tested beforehand to produce only a single amplicon and for a PCR efficiency between 90% and 110%. Amplicons were sequenced to confirm the identity of the

target sequence. Amplicon length varies between the primer-pairs from 114bp to 246bp. All PCR reactions were performed in duplo. For the screening experiment, transcription levels were normalized against RNA polymerase II (*RPII*) transcript levels. To minimize normalization-induced error, the transcription levels in the validation experiment and BIX-01294 treatment experiment where normalized against the geometric mean Cq-value of *RPII*, Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), ß-glucuronidase (*GUS*), and Hypoxanthine-guanine phosphoribosyltransferase (*HPRT*).

Western Blotting

Monocytes were isolated from peripheral blood and *in vitro* differentiated to immature Dendritic Cells (iDCs), with and without BIX-01294 present in the medium, as described on page 73 and harvested after 5 days of culture. From freshly isolated monocytes and iDCs, two independent histone lysates were prepared by acid extraction according to Shechter et al.[24] Lysates were separated by electrophoresis in a 15% Sodium Dodecyl Sulfate (SDS)-Polyacrylamide gel and transferred overnight to a PVDF membrane at 10V at 4°C. This membrane was blocked with 2% w/v Elk (dried skimmed milk; Campina) in PBST buffer consisting of PBS with 1% v/v Tween-20 (Sigma-Aldrich) and probed subsequently with anti-Me2K9H3 antibodies (1:5000, Abcam) and anti-mouse-HRP (1:1000, Dako). Antibodies where diluted in 0.2% w/v Elk-PBST. To correct for the concentration difference in the loading the same blot is stripped at 50°C using a buffer consisting of 2% w/v SDS (Sigma Aldrich), 62.5 mM Tris-HCl (Sigma Aldrich) pH6.8 and 0.8% v/v ß-mercaptoethanol (Sigma Aldrich). After stripping, the blot was rinsed with tap water for 30 min and washed extensively with PBST. Thereafter it was probed again with total-H3 antibody (1:10000, Abcam) followed by anti-mouse HRP (1:1000, Dako).

Separated proteins were visualized using ECL (GE Healthcare) and recorded using radiographic film (Fuji). The films where scanned and multiple exposures of each independent experiment were densitometrically analyzed using the ImageJ software.[25]

Flow Cytometry

Monocytes were isolated from peripheral blood and differentiated to iDCs *in vitro* in the presence of BIX-01294, DZNep, a combination of BIX-01294 and DZNep, or without inhibitor as described on page 73. After 7 days of culture the iDCs

were harvested by scraping of the dish and washed twice in FACS buffer: PBS, supplemented with 1% FCS (PAA) and 0.1% Sodium Azide (LUMC Pharmacy). The cells were kept on ice during the rest of the procedure. Cells were stained after dilution of the antibody in FACS buffer for 45 min. with DC-Sign (Rat Anti-Human CD209 APC; eBioscience; clone eB-h209; dilution 1:80) or the appropriate isotype control (Rat IgG2a, κ Isotype Control APC; eBioscience; clone eBR2a; dilution 1:80). Cells were washed twice in FACS buffer and taken up in 1% paraformaldehyde. Hereafter the cells were kept at 4°C until data acquisition. Flow cytometry data acquisition was performed on a FACSCalibur flow cytometer (Becton Dickinson) using Cell Quest software. Data was analyzed using the FlowJo software package. Live cells were selected based on forward and side scatter properties and analyzed for CD-209 expression.

Statistics

The relative expression of transcripts is represented as mean ± SEM with propagated errors. Initial screening experiments where analysed with a linear model (ANOVA). First, we applied a variance-stabilizing (cube-root) transformation to the expression values. Next, we tested for expression differences between cell types among specific groups of genes (F-test). To correct for multiple testing, we used the inheritance procedure.[26] Briefly, this method is applicable to situations where the hypotheses that are to be tested can be structured in a tree. Starting from the root of the tree (all genes), we proceed to test ever-smaller subsets of genes, until we arrive at the leaves of the tree (single genes). The inheritance procedure, guarantees that the family-wise error rate across all tested gene sets does not exceed 5%. The analysis was performed in the statistical software R (R, http://www.r-project.org). Sequential tests were performed following the tree-model depicted in figure 3–2B. Statistical testing of the validation study was performed using the Graphpad Prism software by means of a Mann–Whitney U test. Differences were considered to be significant if $p < 0.05$ after multiple testing correction.

Results

Transcription levels of epigenetic modifiers

To assess the role of epigenetic processes in monocyte differentiation, we studied the expression profiles of the genes encoding epigenetic regulatory enzymes during monocyte differentiation. Monocytes were isolated from peripheral blood of

three volunteers and were cultured for 24-hours in medium alone or for a total of 7 days in the presence of stimuli to induce differentiation into type 1 macrophages (M1) and type 2 macrophages (M2) or immature dendritic cells (iDCs). To monitor the effect of activation by LPS, after 5 days part of the cell cultures were activated for an additional 2 days by addition of LPS. Quantitative PCR was performed to measure the transcript levels of 51 genes encoding epigenetic regulatory enzymes after extraction of total RNA and cDNA synthesis. These genes represent the different classes of epigenetic proteins i.e. DNA methyltransferases (DNMTs), lysine acetyltransferases (KATs), histone deacetylases (HDACs) and sirtuins, lysine methyltransferases (KMTs) and lysine demethylases (KDMs). Additionally, we studied the expression profile of members of the two Polycomb Repressive Complexes: PRC2 (involved in initiation of gene repression) and PRC1 (involved in maintenance of repression). The relative transcription levels were normalized against RNA polymerase (*RPII*) transcription. Relative transcription levels are shown in figure 3–1 and supplementary figure 3–S1.

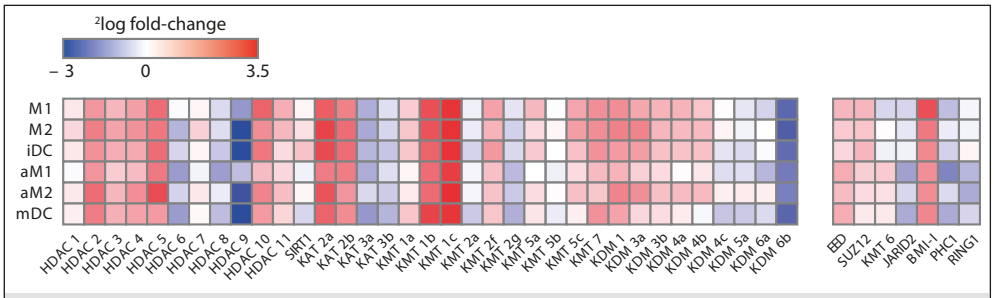

Figure 3–1. Heatmap of transcription levels of epigenetic modifying enzymes in M1, M2 iDC and activated M1, M2, mDC. Monocytes were isolated from PBMCs of three independent donors and differentiated to the depicted cell types in-vitro. All transcription levels were normalized to *RPII* transcription levels. Data is shown as ^{2}log fold-change relative to monocytes cultured for 24h. Polycomb components are shown separately, as these components were also analysedd separately. Data was visualized using GENE-E (http://www.broadinstitute.org/cancer/software/GENE-E/).

Differential deployment of epigenetic modifiers

To aid in the analysis of transcript levels, the analyzed genes were divided into several (sub-)groups based on their biological properties. In a first division, genes were classified according to global substrate specificity (i.e. histone acetylation, histone methylation or DNA methylation). Within the acetylation and methylation groups, genes were then further subdivided into writing or erasing post-translational histone modifications (i.e. HDACs vs. KATs and KMTs vs. KDMs). Finally, the HDACs were further subdivided into the common HDAC classes (i.e. HDAC class I,

II and IV; based on structural properties), and sirtuins of which only *SIRT1* was investigated.[27] A schematic representation of these divisions is shown in figure 3–2A. Genes who are a member of one of the PRCs were analysed separately. The lysine methyltransferase KMT6 is the catalytic subunit of the PRC2 complex (see Figure 3–2A) and is therefore not analyzed together with other KMTs.

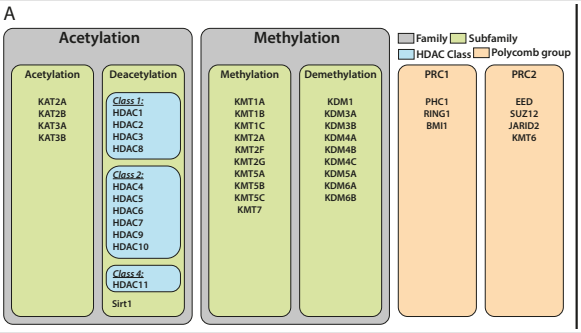

Figure 3–2. (A) Classification of epigenetic modifying enzymes into their respective classes used for statistical testing. (B) All classes showing significant difference in transcription levels ($p<0.05$ after multiple-testing correction) after differentiation are shown in black. The data were tested for expression differences between cell types among specific groups of genes (F-test). Correction for multiple comparisons was done using the inheritance procedure for tree-structured hypotheses.[26]

Following differentiation, the transcription levels of the genes encoding the enzymes involved in histone methylation as well as histone acetylation were significantly altered in M1, M2 and iDC when compared to the expression levels of undifferentiated monocytes that were cultured in medium alone. Transcription levels of PcG genes however were not altered by the differentiation stimulus (Figure 3–2B). Within the group of (de)methylating enzymes both the expression of KMTs and KDMs was significantly altered ($p = 1.3\times10^{-6}$ for KMTs and $p = 1.7\times10^{-5}$ for KDMs). Within (de)acetylating enzymes, the expression of HDACs was significantly altered ($p = 0.015$), but notably not the transcription levels of KATs. (Figure 3–2B) The alteration of transcription of KATs and HDACs could not be pointed to a specific enzyme as none of the individual transcription levels was significantly altered. Although *HDAC9* transcription was decreased dramatically, this decrease did not reach statistical significance (Figure 3–3; $p = 0.08$ by ANOVA, after correction for multiple testing). Within the group of KMTs, *KMT1C* was significantly higher expressed in differentiated monocytes then in monocytes cultured for 24h without cytokines (Figure 3–2B & Figure 3–3; $p = 0.002$ by ANOVA, after correction for multiple testing). Maturation or activation of the differentiated cells by LPS did not have an impact on transcription levels of all the genes tested.

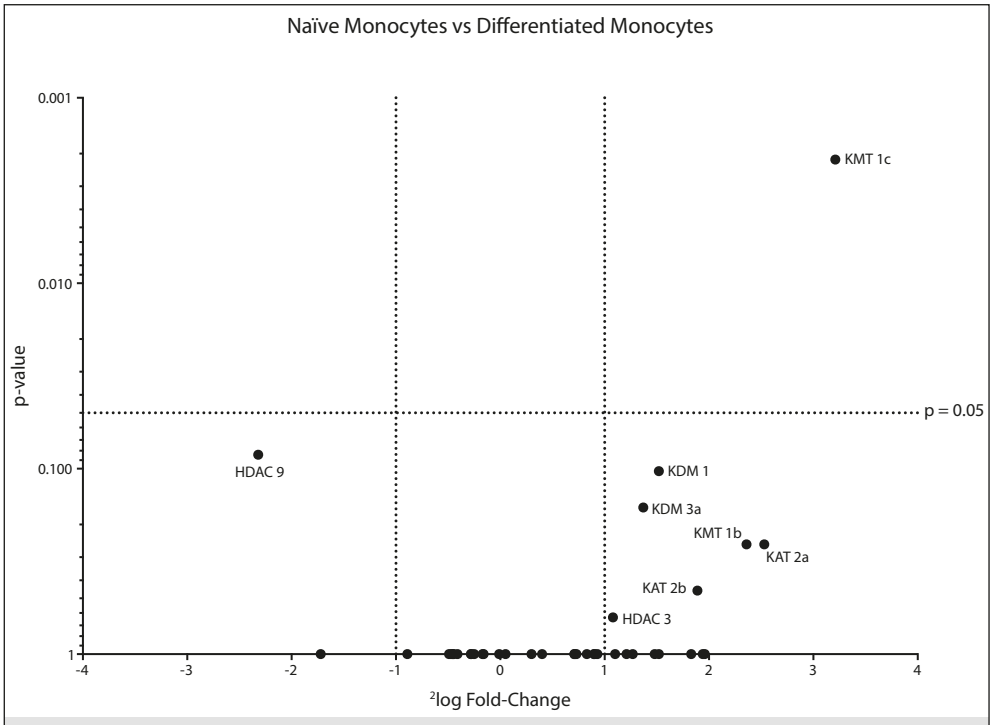

Figure 3–3. Volcano-plot showing transcript level fold-change (as shown in figure 3–1) and their respective p-values. Transcript levels of naïve monocytes were compared to monocytes after differentiation. Data was analysed using a linear model (ANOVA) and correction for multiple comparisons was done using the inheritance procedure for tree-structured hypotheses.[26]

Validation of KMT1C transcription levels

Given the important role of dendritic cells in various diseases,[10] this cell type was used for further validation of the findings above. An additional, unrelated, five healthy individuals were used in a validation experiment. Furthermore, as 24-hours of culture may already induce differentiation of monocytes, we compared immature DCs (iDCs) after 7 days of differentiation to freshly isolated monocytes instead of monocytes that had been cultured for 24 hours.

The transcription levels of *KMT1C* in this validation experiment are shown in figure 3–4. A significant increase of *KMT1C* transcription ($p = 0.0159$; Mann-Whitney U test; M0 median = 24.52, range: 14.42–45.27; iDC median = 49.77, range: 41.24–73.43) was observed in iDCs compared to freshly isolated monocytes. This increase in transcription in iDC is comparable to the increase observed earlier when the transcription profiles were compared to those of 24h cultured monocytes.

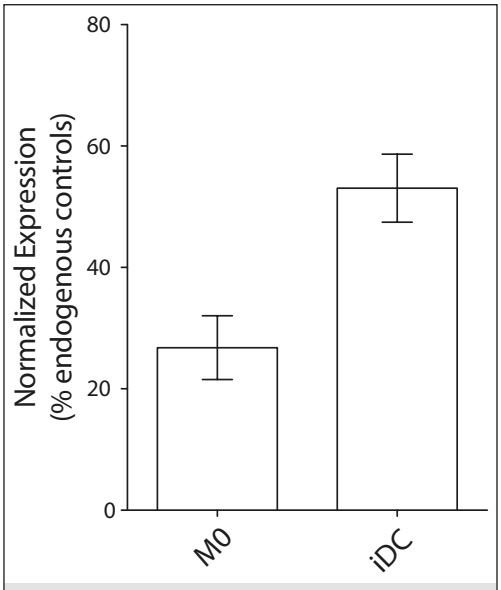

Figure 3–4. Transcription levels of KMT1C in freshly isolated monocytes and iDCs after 7 days of differentiation. There results presented here were obtained in a validation cohort (n=5) to verify the data depicted in figure 3–1 and supplementary figure 3–S1. KMT1C transcription levels are significantly increased in the differentiation process (p=0.0159; Mann-Whitney U test; M0 median=24.52, range: 14.42–45.27; iDC median=49.77, range: 41.24–73.43). Expression levels where normalized to the geometric mean Cq-value of 4 endogenous controls (GAPDH, RPII, GUS and HPRT); error bars indicate SEM with propagated error.

Inhibition of KMT1C impairs monocyte differentiation

KMT1C is described to dimethylate lysine 9 in histone H3 (Me2K9H3).[28] Therefore, to establish whether the increase in *KMT1C* transcription levels also results in altered Me2K9H3 levels, global Me2K9H3 levels were determined by western blot analysis. For this purpose, monocytes were differentiated *in vitro* into iDCs for 5 days. Acid lysates were prepared from freshly isolated monocytes and iDCs as described on page 75. The results of the densitrometric analyses of two independent lysates are shown in figure 3–5A. A clear increase of Me2K9H3 levels can be seen in the iDCs compared to the levels of Me2K9H3 in monocytes.

To determine the functional consequences of this increase in Me2K9H3, monocytes were *in vitro* differentiated into iDCs in presence of the KMT1C-specific inhibitor BIX-01294. BIX-01294 was originally identified as a KMT1C inhibitor during a chemical library screen of small molecules and has previously been used in the generation of induced pluripotent stem cells.[29–31] Although BIX-01294 is generally considered as a KMT1C (G9a) specific inhibitor, it can also inhibit the G9a-like protein (KMT1d).[30] The used concentration of inhibitors allowed for at least 80% of viable cells after 5 days of culturing. After 5 days of culture, acid lysates where prepared to check for Me2K9H3 reduction by western blotting. Addition of BIX-01294 to the culture medium abrogated the increase in Me2K9H3 levels in iDC after monocyte differentiation and resulted in similar Me2K9H3 levels as in freshly isolated monocytes (Figure 3–5).

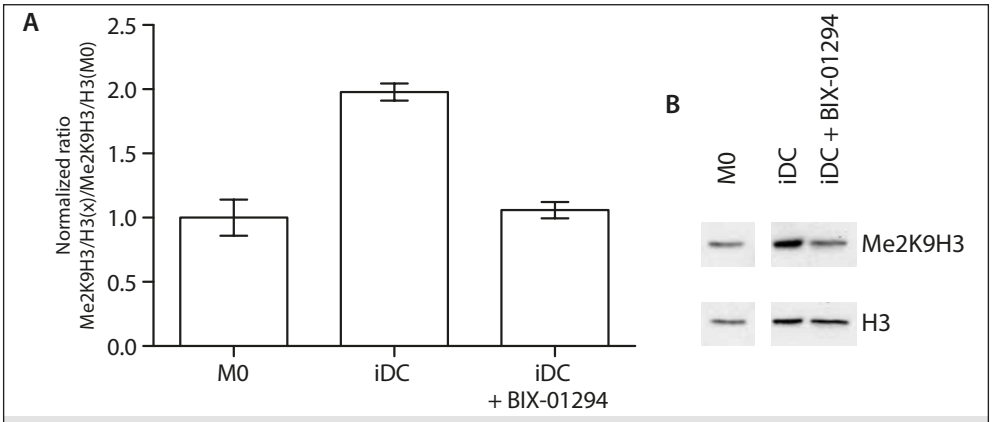

Figure 3–5. (A) Reduction of Me2K9H3 levels measured by densitometric analysis of western blots. Me2K9H3 was normalized to total H3 to correct for any loading differences. The Me2K9H3 ratio was then normalized to the ratio of freshly isolated monocytes. Shown are the results of two independent experiments. Error bars indicate SEM with propagated errors. (B) Representative western blot showing levels of Me2K9H3 and H3.

The transcription levels of the typical DC-marker *DC-SIGN* (*CD209*) were subsequently determined by qPCR analysis. RNA was extracted from freshly isolated monocytes, and iDCs, which were differentiated *in vitro* for 5 days with and without the presence of BIX-01294. Differentiating monocytes into iDCs in the presence of BIX-01294 resulted in a marked reduction of DC-SIGN transcript levels (Figure 3–6). Finally, the surface expression of DC-SIGN of iDCs and iDCs differentiated in presence of BIX-01294 was determined by flow cytometry (Figure 3–7). Although addition of BIX-01294 resulted in a reduction of DC-SIGN transcripts, surface expression of DC-SIGN was not altered by addition of this KMT1C inhibitor.

We also evaluated the impact of inhibition of multiple KMTs considering the fact that transcription of the entire group of KMTs was significantly altered during monocyte differentiation. For the purpose of these investigations we applied the lysine methyltransferase inhibitor (KMTi) 3-Deazaneplanocin A (DZNep) during the differentiation of monocytes into iDC, which is regarded as a general KMTi.[32–34] Monocytes where therefore differentiated into iDCs in presence of DZNep and a combination of DZNep and BIX-01294 and evaluated for cell surface expression of DC-SIGN.

Shown in figure 3–7, DC-SIGN surface expression levels were markedly decreased in the presence of DZNep. By adding both DZNep and BIX-01294 to the cell cultures DC-SIGN expression was almost completely abrogated. Together, this

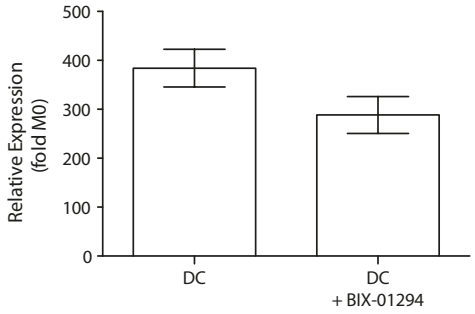

Figure 3–6. Transcription levels of the DC-marker *DC-SIGN*, determined by qPCR. The data is normalized to the geometric mean of 4 endogenous controls (*GAPDH*, *RPII*, *GUS* and *HPRT*) and represent as fold-change relative to monocyte transcription levels. Inhibiting KMT1C activity by addition of BIX-01294 results in lowered *DC-SIGN* transcription levels.

indicates that KMT1C has an important role in monocyte to DC differentiation and seems to act synergistically with the other KMTs in the differentiation of monocyte-derived dendritic cells based on expression of DC-SIGN.

Discussion

The current study shows that transcription of the genes encoding epigenetic modifying enzymes is modulated during monocyte differentiation into macrophages and into dendritic cells. We show that, with the notable exception of KATs and PcG genes, all major classes of the genes encoding epigenetic modifying enzymes are differentially transcribed when comparing monocytes to either macrophages (type 1 and type 2) or to immature dendritic cells. Further stimulation with LPS did not alter these expression patterns. Although the KDM, KMT and HDAC classes as a whole are differentially transcribed during differentiation, on a single gene level, only *KMT1C* was found to have significantly altered transcription levels during differentiation of monocytes into various lineages. Blocking KMT1C activity with BIX-01294 resulted in a reduction of *DC-SIGN* transcripts, while at the same time cell surface expression of DC-SIGN was hardly affected. However, surface expression of DC-SIGN was reduced when applying the general KMTi DZNep. Notably, DC-SIGN surface expression was almost completely lost when both DZNep and BIX-01294 were present in the culture medium. Together, these data indicate that KMT1C plays a role in monocyte to iDC differentiation. Furthermore, the additional studies with DZNep and both DNZep and BIX-01294 show that other KMTs are also involved in monocyte differentiation into iDC and that they might act synergistically with KMT1C in these differentiation processes.

Unfortunately we were unable to correlate the significantly increased levels of *KMT1C* transcripts with protein levels. Of the multitude of commercially available antibodies tested, none showed to be specific for KMT1C. Thus whether the rise in

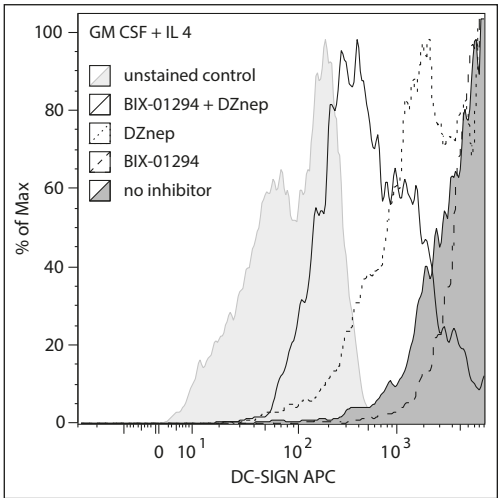

Figure 3–7. Determination of DC-SIGN surface expression on iDCs by flow cytometry. Live cells were selected based on forward and side scatter properties and analyzed for DC-SIGN surface expression. Shown histograms are normalized to peak-cell count (% of Max). Addition of BIX-01294 has little effect on the surface expression of DC-SIGN. However addition of DZNep clearly reduces the surface expression. When DZNep was combined with BIX-01294 surface expression of DC-SIGN was almost completely abrogated.

transcript levels is also associated with a rise in protein levels remains to be established.

Our data, revealing an effect of KMT inhibition on DC-SIGN expression, are in line with previous work, which has established a role for histone methylation at the DC-SIGN promoter.[19] In that study however, only H3K4, H3K9 and H3K20 triple methylation was studied and notably not dimethylation of H3K9.

Using a western blot and densitometry of X-ray film global Me2K9H3 levels were determined after BIX-01294 treatment. X-ray film has limited linearity[35] and the represented data should therefore be regarded as qualitative instead of quantitative. Although global Me2K9H3 levels where lowered after BIX-01294 treatment concomitant with a reduction in DC-SIGN transcript levels, this reduction apparently did not affect levels of DC-SIGN cell surface expression. The reduction in DC-SIGN transcripts also reveals that KMT1C contributes to DC-SIGN gene activation during monocyte differentiation. This co-activator function of KMT1C has been documented previously.[36–38] In addition, it could very well be that in the case of monocyte differentiation, methylation of non-histone proteins involved in DC-SIGN homeostasis are likely to compensate the lowered levels of DC-SIGN transcripts.[39,40] Protein surface expression can therefore be influenced both transcriptionally and post-transcriptionally by KMTs. Furthermore the observed difference in *DC-SIGN* transcription could be due to complex interactions of other regulatory factors involved in *DC-SIGN* transcription instead of directly influencing the *DC-SIGN* promoter. Thus the precise molecular interactions during differentiation remain however to be established.

The results presented here indicate that the transcription of KDM, KMT and HDAC genes are altered during monocyte differentiation. The work performed by Wang et al. is interesting in that respect, as they showed that butyrate functionally inhibited the differentiation of monocytes into DCs.[22] Butyrate is an HDACi with a specificity for class I and IIa (i.e. HDAC-4, -5, -7, -9) HDACs.[41] Both HDAC and KMT transcription levels rise during differentiation and blocking of HDAC or KMT activity seems to impair differentiation of monocytes into DCs. It would therefore also be interesting to see whether KDMi's will influence monocyte differentiation.

After the successful usage of HDACi as anti-epileptics and in the treatment of various cancers,[42–44] it has recently become apparent that epigenetic interference can be useful as well in the field of immunology and for treatment of autoimmune disorders.[45] Recently Immunology and Cell Biology ran a special issue on this emerging field. From the editorial it was evident that research in this field is mainly focused on HDACs and HDACi.[46] The work presented here not only underlines the potential of epigenetic therapy, but also illustrates that we should look beyond the HDAC-horizon.

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Table 3–S1: Primers used for the transcriptional profiling of genes encoding for epigenetic regulatory enzymes and the annealing temperatures used for the primers in qPCR.

Gene	Sequence (5'-3')	T_{ann}	Gene	Sequence (5'-3')	T_{ann}	Gene	Sequence (5'-3')	T_{ann}
HDAC1	F: GGACCAGATTTCAAGCTCCA R: CAGGCAATTCGTTTGTCAGA	61	KDM4A	F: GCTGGGAAGGACAACACAGT R: GGTCTCCTTCCTCTCCATCC	60	KMT5C	F: CGGTGAGAATGACTTCAGCA R: CTCACAGGTGTGGCATTCAC	60
HDAC2	F: CATGGCGTACAGTCAAGGAG R: TTCTTCGGCAGTGGCTTTAT	60	KDM4B	F: GCCCATCATCCTGAAGAAGT R: GCCACTTTGCCGTAGTCAAT	60	KMT6	F: AACACCGGTTGTGGGCTGCAC R: AGCCAGGTAGCACGGGCACT	64
HDAC3	F: TGGCTTCTGCTATGTCAACG R: TCTCTGCCCCGACTTCATAC	60	KDM4C	F: CACCGAAGACATGGACCTCT R: ATCTTGTGGCGAAGAAATGC	66	KMT7	F: TGTCCCCAGCCGCCATGGAT R: TACCCCTCCAGGGTGCTGCC	60
HDAC4	F: TTTCCGTGGAAATTTTGAGC R: AGGCAGTGAGAACTGGTGGT	60	KDM5A	F: CCTCCATTTGCCTGTGAAGT R: CCTTTGCTGGCAACAATCTT	61	EED	F: GCCTGCGGCCAAGAAGCAGA R: TCCTTCCAGGTGCATTTGGCGTG	64
HDAC5	F: CCCAACGAGTCGGCAGATGGGA R: CCCCGTAGCTCCACAGGGCT	60	KDM6A	F: ACCCCAGCTCAGCAGAAGTT R: GGAGGAAAGAAAGCATCACG	60	SUZ12	F: CGTCCAGGCTGACCACGAGC R: GCTGCAAATGAGCTGACAAGCTATGA	60
HDAC6	F: CCGTGTCCTAGCAGACACCT R: GTGATGTCCAGGAGGCCTAA	60	KDM6B	F: CGCCTCATCCCCCTCCTCGT R: TGGGCGCCCCTGGAGCATAA	61	JARID2	F: AACCAGTGCCAAAAAGATGC R: GAACTGGGCTTGTGGTGATT	60
HDAC7	F: AGCGGAGGTGATTCTGAAAA R: TCAGGTTGGGCTCAGAGACT	60	KMT1A	F: AAGAAGATCCGCGAACAGGA R: GGAACTGCTTGAGGATACGC	60	BMI1	F: TTGCCCATTGACAGCGGCGT R: ACAAAGCACACACATCAGGTGGGG	64
HDAC8	F: CGACGGAAATTTGAGCGTAT R: CCAACATCAGACACGTCACC	61	KMT1B	F: CGCTTTGCATCTTTCGAACT R: ACGTGATTCCCTTGTTGTCA	60	PHC1	F: AGCTCAGGGGGCAGCTCTCG R: ATTGTGGCCTGCTGGACGGC	60
HDAC9	F: CAGCAACGAAAGACACTCCA R: CAGAGGCAGTTTTTCGAAGG	60	KMT1C	F: AACATCGATCGCAACATCAC R: TAGAGCTGTAGCCGCACCTT	60	SIRT1	F: ACGCTGGAACAGGTTGCGGG R: AGCGGTTCATCAGCTGGGCA	60
HDAC10	F: ACAGAGGTGTCTGCGGTTGT R: GTGAAAGGTACTCGGGTGGA	60	KMT1D	F: GAGCGCTCTCATCGCCCTCGA R: GGGTCAATTCCGTCCACCAGCAT	60	DNMT1	F: GCTAAGCCTGAACCTTCACC R: TCCTTGATGGACTCATCCGA	65
HDAC11	F: CACCACTGCTCCAGCGACCG R: GCCTGATGGCCTGCTTGGCA	62	KMT2A	F: GAGGAAGACCTCCCACCTTC R: TTTGTACCCCCTTCCTTCCT	60	DNMT3A	F: TGATACGGCTGAGAAGAAAGC R: CTCCTCCACCTTCTGAGACT	61
KAT2A	F: AAGGGGAAGGAGCTGAAGGA R: GCTCAGTCATGGTCTTCAGGT	60	KMT2B	F: TCATCCCCTGAGAGCATCTT R: GGTATCTGGGAAGACTGGGA	60	DNMT3B	F: AAGCCCACTGGGATCGAG R: TCGAGTCTTGTTCTCGTATTTCC	60
KAT2B	F: GGAGCCACTTTAATGGGATG R: CTTTTCCACTCGGTTTCCAG	60	KMT2C	F: AGAGCCAGAAAGAAACCTCG R: TCCACTTCTGCTTCAGCATC	60	RING1	F: CTGAGCCGCCTGCACAACCA R: CCTTCTCCCTCCCCGGGCTC	63
KAT3A	F: CCTATGCTGCTCTCGGACTC R: CGTTCATCAGTGGGTTTGTG	61	KMT2D	F: TTGTGAACCCCGATGGTTTT R: AATGCGGATGGAACCAATGA	60	GAPDH	F: GGTCGGAGTCAACGGATTTG R: ATGAGCCCCAGCCTTCTCCAT	60
KAT3B	F: GGACAAAAAGGCAGTTCCTG R: TTGTGAGCATGCAAAAGGAG	60	KMT2E	F: AGACATCTCATGGGTTTGTGG R: GACGTTCGCCTCTGGTAAAT	60	RPII	F: CAGGAGTGGATCCTGGAGAC R: GGAGCCATCAAAGGAGATGA	60–66
KDM1	F: ATCTGCAGTCCAAAGGATGG R: GCCAACAATCACATCGTCAC	60	KMT2F	F: GGACACAAGCTTCTCCAGCA R: GCTGAAGATGCAGAGAAGTGG	60	GUS	F: GAAAATATGTGGTTGGAGAGCTCATT R: CCGAGTGAAGATCCCCTTTTTA	60
KDM2A	F: TCTTCCGCTACCTCAGCCGCA R: GGTCTTTCAGTCCTGGCAGCCT	61	KMT2G	F: ATCGACGCCACCAAGTGCGG R: GAGTCTGGTGCCGGGGCCTA	60	HPRT	F: GACCAGTCAACAGGGGACAT R: CTTGCGACCTTGACCATCTT	60
KDM3A	F: TGGTGTGTTGCGGGTAGAAGGC R: TCCAAGCAACCTGTCTGTGTGGC	60	KMT5A	F: CGCACCGACGGGGAGAACG R: CATGGCGCTCCGTACTGCGT	61	DC-SIGN	F: TCGAGGATACAAGAGCTTAGCA R: AAGGAGCCCAGCCAAGAG	60
KDM3B	F: CCGGCCACGCAGTGAGACAG R: GCCCCGGGCAGCATGTACCA	60	KMT5B	F: GCACACGGGGAAGGACACCC R: GCTCCTCGAAGGAAAGGCGCTAGT	60			

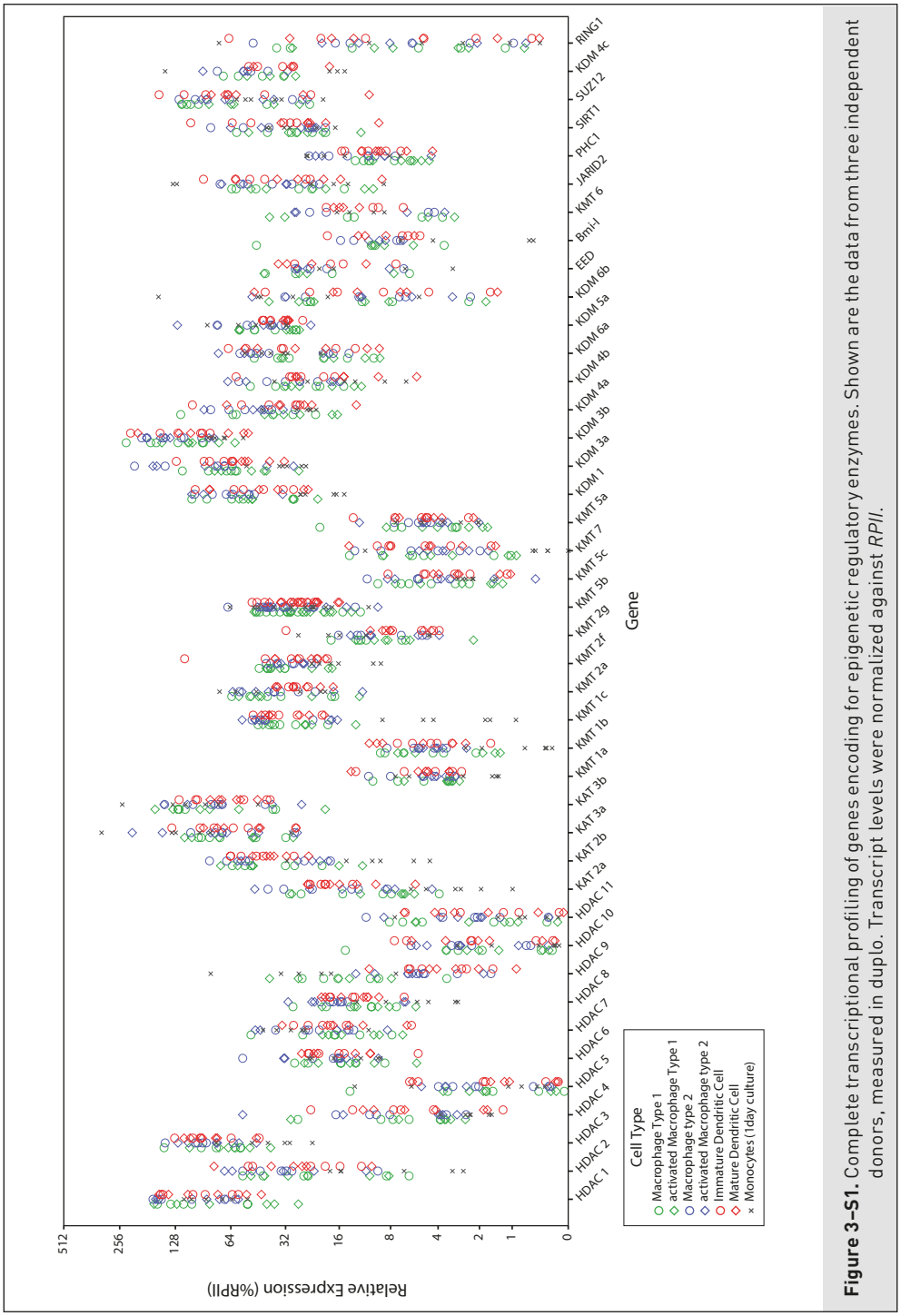

Figure 3–S1. Complete transcriptional profiling of genes encoding for epigenetic regulatory enzymes. Shown are the data from three independent donors, measured in duplo. Transcript levels were normalized against *RPII*.

4

Genetic and Epigenetic Regulation of CCR5 Transcription

Adapted with minor modification from:

Epigenetic control of CCR5 transcript levels in immune cells and modulation by small molecules inhibitors

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4

Abstract

The chemokine receptor CCR5 regulates trafficking of immune cells of the lymphoid and the myeloid lineage (such as monocytes, macrophages and immature dendritic cells) and microglia. Because of this, there is an increasing recognition of the important role of CCR5 in the pathology of (neuro-) inflammatory diseases such as atherosclerosis and multiple sclerosis. Expression of *CCR5* is under the control of a complexly organized promoter region upstream of the gene. The transcription factor cAMP-responsive element binding protein 1 (CREB-1) transactivates the *CCR5* P1 promoter. The cell-specific expression of *CCR5* however is realized by using various epigenetic marks providing a multivalent chromatin state particularly in monocytes. In this chapter the transcriptional regulation of *CCR5* is discussed, with a focus on the epigenetic peculiarities of *CCR5* transcription.

Introduction

The CC chemokine receptor 5 (CCR5) regulates trafficking of lymphoid cells such as memory/effector Th1 lymphocytes, or cells of the myeloid lineage (e.g. monocytes, macrophages, immature dendritic cells) and microglia. As such, CCR5 is implicated in the pathogenesis of various inflammatory diseases such as atherosclerosis and multiple sclerosis.[1–4] Furthermore, CCR5 also functions as a co-receptor for HIV-1.[5–7] Notably, CCR5 expression is markedly upregulated upon T cell activation,[8–11] which allows the activated T cells to migrate towards site(s) of inflammation.[8,12]

Upon encountering a pathogen, antigen presenting cells will present the antigenic peptide to resting naïve T cells which results in the generation and activation of antigen-specific T cells.[13,14] After activation, the T cells migrate to the site of inflammation, guided by chemokine receptors.[15] Similarly, circulating monocytes are also attracted to inflammatory sites by chemokine receptors, where they then can differentiate into e.g. macrophages or microglia.[16–18] Multiple sclerosis and atherosclerosis are greatly characterized by inflammatory lesions, consisting of T cells and macrophages or microglia.[19–21] The chemokine receptor CCR5 has been shown to be implicated in the pathogenesis of both of these diseases.[22–25]

Expression of CCR5 is under the control of a complexly organized promoter region upstream of the gene. The main transcriptional activity of the *CCR5* promoter region is contained within the downstream promoter P1.[9,11,26] A number of transcription factors have been shown to play a role in CCR5 transcriptional regulation (see Wierda et al.[27] and references therein). A graphical representation of the *CCR5* promoter organization and transcription factor binding sites is shown in figure 4–2. We have previously shown however that the transcription factor cAMP responsive element binding protein 1 (CREB-1) is the main transactivating factor for the *CCR5* P1 promoter.[26] However, considering the ubiquitous expression of CREB-1[28], we argued that epigenetic mechanisms are also involved in the cell type-specific regulation of *CCR5* transcription. In line with this notion is the observation that transient promoter-reporter studies in CCR5-deficient Jurkat T leukemia cells revealed that the *CCR5* promoter-reporter was activated upon transfection.[9] This observation infers that Jurkat T leukemia cells contain all the transcription factors required for *CCR5* transcription, and demonstrates that *CCR5* transcription is additionally controlled by epigenetic mechanisms.

Epigenetic mechanisms control the accessibility of DNA for transcription factors and are thought to form the basis for cell-to-cell inheritance of gene expression profiles.[29] Epigenetic mechanisms as such play an essential role in the regulation of gene transcription. Epigenetic modifications include methylation of DNA at CpG residues and posttranslational modifications of histone tails such as acetylation and methylation.[30] Together these modifications form a 'histone code' – like the genetic code – that controls transcription levels of genes.[31] Importantly, modifications to DNA and to histone tails have been shown to be functionally linked.[32]

Well-studied mechanisms that underlie gene repression by histone methylation involve tri-methylation of histone H3 at lysine 9 (H3K9Me3) and at lysine 27 (H3K27Me3), and of histone H4 at lysine 20 (H4K20Me3). These modifications are catalysed respectively by the lysine methyltransferases (KMTases) SUVAR39H1 (hKMT1A), Enhancer of Zeste homolog 2 (EZH2, hKMT6), a subunit of the Polycomb Repressive Complex 2 (PRC-2), and SUV4-20H1/H2 (hKMT5B/C).[33–36] The KMTase hSet1 and the MLL genes (hKMT2A/G) catalyses tri-methylation of K4-H3 (3MeK4H3) and this modification is associated with gene transcription.[36,37]

In this study we show that induction of CCR5 transcription – upon $CD4^+$ T cell activation – correlates with reduced levels of DNA methylation as well as changes in specific histone modifications within the *CCR5* promoter. To establish whether the found epigenetic profiles are T cell specific, we also determined the epigenetic profile in $CD14^+$ monocytes, being of the myeloid instead of the lymphoid lineage. It is shown that the CCR5 chromatin status in primary $CD14^+$ monocytes correlates with the intermediate transcription levels of *CCR5*. Furthermore, the T-lymphoblastic cell lines studied (Jurkat, Molt-4, HSB-2) do not express CCR5 and show a transcriptionally repressive chromatin environment. Moreover, we show that pharmacological interference in these epigenetic silencing mechanisms in the CCR5-deficient T leukemia cell lines results in the induction of CCR5 expression. Together, these data reveal that epigenetic mechanisms play a pivotal role in the control of *CCR5* transcription.

Materials & Methods

Cell culture and activation

Naïve human $CD4^+$ T cells were sorted from freshly isolated PBMC using a FACSAria Flow Cytometer (Becton Dickinson). Sorted cells were directly used for

chromatin immunoprecipitation (ChIP) analysis, RNA extraction and DNA isolation for bisulphite analysis. Naïve CD4+ T cells were also activated *in vitro* as described earlier.[38] In brief, naïve CD4+ T cells were stimulated with 1 µg/mL phytoheamagglutinin (PHA, Remel Europe Ltd.) and 20 U/mL IL-2 in the presence of irradiated allogeneic PBMCs (3000 Rad). After 11 days of culture, cells were restimulated the same way and after 12 days cells were harvested for ChIP analysis and bisulphite sequencing analysis. For RNA-extraction naïve CD4+ T cells were stimulated with anti-CD3 and anti-CD28 for 30 min. Thereafter CD4+ T cells were cultured for 48h in CFU-EC medium (Stemcell technologies). RNA was isolated with the RNA-Bee extraction method (see page 98).

The leukemic T cell lines Jurkat (Clone E6-1; American Type Culture Collection (ATCC)) and MOLT-4 (ATCC) were cultured in RPMI-1640 medium (Gibco, Invitrogen) supplemented with 10% heat-inactivated foetal calf serum (FCS; PAA), 100 IU/mL streptomycin, 100 IU/mL penicillin (both Lonza) and 2 mM L-glutamine (Gibco). The HSB-2 cell line was cultured in Iscove's modified Dulbecco's medium (IMDM; Lonza), supplemented with 10% heat-inactivated FCS, 100 IU/mL streptomycin, 100 IU/ml penicillin, and 2 mM L-glutamine.

To obtain CD14+ monocytes, PBMCs were freshly isolated from the blood of healthy volunteers by density gradient centrifugation using Ficoll-Paque™ PLUS (GE Healthcare). Monocytes where enriched from the PBMC fraction by magnetic separation with CD14 magnetic beads (MACS; Miltenyi Biotec).

Flow cytometry

CCR5 expression on Jurkat, HSB-2, Molt-4 and primary T cells was determined by flow cytometry, using the mouse monoclonal antibody MC-5 (kind gift of Prof. M. Mack, University of Regensburg, Regensburg, Germany) and a PE-conjugated anti-mouse IgG secondary antibody (Becton Dickinson) and the appropriate controls. Data acquisition was performed on a FACSCalibur flow cytometer (Becton Dickinson) using Cell Quest programming. Data was analyzed using the FlowJo software package.

Bisulphite sequencing

Total genomic DNA was isolated from naïve and activated T cells, Jurkat T leukemia cells, and CD14+ monocytes. One µg of genomic DNA was used to bisulphite convert unmethylated CpGs using the EZ DNA Methylation kit (Zymo Research).

CCR5 promoter DNA was then amplified using primer sets for specific CpG containing regions (Table 4–1, Figure 4–2). PCR products were purified using the NucleoSpin Extract II kit (Macherey-Nagel), cloned into pGEM-T easy vector (Promega), and individual clones were sequenced at the Leiden Genome Technology Center. Results of at least 10 individual clones are represented as pie charts for each CpG analyzed. The percentage of methylated clones is depicted in black.

Table 4–1: Primers used for ChIP, bisulphite sequencing and qPCR.

Gene	Promoter region	Region spanning, relative to CDS	Primer sequence, 5'-3'	Application
CCR5	B1	-3509 to -3090[a]	F: TGTTATTGAGTTTTGTTGTAGTATAGATA R: ACCAAACTTAAAACCTATCTTACCC	Bisulphite
	B3	-2625 to -2434[a]	F: TTTAGAAAAAGATGGGAAATTTGTT R: TCCTAAACTTCACATTAACCCTATATC	
	B4/5	-2210 to -1866[a]	F: TTAATAGATTTTGTGTAGTGGGATGAGTA R: CTCATCTCAAAAACTAACTAACAAAC	
		-2277 to -1932[a]	F: TGTGGGCTTTTGACTAGATGA R: TAGGGGAACGGATGTCTCAG	ChIP
		-47 to +188[b]	F: CTGAGACATCCGTTCCCCTA R: GCTCTTCAGCCTTTTGCAGT	qPCR
RPII		+3993 to +4172[c]	F: CAGGAGTGGATCCTGGAGAC R: GGAGCCATCAAAGGAGATGA	qPCR
CREB-1		+276 to +609 (isoform a)[d] +276 to +659 (isoform b)[e]	F: AACCAGCAGAGTGGAGATGCAGCT R: CTGTAGGAAGGCCTCCTTGAAAGA	semi-quantitative PCR
ICER		+150 to +750[f]	F: CAGATCCGAGCTCCTACTGC R: CAACTCGGCTCTCCAGACAT	semi-quantitative PCR

a: Based on accession number NC_000003.10
b: Based on accession number NM_000579.3
c: Based on accession number NM_000937.2
d: Based on accession number NM_004379.3
e: Based on accession number NM_134442.3
f: Based on accession number NM_182717.1

Chromatin immunoprecipitation (ChIP)

ChIP was performed as described earlier.[26] One µg of cross-linked DNA was immunoprecipitated with antibodies (5 µg) directed to specific histone modifications (Table 4–2), or no antibody as background control. Quantitative PCR (qPCR) of the immune-precipitated chromatin was performed using the primer pairs shown in table 4–1.

Table 4–2: Antibodies used for ChIP.

Antibody reactivity	Manufacturer	Catalogue #:
H3Ac	Millipore	06-599
3MeK4H3	Cell Signalling Technology	97510
H3K9Me3	Abcam	ab8898
H4K20Me3	Abcam	ab9053
H3K27Me3	Millipore	07-449
CREB-1	Rockland	100-401-195; [62]
RNA pol II	Santa Cruz	sc899x

Zebularine, DZNep and MS275 treatment

For induction of expression of CCR5, Jurkat, HSB-2 and Molt-4 cells were exposed to 100 µM of Zebularine (V.E. Marquez) for 96 hours followed by an additional treatment with 2 µM of 3-Deazaneplanocin A (DZNep, V.E. Marquez) for 72 hours and 0.5 µM MS275 (Sigma-Aldrich) for 48 hours in IMDM (HSB-2) or RPMI-1640 (Jurkat and Molt-4) with supplements as described above.

RNA isolation and (quantitative) RT-PCR

Total RNA was isolated using the RNA-Bee extraction method (TelTest) from naïve and activated $CD4^+$ T cells, from $CD14^+$ monocytes and from Jurkat, HSB-2 and Molt-4 cells prior to and after treatment with Zebularine, DZNep and MS275. From 1µg of RNA, cDNA was synthesized using 250ng random hexamers (Promega) and Superscript III reverse transcriptase (Invitrogen).

CCR5 and *RNA polymerase II (RPII)* transcripts were quantified on an iCycler IQ system (BioRad Laboratories) using the IQ SYBR Green Supermix (BioRad Laboratories). Relative transcript levels of *CCR5* were calculated with the comparative Ct method (or ΔΔCt method) and related to *RPII* transcript levels. The induced levels of *CCR5*, after treatment of Jurkat, Molt-4 and HSB-2 cells with Zebularine, DZNep and MS275, are also depicted relative to the *CCR5* expression level in *in vitro* activated primary T cells. The primers used in the qPCR reactions are shown in table 4–1.

CREB-1 and *inducible cAMP early repressor* (*ICER*), the inducible isoform of *cAMP-responsive element modulator* (*CREM*) transcripts were analyzed in triplicate by semi-quantitative PCR as previously described.[26] PCR products were separated

by gel electrophoresis on a 1.5% agarose gel, run at 90V for 45min, and visualized by ethidium bromide staining. Densitrometric analysis was performed in ImageJ (U.S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/).

Results

DNA methylation patterns of the CCR5 P1 promoter.

Using flow cytometry we found that only a few naïve primary CD4⁺ T lymphocytes express low levels of CCR5 at the cell surface, whereas CCR5 cell surface expression is markedly upregulated after *in vitro* activation of these cells (Figure 4–1A). The CCR5 cell surface expression pattern of activated CD4⁺ T cells is accompanied by relatively high levels of *CCR5* transcripts (Figure 4–1B). In naïve T cells

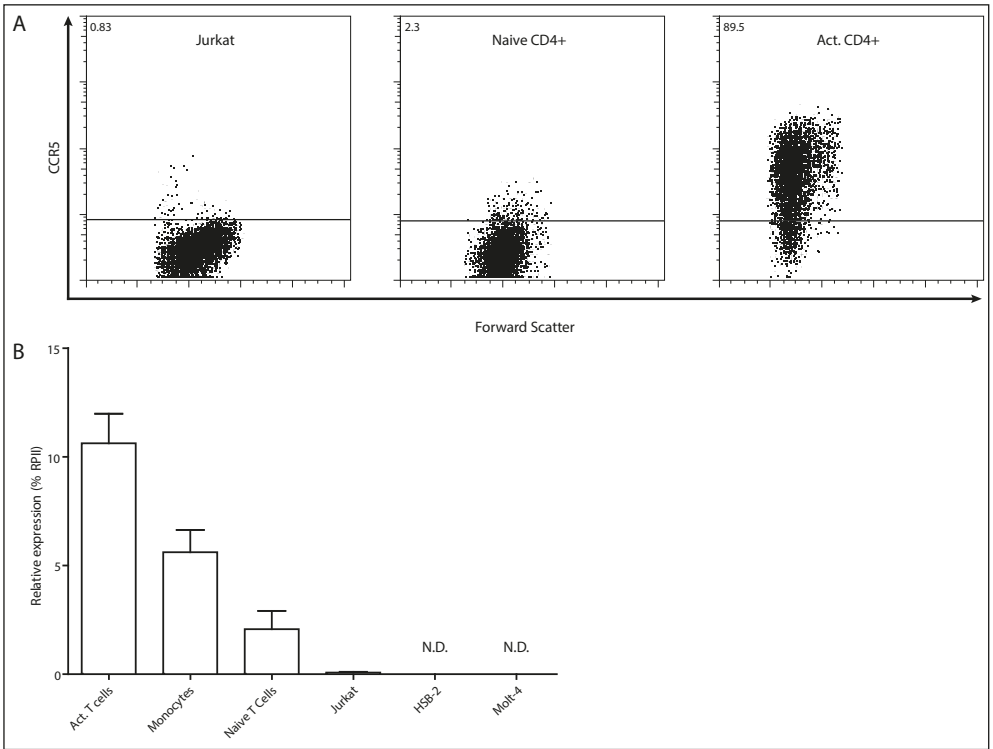

Figure 4–1. (A) Cell surface expression of CCR5 in Jurkat T leukaemia cells, naïve and activated CD4⁺ T cells as determined by flow cytometry. Numbers indicate percentage of CCR5 positive cells. (B) Relative transcript levels of *CCR5* in various cells types. Numbers indicate expression percentage relative to *RNA polymerase II* (*RPII*). N.D. indicates transcript levels were below the detection threshold.

CCR5 transcripts were detected at low levels (Figure 4–1B). Myeloid cells, such as monocytes express CCR5 at low to intermediate levels.[39] When compared with activated and naïve CD4⁺ T cells, CD14⁺ monocytes indeed show intermediate levels of *CCR5* transcripts (Figure 4–1B). In contrast, most established tumour T cell lines completely lack CCR5 surface expression, including the human CD4⁺ leukemic T cell lines Jurkat, Molt-4 and HSB-2 (Figure 4–1A and Figure 4–5A). Furthermore, these leukemic T cell lines show only very low or undetectable CCR5 transcript levels (Figure 4–1B).

Evaluating the role of epigenetic mechanisms in the regulation of *CCR5* expression we first assessed the CpG methylation status of three subregions regions of the *CCR5* downstream promoter P1 (Figure 4–2). The most downstream subregion (B4/5), which is known to be transactivated by CREB-1,[26] appears to be mostly unmethylated and displays only marginal differences in DNA methylation between the various cell types (Figure 4–2). The upstream subregions B1 and B3 display remarkable differences in DNA methylation status. In activated T cells, the CpG residues in these subregions of the P1 promoter display low levels of DNA methylation. In monocytes, which express intermediate levels of CCR5, the promoter subregions B1 and B3 are highly methylated, while the B4/5 region displays low levels of DNA methylation (Figure 4–2). By contrast, in naïve CD4⁺ T cells these subregions are mainly methylated and almost completely methylated in Jurkat T cells. Together, these data reveal that the intermediate, low and lack of *CCR5* transcription levels, in monocytes, unstimulated CD4⁺ T cells and in Jurkat T leukemia cells

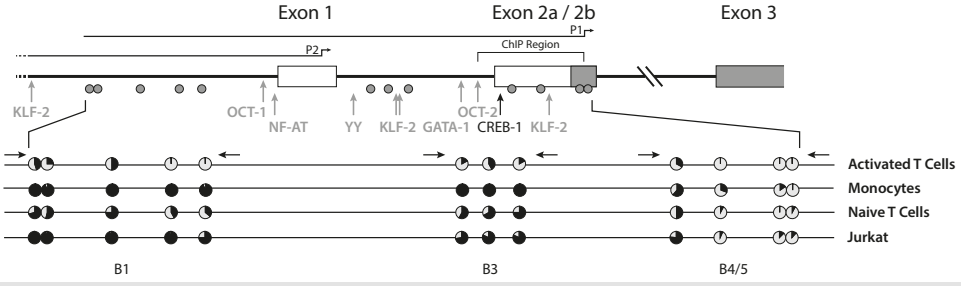

Figure 4–2. Methylation analysis of the *CCR5* promoter of several cell types. Each circle represents a single CpG residue. The percentage of clones methylated at a specific residue is indicated by the black colour. The distance between each circle represents the relative distance between CpG residues on the genomic sequence. Horizontal arrows indicate the relative position of primers used to amplify bisulphite modified DNA. The CREB-1 binding site most likely involved in *CCR5* transactivation is indicated with a vertical arrow.[26] Other transcription factors that have been attributed to *CCR5* transcription regulation are indicated with grey arrows. The region investigated by chromatin immunoprecipitation is annotated as "ChIP region".

respectively, are associated with high levels of DNA methylation in the subregions B1 and B3 of the P1 promoter but not in the B4/5 subregion.

Histone modifications of the CCR5 P1 promoter

Next we determined the association of specific histone acetylation and methylation modifications within chromatin of the *CCR5* P1 promoter by chromatin immunoprecipitation (ChIP) (Figure 4–2 and Figure 4–3A–C). CCR5 expressing, activated, $CD4^+$ T cells display relative high levels of H3Ac (Figure 4–3A). Interestingly, monocytes display H3Ac levels in chromatin of the *CCR5* P1 promoter, which are similar to activated T cells (Figure 4–3A). This is in contrast to the non-CCR5 expressing naïve T cells and Jurkat T cells, which display markedly lower levels of H3Ac in *CCR5* P1 chromatin.

CCR5-expressing activated T cells display relatively high levels of the permissive 3MeK4H3 mark in *CCR5* P1 chromatin. Interestingly, naïve T cells expressing low levels of CCR5 show similar levels of the permissive 3MeK4H3 mark (Figure 4–3A). In contrast, CCR5-deficient Jurkat T cells display low levels of the permissive 3MeK4H3 modification (Figure 4–3A).

The repressive marks H3K9Me3 and H3K27Me3 are only present at very low levels in chromatin of low CCR5-expressing naïve T cells (Figure 4–3B). In contrast, the repressive mark H4K20Me3 is highly enriched at the *CCR5* P1 promoter region

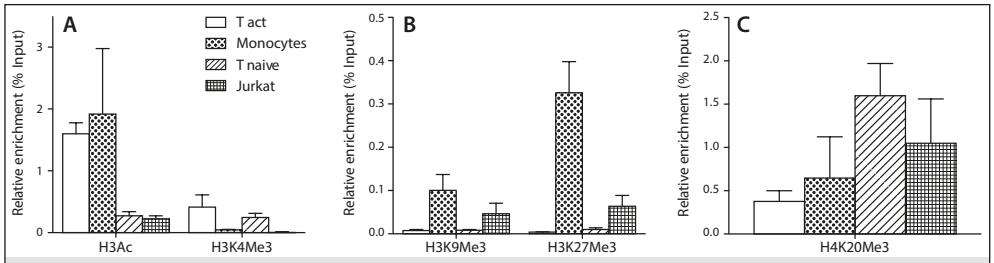

Figure 4–3. Chromatin environment at the *CCR5* promoter as determined by ChIP analysis. CCR5 expressing activated $CD4^+$ T cells clearly show higher levels of transcriptionally permissive chromatin marks H3Ac and H3K4Me3 (A) whereas there is an opposite association with transcriptionally repressive chromatin marks H3K9Me3, H3K27Me3 (B) and H4K20Me3 (C). These non-permissive marks are clearly present in higher amounts in CCR5 non-expressing cells (Jurkat T leukaemia cells, naïve $CD4^+$ T cells) versus expressing activated $CD4^+$ T cells. Naïve T cells and $CD14^+$ monocytes show a poised chromatin state, encompassed by both transcriptionally permissive and non-permissive marks. Whereas naïve T cells show relatively low levels of H3Ac, monocytes have high levels of H3Ac.

of naïve T cells (Figure 4–3C). The presence of both an activating mark (3MeK4H3) and a repressive mark (H4K20Me3) indicates a bivalent, so-called 'poised' state of the *CCR5* promoter chromatin of naïve CD4$^+$ T cells.

Activated CD4$^+$ T cells show a twofold higher *CCR5* transcription level as compared to monocytes. Assessing the chromatin status of CD14$^+$ monocytes, we observe the presence of relative high levels of the repressive marks H3K9Me3 and H3K27Me3 in the monocytic *CCR5* P1 promoter (Figure 4–3B). Conversely, the repressive mark H4K20Me3 is only slightly enriched in monocytes as compared to activated T cells (Figure 4–3C). Furthermore, hardly any of the permissive 3MeK4H3 mark could be detected, yet monocytes show high levels of H3Ac in the *CCR5* promoter (Figure 4–3A). This indicates that also monocytes display a chromatin state in which repressive and permissive histone modification marks co-exist. Compared to naïve CD4$^+$ T cells however the chromatin state of CD14$^+$ monocytes, is markedly different, permitting transcription of *CCR5*.

CCR5-deficient Jurkat T cells show relative high levels of the repressive H3K9Me3 and H3K27Me3 histone marks, when compared with naïve and activated T cells (Figure 4–3B). Like naïve T cells, Jurkat T leukemia cells also show higher levels of the repressive H4K20Me3 modification when compared to activated T cells (Figure 4–3C). The presence of these repressive marks in the absence of activating histone modifications clearly shows a repressive chromatin conformation encompassing the *CCR5* P1 promoter in Jurkat T cells.

Taken together, these data show that there is a differential pattern of chromatin conformation of the *CCR5* P1 promoter region in the different cell populations investigated in this study. Our observations also indicate that the *CCR5* transcription profiles could not be explained by a single epigenetic modification, but rather the sum of modifications appears to determine the level of *CCR5* transcripts in the various cell types investigated.

Re-expression of CCR5 through pharmacologic interference in epigenetic mechanisms in Jurkat, Molt-4 and HSB-2 T cell lines

To show that DNA methylation, and histone acetylation/methylation mechanisms control *CCR5* transcription, we aimed to induce *CCR5* transcription in non–CCR5-expressing cells through pharmacologic interference in the catalytic activities of the various enzymes involved in these epigenetic regulatory processes. Figure 4–4 presents a schematic overview of the working mechanisms of the agents used for

this purpose. Zebularine is a potent inhibitor of DNA-methylation showing much lower toxicity then the widely used inhibitor 5-Aza-dC.[40,41] First recognized as an inhibitor with specificity for the KMTase EZH2, DZNep is now regarded as a more general lysine methyltransferase inhibitor, with a high affinity for the enzymes that triple-methylate K20H4 and K27H3 (Miranda *et al.* (2009),[42] Tan *et al.* (2007)[43] and own observations). Finally, MS275 is a potent inhibitor of histone deacetylase activities (HDACs), with high affinity for the class I HDACs 1 and 3.[44]

Originally we found that inhibition of DNA-methylation by 5-Aza-dC treatment resulted in only a modest and time-dependent induction of CCR5 mRNA expression levels in Jurkat cells (results not shown). However, combining inhibition of DNA and histone methylation by inclusion of DZNep resulted in a clear synergistic induction of CCR5 mRNA expression, whereas inhibition of histone methylation alone was found only marginally effective (results not shown). Additional treatment with the HDAC inhibitor MS275 mainly potentiated the effect obtained by the other inhibitors (results not shown).

We therefore combined all of the above-mentioned inhibitors to induce CCR5 expression in Jurkat, Molt-4 and HSB-2 T leukemia cells and included Zebularine rather than 5-Aza-dC for the aforementioned reasons. After treatment with Zebularine, in combination with DZNep and MS275, 67.7% of Jurkat cells are CCR5

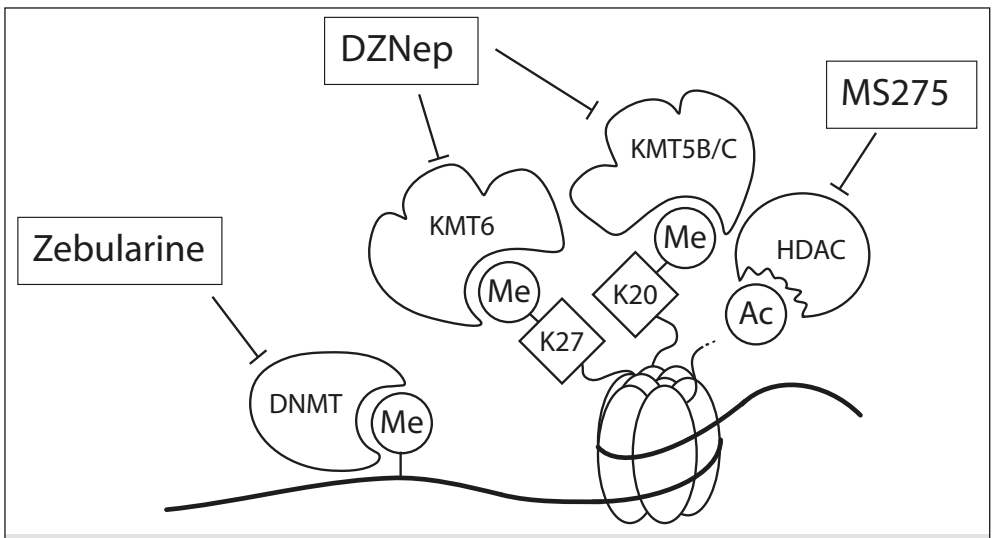

Figure 4–4. Schematic representation of the working mechanism for the pharmacological intervention in *CCR5* transcription. Zebularine inhibits DNMTs, whereas MS275 inhibits HDACs. DZNep is a more general KMTase inhibitor.[42]

positive as determined by flow cytometry (Figure 4–5A). In untreated Jurkat cells, only 0.83% of the cells stain positive for CCR5 (Figure 4–1A). Correspondingly, after treatment the levels of *CCR5* transcripts found in Jurkat T cells increased to 43% of the *CCR5* transcript levels found in activated CD4⁺ T cells (Figure 4–5B). HSB-2 and Molt-4 were more refractory to this combined epigenetic treatment, however still 49.4% and 18.2% of the cells respectively were expressing CCR5 at the cell surface after treatment (Figure 4–5A), whereas transcript levels were 20% and 4.8% relative to activated T cell transcript levels in HSB-2 and Molt-4 respectively (Figure 4–5B).

Next we evaluated the effect of the epigenetic drug treatment on the expression characteristics of CREB-1 and ICER in Jurkat cells by semi-quantitative RT-PCR as we have previously explored.[26] ICER, the inducible cAMP early repressor, which is induced by forskolin, competes with CREB-1 for DNA binding. We and more recently also others have shown that induction of ICER by forskolin treatment indeed reduces CCR5 expression.[26,45] In figure 4–6 it is shown that pharmalogical induction of CCR5 expression did neither result in the induction of CREB-1, nor in a reduction

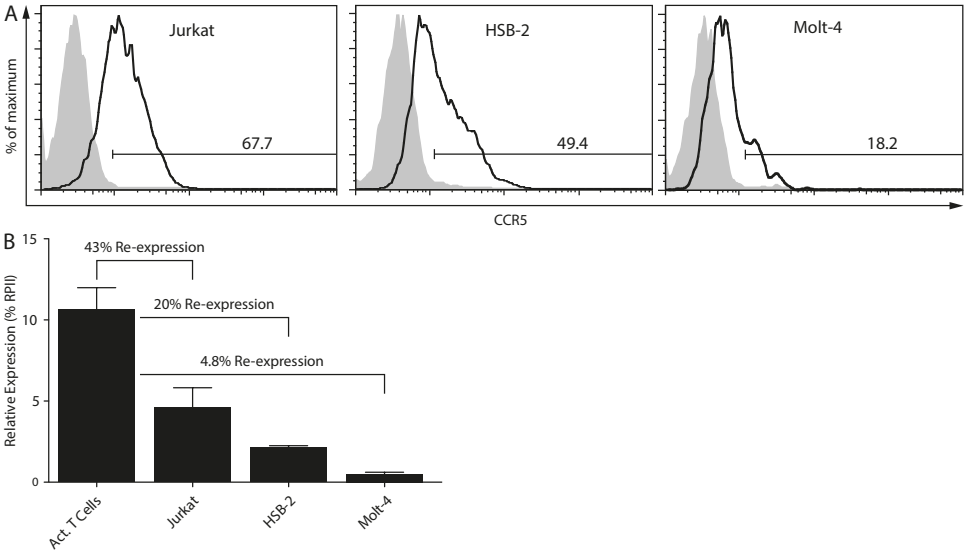

Figure 4–5. (A) Restoration of CCR5 expression in Jurkat, HSB-2 and Molt- 4 T leukaemia cell lines following exposure of cells to Zebularine, DZNep and MS275 determined by flow cytometry. Numbers indicate the percentage of CCR5 positive cells; filled histograms represent the non-treated cells and open histograms the treated cells. (B) Levels of *CCR5* transcripts in treated Jurkat, HSB-2 and Molt-4 T leukaemia cells relative to *CCR5* transcript levels in activated CD4⁺ T cells. The transcript level data of activated T cells are the same as shown in figure 4–1B.

of ICER in Jurkat T cells. Notably, when compared with naïve or activated $CD4^+$ T cells, Jurkat cells do express CREB-1, but hardly any ICER could be detected. In contrast, naïve T cells show low levels of CREB-1, with relatively high levels of ICER. Upon activation, the levels of ICER are reduced while on the other hand CREB-1 levels are induced (Figure 4–6). These observations indicate that in Jurkat T cells induction of CCR5 expression most likely is not resulting from alterations in the interplay of CREB-1 and ICER.

We also investigated whether the pharmalogical induction of CCR5 expression was associated with alterations in the histone acetylation/methylation profile and recruitment of CREB-1 and RNA polymerase II in CCR5 promoter chromatin. As shown in figure 4–7A there is a clear increase in the H3Ac mark (associated with gene expression) after treatment, whereas histone marks associated with gene repression appear to be more resistant to the treatment. Shown in figure 4–7B is that the permissive *CCR5* chromatin structure in activated T cells (Figure 4–3) results in increased recruitment of CREB-1 and RNA polymerase II into CCR5 promoter chromatin when compared with naïve T cells. Similarly, the induction of CCR5 expression after epigenetic treatment of Jurkat cells is also accompanied by an

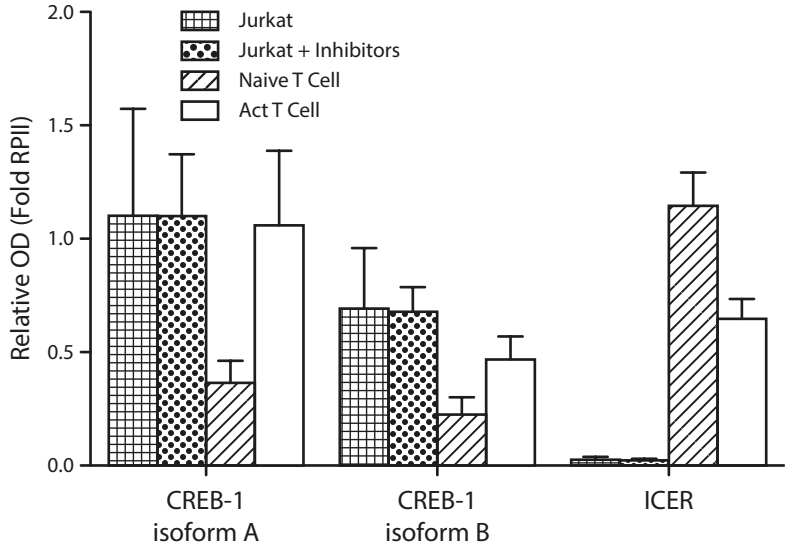

Figure 4–6. Semi-quantitative RT-PCR for CREB-1 isoforms and ICER were performed in triplicate. Activated T cells show higher levels of both CREB-1 isoforms, when compared to naïve T cells, whereas naïve T cells show higher levels of ICER then activated T cells. Jurkat T leukaemia cells show virtually undetectable levels of ICER. Treatment of Jurkat cells with Zebularine, DZNep and MS275 does not influence CREB-1 or ICER transcript levels.

increase in the recruitment of CREB-1 and RNA polymerase II into CCR5 promoter chromatin (Figure 4–7B). Together, the pharmalogical inhibition of the activities of the various epigenetic enzymes that account for the repressive chromatin state of CCR5 in Jurkat T cells has resulted in a shift into a more open chromatin structure.

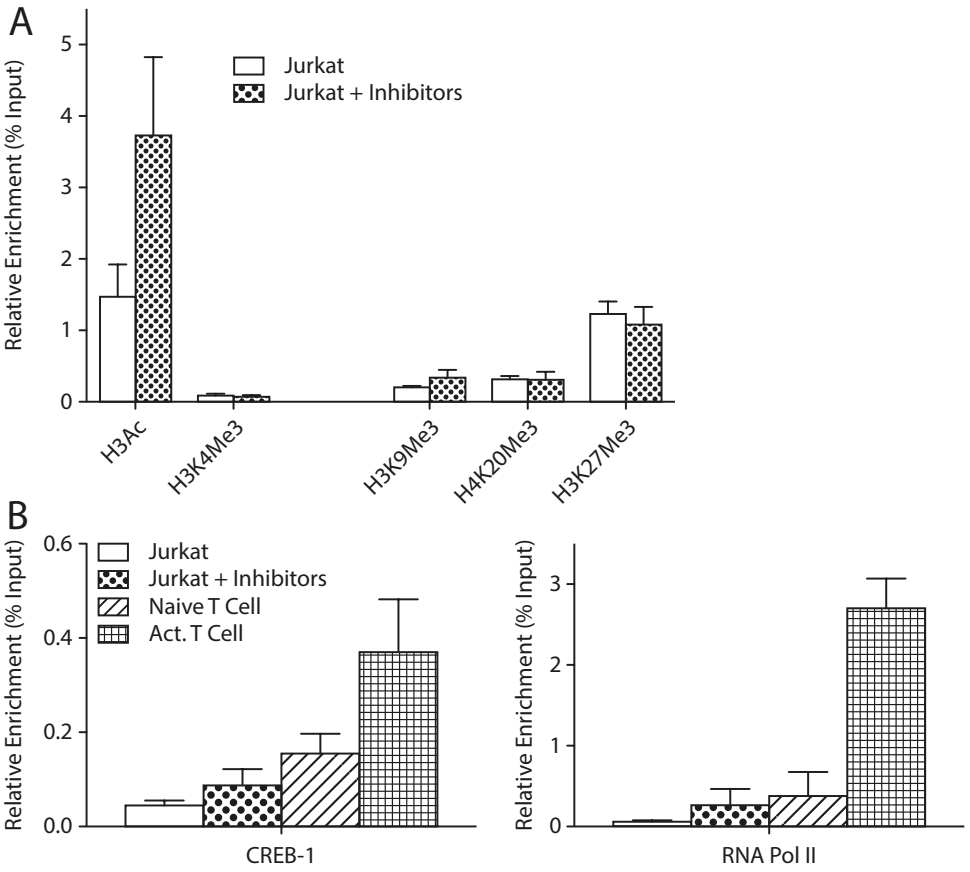

Figure 4–7. (A) ChIP analysis of histone modification at the *CCR5* promoter in Jurkat cells, after treatment with Zebularine, DZNep and MS275. The treatment of Jurkat cells results in an increase of H3Ac at the *CCR5* promoter. Repressive marks at the *CCR5* chromatin are not influenced much by the treatment, although a minor decrease in H3K27Me3 can be noted. (B) ChIP analysis of the *CCR5* promoter for CREB-1 and RNA polymerase II after treatment with SMIs in Jurkat, compared to both naïve and activated T cells. Treatment of Jurkat cells with Zebularine, DZNep and MS275 slightly increases CREB-1 in chromatin of the *CCR5* promoter. In both naïve and activated T cells higher levels of chromatin-associated CREB-1 can be found. Compared to naïve T cells, there is an increase of CREB-1 in activated T cells. Treatment of Jurkat cells with SMIs increases RNA polymerase II recruitment to the *CCR5* promoter to levels similar of naïve T cells. In comparison to activated T cells, the levels of RNA polymerase II in the *CCR5* promoter region of treated Jurkat cells are modest.

This is accompanied by an increase in promoter association of the transcription factor CREB-1 and recruitment of RNA polymerase II.

Discussion

This study reveals that epigenetic mechanisms involving DNA methylation, histone acetylation and methylation modifications all contribute to the transcriptional regulation of CCR5 expression. In CCR5-deficient T leukemia cells we show that the promoter region is mainly characterized by repressive histone marks in the presence of methylated DNA. In CCR5-expressing activated T cells this region is mainly associated with activating histone marks and low levels of DNA methylation. Interestingly, the B4/5 region in the CCR5 promoter, which was previously attributed to CREB–1-mediated transactivation is mostly unmethylated both in Jurkat and activated T cells.

Intermediate or low CCR5-expressing monocytes and naïve T cells respectively are characterized by both repressive histone methylation marks and permissive histone acetylation marks. In naïve T cells an intermediate level of DNA methylation accompanies these histone modifications. However, in monocytes the level of DNA methylation is markedly higher as compared to naïve T cells, with the B4/5 region in a mostly unmethylated state in both cell types. Together, the cell types investigated here show that the B4/5 region is mostly unmethylated, irrespective of CCR5 transcription. This suggests that the B1 and B3 regions could contribute to the transcriptional regulation of CCR5 as has been argued previously.[26,46,47]

Notably, monocytes and naïve $CD4^+$ T cells represent a poised state recognized by the presence of both repressive and permissive histone marks. Considering the various histone triple-methylation modifications investigated, we conclude that acetylation of histone H3 is essential for CCR5 expression as is illustrated in naïve T cells and in monocytes. The dominant role of histone modifications is further underscored by the fact that monocytes show high levels of DNA methylation. Although DNA methylation is usually interpreted as a repressive chromatin mark, this study as well as some recent other studies show that DNA methylation in the absence of repressive histone marks permits active gene transcription.[48–54] This is also in line with previous studies showing that the presence of the H3K27Me3 histone modification correlated with lack of transcription despite absence of DNA methylation.[50,55,56] Interestingly, the monocyte population presented in this study shows transcription in presence of DNA methylation, H3K27Me3, H3K9Me3 and H3Ac, but notably low levels of H4K20Me3. This underscores – as has been previously

noted[48] – that not all epigenetic histone marks contribute equally to a specific chromatin status. Rather, the sum of epigenetic modifications, or "epigenetic profile", is more important than individual modifications to allow gene transcription.

The role of epigenetic regulatory mechanisms in the control of CCR5 transcription is also underscored by the pharmacological interference in the identified components of epigenetic regulation. Since the epigenetic modifications were observed in both DNA and in histones encompassing the CCR5 promoter, we combined the various inhibitors to induce re-expression. This intervention resulted in the re-expression of CCR5 in Jurkat, HSB-2 and Molt-4 T leukemia cells, albeit that the levels of re-expression differ between the cell lines investigated and were never on par with activated T cells. Although the individual epigenetic inhibitors allowed marginal induction of CCR5 transcripts (data not shown), combination of inhibitors induced much higher transcription levels.

Changing the DNA methylation status through pharmacological disruption with Zebularine requires incorporation of Zebularine into the DNA.[40,41] Demethylation through usage of Zebularine thus requires replication of DNA and therefore proliferation of cells. Jurkat, HSB-2 and Molt-4 cell lines show different doubling times. The difference in re-expression levels of CCR5 after combined epigenetic therapy can therefore be explained by this difference in cell doubling times. Furthermore the relative toxicity of MS275 and DZNep may lower the proliferative capacity of the cells, thereby influencing the efficacy of Zebularine treatment. Especially since DNA methylation and histone modifications are intimately linked,[32,57] this may result in a situation where 100% re-expression of the gene of interest might prove to be a challenge. Yet despite these drawbacks, interference in the epigenetic machinery still results in a dramatic rise of CCR5 transcripts in T leukemia cells.

Together, these data strongly indicate that histone acetylation and methylation modification mechanisms contribute to the transcriptional control of CCR5. In addition, we show that chromatin in a bivalent state allows for the fine-tuning of transcription levels, as has been shown before for other genes.[32,58] Moreover, our data suggest that epigenetic deregulation could be one of the mechanisms leading to enhanced CCR5 expression as observed in a variety of inflammatory conditions. Although we demonstrate in this study the re-expression of CCR5, it could be envisioned that the use of lysine acetyltransferase inhibitors (e.g. curcumin or garcinol[59,60]) may have the opposite effect. As such, CCR5-mediated trafficking of lymphoid and myeloid cells is a possible target for pharmacological intervention.

Interference in these deregulated epigenetic processes may therefore be a promising therapy for the treatment of inflammatory diseases.

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Authorship Contributions

RJW, HFK, MCJAvE, AB, JCvL, SC, and SBG performed experiments. RJW and HFK wrote the paper. VEM provided essential reagents. JWJ, PHAQ, and PJvdE critically discussed and reviewed the paper. PJvdE supervised the project.

Conflict-of-interest disclosure

The authors confirm that there are no conflicts of interest.

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The PCAF –2481C/G polymorphism in the upstream promoter region does not modulate constitutive PCAF transcription in monocytes or HUVECs

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Abstract

Recently we described that the C-allele of a polymorphism (rs2948080) located 2481bp upstream of the transcription start-site of the KAT P300/CBP-associated factor (*PCAF*, or *KAT2B*) is associated with a significant reduction in vascular morbidity and mortality. Given the localization of this polymorphism in the *PCAF* promoter, we have investigated whether this polymorphism has an impact on the constitutive transcript levels of *PCAF*. We therefore analysed *PCAF* transcription levels in two cell types relevant for human vascular disease: monocytes and Human Umbilical Cord Endothelial Cells (HUVECs). No significant differences were observed in constitutive *PCAF* transcript levels with regards to the genotype of either the monocytes or HUVECs in this cohort of investigated individuals. Therefore, the exact mechanism by which the –2481G/C SNP protects from vascular morbidity and mortality remains to be elucidated.

Introduction

Epigenetic processes hereditably influence gene expression without modifying the actual genetic code. It is commonly thought to provide an essential linkage between environmental factors and gene expression. Given the impact of environmental factors on atherosclerosis, in recent years the field of epigenetics is intensively investigated and evidence for epigenetic contribution to diseases is slowly accumulating.[1,2] Epigenetics is commonly thought to provide an essential linkage between environmental factors and gene expression.[3] In recent years, many advances have been made in the field of epigenetics and evidence for epigenetic contribution to diseases is slowly accumulating.[1]

A myriad of cellular processes (e.g. DNA methylation and histone modifications) are responsible for epigenetic regulation. 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.[4,5] Modifications of histone tails include (amongst others) acetylation and methylation of lysine residues. Whereas acetylation of histone tails is correlated with gene activation,[6–8] the influence of histone methylation depends on the exact residue methylated and the number of added methyl groups.[9–11] Post-translational modifications made to the histone-tails are reversible. Acetylation at lysine residues in the histone N-terminal tails is established by lysine acetyltransferases (KATs). KATs are counteracted by histone deacetylases (HDACs) and Sirtuins (SIRT). Likewise, histone methylations established by lysine methyltransferases (KMTs), can be counteracted by lysine demethylases (KDMs). In this way these enzymes promote a return to respectively repressive or active chromatin structure.[12–14]

Recently we described that the C-allele of a polymorphism (rs2948080) located 2481bp upstream of the transcription start-site of the KAT P300/CBP-associated factor (*PCAF*, or *KAT2B*) is associated with a significant reduction in vascular morbidity and mortality.[15] Besides that the –2481 SNP was associated with survival, *PCAF* transcription levels were significantly altered in a mouse model of reactive stenosis, providing an indication that *PCAF* in involved in vascular remodelling. In an electrophoretic mobility shift assay, we found differential protein binding to the different alleles. This study underscored the concept that epigenetic processes are also under genetic control. Given the localization of this polymorphism in the *PCAF* promoter, we have investigated whether this polymorphism has an impact on the constitutive transcript levels of *PCAF*. We therefore analysed *PCAF* transcription

levels in two cell types relevant for human vascular disease: monocytes and Human Umbilical Cord Endothelial Cells (HUVECs).

Materials and Methods

Study subjects

Per genotype 5 patients were selected from the GENDER study population. This study has been described previously.[16] In brief, the GENetic DEterminants of Restenosis project (GENDER) was a multicenter follow-up study designed to study the association between various gene polymorphisms and clinical restenosis. Patients eligible for inclusion in the GENDER study were treated successfully for stable angina, non-ST–elevation acute coronary syndromes or silent ischemia by PCI in 4 out of 13 referral centres for interventional cardiology in The Netherlands. Patients treated for acute ST-elevation myocardial infarction were excluded. Written informed consent was obtained from all participants. The study protocol conforms to the Declaration of Helsinki and was approved by the ethics committees of each participating institution.

Cell isolation and culturing

Blood was drawn into citrate tubes (Greiner) and PBMCs where isolated by density gradient centrifugation using Ficoll Isopaque plus (GE healthcare). Five parts of blood where diluted with one part of buffer consisting of PBS (LUMC Pharmacy) + 10% v/v GPO (Sanquin) + 10% v/v Sodium Citrate (LUMC Pharmacy). PBMC fraction was recovered and washed tree times in buffer. Monocytes where isolated from the PBMC fraction by magnetic separation with CD14 magnetic beads (MACS; Milteny Biotech).

HUVECs from 10 umbilical cords, were isolated following established protocols,[17] and cultured until confluence in EGM-2 medium (Lonza) in T75 culture flasks coated for 30 min at 37°C in 5% CO2, with 1% w/v gelatine. Cells were harvested using a cell scraper and washed two times in cold PBS then RNA and genomic DNA (gDNA) was extracted (see page 121). Following gDNA extraction, the genotype of the HUVECs was assessed using Sanger sequencing (performed by the Leiden Genome Technology Center). Primers used for qPCR and sequencing are shown in table 5–1

Table 5–1: Primers used for qPCR and sequencing analysis.

Target	Application	Sequence (5'—3')
RPII	qPCR	F: CAGGAGTGGATCCTGGAGAC R: GGAGCCATCAAAGGAGATGA
PCAF	qPCR	F: TCATCCCCTGAGAGCATCTT R: GGTATCTGGGAAGACTGGGA
	Sequencing	F: CCACAAGTAACCTCCAGTGG R: CAAGCGTCCCTCAGAATTCA

The cell lines used for the preparation of nuclear extracts were obtained through the ATCC and were cultured in Iscove's modified Dulbecco's medium (IMDM; BioWhittaker Europe, Verviers, Belgium) supplemented with 10% (v/v) heat-inactivated foetal calf serum (FCS; Greiner, Alphen a/d Rijn, The Netherlands), 100 IU/ml streptomycin and 100 IU/ml penicillin. For interferon-γ (IFN-γ) inductions, cells were treated with 500U/ml of IFN-γ (Boehringer-Ingelheim, Alkmaar, The Netherlands) for 4 hours; hereafter nuclear extracts were prepared (see below).

For the preparation of nuclear extracts HUVECs were isolated as described on page 119 and cultured in Medium 199 with Earl's Salt and L-glutamine (Life Technologies, Breda, The Netherlands), supplemented with 20% (v/v) FCS (PAA, Pasching, Austria), 100 IU/ml streptomycin and 100 IU/ml penicillin, 10 IU/ml heparin (Leo Pharma, Breda, The Netherlands) and 25 mg Bovine Pituitary Extract (BPE; Life Technologies, Breda, The Netherlands).

For IFN-γ inductions of HUVECs, cells were treated with 500 U/ml of IFN-γ (Boehringer-Ingelheim, Alkmaar, The Netherlands) for 4 hours; hereafter nuclear extracts were prepared (see below).

Transcription factor binding site search

Potential transcription factor binding sites were identified using the TFSEARCH program (http://www.cbrc.jp/research/db/TFSEARCH.html), which searches the TRANSFAC database.[18,19] Cut-off was set at 75% of the consensus TF binding site.

Nuclear extracts and electrophoretic mobility shift assay (EMSA)

Nuclear extracts and EMSAs were performed as described previously.[20] In brief, 2 µl of nuclear extracts in binding buffer were incubated for 30 minutes on ice, with 2 ng of a [^{33}P]-labelled double stranded DNA probe. The probe

sequences were similar to either the C or G promoter variants of the *PCAF*-gene (PCAF-C: 5'–GCAATAAGCCTCCTCAATCCTTTGCCCTTG–3'; PCAF-G: 5'–GCAATAAGCCTCCTGAATCCTTTGCCCTTG–3'). Probe sequences for transcription factors MZF1 and GATA1-3 were similar to their previously described consensus sequence (MZF1 (zinc fingers 1–4): 5'–GATCTAAAAGTGGGGAGAAAA–3'; MZF1 (zinc fingers 5–13): 5'–GATCCGGCTGGTGAGGGGGAATCG–3'; GATA: 5'–GGACCTTGATCTTATCTT–3').[21,22]

In the case of competition assays, nuclear extracts were incubated with unlabelled oligonucleotides in 100-fold excess for 30 min on ice, prior to incubation with the labelled probe. In case of interferon-γ treated samples, cells were stimulated with interferon-γ (500U/ml; Boehringer-Ingelheim, Alkmaar, The Netherlands) for 24-hours prior to preparing nuclear extracts. Samples were run on a 6% polyacrylamide gel in 0.25x TBE-buffer for 2 hours at 200 V. Gels were densitometrically analysed using the ImageJ software.[23]

RNA/DNA isolation and cDNA synthesis

From 1×10^6 $CD14^+$ monocytes total RNA was isolated. After harvesting HUVECs, half of the cells were used for RNA isolation and the other half was used for genomic DNA (gDNA) isolation. Cells were centrifuged for 5 min at 500×g and lysed in RNAbee according to manufacturer's instructions. Lysates were stored at -80°C until further processing. Total RNA was then extracted according to manufacturer's instructions. Subsequently from 1 µg total RNA, cDNA was synthesized using SuperScript III (Invitrogen), with random hexamers (Promega), at 50°C for 1 hour according to manufacturer's instructions. Genomic DNA was extracted by standard chloroform/phenol extraction.[24]

qPCR

Quantitative PCR was performed on a Bio-Rad iCycler 5 with SYBR Green supermix (Bio-Rad) For each reaction, 125 ng of cDNA and 5 pmol of forward and reverse primer (Table 5–1) were used in a total volume of 25µL. Transcription levels were normalized against RNA Polymerase II (*RPII*). Both primer-pairs produce a single amplicon. Amplicons were sequenced to confirm the identity of the target sequence. The PCR-program used was: 95°C for 3 min, followed by 40 cycles [30 sec 95°C; 30 sec 60°C; 30 sec 72°C] and was concluded by melting curve analysis to confirm target specificity.

Statistics

Data was analysed using an ANOVA and corrected for multiple testing using the Bonferroni method with the in SPSS software package (version 17). Data was considered significant when $p < 0.05$ after multiple testing correction.

Results

In a previous study, the –2481 polymorphism was correlated to decreased vascular morbidity and mortality.[15] Using the EMSA technique we tested whether the observed polymorphism would lead to differential protein binding in vitro. Using nuclear extracts of different cell types, we studied complex formation at the –2481 region of *PCAF*. We could detect constitutive protein binding to both *PCAF* C and G-variants (Figure 5–1A). In both HUVEC and U251 cells nuclear factor binding is slightly enhanced by IFN-γ stimulation. Densitometric analysis revealed that the *PCAF* G-variant exhibits slightly stronger protein binding in some cell types than the C-variant, although these differences are not statistically significant. Competition assays also suggest a modest difference in binding affinity (Figure 5–1B).

Using the TFSearch program,[19] we identified possible binding sites for MZF1 and GATA1-3 in the *PCAF* C-variant promoter. However, ds-oligonucleotides representing the consensus binding sites for MZF1 and GATA1-3 did not compete with factor binding to the *PCAF* C promoter variant (data not shown).

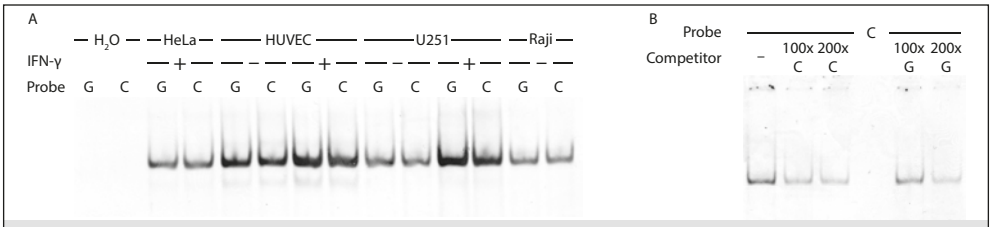

Figure 5–1. Protein binding to the C and G-variant of the –2481 region. (A) Electrophoretic mobility shift assays (EMSA) showing binding of protein to the C and G-variant of the –2481 region using nuclear extracts of various cell-lines. EMSA suggests slightly stronger binding of protein to the G-variant of the –2481 region, most pronounced in human umbilical vein endothelial cells (HUVEC) and U251 cells. (B) Competition assay with nuclear extracts from HeLa cells stimulated with IFN-γ also suggests slightly increased binding affinity for the –2481G-variant at high concentrations. The difference is best observed when unlabelled probe is added in 200-fold excess, indicating that the observed difference in affinity is weak. Shown are representatives of multiple independent experiments. From: Pons, D. et al. *Heart (2011)*; **97**, 143–50. doi:10.1136/hrt.2010.199927

Mouse models of reactive stenosis showed that *PCAF* transcription was modulated after placing a non-restrictive cuff around the femoral artery.[15] Given the location of this polymorphism in the *PCAF* promoter we have investigated whether this polymorphism has an impact on the constitutive transcript levels of *PCAF*. We therefore analysed *PCAF* transcription levels in two cell types relevant for human vascular disease: monocytes and Human Umbilical Cord Endothelial Cells (HUVECs). Monocytes were obtained from the previously investigated GENDER population. For each genotype 5 patients were randomly selected. Monocytes were freshly isolated from peripheral blood and total RNA was extracted directly after isolation. After cDNA synthesis, *PCAF* transcription levels were then determined by qPCR and normalized to transcript levels of RNA Polymerase II (*RPII*). The normalized *PCAF* transcript levels in monocytes are shown in figure 5–2. No significant differences ($p < 0.05$) could be observed in *PCAF* transcript levels with regards to the genotype of the monocytes in this cohort of investigated patients.

In addition to monocytes, *PCAF* transcription levels were also studied in HUVECs from 10 umbilical cords. HUVECS were isolated as previously described by Jaffe et al.[17] After isolation cells were grown to confluence and subsequently gDNA and RNA was isolated. *PCAF* transcript levels were determined and subsequently the genotype was determined using Sanger sequencing. The relative transcription levels of *PCAF* in HUVECS are shown in figure 5–3. Only C/G and G/G genotypes were found in the isolated HUVECs. Also in HUVECs no differences were found in *PCAF* transcript levels between the various genotypes.

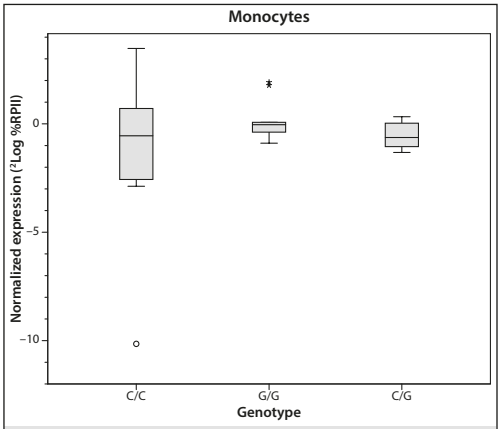

Figure 5–2. Boxplot of transcription levels of *PCAF* for various genotypes of the –2481C/G polymorphism in peripheral blood monocytes. Transcript levels are normalized to *RPII* transcription level. Numbers are shown as ^{2}log relative expression. Values between > 1.5 × IQR (inter quartile range) and < 3 × IQR are marked by an asterisk (*); values > 3 × IQR is marked by an open circle (o)

Discussion

Single Nucleotide Polymorphisms (SNPs) in promoter regions, although associated with some disease or mortality, can prove to be hard to link to actual biological processes.

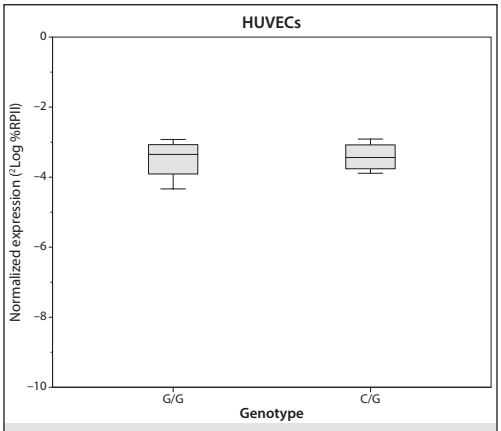

Figure 5–3. Boxplot of transcription levels of *PCAF* for various genotypes of the –2481C/G polymorphism in HUVECs. Transcript levels are normalized to *RPII* transcription level. Numbers are shown as ^{2}log relative expression. No C/C genotypes were encountered in the isolated HUVECs.

Although risk genes for various diseases have been found in Genome-Wide Association Studies (GWAS), many of these failed to link functional implications to the reported risk gene.[25,26] When the –2481G/C polymorphism upstream of the epigenetic modifying enzyme PCAF was identified in a GWAS as a cardiovascular disease risk factor, we performed some initial experiments to find biological implications of this SNP.[15] Through the use of an electrophoretic mobility shift assay, we could demonstrate protein binding to an oligonucleotide encompassing the SNP. Since this protein binding showed some minor differential binding between the C/C and G/G allele, we hypothesized that this may result in differential PCAF expression.

Using quantitative PCR we measured the transcription levels of *PCAF* in cell types relevant to cardiovascular disease. Nor in monocytes, nor in HUVECs were we able to demonstrate a difference in *PCAF* transcript levels related to the –2481G/C polymorphism. Thus the functional implication of the SNP association to vascular mortality does not seem to be caused by a clear difference in basal *PCAF* transcription levels. From these results it cannot be excluded though, that the –2481G/C polymorphism influences the regulation of PCAF expression induced by environmental stimuli. Given the fact that multiple *PCAF* SNPs have been described and investigated for their association with coronary restenosis, the apparent lack of differences in the constitutive transcript levels of *PCAF* could reflect complex interactions of the described genetic variations that determine the overall levels of gene expression. The apparent lack of correlation between a promoter polymorphism and transcript levels has also been observed in other genetic systems of which the TNF promoter polymorphisms were elaborately studied.[27]

The functional implication of the SNP might not necessarily be differential transcription of the *PCAF*. The SNP could also function in e.g. structural integrity and long-range gene interaction. As already noted in an editorial comment, the

–2481G/C polymorphism could just be a proxy marker of a functional SNP located elsewhere.[28] Since we do not observe significant differences in the levels of *PCAF* transcripts the exact mechanism by which the –2481G/C SNP protects from vascular morbidity and mortality remains to be elucidated.

Interesting to note in this respect is that the –2481G/C polymorphism was not reported in a systematic testing of literature reported variations associated with coronary restenosis.[29] This systematic testing was performed on the GENDER study in which the –2481G/C polymorphism was originally reported.[15] Although the authors do not report the –2481G/C polymorphism in the results, the *PCAF* gene is listed as one the top 6 most significant associated genes. This could indicate that a biological pathway involving PCAF or the *PCAF* locus is functionally involved in the associated survival rather than PCAF by itself.

Many GWAS have been published in the last decade and hundreds of SNPs have been associated with a wide variety of diseases.[30] It has been argued before that these studies by themselves are of limited value unless in subsequent steps the biological relevance or pathogenic mechanisms are elucidated.[30] Furthermore, the number of discovered variants by GWAS is strongly correlated with the experimental sample size.[31] This correlation roughly predicts that after a certain sample-size threshold a doubling in sample-size leads to a doubling in associated SNPs.[31]

The data presented here illustrates that GWAS data by itself is of limited use, but can serve as a good starting point for further analysis. Indeed there are some well-studied examples were GWAS data provided a starting point for taking our knowledge on the mechanisms involved in the pathogenesis of certain diseases a step further. However this also illustrates that the real challenge in interpreting GWAS data is in finding the biological significance.

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6

Summary & General discussion

Parts published in:

Genetic and Epigenetic Regulation of CCR5 Transcription

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Epigenetic contribution to atherosclerosis

When combining all the currently available reported data, there is evidence in support of the notion that epigenetic mechanisms contribute to atherosclerosis pathogenesis.[1–3] Strong indications can be found in the fact that atherosclerosis pathology is characterised by many environmental risk factors.[4] In some cases, there has been a direct link between risk factors for atherosclerosis and epigenetic control of gene expression.[5,6] Besides the risk factors contributing to disease development, many examples are known of the epigenetic regulation of specific genes involved in atherosclerosis pathology. Much of this data is summarized in **chapter 1**.

Risk factors aside, susceptibility to coronary heart disease (CHD) may (in part) be a consequence of events in early life. In 1992, Hales and Barker formed the thrifty phenotype hypothesis (also referred to as the Barker-hypothesis or foetal-origins hypothesis).[7] The hypothesis states that adaptation to an unfavourable maternal environment is beneficial to the developing embryo in utero.[7,8] When environmental conditions are changed in later life, this may lead to development of chronic disease, such as cardiovascular disease. The observations made by Barker cannot be correlated to alterations in the DNA sequence, hence epigenetic regulation is thought of as the underlying mechanism. This notion is strengthened by the altered DNA methylation patterns involving the *IGF2* locus observed in the Dutch Hunger Winter cohort,[9] but also in more controlled mouse-studies.[10]

Barker's hypothesis has been much criticized. For example: it is claimed that Barker never properly tested his hypothesis,[11] and has not corrected for confounding.[12,13] More importantly, the hypothesis formulated by Barker does not account for environmental factors and stochastic changes that occur during a lifetime. This 'acquired' epigenetic contribution is indicated by e.g. the incomplete concordance for autoimmune diseases between genetically identical (monozygotic) twins, in conjunction with the finding of epigenetic drift during aging in twin cohorts.[14,15] An alternative hypothesis, the accumulation of risk hypothesis, takes into account genetic factors, the in-utero environment, and life-acquired 'epimutations'.[16] From monozygotic twin studies it became apparent how acquired epimutations may change phenotype and disease susceptibility.[14,17] Importantly, when epimutations are acquired during a lifetime, 'epitherapy' might be able to reverse these epigenetic alterations.

Understanding of the mechanism how epigenetic gene regulation influences the formation of atherosclerotic plaques may help us in developing new therapies and prevention strategies. A comprehensive view on how our environment influences epigenetic regulation and how in turn this epigenetic regulation affects atherosclerosis development is very valuable in this respect. In the research presented in this thesis an attempt was made to provide an integrative approach. Starting with identifying epigenetic components in human atherosclerotic plaques (**chapter 2**) and then cascading down to an individual gene level (**chapters 4 & 5**).

Identification of epigenetic components in plaques

The clinical manifestation of atherosclerosis is present in the form of plaques in the arterial intima.[18] Therefore, to evaluate how epigenetics is contributing to this disease, the epigenetic components of the plaque where examined in **chapter 2**. Using immunohistochemistry, the aim was to identify aberrant epigenetic patterns that could be correlated with plaque stage. Using this method a reduction of H3K27Me3 was found in the *tunica media* of late atherosclerotic plaques. Although a significant reduction was found, the results found is this study should be further validated given the inherent difficulties of analysing IHC data in a quantitative manner.

The quantitative analysis of this study is mainly hampered by two factors. Firstly, staining intensity is not always correlated with protein concentration.[19] Furthermore DAB staining does not follow Lambert Beers Law, complicating quantification. Secondly, DAB and haematoxylin are hard to spectrally unmix by image processing.[20] In the data presented in **chapter 2**, the haematoxylin staining is often overpowered by strong DAB intensity. Although these factors can be mostly overcome by choosing an immunofluorescence method over IHC, this does not imply that staining intensity can be correlated with protein concentration when using immunofluorescence.

Besides these methodological considerations, the staining pattern of the positive control (total H3) raises some questions that should be addressed. In stainings directed to total H3 not every nucleus stains positive. This can potentially be explained by the fact that a limiting dilution is used on the primary antibody. However, this can also be explained by an accessibility artefact, either due to the origin of the sample (FFPE material) or by the antigen retrieval-method used. Although this lack of staining has been observed in numerous publications, it justifies the need for additional validation.

In **chapter 2**, it was observed, that the reduction in in H3K27Me3 staining was not accompanied by a reduction in the H3K27Me3 associated proteins JMJD3, EZH2 and BMI1. Whether the reduction of H3K27Me3 is the result of altered targeting of H3K27Me3 modifying enzymes, or due to reduced activity of these enzymes, remains to be established. Additionally, in **chapter 2**, it was observed that the *tunica adventitia* shows a higher percentage of positive nuclei, regardless of the staining. It needs to be confirmed whether this is a reflection of cell types residing in the *tunica adventitia*.

Epigenetic components in monocyte differentiation

Even though FFPE material is available to study plaque composition, it is unfortunately impossible to follow the dynamic process of plaque formation *in vivo*. Neither is it possible to simulate plaque formation *in vitro*. As such only certain components of the plaque formation process can be studied.

In the tunica media reside many infiltrating cell types, including one of the main precursor cells to plaque formation: monocytes. As reviewed in **chapter 1**, monocytes infiltrate the subendothelium and there differentiate into macrophages. Since this differentiation process is a key element in plaque formation it was extensively studied in **chapter 3**.

By using quantitative PCR the transcript levels of genes encoding for epigenetic modifying enzymes were evaluated during the differentiation of monocytes into macrophages or dendritic cells (DCs). With the notable exception of KATs and PcG genes, all major classes of the genes encoding epigenetic modifying enzymes are differentially transcribed when comparing monocytes to either macrophages (type 1 and type 2) or to immature dendritic cells. Further stimulation with LPS did not alter these expression patterns. On a single gene level, only *KMT1C* was found to have significantly altered transcription levels during differentiation of monocytes into various lineages. Blocking KMT1C activity with BIX-01294 resulted in a reduction of *DC-SIGN* transcripts, while at the same time cell surface expression of DC-SIGN was hardly affected. However, surface expression of DC-SIGN was reduced when applying the general KMTi DZNep. This reduction in surface expression of DC-SIGN was even more pronounced when BIX-01294 and DZNep were combined.

Previous work published on epigenetic regulation involving monocytes focuses mainly on the epigenetic regulation of single genes, including following the response to interferon stimulation. For instance, histone H3-Lysine 9 methylation

has been shown to be involved in the regulation of single genes following monocyte differentiation.[21,22] In **chapter 2** a reduction in H3K27Me3 levels was identified in late-stage atherosclerotic plaques. This specific post-translation histone modification has been shown before to be involved in the regulation of *CD14* and *CD209* (*DC-SIGN*) transcription during monocyte differentiation.[23] Epigenetic modifying enzymes putting the H3K27Me3 mark in effect however were neither identified as being transcriptionally altered (**chapter 3**) nor did they show altered expression (**chapter 2**) during monocyte differentiation. It is likely that the epigenotype switching at the *CD14* and *CD209* loci is the result of differential targeting of epigenetic modifying enzymes without an alteration in transcript or protein expression levels.

Recently, a large advancement in understanding monocyte differentiation has been made by the first genome-wide epigenetic profiling of monocyte to macrophage differentiation.[24] However, from an epigenetic point of view, monocyte differentiation is not yet as well understood as T cell differentiation is. The differentiation of naïve T cells into Th1 or Th2 is determined by the cytokines IL-12 and IL-4, respectively. In response to these signals, transcription is initiated of lineage specific cytokine genes including *IFN-γ* and *IL-4*.[25] The *IFN-γ* and *IL-4* loci are maintained in a 'poised' state in naïve T cells—i.e. they show both repressive and activating epigenetic marks—allowing rapid, early transcription. Such a poised state resembles the multivalent states observed for *CCR5*, as described in **chapter 4**. The multivalent state for *CCR5* however is used for fine-tuning of transcription levels instead of rapid transcription upon induction. It has been suggested that conclusions made from studies of T cells are broadly relevant to differentiation in other cell types and tissues.[26] In particular, the concepts of transcriptional poising and promoter bivalency as mechanisms that regulate fate decisions are pertinent during the differentiation of stem cells and less primitive tissues.[26] Similar mechanisms are observed in the transcriptional regulation of *CCR5* as described in **chapter 4**.

Studying epigenetic processes in T cell differentiation has led to interesting insights into disease pathogenesis of autoimmune diseases and of haematological malignancies. For instance, systemic lupus erythematosus (SLE) T-helper cells exhibit increased and prolonged expression of cell-surface CD154, spontaneously overproduce interleukin-10 (IL-10), but underproduce IFN-γ.[27] In human SLE T cells, the HDAC inhibitor trichostatin A (TSA) reverses the skewed expression of CD154, IL-10 and IFN-γ products.[27] This illustrates the potential use of Small Molecule Inhibitors (SMI) as a therapy for certain diseases (see page 138). T cells from patients with SLE and RA were found to exhibit globally hypomethylated DNA.[28]

Widespread hypomethylation has been described in other disease contexts (e.g. T cell lymphomas or chronic lymphocytic leukemia cells), in which it is associated with gene activation and chromosomal instability.[15,29] Interestingly, global DNA hypermethylation has been linked with a predisposition to, and natural history of atherosclerosis.[2]

Regulation at the single gene level

Although any change identified in the expression of epigenetic modifying enzymes is likely to be of importance in the pathogenesis of atherosclerosis, these enzymes are not solely responsible for cellular activity. Quite a few other molecules (e.g. chemokines and their receptors) are known to play a role in atherosclerosis.[30] To get a proper understanding on the transcriptional regulation of these genes, epigenetic regulation must be taken into account as well. In **chapter 4**, the regulation of *CCR5* was extensively studied, covering both classical regulation as well as epigenetic regulation.

In the initial characterization of the *CCR5* promoter, it was suggested that *CCR5* transcription could be upregulated by NF-κB.[31,32] Indeed, several potential binding sites for NF-κB have been found in the *CCR5* P1-promoter.[32,33] However, the results of the study by Kuipers et al. indicate that CCR5 expression is neither induced nor modulated by NF-κB.[33] In addition, these authors also found binding sites for interferon regulatory factors (IRFs) and CREB-1 in the *CCR5* P1– and P2-promoters. Like for NF-kB and in contrast to CREB-1, Kuipers et al. could not establish a role for the IFNγ induced regulatory pathway in *CCR5* transcription. By using various reporter assays, as well as by competition for CREB-1 binding-sites by inducible cAMP early repressor (ICER), which is induced by forskolin treatment, the authors concluded that *CCR5* transcription is regulated by CREB-1.[33] More recently, Banerjee et al. also showed that in the TF-1 human bone marrow progenitor cell line, *CCR5* is regulated at the transcriptional level by the cAMP/PKA/CREB pathway.[34]

Transcriptional regulation of *CCR5* cannot be explained by the sole action of transcription factors. Expression of CREB-1, however, is ubiquitous whereas *CCR5* transcription is not. The extensive study on epigenetic regulation of *CCR5* in **chapter 4**, showed the complex nature of epigenetic regulation and how this is used to fine-tune transcription levels. The work presented in **chapter 4** illustrates that transcription, besides the activity of transcription factors, is the result of the sum of chromatin marks. This is exemplified by the chromatin state of *CCR5* in monocytes. Here, high levels of permissive and non-permissive histone modifications

are found together. Furthermore, the *CCR5* promoter in monocytes is densely methylated at the DNA level. This results in intermediate *CCR5* transcription levels in monocytes.

The epigenetic regulation of *CCR5* transcription is determined by the interplay of DNA methylation and histone modifications. The mode of regulation is highly similar to the regulation of *HLA-G*. *HLA-G*, similar to *CCR5*, is transactivated by CREB-1. This cannot explain the tissue-restricted expression of HLA-G, which also suggests the involvement of epigenetic mechanisms in the regulation of *HLA-G* transcription.[35]

Traditionally it was thought that chromatin was either marked by permissive or non-permissive marks (Figure 6–1, 'classical' regulation).[36–38] The original histone code hypothesis, with an one-mark-to-one-module type of decoding, has received some criticism over the years (Ruthenburg et al.[39] and references therein). Later work, however, showed that both permissive and non-permissive chromatin marks could coexist.[40,41] Interestingly, activating and repressive modifications can even occur on the same nucleosome (Figure 6–1, 'non-classical' regulation).[41]

It has been proposed that a bivalent domain (characterized by the presence of H3K4Me3 and H3K27Me3) is merely an intermediate state.[39] An idea strengthened by the finding that the 'eraser' of H3K9Me3 (considered as a repressive chromatin mark) is recruited by binding H3K4Me3 (considered as a permissive chromatin mark).[42,43] Additionally, removal of the H3K27Me3 (considered as a repressive chromatin mark) facilitates the recruitment of a H3K4 methyltransferase.[44] However, in the context of cellular differentiation, co-localization of opposing epigenetic marks is also employed to poise genes for rapid activation or repression.[40]

Proximal modifications that constitute a putative 'code' need not be restricted to a single histone tail as originally anticipated,[36] but may span two or more tails on a given nucleosome, adjacent nucleosomes, or nucleosomes that are discontinuous in primary DNA sequence but spatially co-localized in a chromatin territory.[45] Furthermore, the multivalency of chromatin marks may also be thermodynamically more favorable then monovalent histone marks.[39] As reviewed by Rothbart and Strahl, multivalent chromatin marks may be far more common than originally appreciated and the one-mark-to-one-module type of decoding is a too simplistic view.[46]

The reduction in H3K27Me3 found in atherosclerotic plaques, as described in **chapter 2** could be partially explained by the regulation seen at the *CCR5* locus. In later stages, CCR5 expressing T cells are found in atherosclerotic plaques. It remains to be established however, whether the reduction at a single locus would be enough to be detected by IHC. Furthermore, the reduction in H3K27Me3 as a result of T cell influx may be counterbalanced by the monocytes present, which show high levels of H3K27Me3.

In the context of atherosclerosis pathogenesis the demonstrated interference in epigenetic regulation is probably of more relevance. By adding inhibitors of epigenetic regulating enzymes, *CCR5* transcription and CCR5 expression could be restored in non-CCR5 expressing cell-lines. This indicates that the epigenetic regulatory mechanism could form a potential target for pharmacological intervention. CCR5 has been shown previously to be involved in atherosclerotic lesion formation.

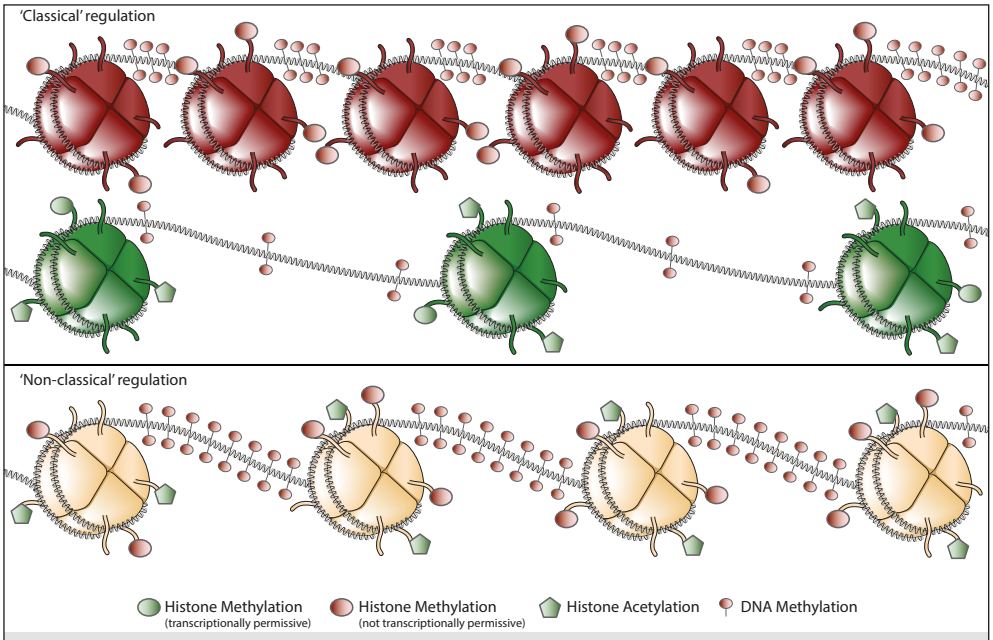

Figure 6–1. Schematic representation of chromatin states encountered in the *CCR5* locus. Chromatin can be marked by mainly repressive or mainly permissive marks, regarded as the classical euchromatin (green) and heterochromatin (red) states ("classical" regulation). Nowadays it is widely appreciated that more complex forms of chromatin exist, hallmarked by both repressive and permissive marks in the same locus ("non classical" regulation). Note: For clarity DNA-methylation is drawn on the internucleosomal-DNA, whereas it has been shown that methylated DNA co-localizes also with nucleosomes.[47] From: Wierda, R.J. et al. *Biology (2012)*; **1**, 869–79. doi:10.3390/biology1030869

Altering CCR5 expression by use of these SMI could have a beneficiary effect on disease progression.

Genetics of epigenetics

Previously it was reported that transcription levels of the epigenetic regulatory enzyme PCAF (KAT2B) were significantly altered in a mouse model of reactive stenosis. In addition it was found that the C-allele of a polymorphism 2481bp upstream of the transcription start-site of *PCAF* (rs2948080) is associated with a significant reduction in vascular morbidity and mortality. Given the location of the SNP it was investigated whether this SNP has an impact on the constitutive transcript levels of *PCAF* in **chapter 5**. No significant differences were observed in constitutive *PCAF* transcript levels with regards to the genotype of either the monocytes or the HUVECs in the cohorts of investigated individuals.

The apparent lack of correlation between a promoter polymorphism and transcript levels has also been observed in other genetic systems of which the TNF promoter polymorphisms were elaborately studied.[48] Similarly, the HLA-region has been known as a risk factor for Multiple Sclerosis for around 40 years.[49] Although the risk factor has been refined through the years to the DRB1*1501 allele, molecular mechanisms underlying the risk factor have not yet been completely elucidated.[49] Astonishingly, GWA studies have identified hundreds of susceptibility genes for a large number of human conditions and quantitative traits.[50] Deletion of the corresponding stretch of DNA in mice has shown that this part of the chromosome regulates cardiac expression of two genes approximately 100 kb away from the site of the variation.[55] Yet in large human cohorts no association of the 9p21.3 MI/CAD risk-variant with the expression of the two genes was found. Thus, "the mystery of the 9p21.3 locus remains wide open".[56] In the case of the *PCAF* -2481G/C polymorphism it may turn out that the identified SNP is influencing genomic regions located far away from the identified SNP, or even on different chromosomes. Even though it remains to be elucidated exactly how the *PCAF* -2481G/C polymorphism affects vascular morbidity and mortality, this SNP is illustrative of the complex interactions between genetic and epigenetic regulation. When considering epigenetic modifying enzymes, such as PCAF, as pharmacological targets it is important to realize that also in epigenetic therapy pharmacogenomics is in play.

Epigenetic regulation as a pharmacological target

SMI can influence the activity of epigenetic regulatory enzymes. Many of these compounds are relatively well tolerated as demonstrated in various clinical trials of these compounds in cancer therapy. As demonstrated in **chapter 4** and in other studies,[27] these SMIs can be used to influence gene expression. In atherosclerosis treatment, these inhibitors may be beneficial as well.

Epigenetic regulation is essential in keeping cells in their terminally differentiated form. De-differentiation, or slowing down differentiation, can be beneficiary in atherosclerosis for instance by preventing foam cell formation. As described in **chapter 3**, KMTs play a role in the differentiation of monocytes into various lineages. Treatment with KMTi's (such as DZNep and BIX-01294) could perhaps reduce the formation of foamcells in the vessel wall. Similarly, as shown in **chapter 4**, CCR5 can be modulated by SMI. By targeting CCR5 or other chemokine (receptors) the flux of immune cells to the vessel wall could potentially be modulated.

Although demonstrated in this thesis that the expression profile of a single gene could be altered by SMI, the potential use of SMI as curative agent requires careful consideration. Of high importance in this respect is the targeting of the SMI in order not to cause side effects elsewhere in the body. This is especially important given the fundamental gene regulation mechanism these inhibitors act upon. In principle these reactions can be avoided by targeted delivery and dosage. Careful selection of the SMI used based on its working mechanism can help to prevent these side effects as well. For instance Zebularine, a DNA methylation inhibitor, requires incorporation into the DNA and therefore may affect dividing cells stronger than non-dividing cells.

Thorough understanding of interactions within the cell is also necessary to predict potential side effects in SMI-therapy. Epigenetic regulation is part of a complex network of interactions. Most, if not all genes, are under epigenetic control. Intervention in this mechanism may result in unwanted deregulation of certain genes. This can be caused either as a result of direct intervention in transcription or as a result of disruption of long-range interactions within the nucleus.

Recommendations for future research

Determining the onset of complex multifactorial diseases such as atherosclerosis might be best investigated in a multi-disciplinary approach. Starting at a

demographic or epidemiological approach and then cascading down to individual molecular pathways. From an epidemiological point of view quite some knowledge has been gained on atherosclerosis in the past years. Many potential risk factors have been found, and many GWA studies have found potentially interesting genomic targets. However, these findings are often hard to translate to molecular underpinnings as has been demonstrated in **chapter 5**.

On the other hand, *in vitro* studies or even mouse studies are often hard to translate to clinical settings. Much can therefore be gained by focusing research on clinically relevant materials, e.g. tissue and biological fluids present in various bio banks. In a similar approach as in **chapter 2**, it would be possible to identify potential targets for epigenetic regulation by using ChIP-Seq strategies on plaque material. The results of such studies could provide fuel for further *in vitro* studies.

Complicating such an approach however is the lack of availability of such material. Materials acquired are almost always post-mortem, which may influence the results obtained. As a result research is often performed on more easily available materials or models such as peripheral blood, or *in vitro* and mouse models. Replicating plaque formation could advance the field much in this respect. Similar attempts have been undertaken in creating *in vitro* skin, but is must be noted that plaque material is much more complicated in composition then the epidermis. In case successful plaque models can be created *in vitro*, these could be used to test the molecular mechanisms underlying plaque development.

Furthermore, a better understanding of the mechanisms underlying differentiation of monocytes, such as presented in **chapter 3**, and other cell types may help us in understanding the pathology of plaque formation. A better understanding of these fundamental processes may also be of benefit in the understanding of other diseases with an inflammatory component, such as MS and diabetes. Genome-wide epigenetic profiling may lead to new insights on loci involved in differentiation. As illustrated by the work in **chapter 4** it is important to investigate the broadest repertoire of epigenetic marks possible. Such genome-wide studies should be accompanied with more detailed mechanistic studies. Just as is the case with GWA studies, genome-wide epigenetic profiling may provide us with data that is hard to link to various cellular processes.

Conclusions

The work presented in this thesis provides a strong indication that H3K27Me3 levels are lowered in more advanced plaques. This is not accompanied by any change in enzymes involved in establishing or making the H3K27Me3 mark into effect. These observations, however, require further validation by other methods. Furthermore it was shown that KMTs are involved in the differentiation of monocytes into DCs and that this process might be potentially steered by using SMI.

In addition, the complexity of epigenetic regulation is demonstrated at the *CCR5* locus. The regulation of *CCR5* shows that transcription levels are the result of the sum of epigenetic modifications in addition to classical gene regulation by transcription factors. The interplay between classical genetic regulation and epigenetic regulation is underlined by the SNP (rs2948080) found in the *PCAF* gene. Although this SNP does not have a direct influence on *PCAF* transcription, the SNP itself is associated with higher vascular mortality. Potentially this is the result of higher-order or long-range genomic interactions. Finally, the work presented on monocyte differentiation and *CCR5* regulation shows the potential of 'epigenetic therapy' by using SMI.

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N

Nederlandse Samenvatting

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Hart- en vaatziekten zijn momenteel doodsoorzaak nummer één in ontwikkelde westerse landen. De primaire oorzaak van coronair vaatlijden en hartinfarct is atherosclerose. In de volksmond, onterecht, wellicht beter bekend als aderverkalking. De klinische manifestatie van atherosclerose is het ontstaan van zogenaamde plaques in de vaatwand. Het ontstaan van deze plaques wordt waarschijnlijk veroorzaakt door geoxideerd LDL cholesterol (OxLDL). Onder invloed van deze stof migreren een bepaald type cellen (monocyten) uit het bloed de vaatwand in. Daar differentiëren deze cellen in macrofagen, die proberen de OxLDL op te ruimen. Macrofagen kunnen OxLDL echter niet verteren, maar blijven deze stof wel continu opnemen. Hierdoor ontstaan opgezwollen macrofagen, vol met OxLDL; de zogenaamde schuimcellen. De aanwezigheid van deze schuimcellen is wat de plaques vormt in de vaatwand. Monocyten en macrofagen zijn onderdeel van ons immuunsysteem. De voortdurende activatie van het immuunsysteem door OxLDL zorgt voor een chronische ontsteking in de vaatwand. De vaatwand zal onder druk van de groter wordende plaque initieel verwijden, maar uiteindelijk vernauwd de vaatwand zich. Als een vat te sterk vernauwd is, of door het scheuren van een plaque verstopt, stopt de bloedsomloop hetgeen een infarct veroorzaakt.

Het ontstaan van atherosclerose wordt gekenmerkt door vele omgevingsfactoren, zoals voeding en roken. Daarom wordt gedacht dat epigenetische regulatie een belangrijke rol speelt in het ontstaan van plaques, de klinische manifestatie van atherosclerose. Epigenetische regulatie is een proces dat cellen gebruiken om genexpressie te reguleren. In tegenstelling tot vele andere manieren van regulatie is epigenetische regulatie stabiel en blijft het behouden door celdivisies heen, maar zelfs door generaties heen kunnen epigenetische kenmerken bewaard blijven. Epigenetische regulatie bestaat uit het aanbrengen van chemische modificaties (methylgroepen) op het DNA, zonder dat daarbij de DNA-sequentie veranderd wordt. Daarnaast worden de eiwitten waaromheen het DNA opgewonden zit (histonen) voorzien van chemische modificaties. Of een gen tot expressie komt of niet wordt bepaald door de (combinatie van) modificaties die zijn aangebracht. In dit proefschrift wordt gekeken naar de invloed van epigenetische genregulatie op het ontstaan van atherosclerose.

Hoofdstuk 2 van dit proefschrift beschrijft het onderzoek naar de aanwezigheid van epigenetische modificaties in plaque-materiaal. Dit materiaal is verkregen van orgaandonoren en vervolgens in dunne (4µm dik) plakjes gesneden. Door middel van immunohistochemie is het mogelijk de chemische modificaties van histonen,

die de basis vormen van epigenetische regulatie, aan te kleuren. Door middel van de computertechniek beschreven in **hoofdstuk 2** werden het aantal cellen met en zonder epigenetische kenmerken geteld. Daarna is een correlatie gemaakt tussen het plaque stadium (1 = vroeg tot 6 = laat stadium). Er werd een zwakke, maar significante correlatie gevonden in één van de epigenetische markers (H3K27Me3). De eiwitten die deze modificatie lezen, aanbrengen of verwijderen laten echter geen veranderingen in expressie zien. Hierbij moet echter aangemerkt worden dat deze resultaten mogelijk vertekend zijn door de gebruikte technieken en de gevoeligheid daarvan.

In **hoofdstuk 3** wordt de differentiatie van monocyten in andere celtypen op moleculair niveau beschreven. Monocyten, geïsoleerd uit het bloed van gezonde vrijwilligers, worden *in vitro* gedifferentieerd tot dendritische cellen en macrofagen (type 1 en type 2). Door middel van kwantitatieve PCR is in deze cellen gekeken naar de RNA transcriptieniveaus van 59 genen betrokken bij epigenetische regulatie. Het blijkt dat van de vier verschillende klassen van enzymen er drie verschillen in transcriptie tussen monocyten en gedifferentieerde monocyten: KMTs, KDMs en HDACs. Van alle bekeken genen is er alleen bij *KMT1C* sprake van significant verschillende transcriptie. Wanneer tijdens de differentiatie van monocyten naar dendritische cellen (DCs) dit enzym geblokkeerd wordt door BIX-01294, wordt de transcriptie van *DC-SIGN* (typerend voor DCs) verlaagd. Op eiwitniveau heeft BIX-01294 geen effect op *DC-SIGN* transcriptie. Echter wanneer KMT-activiteit in het algemeen geremd wordt door DZNep is er ook minder DC-SIGN aanwezig op het celoppervlak. Dit effect wordt versterkt wanneer DZNep en BIX-01294 gecombineerd worden. Dit toont aan dat KMT1c een rol speelt bij de differentiatie van monocyten naar DCs.

In **hoofdstuk 4** wordt in detail beschreven hoe epigenetische regulatie de expressie van een gen beïnvloed. Het *CCR5* gen, wat betrokken is bij het ontstaan van atherosclerose komt in verschillende niveaus tot expressie in verschillende celtypen. Klassieke genregulatie, door middel van transcriptiefactoren die binden op het DNA kan deze verschillen niet verklaren. *CCR5* wordt slechts door de transcriptiefactor CREB-1 gereguleerd, maar deze transcriptie factor is vrijwel continu aanwezig in alle cellen. Het verschil kan wel verklaard worden door de 'epigenetische status' van het *CCR5* gen. In cellen waar CCR5 niet tot expressie komt zit het gen ook epigenetisch 'op slot', waarbij in cellen die CCR5 tot expressie brengen de epigenetische status daartoe ook gunstig is. Dit is compleet in lijn met de huidige theorie over epigenetische regulatie. Wat echter opvalt, is dat de epigenetica hier ook de mate van transcriptie bepaald, iets wat tot nu toe maar in een paar gevallen

beschreven is. Belangrijk bij het bestuderen van epigenetische regulatie is dus om alle factoren zowel, positief als negatief regulerend, in ogenschouw te nemen.

In **hoofdstuk 5** wordt een zogenaamd polymorfisme in de promotor van een epigenetisch gen beschreven: *PCAF*. In een eerdere studie werd dit polymorfisme gecorreleerd met een grotere vatbaarheid voor atherosclerose. In diezelfde studie werd eveneens aangetoond dat op de plek van het polymorfisme eiwitten kunnen binden en dat deze binding afhankelijk is van de aanwezige base in het DNA. In het onderzoek beschreven in dit hoofdstuk is vervolgens gekeken of deze eiwitbinding ook leidt tot een verschil in expressie. Deze expressie is gecontroleerd in cellen die aanwezig zijn in de vaatwand (HUVECs) en in de eerder beschreven monocyten. Er kon geen verschil aangetoond worden in expressie tussen de verschillende genotypen van het polymorfisme. Het verschil in vatbaarheid voor atherosclerose moet dus op een andere manier verklaard worden dan door een verschil in expressie van het *PCAF* gen.

Tot slot worden de gevonden resultaten in **hoofdstuk 6** samengevat en bediscussieerd. Aan het einde van dit hoofdstuk worden aanbevelingen gedaan voor toekomstig onderzoek. Met name wordt aanbovelen om meer epigenetisch gereguleerde loci te vinden middel van CHiP-Sequencing en de uitkomsten hiervan combineren met mechanistische studies.

C

Curriculum Vitae

Curriculum Vitae

Rutger Jeen Wierda werd geboren op 17 juli 1983 in Santpoort-Zuid (gemeente Velsen). In 2001 behaalde hij zijn VWO diploma aan het Kennemer Lyceum te Overveen. In datzelfde jaar begon hij aan zijn opleiding Life Science & Technology aan de TU Delft en de Universiteit Leiden. Wetenschappelijke ervaring werd opgedaan tijdens een tweetal stages. De eerste onder leiding van prof. dr. R.C. Hoeben, bij de vakgroep virologie aan de afdeling Moleculaire Celbiologie van het Leids Universitair Medisch centrum waar onderzoek werd gedaan naar de gly-ala repeat van het Epstein-Barr virus. Daarna werd een stage voltooid op de afdeling virologie op het ErasmusMC in Rotterdam onder leiding van prof. dr. R.A.M. Fouchier en afdelingshoofd prof. dr. A.D.M.E. Osterhaus. Tijdens deze laatste stage bestudeerde hij het fusie-eiwit van het humane metapneumovirus in een poging een vaccin te ontwikkelen tegen dit virus. In december 2007 behaalde hij zijn masterdiploma. In 2008 werd begonnen aan het promotieonderzoek waarvan de resultaten zijn beschreven in dit proefschrift. Het onderzoek beschreven in dit proefschrift is uitgevoerd bij de sectie moleculaire biologie van de afdeling Immunohematologie en Bloedtransfusie van het LUMC onder leiding van prof. dr. P.J. van den Elsen. Sinds juni 2012 is Rutger werkzaam als Field Application Scientist bij Thermo Fisher Scientific waar hij wetenschappelijk ondersteuning biedt op het gebied van kwantitatieve PCR.

P

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D

Dankwoord

Dankwoord

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A

Afkortingen

Lijst met afkortingen

APC	Antigen Presenting Cell
ATCC	American Type Culture Collection
BMI1	B Lymphoma Mo-MLV Insertion Region 1 Homolog
CAD	Coronary Artery Disease
cAMP	Cyclic Adenosime Mono Phosphate
CCR5	CC-Motif Chemokine Receptor 5
ChIP	Chromatin Immunoprecipitation
CIITA	Class II Transactivator
COX-2	Cyclooxygenase-2
CREB	Camp-Responsive Element Binding Protein
CREM	cAMP-Responsive Element Modulator
CRP	C-Reactive Protein
CVD	Cardiovascular Disease
DAB	3–3' Diaminobenzidine Tetrachloride
DC	Dendritic Cell
DNMT	DNA Methyltransferase
EC	Endothelial Cells
ECM	Extracellular Matrix
EMSA	Electrophoretic Mobility Shift Assay
ER	Oestrogen Receptor
EZH2	Enhancer of Zeste Homolog 2
FFPE	Formalin-Fixed Paraffin Embedded
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HDAC	Histone Deacetylase
HDL	High Density Lipoprotein
HS	Human Serum
HUVEC	Human Umbilical Vein Endothelial Cell
ICER	Inducible Camp Early Repressor
IFN-γ	Interferon gamma
IHC	Immunohistochemistry
IL	Interleukin
IRF	Interferon Regulatory Factor
JMJD3	Jumonji Domain Containing 3
KAT	Lysine Acetyltransferase
KDM	Lysine Demethylase
KMT	Lysine Methyltransferase
LDL	Low Density Lipoprotein
LSD1	Lysine-Specific Histone Demethylase 1
M-CSF	macrophage colony- stimulating factor
MHC	Major Histocompatibility Complex
Mφ	Macrophage
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NK Cell	Natural Killer Cell
NKT Cell	Natural Killer T Cell
NOS	Nitric Oxide Synthetase
OxLDL	Oxidized Low-Density Lipoprotein
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PcG	Polycomb Group
PCI	Percutaneous Coronary Intervention
PRC	Polycomb Repressive Complex
qPCR	Quantitative Polymerase Chain Reaction
SAHA	Suberoylanilide Hydroxamic Acid
siRNA	Small Interfering RNA
SIRT	Sirtuin
SLE	Systemic Lupus Erythematosus
SMC	Smooth Muscle Cells
SMI	Small Molecule Inhibitor
SNP	Single Nucleotide Polymorphism
TF	Tissue Factor
TGF-β	Transforming Growth Factor Beta
TNF-α	Tumour Necrosis Factor Alpha
TSA	Trichostatin A
VALT	Vasculature-Associated Lymphoid Tissue
vEC	Vascular Endothelial Cells
VPA	Valproic Acid
vSMC	Vascular Smooth Muscle Cell