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Genetic and Epigenetic Regulation of CCR5 Transcription

Rutger J. Wierda & Peter J. van den Elsen

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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. 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. Furthermore DAB staining does not follow Lambert Beers Law, complicating quantification. Secondly, DAB and haematoxylin are hard to spectrally unmix by image processing. 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.\textsuperscript{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 \textit{CD14} and \textit{CD209} (\textit{DC-SIGN}) transcription during monocyte differentiation.\textsuperscript{23} Epigenetic modifying enzymes putting the H3K27Me3 mark in effect however were neither identified as being transcriptionally altered \textit{(chapter 3)} nor did they show altered expression \textit{(chapter 2)} during monocyte differentiation. It is likely that the epigenotype switching at the \textit{CD14} and \textit{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.\textsuperscript{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-\gamma and IL-4.\textsuperscript{25} The IFN-\gamma 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 \textit{CCR5}, as described in chapter 4. The multivalent state for \textit{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.\textsuperscript{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.\textsuperscript{26} Similar mechanisms are observed in the transcriptional regulation of \textit{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-\gamma.\textsuperscript{27} In human SLE T cells, the HDAC inhibitor trichostatin A (TSA) reverses the skewed expression of CD154, IL-10 and IFN-\gamma products.\textsuperscript{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.\textsuperscript{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.\cite{15,29} Interestingly, global DNA hypermethylation has been linked with a predisposition to, and natural history of atherosclerosis.\cite{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.\cite{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.\cite{31,32} Indeed, several potential binding sites for NF-κB have been found in the *CCR5* P1-promoter.\cite{32,33} However, the results of the study by Kuipers et al. indicate that CCR5 expression is neither induced nor modulated by NF-κB.\cite{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-κB 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 represor (ICER), which is induced by forskolin treatment, the authors concluded that *CCR5* transcription is regulated by CREB-1.\cite{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.\cite{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.\textsuperscript{35}

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

It has been proposed that a bivalent domain (characterized by the presence of H3K4Me3 and H3K27Me3) is merely an intermediate state.\textsuperscript{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).\textsuperscript{42,43} Additionally, removal of the H3K27Me3 (considered as a repressive chromatin mark) facilitates the recruitment of a H3K4 methyltransferase.\textsuperscript{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.\textsuperscript{40}

Proximal modifications that constitute a putative ‘code’ need not be restricted to a single histone tail as originally anticipated,\textsuperscript{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.\textsuperscript{45} Furthermore, the multivalency of chromatin marks may also be thermodynamically more favorable then monovalent histone marks.\textsuperscript{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.\textsuperscript{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. 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 foam cells 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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