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Review

Epigenetics of Atherosclerosis: Emerging Mechanisms and Methods

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Atherosclerosis is a vascular pathology characterized by inflammation and plaque build-up within arterial vessel walls. Vessel occlusion, often occurring after plaque rupture, can result in myocardial and cerebral infarction. Epigenetic changes are increasingly being associated with atherosclerosis and are of interest from both therapeutic and biomarker perspectives. Emerging genomic approaches that profile DNA methylation, chromatin accessibility, post-translational histone modifications, transcription factor binding, and RNA expression in low or single cell populations are poised to enhance our spatiotemporal understanding of atherogenesis. Here, we review recent therapeutically relevant epigenetic discoveries and emerging technologies that may generate new opportunities for atherosclerosis research.

Introduction

Atherosclerosis is characterized by a pathological build-up of plaque within arterial vessel walls and contributes to cardiovascular diseases, the leading cause of death in developed nations (reviewed in [1]). Recent findings suggest that the pathogenesis of atherosclerosis involves dynamic changes in epigenetic modifications and gene expression in a cell type- and stage-specific manner [2,3]. Epigenetics can be defined as the processes whereby a cell retains a memory of past cellular states and perturbations without altering the DNA sequence itself (reviewed in [4]). Much remains to be discovered regarding the epigenetic mechanisms that are operative in atherosclerosis, and this is due in part to the difficulty in analyzing specific cell types in the complex milieu of the plaque (see Outstanding Questions). However, emerging technological advances (Table 1) and multiple complementary experimental systems for studying atherosclerosis (Box 1) are rapidly transforming the approaches that can be utilized to understand the molecular basis of this disease. Here, we explore the known roles of epigenetics in the etiology of atherosclerosis and provide a guide to researchers and clinicians regarding emerging technologies for epigenetic profiling. Finally, we discuss how epigenetic pathways might be harnessed or antagonized for the development of therapeutics.

Atherosclerosis: A Chronic Inflammatory Disease Involving Multiple Cell Types

In humans, atherosclerosis can affect multiple vascular beds, within the heart [**coronary artery disease** (CAD), see Glossary], the brain (**cerebrovascular disease**) and the periphery (**peripheral artery disease**), among others. Risk factors for atherosclerosis include elevated circulating **low-density lipoprotein** (LDL) and triglyceride levels, smoking, obesity, and aging. In response to these pathological stimuli, vascular endothelial cells (ECs), the cells of the inner lining of blood vessels, become activated and recruit monocytes from the circulation. Upon

Trends

Recent studies have implicated epigenetic regulation in the etiology of atherosclerosis, but how epigenetic pathways are altered in specific cell types in the complex milieu of the plaque is not known.

Emerging genomic technologies are circumventing the issue of limited material and can be used to resolve the epigenome in atherosclerosis at a single cell level.

Epigenetic changes in atherosclerosis models as well as systems genetics approaches are providing insight into the effect of human genetic variations on atherosclerotic disease, especially variation within noncoding regions of the genome.

Drugs that modify epigenetic pathways (e.g., DNA methylation, histone acetylation/methylation, and enhancer regulation) have shown promise in preclinical models of atherosclerotic disease. A further understanding of epigenetic mechanisms will open new avenues for treatment.

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entering the vascular wall, monocytes differentiate into macrophages and, following uptake of lipids, can further differentiate into **foam cells**, which are retained underneath the endothelial layer (reviewed in [1]). Over decades, the amassing of foam cells in the **neointimal layer**, coupled with defective clearance of cellular debris (i.e., **efferocytosis** [5]), result in the growth of an atherosclerotic plaque. The dedifferentiation, migration, and proliferation of **vascular smooth muscle cells** (vSMCs) initially stabilize the plaque through deposition of extracellular matrix proteins, such as collagen (reviewed in [1]). However, during late stages of atherogenesis, plaque destabilization through the activity of **metalloproteases** can lead to plaque rupture, resulting in myocardial and cerebral infarctions, which significantly contribute to the morbidity and mortality from atherosclerotic disease (reviewed in [1]). Recent evidence reveals that vSMCs can also differentiate into macrophage- and mesenchymal stem cell-like cells that promote lesion expansion and instability [6].

Atherosclerosis and Epigenetics: Biology and Biomarkers

DNA sequences encode sufficient information to direct transcription factor (TF) binding, histone post-translational modifications (PTMs), and DNA methylation at ***cis*-regulatory elements**, such as promoters, **enhancers**, and **boundary elements** [7–10]. Once epigenetic modifications, such as DNA methylation and histone PTMs, are established, these marks can serve to propagate cellular memory. Importantly, epigenetic marks are not static, because they can be altered by environmental stimuli. Such alterations have the potential to modify the dynamics of subsequent TF binding, gene expression, and cell phenotype [11–13]. For example, transplantation of macrophages into a new tissue environment in mice can alter epigenetic profiles and gene expression to more closely match those of tissue-resident macrophages [14,15]. Genome-wide epigenomic mapping techniques are now being applied to tissues from a diverse set of healthy and pathological adult and fetal mammalian samples [16–18]. In this manner, various conditions can be analyzed, including inflammatory diseases, thereby providing a global view of the epigenetic changes that might be associated with pathological conditions *in vivo* [16–18]. These genome-wide epigenetic maps are providing base-pair resolution insight into the regulatory landscape of tissues, and are identifying regions in the genome where DNA variants may impact disease processes [19] (Boxes 2 and 3).

Multiple layers of epigenetic regulation (e.g., DNA methylation, histone PTMs, and noncoding RNA) cooperate to control gene expression. Through the activity of **DNA methyltransferases** (DNMTs), methyl groups are added to cytosines, primarily on CpG dinucleotide sequences, and this is typically associated with low levels of transcription (i.e., transcriptional repression) [20]. DNA can also be hydroxymethylated, with recent studies revealing high levels of this modification in gene bodies and enhancers of actively transcribed genes [21]. Histone modifications are dynamically governed by various **chromatin-remodeling complexes** (reviewed in [22]). Respectively, '**writer**' and '**eraser**' complexes deposit or remove covalent modifications, such as methyl, acetyl, or ubiquitin marks, onto lysine (or, in some cases, arginine) residues of histone proteins. For example, trimethylation of lysine 27 of histone 3 (**H3K27me3**) is a repressive mark that is deposited by the **polycomb group (PcG) repressive complex 2** (PRC2) and erased by the **Jumonji C domain-containing** (JMJD) **histone demethylases** [22]. Furthermore, establishment of active *cis*-regulatory elements through acetylation of histones [e.g., histone 3 at lysine 27 (**H3K27ac**)] is accomplished by the dynamic activity of **histone acetyltransferases** (HATs), such as p300 (EP300) and **histone deacetylases** (HDACs) [23]. Histone PTMs are subsequently read by '**reader**' proteins, such as **bromodomain-containing proteins**, which bind acetyl groups [22]. In many cases, TFs guide histone writers, erasers, and/or readers to target sites in chromatin. For example, p300 is recruited by the proinflammatory transcription factor complex, **nuclear factor κ-light-chain enhancer of activated B cells** (NF-κB) to inflammation-induced enhancers in cultured human ECs. This in turn directs the hyperacetylation of chromatin that serves as an anchor for

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Box 1. Experimental Systems for Studying Atherosclerosis

Assessing the molecular mechanisms involved in human atherogenesis is complicated by the length of time the disease takes to manifest, and the complex cellular and microenvironmental components that directly contribute to disease progression. Epidemiological studies and **genome-wide association studies** (GWAS) in humans have revealed multiple risk factors and >200 candidate genes related to CAD [112–115]. System genetics approaches that combine experimental, computational, and statistical approaches to integrate existing genetic variation in populations, in combination with epigenetic, gene expression, and proteomic profiling in relevant cell types, are an increasingly powerful way to link genes to phenotypes [19,116,117].

Human cell culture strategies are essential for testing the effect of human genetic variation on gene expression, despite known cell culture-induced effects on gene expression (e.g., [15,118,119]). Technological advances have dramatically increased the power of human cell-based research to deliver both mechanistic and therapeutic insights (Table 1, main text). These advances include utilizing systems that exploit **induced pluripotent stem cell-derived cells**, **tissue-engineered blood vessels**, **CRISPR/Cas9** genome and epigenome editing, and microfluidic-based 2D and 3D cell culture methods.

Nevertheless, animal models, in particular mouse models of atherosclerosis, continue to be instrumental in deciphering atherosclerosis-related disease mechanisms (reviewed in [120]). The most widely used atherosclerosis models rely on hyperlipidemia, which can be induced by deletion of the genes encoding Low-density lipoprotein receptor (*Ldlr*) or Apolipoprotein E (*Apoe*) in mice. New approaches include the use of adeno-associated viral constructs to express gain-of-function proprotein convertase subtilisin/kexin type 9 (Pcsk9) protein in mice, which also drives hypercholesterolemia [121]. In these mouse models, atherosclerosis develops rapidly and reproducibly, primarily at regions of disturbed blood flow, such as vessel curvatures and branch points.

However, certain aspects of human disease cannot be adequately modeled in mice, rendering large animal models an integral part of preclinical research (reviewed in [122]). Finally, epigenetic studies of human atherosclerotic lesions themselves are yielding new biological insights and putative biomarkers to follow disease progression [26].

bromodomain-containing protein 4 (BRD4), a member of the BET family of bromodomain-containing proteins, facilitating transcriptional elongation [2].

DNA Methylation: Genome-Wide Hypermethylation Is Associated with Atherogenesis

Human and mouse studies have noted global DNA hypermethylation of cytosines in the context of CpGs as an accompanying feature of atherosclerosis [3,24,25]. Indeed, a positive correlation between DNA methylation and atherosclerotic lesion grade was discovered by using genome-wide DNA methylation sequencing (i.e., **bisulfite sequencing**) of healthy and atherosclerotic human aortas [26]. Differentially methylated regions within loci of cardiovascular disease-associated genes in ECs isolated from atherosusceptible regions of porcine aortas were also discovered using **methylated DNA immunoprecipitation sequencing** (meDIP-seq) [27]. These findings demonstrated that DNA methylation profiling could reveal atherosclerosis biomarkers, suggesting a potential role for DNA methylation in disease progression. Recent advances in low or single cell techniques (Table 1) will facilitate further characterization of DNA methylation in atherosclerotic plaques of humans and atherosclerotic models.

Evidence from *in vitro* studies suggests that DNA methylation is regulated by inflammatory signaling pathways (Figure 1). For example, treatment of human umbilical vein ECs (HUVECs) with proinflammatory stimuli, such as oxidized LDL (oxLDL), was shown to upregulate DNMT1 and lead to methylation of the promoter of the gene encoding Krüppel-like factor 2 (*KLF2*) [28]. KLFs are a family of anti-inflammatory TFs (reviewed in [1]), and the resulting repression of *KLF2* increases proatherogenic endothelial inflammation [28]. The effect of oxLDL on *KLF2* methylation could be reversed by treating ECs with the DNMT inhibitor, 5′-azacytidine (5-AZA), a chemical analog of cytosine that blocks DNMT activity [28]. Moreover, proatherogenic-disturbed blood flow (the non-uniform flow of blood at arterial curvatures and branches) has a role in DNA methylation. ECs exposed *in vitro* to disturbed blood flow patterns displayed increased levels of DNMT1 and, consequently, presented DNA hypermethylation of their genome [29].

Glossary

Assay for transposase-accessible chromatin using sequencing (ATAC-seq): next-generation sequencing (NGS) technique for mapping genome-wide chromatin accessibility using transposition reaction.
Bisulfite sequencing: NGS technique for the identification of DNA methylation patterns where bisulfite treatment is used to convert cytosine to uracil without affecting 5-methylcytosine.
Boundary element: genomic element (such as an insulator) that blocks interaction between distal DNA regions, such as enhancers and promoters. This can be achieved through the activity of repressor proteins, such as CTCF.
Bromodomain-containing proteins: protein complexes that function as epigenetic readers, which can recognize and bind acetylated lysines on histones.
Capture Hi-C: Hi-C method that utilizes target sequence enrichment for the genome-wide detection of enhancer–promoter interactions.
Cerebrovascular disease: a group of conditions that impact the supply of blood and oxygen to the brain; examples include stroke and aneurism.
Chromatin immunoprecipitation sequencing (ChIP-seq): a commonly used epigenomic assay for surveying TF binding or histone modification throughout the genome using chromatin pull-down with an antibody of interest followed by NGS.
Chromatin remodeling complexes: groups of proteins responsible for the packaging of chromatin and regulating gene expression.
***Cis*-regulatory elements:** noncoding DNA sequences, such as enhancers and promoters, that are bound by TFs and regulate gene expression.
Clustered regularly interspaced short palindromic repeats/ CRISPR associated protein9 (CRISPR/Cas9): a bacterial defense system against viruses that has been exploited for genome engineering.
Coronary artery disease (CAD): condition where the arteries of the heart have a build-up of plaque, which decreases blood supply to the heart.

Box 2. Human Genetic Variation and Epigenetics of Inflammatory Disease

Over 90% of genetic variants identified through GWAS fall within regions of noncoding DNA, complicating the functional interpretation of mutations in complex diseases [123]. Indeed, more than 85% of single nucleotide polymorphisms (SNPs) contributing to CAD reside within intronic and intergenic sequences, and many of these SNPs are located in the vicinity of genes implicated in the pathogenesis of atherosclerosis [124].

Integration of GWAS results with epigenomic and transcriptomic data is crucial for the biological characterization of noncoding disease variants. Not only does linking a noncoding variant to the expression of a CAD gene (or genes) elucidate biological pathways, but it is also a requisite for functional studies *in vivo*. For example, new atherosclerosis-causing endothelial mechanotransduction pathways involving a CAD-associated gene [encoding Phosphatidic Acid Phosphatase Type 2B (*PPAP2B*)], were discovered in human aortic ECs in this manner [115].

Chromatin conformation assays are also increasingly being used to link noncoding GWAS variants associated with inflammatory diseases to distal enhancers and to identify new candidate regions and regulated genes [125–127]. For instance, a promoter of a cytokine receptor gene encoding interleukin 20 receptor subunit alpha (*IL20RA*) was recently shown to contact a distal genetic locus (i.e., 6q23) associated with multiple inflammatory diseases, such as rheumatoid arthritis and systemic lupus erythematosus, as identified using **capture Hi-C** in human lymphoblastoid cell lines [126].

The enrichment of GWAS SNPs in **DNase I hypersensitive sites** [123], enhancer–promoter contact regions [128], active chromatin marks [129], and **expression quantitative trait loci** (eQTLs) [129] imply that disease variants alter *cis*-regulatory elements. For example, a noncoding variant (rs12740374) associated with CAD and serum levels of LDL cholesterol was shown to alter the expression of Sortilin 1 (*SORT1*) in human liver eQTL analyses [114]. Subsequent analysis in cultured human hepatocytes revealed that this SNP modified the binding site of a liver-enriched transcription factor, CEBPA [114]. Moreover, a noncoding myocardial infarction risk allele (rs9349379) has been predicted to affect MEF2 binding *in silico* and deletion of this allele using CRISPR/Cas9 resulted in reduced MEF2 binding *in vitro* as well as decreased expression of the gene encoding Phosphatase and Actin Regulator 1 (*PHACTR1*) in human ECs [130].

Noncoding GWAS SNPs associated with CAD risk have also been shown to disrupt the binding of Signal transducer and activator of transcription 1 (STAT1), enhancing the expression of the lncRNA *ANRIL* in human lymphoblastoid cell lines [131]. NF-κB-binding regions in human lymphoblastoid cell lines are also enriched with SNPs associated with inflammation diseases that affect NF-κB binding and gene expression [54], and a GWAS-identified risk locus that has been associated with systemic lupus erythematosus results in diminished NF-κB binding and reduced expression of the gene encoding TNF Alpha Induced Protein 3 (*TNFAIP3*); this protein is a negative regulator of NF-κB signaling [132]. It has also been noted in human lymphoblastoid cell lines that a single variant can disrupt binding of multiple TFs that act cooperatively and facilitate each other's recruitment, such as NF-κB and JUND, which are both involved in the inflammatory response [133,134].

Similarly, DNMT3A was found to be upregulated *in vitro* in human aortic ECs exposed to disturbed blood flow and, *in vivo,* in regions of the porcine aorta experiencing disturbed flow [30]. DNMT3A upregulation was accompanied by methylation of the *KLF4* promoter, decreased KLF4 levels, and increased vascular inflammation [30] (Figure 1). Furthermore, global DNA hypermethylation has been observed in peripheral lymphocytes of patients with CAD, and may be linked to the inflammatory activity of innate immune cells [31]. In *Ldlr*$^{-/-}$ and *Apoe*$^{-/-}$ mouse models, intraperitoneal injections of 5-AZA reduced the atherosclerotic lesion burden, as measured by **Oil red-O** staining and macrophage accumulation in plaques [32,33]. Thus, targeting DNA methylation pathways may represent a promising avenue for therapy in atherosclerosis, similar to current clinical uses of DNA-hypomethylating agents in leukemia [34] (Figure 2). However, further work is needed to decipher cell- and gene-specific DNA methylation changes (especially in humans) and to determine whether treatment with DNMT inhibitors has disparate effects at distinct stages of disease.

Histone PTMs: Complex Cell Type-Dependent Effects on Atherogenesis

The role of histone 3 (H3) methylation in atherosclerosis, which mediates transcriptional activation or repression depending on the modification, is only just emerging. While one study probing the H3K27me3 repressive mark using immunohistochemistry found no global changes in aortic ECs of *Apoe*$^{-/-}$ mice, levels were decreased in vSMCs [35]. In addition, a separate

Curcumin: bright-yellow chemical found in turmeric that acts to decrease inflammation and is a known p300 histone acetyltransferase inhibitor.
DNA methyltransferases (DNMTs): protein complexes that act as epigenetic writers that add methyl marks to cytosines, particularly at CpG sites.
DNase I hypersensitive sites: open chromatin regions with exposed DNA that are sensitive to deoxyribonuclease I (DNase I) cleavage.
DNase I hypersensitive sites sequencing (DNase-seq): NGS technique for mapping chromatin accessibility using DNaseI.
Efferocytosis: the process by which macrophages and other phagocytic cells take up and clear apoptotic cells.
Enhancer: region of TF binding in the genome that influences the expression of genes. The mechanism of action often involves chromatin looping to allow for enhancer–promoter interactions.
Eraser: a type of chromatin remodeling complex that is responsible for removing histone marks.
Expression quantitative loci (eQTLs): DNA sequence variants associated with differential gene expression levels.
Fixed tissue ChIP-seq (FiT-seq): ChIP-seq method that enables extraction of soluble chromatin from formalin-fixed paraffin-embedded (FFPE) tissue samples.
Fluorescence-activated sorting of fixed nuclei (FAST-FIN): method that isolates cell type-specific nuclei from a fixed tissue while preserving post-translational protein modifications.
Foam cell: type of macrophage that takes up LDL and accumulates within atherosclerotic plaques.
Genome-wide association studies (GWAS): studies linking particular phenotypes with regions of the genome. In human populations, these can be used to identify genetic variations that are associated with a disease of interest.
H3K27ac: acetylation of the 27th lysine on histone 3. This histone mark can be indicative of active enhancers.
H3K27me3: trimethylation of the 27th lysine on histone 3. This histone mark is typically repressive, but can

Box 3. Clinician's Corner

Epigenetics encompasses changes in gene expression without altering the underlying DNA sequence. Principally, epigenetic changes occur through covalent modification of DNA and post-translational modification of histones. Epigenetic dysregulation has been implicated in multiple diseases, and is emerging as a powerful biomarker, rendering it an important area of study.

The investigation of epigenetic changes in atherosclerosis is still in its infancy. Distinguishing which epigenetic changes are caused by the disease, and which ones contribute to disease, is an area of active research. Changes in DNA methylation, histone methylation, and histone acetylation between healthy and atherosclerotic tissue have been documented. However, these studies have relied principally on global assessment of the levels of these marks. Most insights into the mechanistic role of epigenetics in atherosclerosis have come from *in vitro* work.

Recent developments in genomics technologies, especially those that dissect DNA methylation, open chromatin, or RNA expression at the single cell level, will facilitate the study of epigenetic changes in clinical atherosclerotic samples. It is even becoming possible to carry out some of these studies using fixed samples embedded in paraffin. Hence, it will be important to collect and catalogue human atherosclerotic samples for future analyses.

Given the success of epigenetic drugs in the treatment of cancer, studying epigenetic changes in atherosclerosis could aid drug development. It will be important to determine how these drugs affect the cells that participate in atherosclerosis and to understand their impact at distinct stages of the disease.

Studying the epigenetics of atherosclerosis will also help fine map genetic variations that associate with disease susceptibility. Insight into the function of these polymorphisms will be crucial for assessing a patient's risk of developing atherosclerosis. This could guide early life-style interventions alongside prophylactic treatment.

study also observed decreased levels of H3K27me3 in vSMCs from the medial layer of advanced human atherosclerotic plaques [36]. Interestingly, levels of the chromatin remodeler erasing H3K27me3 marks, JMJD3, were increased in mouse macrophages in response to lipopolysaccharide (LPS), a potent inflammatory stimulus [37]. JMJD3 is known to bind gene targets that are normally repressed by PcG complexes, and can also mediate the removal of H3K27me3, modulating gene activation [37]. Additionally, treatment with the cell permeable prodrug GSK-J4 (which is hydrolyzed to GSK-J1, a potent inhibitor of JMJD3 activity) decreased the expression of proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), in LPS-stimulated primary human macrophages *in vitro* [38] (Figure 2). However, further work is necessary to test whether chemical inhibition of JMJD3 has a protective effect in atherosclerotic mouse models. As described in Table 1, technologies to probe H3K27me3 and other histone PTMs genome-wide in small samples will be vital to fully understand the cause and consequence of epigenetic changes that occur during atherogenesis.

Histone acetylation is dynamically regulated, and has an important role in the establishment and maintenance of transcription. Several studies have assessed the involvement of HDACs and HATs in atherosclerosis, often with conflicting results (Figures 1 and 2). On the one hand, HDAC3, HDAC5, and HDAC7 can repress *KLF4* expression in HUVECs, and their overexpression leads to a proatherogenic phenotype [39]. In mice, *Hdac3* deletion in macrophages could increase the expression of IL-4-activated genes, resulting in an alternatively activated anti-inflammatory phenotype attributed to a 'reprogramming'-like effect [40]. *Hdac9* deletion in mice also decreased macrophage inflammatory gene expression and cytokine secretion in response to LPS [41]. In rats, HDACs are upregulated in aortic SMCs under mitogenic stimulation and inhibition of HDACs alters the expression of cell cycle genes, resulting in diminished SMC proliferation, which might be expected to be atheroprotective [42,43] (Figure 1). On the other hand, HDACs appear to be protective in atherosclerosis models. Indeed, *Apoe*$^{-/-}$ mice in which *Hdac3* was knocked down in ECs, exhibited increased neointimal formation, suggesting an atheroprotective role of endothelial HDAC3 [44]. Likewise, *Ldlr*$^{-/-}$ mice given intraperitoneal injections of the HDAC inhibitor, Trichostatin A, exhibited increased plaque size and enhanced macrophage infiltration into plaques [45]. This effect was attributed

also be indicative of poised enhancers.

Heterogeneous nuclear ribonucleoproteins (hnRNP): protein and RNA complexes that have a role in gene transcription and post-transcriptional modification of recently transcribed mRNA.

Hi-C: chromosome conformation capture method for genome-wide detection of spatial chromatin organization

Histone acetyltransferases (HAT): groups of epigenetic writers responsible for adding acetyl groups to the lysines of histones. Their function is antagonistic to histone deacetylases.

Histone deacetylases (HDAC): groups of epigenetic erasers responsible for removing acetyl groups from the lysines of histones. Their function is antagonistic to histone acetyltransferases.

Indexing-first ChIP-seq (iChIP-seq): ChIP-seq method on a small number of cells where sonicated chromatin from multiple cell populations is barcoded and pooled together for ChIP and library preparation.

Induced pluripotent stem cell-derived cells: cells derived from induced pluripotent stem cells through the process of differentiation.

Inflammasome: system of receptors and sensors that regulate caspase-1 activation and activate an innate immune response to clear infections and respond to microbe-derived molecules.

Isolation of nuclei tagged in specific cell types (INTACT): method for isolating cell type-specific nuclei from tissues where transgenic expression of biotinylated nuclear membrane protein in a cell type of interest tags nuclei for affinity isolation with streptavidin magnetic beads.

Jumonji C domain containing (JMJD) histone demethylases: group of epigenetic erasers responsible for removing methyl marks from the lysines of histones. Their function is antagonistic to that of polycomb group proteins.

Laser capture microdissection: (LCM) method for isolation of specific cells where a laser coupled to a microscope is used to cut out cells of interest from a tissue.

Lineage-determining TFs: pioneer TFs that define lineage-specific gene expression patterns.

to the upregulation of **scavenger receptor CD36**, which in turn could increase macrophage oxLDL uptake and foam cell formation [45]. Taken together, these studies illustrate the complex role of individual HDACs in modulating atherosclerosis, and suggest that cell type-specific roles exist at different times during atherogenic progression.

Histone acetylation by HATs appears to have a proatherogenic role, regulated in part, through inflammatory transcriptional pathways. For example, **curcumin**, a p300 HAT inhibitor, has been shown to enhance macrophage cholesterol efflux in human and mouse macrophages *in vitro* and exerts an anti-inflammatory effect via the downregulation of NF-κB activity [46–48] (Figure 2). Immunohistochemistry analysis of early and advanced human atherosclerotic plaques revealed increased staining for active H3K9ac and H3K27ac marks compared with healthy controls [49]. This enhanced histone acetylation was found to localize to ECs, macrophages, and vSMCs, and could indicate enhanced global transcriptional activity [49]. The impact of p300-mediated regulation of NF-κB signaling is one potential mechanism linking histone acetylation with atherosclerosis. Upon receipt of an inflammatory stimulus, p300 interacts with the p65 (RELA) subunit of NF-κB in the nucleus to displace repressive p50–HDAC1 interactions [50]. This results in increased acetylation of both p65 itself and histones in the vicinity of NF-κB binding, driving NF-κB-induced transcription by prolonging its nuclear retention and establishment of enhancer regions [2,51]. The p65–p300 interaction has been found to be critical for the inflammatory activation of both mouse macrophages and HUVECs [2,52].

Overall, the conflicting results surrounding the activity of HDACs/HATs is likely due, at least in part, to the broad effects that HDAC/HAT inhibitors can have on multiple protein family members, resulting in the modulation of genome-wide transcriptional pathways in multiple cell types (Figure 2). New genomic technologies that profile gene expression and DNA methylation in single cells, and histone modifications in low cell populations (Table 1) should allow for the unprecedented molecular dissection of the functional impact of epigenetic modifications during atherogenesis.

Inflammation-Induced NF-κB Signaling: 'A View From the Genome'

Only recently has the genome-wide binding of NF-κB to chromatin been elucidated in cell types of relevance to atherosclerosis, such as human ECs [2] and mouse macrophages [53]. The functional importance of these *cis*-regulatory elements in inflammatory diseases is illustrated by the finding that common single nucleotide polymorphisms (SNPs) associated with inflammatory disease conditions, such as myocardial infarction and rheumatoid arthritis, are enriched in NF-κB-bound elements [54]. It is now appreciated that NF-κB either binds to regions of the genome that are maintained in an accessible chromatin state through the activity of **lineage-determining TFs** or, alternatively, directs the formation of *de novo* enhancers through chromatin remodeling. In macrophages, most enhancers that NF-κB binds to following inflammatory activation are already prebound by a hematopoietic lineage factor, PU.1 (SPI1), in a cell type-specific manner [53]. The *de novo* formation of latent NF-κB enhancers depends on **SWI/SNF chromatin-remodeling complexes**, since these regions are highly occupied by **nucleosomes** [11]. The assembly of *de novo* NF-κB enhancers in activated macrophages involves collaborative binding of NF-κB and lineage-determining TFs, PU.1, and CCAAT/enhancer-binding protein (CEBPA) to closed chromatin; this is followed by the *de novo* establishment of enhancers through recruitment of HATs and RNA polymerase II [52]. Most *de novo* enhancers do not return to their previous ground epigenetic state once stimulation ends, but instead maintain enhancer marks that can mediate quick and robust responses once cells are restimulated [11]. The presence of such epigenomic memory might explain some of the epigenetic basis of chronic inflammatory disease conditions, although this area remains relatively unexplored.

Linear DNA amplification ChIP-seq (LinDA-ChIP-seq): ChIP-seq method for limited amount of cells where a single-tube T7 RNA polymerase linear amplification is used to amplify small quantities of ChIP DNA.
Low density lipoprotein (LDL): class of lipoproteins responsible for transporting cholesterol through the bloodstream. Elevated blood levels of LDL have been associated with atherosclerosis.
Low-input DNase I sequencing (liDNase-seq): method for assessing chromatin accessibility in small numbers of cells where loss of DNA is reduced by using circular carrier DNA as well as modifications to the scDNase-seq protocol, such as reduced purification steps before adaptor ligation and use of solid-phase reversible immobilization beads for size selection.
Metalloproteases: proteases that require metals for their catalytic activity.
Methylated DNA immunoprecipitation sequencing (meDIP-seq): NGS technique for mapping genome-wide DNA methylation patterns that is also able to distinguish 5-hydroxymethylcytosine (5hmC) from 5-methylcytosine (5mC) by using antibodies against 5mC.
Multiplexed Indexed T7 ChIP-seq (mint-ChIP-seq): method optimized to work on small cell numbers (~500 cells) where barcoded samples are pooled and then split for parallel ChIPs. The ChIP DNA is then linearly amplified using T7 RNA polymerase.
Neointimal layer: newly formed layer of the intima observed in pathologies such as atherosclerosis and restenosis. It is formed by vSMCs migrating from the medial region, recruitment of monocytes from the circulation and macrophage proliferation.
Nuclear factor κ-light-chain enhancer of activated B cells (NF-κB): transcription factor family that drives the expression of inflammatory genes and cell survival. Nuclear translocation of NF-κB subunits occurs in response to inflammatory stimuli.
Nucleosome: functional subunit of eukaryotic chromatin comprising DNA wrapped around core histones.
Oil Red-O: fat-soluble dye used to stain lipids and neutral triglycerides; commonly used in atherosclerosis

Recent studies have begun to uncover the genome-wide function of NF-κB in ECs, as well as the potential therapies that might stem from targeting these pathways. For instance, activation of human ECs with the proinflammatory cytokine TNF-α results in the formation of large NF-κB enhancer clusters, recently termed '**super-enhancers**', which are highly occupied by BRD4, and are capable of driving proinflammatory gene expression [2]. Although the individual enhancers within super-enhancers may be indistinguishable from other enhancers [55,56], their high cofactor occupancy and cell type specificity make them intriguing genomic entities [57,58]. Interestingly, following TNF-α stimulation, super-enhancers around basal EC identity genes, such as *SOX18*, are lost, while new NF-κB super enhancers are formed *de novo* around proinflammatory genes, such as that encoding chemokine (C-C motif) ligand 2 (*CCL2*; also known as monocyte chemoattractant protein 1) [2]. In this study, the loss and formation of super-enhancers was attributed to a global redistribution of BRD4 from endothelial cell identity super-enhancers to NF-κB-driven inflammatory super-enhancers [2]. This highlights the functional importance of these NF-κB- and BRD4-driven *cis*-regulatory elements, which appear to shift the cellular state from a basal to a proinflammatory state. A similar redistribution of enhancers also occurs in LPS-induced mouse macrophages [59] and TNF-α-induced human adipocytes [60].

Inflammation-induced super-enhancers established through NF-κB and BRD4 appear to be exquisitely sensitive to perturbations in components of the super-enhancer, as shown by their responsiveness to BRD4 inhibitors in human ECs and adipocytes [2,60]. Thus, it is possible that BRD4 could be a promising therapeutic target in inflammation and cardiovascular disease [2,61–63] (Figure 2 and Box 3). Importantly, clinical trials have recently been initiated for this class of inhibitors for the treatment of cardiovascular diseases [63] and various cancers [64]. The cell permeable small-molecule BET inhibitor known as JQ1 binds to bromodomains of BRD4 and preferentially depletes BRD4 at established inflammatory super-enhancers in human ECs, resulting in impaired transcription of proinflammatory genes, such as *CCL2* and that encoding vascular cell adhesion molecule 1 (*VCAM1*) [2]. This, in turn, has been reported to lead to suppression of leukocyte extravasation through the endothelium, as well as decreased atherosclerotic plaque formation in $Ldlr^{-/-}$ atherogenic mouse models [2]. JQ1 has also been reported to decrease the transcription of proinflammatory genes, such as *TNFA* and that encoding interleukin-6 (*IL6*) in fibroblast-like synoviocytes of patients with rheumatoid arthritis, and *Il17a* and *Il22* in mouse models of **psoriasis** (reviewed in[61]). Another similar BET inhibitor, known as I-BET, decreases the transcription of proinflammatory genes, such as *Il6* and *Il1b,* in LPS-induced mouse macrophages, and injection into mouse models of severe sepsis has been found to protect against death (reviewed in [61]). Thus, BET inhibitors appear to exert a broad anti-inflammatory effect.

Long Noncoding RNAs: Modulators of Inflammatory Signaling in Atherosclerosis

Long noncoding RNAs (lncRNAs) are RNA transcripts >200 nucleotides in length that do not encode a functional protein. While our understanding of their biology is still preliminary, growing evidence has identified a subset of lncRNAs that recruit chromatin-modifying factors, TFs, and **heterogeneous nuclear ribonucleoproteins** (hnRNPs) to DNA, thus controlling epigenetic and/or transcriptional processes. Moreover, lncRNAs can act post-transcriptionally by regulating translation, splicing, and mRNA stability (reviewed in [65]). Several lncRNAs have been identified as epigenetic regulators in vascular diseases (Figure 3). For example, the lncRNA antisense noncoding RNA in the Ink4 locus (*ANRIL*) is transcribed from the human 9p21.3 locus and its levels are affected by DNA polymorphisms in this region; its expression is highly associated with the incidence of CAD [66,67]. *ANRIL* promotes the proliferation of human vSMCs by recruiting repressive PcG protein complexes to its neighboring cell cycle inhibitor genes, *CDKN2A/B* [68,69]. Additionally, *ANRIL* is NF-κB inducible in ECs and upregulates the

studies to measure lipid burden in plaques.

Pathology Tissue ChIP-seq (PAT-ChIP-seq): ChIP-seq method on paraffin-embedded pathology tissues.

Peripheral artery disease: conditions where there is reduced blood supply to the extremities, such as the legs. The narrowing of blood vessels typically causes these conditions.

Polycomb group repressive complex 2 (PRC2): type of chromatin writer that is responsible for the addition of the repressive H3K27me3 mark.

Psoriasis: chronic inflammatory skin condition characterized by a rapid growth of skin cells.

Reader: type of chromatin remodeling complex that can recognize and bind to specific histone modifications.

Scavenger receptors: group of receptors that recognize acetylated or oxidized LDLs to increase their uptake.

Shear stress: frictional force exerted by blood flow at the blood vessel luminal surface.

Single-cell DNase-seq (scDNase-seq): method used on small cell numbers and single cells where circular carrier DNA is used to prevent loss of DNase I hypersensitive DNA, which is then PCR-amplified in two steps.

Super-enhancer: cluster of *cis*-regulatory elements with high co-activator occupancy.

SWI/SNF chromatin remodeling complexes: responsible for chromatin remodeling and mobilization of nucleosomes with the use of energy from ATP hydrolysis.

Tissue-engineered blood vessels: the process of incorporating SMCs and ECs to a tubular scaffold to establish an engineered blood vessel that has a similar architecture to a physiological blood vessel.

Ultra-low-input micrococcal nuclease-based native ChIP-seq (ULI-NChIP-seq): ChIP method utilizing micrococcal nuclease (MNase) for chromatin fragmentation to study histone modifications in native (noncross-linked) chromatin in rare cells.

Vascular smooth muscle cells (vSMC): critical component of the outer layer of arteries, providing structure to arteries, and regulating

their diameter to modulate blood pressure.
Writer: type of chromatin remodeling complex responsible for adding histone marks.

expression of the inflammatory genes *IL6* and *IL8* via interaction and recruitment of the transcription factor, YY1 [70]. Another lncRNA implicated in atherosclerosis, *lincRNA-p21*, is downregulated in atherosclerotic plaques of *Apoe*$^{-/-}$ mice and human coronary arteries [71]. Moreover, knockdown of *lincRNA-p21* has been shown to enhance the expansion of the neointima in a mouse model of carotid artery vascular injury, dependent in part, on the regulation of p53-dependent apoptotic genes in vSMCs [71].

In the context of inflammation and innate immunity, multiple lncRNAs have been characterized. In mouse bone marrow-derived macrophages stimulated with LPS, *lncRNA-Cox2* was induced and shown to regulate hundreds of genes, with a significant portion being inflammatory genes [72]. Although the full mechanism remains to be determined, *lncRNA-Cox2* can interact with hnRNP A/B and hnRNP A2/B1, which may mediate transcriptional repression [72]. Additionally, *lincRNA-EPS* was found to be downregulated in activated mouse bone marrow-derived macrophages [73]. Genetic deletion and ectopic overexpression experiments highlighted the importance of *lincRNA-EPS* in restraining LPS-induced expression of a vast number of

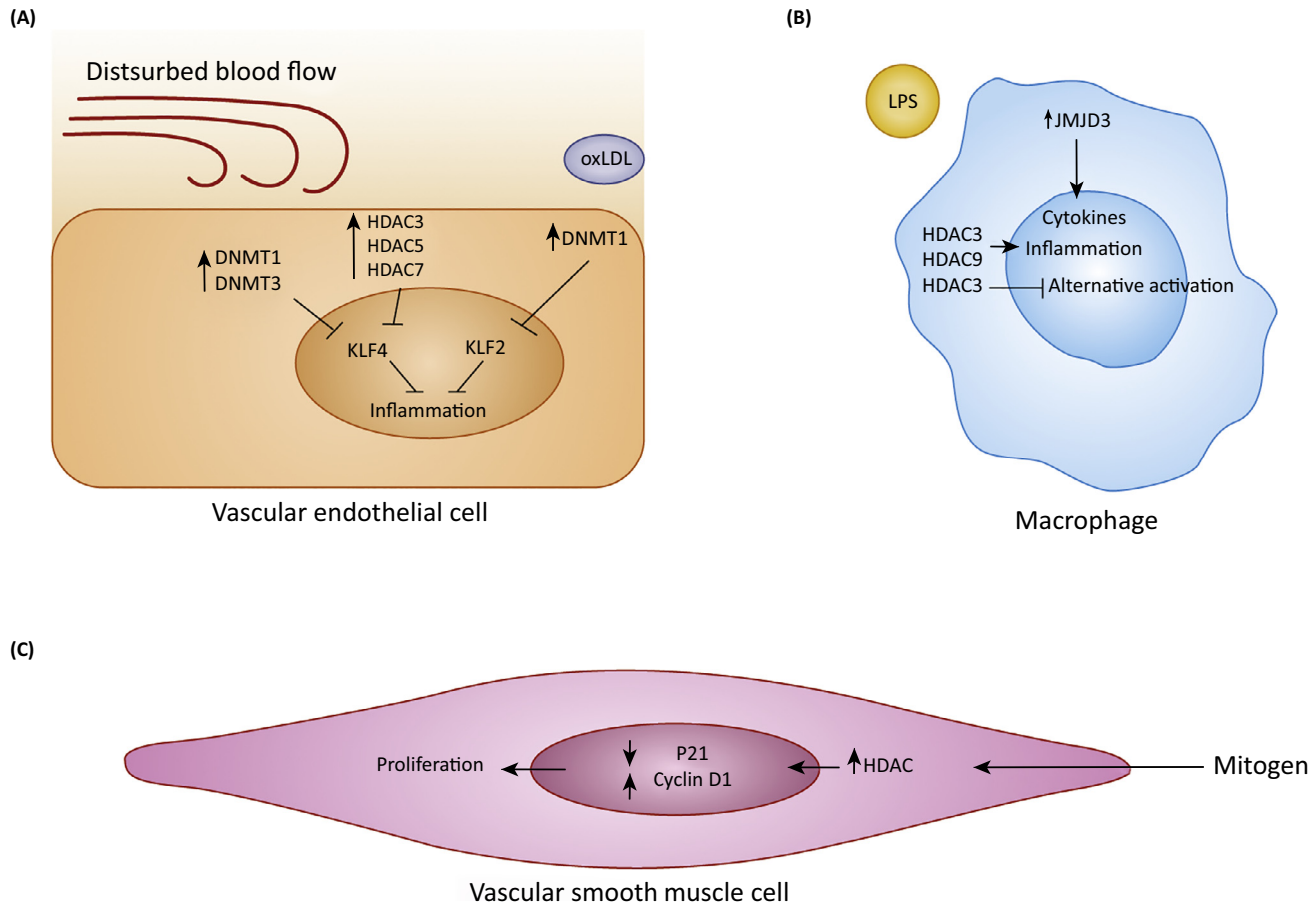

Figure 1. Cell Type-Specific Epigenetic Changes in Atherosclerosis. (A) In vascular endothelial cells, disturbed blood flow and oxidized low density lipoprotein (oxLDL) upregulate DNA methyltransferases (DNMTs) and histone deacetylases (HDACs), which then repress Krüppel-like factor 4 and 2 (*KLF4* and *KLF2*). (B) Lipopolysaccharide (LPS) induction in macrophages increases JMJD3 expression, which then enhances the expression of several cytokines. Furthermore, HDACs have multiple roles in macrophages in controlling inflammation and alternative activation. (C) The regulation of vascular smooth muscle cell proliferation is controlled by HDACs. Upon exposure to a mitogen, HDAC levels are elevated and then act to increase proliferation by increasing Cyclin D1 levels and decreasing P21 levels. Abbreviation: Jmjd, Jumonji C domain-containing.

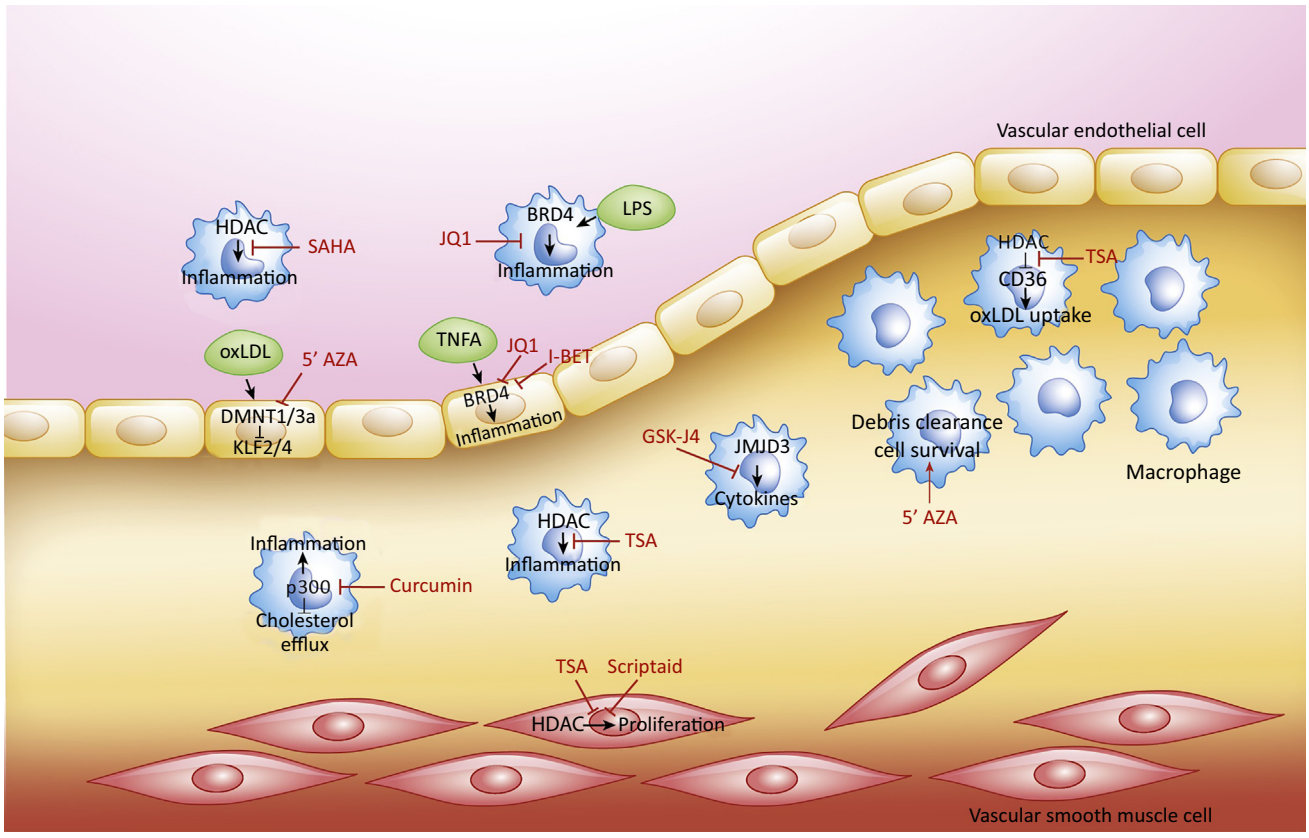

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Figure 2. Potential Epigenetic Pharmacological Interventions in Atherosclerosis. The effect of inhibiting epigenetic machinery in different cell types implicated in atherosclerosis is shown. These inhibitors can be used to diminish inflammation in vascular endothelial cells and macrophages, modulate uptake of oxidized low density lipoprotein (oxLDL) and cholesterol efflux in plaque macrophages, and control cell survival and proliferation of macrophages and vascular smooth muscle cells.

immune response and **inflammasome** component genes [73]. The mechanism was suggested to involve *lincRNA-EPS* binding to hnRNPL [73]. Apart from interacting with hnRNPs, two lncRNAs have been shown to directly interact with NF-κB subunits. *Lethe* is a TNF-α-inducible lncRNA in mouse embryonic fibroblasts. By physically binding to p65 and preventing its interaction with chromatin, *Lethe* participates in a negative feedback loop to decrease NF-κB signaling [74]. Conversely, an LPS-inducible lncRNA, p50-associated Cox-2 extragenic RNA (*PACER*), binds the repressive p50 NF-κB subunit in a human monocyte cell line [75]. By blocking p50 interaction with chromatin, *PACER* has been reported to increase binding of p300 and open chromatin formation to promote expression of the neighboring gene encoding cyclooxygenase 2 (*COX2*), which is involved in the production of inflammatory prostaglandins [75]. Although promising, lncRNA biology is only beginning to be investigated in cells that are relevant for atherosclerosis. Being able to profile lncRNAs in single cells isolated *in vivo* from human tissue and animal models of atherosclerosis represents an opportunity to identify cell type-specific lncRNAs involved in atherogenesis. Such knowledge would inform therapeutic strategies that might target these molecules.

Challenges and Solutions for Studying Epigenetics during Atherosclerosis *In Vivo*

Atherosclerosis is a complex disease that takes decades to progress to the point of clinical symptom manifestation. Plaque development involves the interaction of multiple cell types, as well as the integration of multiple proatherogenic stimuli, including LDL, proinflammatory

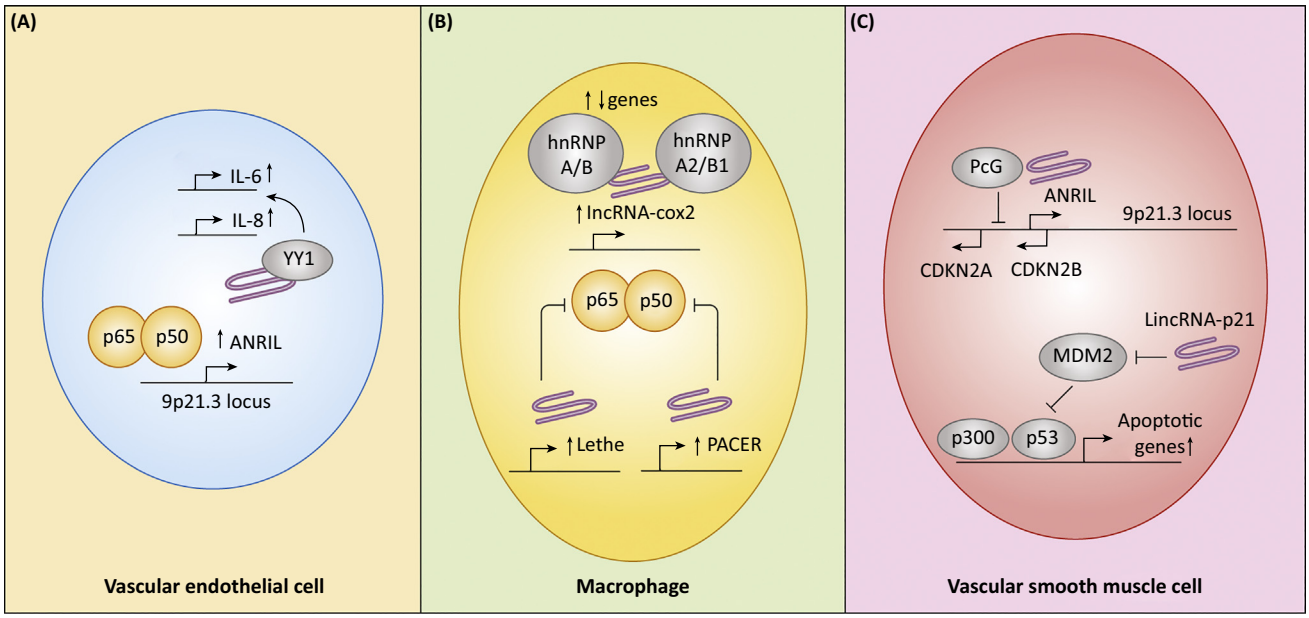

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Figure 3. Long Noncoding RNAs Implicated in Inflammatory Signaling and Cell Survival. In vascular endothelial cells, antisense noncoding RNA in the Ink4 locus (*ANRIL*) is induced by nuclear factor κ-light-chain enhancer of activated B cells (NF-κB) and acts to increase interleukin 6 and 8 (*IL6* and *IL8*) expression. In macrophages, multiple long noncoding RNAs (lncRNAs) are regulated by NF-κB signaling. One of the upregulated lncRNAs, *lncRNA-Cox2*, acts to modulate the expression of inflammatory genes through interaction with heterogeneous nuclear ribonucleoprotein (hnRNP) A/B and hnRNP2 A/B. Another two upregulated lncRNAs, *Lethe* and p50-associated Cox-2 extragenic RNA (*PACER*), interact with p65 and p50 subunits, respectively. In vascular smooth muscle cells, *ANRIL* regulates cell proliferation by interacting with the Polycomb Group Protein (PcG) complex to decrease the expression of the cell cycle genes, cyclin-dependent kinase inhibitor 2 A and B (*CDKN2A* and *CDKN2B*). In addition, *lincRNA-p21* increases expression of apoptotic genes by binding to mouse double minute 2 homolog (MDM2) and inhibiting its activity, which relieves p53 from MDM2-dependent repression.

cytokines, and physical forces of blood circulation, such as **shear stress**. Therefore, defining the role of epigenetics in vascular pathogenesis using human atherosclerotic tissue or animal atherosclerotic models is complicated by the dynamic nature of the disease as well as by tissue heterogeneity. Indeed, tissue heterogeneity might mask cell type-specific epigenetic changes. In addition, access to *in vivo* material is limited. To characterize the epigenetic and gene expression changes that occur during atherogenesis in a robust and cost-effective manner, it is necessary to perform epigenetic and RNA profiling in relevant cell types (preferably single cell analysis), as well as profile numerous individual cells, time points (e.g., longitudinal analysis), experimental conditions, and individuals (Table 1).

Purification methods have traditionally relied on microdissection, fluorescence-activated cell sorting (FACS), or **laser capture microdissection** (LCM) (reviewed in [76]). However, new methods, such as **fluorescence-activated sorting of fixed nuclei** (FAST-FIN) [77] or **isolation of nuclei tagged in specific cell types** (INTACT) [78,79], are promising approaches for the isolation of nuclei from specific cell types of complex tissues, which can then be used for epigenetic profiling. Advances in microfluidic devices and droplet-based technologies have further enabled large-scale single cell purification [80,81].

Epigenomic profiling techniques have recently been developed to utilize low numbers of cells and, in some cases, single cells (Table 1). For example, genome-wide DNA methylome maps can now be generated from single cells [82]. Modifications to ChIP-seq, such as **linear DNA amplification ChIP-seq** (LinDA-ChIP-seq) [83] and **ultra-low-input micrococcal nuclease-based native ChIP-seq** (ULI-NChIP-seq) [84], have been shown to work with inputs as

Table 1. Next-Generation Sequencing Techniques for Epigenomic Profiling of Limited Numbers of Cells

Epigenomic feature	Technique modified for low cell input	Modification to original technique	Refs
DNA methylation	Small cell population whole-genome bisulfite sequencing (μWGBS) and single cell WGBS (scWGBS)	Bisulfite treatment performed before library preparation	[103]
	Single cell reduced representation bisulfite sequencing (scRRBS)	Purification steps before bisulfite treatment are eliminated	[104]
TF binding and histone modifications	Carrier chromatin immunoprecipitation sequencing (cChIP-seq)	Chemically modified histone is used as a chromatin carrier	[105]
	Nano-ChIP-seq	Sequenase is used to prime a trace amount of ChIP DNA, which is then PCR amplified and ligated with adaptors	[106]
	ChIPmentation	Bead-attached chromatin is tagmented using Tn5	[107]
	Linear DNA amplification ChIP-seq (LinDA-ChIP-seq)	T7 RNA Pol-based linear amplification of ChIP DNA	[83]
	Ultra-low-input micrococcal nuclease-based native ChIP-seq (ULI-NChIP-seq)	MNase treatment of cells sorted into lysis buffer	[84]
	Indexing-first ChIP-seq (iChIP-seq)	Chromatin is barcoded and pooled from multiple samples, reducing DNA loss	[85]
	Multiplexed Indexed T7 ChIP-seq (mintChIP-seq)	Barcoding, pooling, and splitting of lysed chromatin for ChIP, followed by T7 RNA Pol-based linear amplification	[86]
	Recovery via protection ChIP-seq (RP-ChIP-seq); favored amplification RP-ChIP-seq (FARP-ChIP-seq)	Yeast DNA and biotinylated DNA are used as carriers to protect loss of ChIP DNA	[108]
	Micro-scale ChIP-seq (μChIP-seq)	Nonspecific binding is reduced using histones and IgG followed by rigorous wash steps	[109]
	Single cell ChIP-seq (scChIP-seq)	Microfluidic droplets containing lysed single cells are merged with DNA barcode droplets	[110]
	Single cell DNA adenine methyltransferase identification (scDamID)	Single cells expressing DNA methyltransferase fused to protein of interest are processed without intermediary purification steps	[111]
	Microfluidic oscillatory washing-based ChIP-seq (MOWChIP-seq)	Sonicated chromatin is immunoprecipitated in microfluidic chamber followed by oscillatory washing	[81]
Chromatin accessibility	Assay for transposase-accessible chromatin using sequencing (ATAC-seq)	Cells are lysed, transposased using Tn5, PCR amplified, and barcoded	[90]
	Single cell ATAC-seq (scATAC-seq)	Microfluidic device is used to capture single cells or a combinatorial indexing is used on nuclei from thousands of single cells	[92,93]
	Single cell DNase I hypersensitive sites sequencing (scDNAse-seq)	Plasmid DNA is used to prevent loss of DNase I hypersensitive DNA	[95]
	Low-input DNase-seq (liDNase-seq)	Plasmid DNA is used as a carrier, purification steps before adaptor ligation are eliminated, and solid-phase reversible immobilization beads are used for size selection	[94]
Chromatin organization	Single cell Hi-C	Biotin fill-in and ligation steps are performed in nuclei followed by selection of individual nuclei under microscope (before nuclear lysis)	[97]
		Combinatorial cellular indexing is used where single nuclei are tagged by a unique combination of barcodes	[98]

low as 1000 cells. The recent development of **Indexing-first ChIP-seq** (iChIP-seq) [85] and **multiplexed indexed T7 ChIP-seq** (mint-ChIP-seq) [86] methodologies rely on multiplexing of low-input samples, which enables cost-effective experimental designs. **Pathology tissue ChIP-seq** (PAT-ChIP-seq) [87] and **fixed tissue ChIP-seq** (FiT-seq) [88] can be used to map epigenomes of cross-linked paraffin-embedded tissue samples, potentially facilitating the study of histone PTMs of clinically annotated samples in pathology archives [88,89].

Genome-wide chromatin accessibility maps can be obtained using **DNase I hypersensitive sites sequencing** (DNase-seq) and **assay for transposase-accessible chromatin using sequencing** (ATAC-seq). ATAC-seq is becoming a widely used assay due to its low cell requirements and fast protocol involving a Tn5 transposase that cuts open chromatin regions and ligates adaptor sequences for PCR amplification in a single step [90]. Of note, freezing is known to disrupt nuclear integrity and adversely affect ATAC-seq read-outs [90]. Therefore, a recently developed cell freezing protocol for ATAC-seq that uses a slowly cooling cryopreservation method now enables high-quality transposition of frozen cell samples that is comparable to analyzing fresh samples [91]. It will be important to test such protocols on human cryopreserved clinical samples. Recently, two distinct single cell ATAC-seq methods have been described that identify cell type-specific chromatin accessibility maps [92,93]. Moreover, analyses of DNase I accessibility on low cell numbers, such as **low-input DNase I sequencing** (liDNase-seq) [94] or **single cell DNase-seq** (scDNase-seq) [95], have also been developed. Chromatin accessibility maps obtained from rare population of cells can be used to predict cell, tissue, and disease types from chromatin accessibility data of heterogeneous tissues [96]. They can also be used to predict long-range interactions between enhancers and promoters [92,93]. To confirm such enhancer–promoter predictions, single cell **Hi-C** methodologies are now available [97,98].

Single cell RNA-seq (scRNA-seq) profiling reveals cell type-specific markers and gene regulatory networks underlying disease pathology. For example, scRNA-seq was recently used to discover pathogenic gene expression signatures in autoimmune Th17 lymphocytes in autoimmune encephalomyelitis (EAE) murine models of multiple sclerosis [99]. Genome-wide methods that profile RNA expression and DNA methylation simultaneously have been described [100], and single cell proteomic approaches are rapidly evolving [101]. ScRNA-seq is becoming increasing accessible to investigators and has enormous, untapped potential for increasing our understanding of atherogenesis.

The rapid and powerful advances in single cell 'omic' technologies will undoubtedly prove to be fruitful for atherosclerosis research. For example, the *in vivo* characterization of epigenomes in scarce and diseased tissues, such as atherosclerotic arteries, will give insight into the regulation of the epigenome under pathological conditions. Moreover, disease-stage specific markers revealed by scRNA-seq will enable the purification of specific cell populations for epigenetic profiling. These 'omic' technologies, together with emerging computational strategies that are required to analyze and integrate the data generated [102], will enable the study of cell type-specific gene regulatory networks within the complex ecosystem of an atherosclerotic plaque.

Concluding Remarks

The complexity of atherosclerosis lies in the interplay of multiple cell types and decades of dynamic physiological changes. Our understanding of epigenetic changes during human atherosclerosis has been largely restricted to measuring global changes and reliance on *in vitro* experiments (see Outstanding Questions). Discovering cell- and stage-specific epigenetic changes of atherosclerosis *in vivo*, made possible through new technologies, will be invaluable in understanding the molecular mechanisms involved in disease progression. Similarly, advances in human cell models and systems genetics may enable the functional dissection of genetic

Outstanding Questions

What are the cell type-specific epigenetic changes that occur during atherogenesis? Atherosclerosis involves the interplay of multiple cell types, predominantly ECs, macrophages, and vSMC. These cell types serve diverse roles in disease progression and are responsive to distinct signaling pathways. Considering that current epigenetic drugs target all cell types indiscriminately (as well as a host of other cells throughout the body), understanding cell type-specific epigenetic changes will aid in rational drug development.

How do epigenetic changes evolve through different stages of atherosclerosis progression? Developing over decades, atherosclerosis encompasses a multitude of protective and deleterious changes. Hence, it is important to understand which epigenetic changes should be targeted for therapeutic treatment, since they may have different functions at distinct stages.

How do epigenetic changes translate from atherosclerosis mouse models to human disease? While mouse models have been valuable research tools that have provided insight into potential therapies, studying human atherosclerotic plaques will undoubtedly provide information that can improve therapeutic design.

Which genomic variations affect susceptibility to atherosclerosis and how are they linked to epigenetic changes? SNPs associated with atherosclerosis susceptibility should be considered together with epigenetic changes discovered by studying differences between healthy and diseased tissue. This integrative approach will likely guide experiments that fine map causal SNPs, and potentially even steer the use of prophylactic treatments.

What are the clinical implications of BET inhibitors that target super-enhancers established through NF-κB and BRD4 in atherosclerotic lesions? What other classes of epigenetic-modifying drug will be effective in preventing and/or treating human atherosclerosis?

variants and the identification of causal genes that might be tested in preclinical models (Boxes 1 and 2). Finally, promising findings in preclinical models of atherosclerosis have suggested that drugs that modulate epigenetic pathways (e.g., DNA methylation, histone acetylation/methylation, and enhancer formation) may be utilized in the future (Box 3 and Figure 2) to treat patients with various types of cardiovascular disease.

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