Vascular smooth muscle cells (SMCs), like all cells, acquire a cell-specific epigenetic signature during development that includes acquisition of a unique repertoire of histone and DNA modifications. These changes are postulated to induce an open chromatin state (referred to as euchromatin) on the repertoire of genes that are expressed in differentiated SMC, including SMC-selective marker genes like Acta2 and Myh11, as well as housekeeping genes expressed by most cell types. In contrast, genes that are silenced in differentiated SMC acquire modifications associated with a closed chromatin state (ie, heterochromatin) and transcriptional silencing. Herein, we review mechanisms that regulate epigenetic control of the differentiated state of SMC. In addition, we identify some of the major limitations in the field and future challenges, including development of innovative new tools and approaches, for performing single-cell epigenetic assays and locus-selective editing of the epigenome that will allow direct studies of the functional role of specific epigenetic controls during development, injury repair, and disease, including major cardiovascular diseases, such as atherosclerosis, hypertension, and microvascular disease, associated with diabetes mellitus. (Arterioscler Thromb Vasc Biol. 2015;35:2508-2516. DOI: 10.1161/ATVBAHA.115.305044.)

**Key Words:** atherosclerosis ■ cardiovascular diseases ■ differentiation ■ embryonic stem cells ■ histone modifications ■ vascular injury ■ vascular smooth muscle cells

The vascular smooth muscle cell (SMC) is a highly specialized cell whose principal functions are contraction and regulation of blood pressure and blood flow distribution. Mature differentiated SMCs express a unique repertoire of proteins required for their contractile function. Importantly, a major subset of this SMC-specific repertoire, including the SMC differentiation marker genes, smooth muscle myosin heavy chain (Myh11), smooth muscle α-actin (Acta2), and SM22 α (Tagln) are transcriptionally regulated in a SMC-specific fashion by the CArG box/serum response factor (SRF)/myocardin (or myocardin-related transcription factor) complex. Myocardin (Myocd) is a potent SRF coactivator that is exclusively expressed in SMC and cardiomyocytes that binds to SRF on CArG regions of the SMC marker genes, but not to other SRF-dependent genes, such as c-fos. This highly specialized transcriptional regulation leads to a cell-specific and coordinate activation of most although not all of the repertoire of SMC marker genes. There is considerable evidence that epigenetic mechanisms contribute to SMC-specific transcriptional regulation in mature and phenotypically modulated SMC. Epigenetic regulation is classically defined by mechanisms controlling the heritability of traits or phenotypes from mother to daughter cells without modification of the DNA sequence but rather by modification of DNA bases (methylation and hydroxymethylation), post-translational modifications of the histone tails (methylation, acetylation, phosphorylation, and ubiquitinylation), histone subunit variants, and non-coding RNA. Because of space constraints and the recent publication of several excellent reviews on epigenetic regulation of SMC differentiation and phenotypic switching, we choose to focus this review on epigenetic programming associated with histone and DNA modifications that dynamically control the chromatin structure and thereby contribute to gene activation or repression in SMC during development, tissue repair/remodeling, and cardiovascular disease.

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**Vascular SMC Acquires a Unique Cell-Specific Epigenetic Signature During Development**

There is evidence suggesting that SMC-selective changes in chromatin remodeling contribute to the coordinate activation of...
CArG-dependent SMC marker genes during differentiation of these cells from embryonic stem cells (ESC). Indeed, studies by our laboratory have shown that binding of the SRF/myocardin complex to CArG regions within the SMC marker genes is developmentally regulated and SMC selective. Key observations include the following. First, chromatin remodeling characterized by histone acetylation, a potent mechanism of gene activation, occurs on Myh11 and Acta2 early in the process of SMC differentiation from SMC precursors in response to treatment with retinoic acid. Interestingly, despite being expressed in both undifferentiated and retinoic acid–differentiated SMC precursors, SRF binds only to the SMC marker genes that have been enriched in histone acetylation in retinoic acid–treated SMC precursor cells. Although correlative, these data suggest that changes in chromatin accessibility, mediated at least in part by histone modifications, render SMC marker genes permissive for subsequent activation by the SRF/myocardin complex during SMC differentiation. Moreover, inhibition of histone acetyl transferases or expression of histone deacetylases (HDACs) leads to decreased SMC marker gene promoter activity in cultured SMC. Second, there is marked enrichment of the histone modification H3K4me2 on SMC marker genes, including Myh11, Acta2, Myocd, and SRF, and a correlated decrease in H3K4me2 on CArG-wild type or CArG-mutant promoters demonstrated that H3K4me2 enrichment occurs on the absence of binding of SRF to CArG elements, suggesting that it is regulated through an upstream CArG-SRF independent process. Fourth, there is a selective tethering of myocardin to H3K4me2-modified histone tails. We postulate that this helps stabilize binding of the SRF–myocardin complex to the CArG regions which in the case of many SMC promoters are degenerate and exhibit reduced SRF–binding affinity.

These histone modifications are believed to alter the higher order chromatin structure through attraction or repelling of charged histone tails thereby regulating nucleosome density and the accessibility of various cis promoter–enhancer control elements. However, covalent modifications of DNA nucleotides have also been implicated in gene regulation. For example, methylation of cytosine residues has long been recognized as a stable mechanism of gene repression and chromatin compaction, and for many years, it was incorrectly thought to be relatively irreversible. Indeed, identification of mechanisms that mediate demethylation of DNA at discrete gene loci with associated gene derepression is currently an area of intense interest in the epigenetic field. For example, it has recently been shown that conversion of 5-methylcytosine (5mC) into 5-hydroxymethylcytosine (5hmC) by ten-eleven translocation (TET) enzyme–mediated oxidation seems to be an important mechanism of reactivation of genes. The most notable example of the latter occurs during reprogramming of somatic cells into induced pluripotency stem cells for which Yamanaka received the 2012 Nobel Prize albeit this particular example is induced by artificial hyperphysiological overexpression of the induced pluripotency stem cell reprogramming factors Oct4, Sox2, c-myc, and Klf4. However, it has also been shown that 5hmC plays a major role in maintenance of pluripotency, self-renewal, and proper differentiation of ESC during early development. The role of 5hmC in somatic cells is poorly understood. However, several studies suggest that 5hmC promotes cell-specific differentiation programs in somatic cells via the enzyme TET2. Recent innovative studies by Liu et al provide evidence that 5hmC also plays a key role in regulating SMC-selective gene expression during development and phenotypic switching. TET2 expression is higher in mature SMC compared with ESC and is upregulated after treatment with rapamycin in vitro. TET2 is also highly expressed in medial SMC from healthy aortas. Moreover, knock down of TET2 by small hairpin RNA induces repression of SMC-specific genes, including Myh11, Acta2, Myocd, and SRF, and a correlated decrease in enrichment in 5hmC on these genes in cultured SMC.

Vascular SMC Is Not Terminally Differentiated and Undergoes Marked Changes in Phenotype During Vascular Injury Repair and in Disease States

It has long been assumed that the SMC is not terminally differentiated and that it can undergo rapid changes in phenotype in response to vascular injury or in various cardiovascular diseases, including atherosclerosis. However, until recently, the evidence for this was nearly completely based on inferences from studies in cultured SMC, including demonstrating by our group and many others that treatment of cultured SMC with various growth factors, such as platelet derived growth factor or basic fibroblast growth factor, oxidized phospholipids, or proinflammatory cytokines, resulted in coordinate reductions in expression of SMC marker genes, as well as increased migration, proliferation, and secretion of various extracellular matrix proteins and cytokines, a process collectively referred to as phenotypic modulation or switching. That said, our understanding of phenotypic modulation and the fate of phenotypically modulated SMC in vivo are highly confounded by the fact that this process is characterized by downregulation of the SMC marker genes like Acta2 and Myh11 making their identification in vivo unreliable without rigorous lineage tracing systems. As such, the paradigm of SMC phenotypic switching during vascular injury and disease has been challenged by studies, suggesting that a significant subset, if not the majority, of lesion and neointimal cells are of myeloid or adventitial origin rather than SMC. The possibility that adventitial stem cells give rise to intimal lesion cells is intriguing, but at present there is no direct evidence that they do so because of the lack of model systems with which to define lineage trace these cells in vivo. Moreover, initial claims that the majority of SMC-like cells within lesions

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**Nonstandard Abbreviations and Acronyms**

- ChIP: chromatin immunoprecipitation
- ESC: embryonic stem cells
- HDACs: histone deacetylases
- ISH–PLA: in situ hybridization–proximity ligation assay
- SMC: smooth muscle cell
- SRF: serum response factor
- TET: ten-eleven translocation

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are of myeloid origin by Nagai et al.\textsuperscript{48} were subsequently directly refuted in a series of bone marrow transfer studies by Bentzon et al.\textsuperscript{51,52} who claimed that myeloid cells fail to express Acta2. In direct contrast, a follow-up article by the Iwata et al.\textsuperscript{53} using a combination of bone marrow transfer studies and a Myh11 promoter reporter system, refuted their earlier studies\textsuperscript{48} by showing that the majority of SMC marker positive cells within lesions are not of myeloid origin, but that some myeloid cells can express early (Acta2, Tagln) but not late (Myh11 and Cnn1) SMC differentiation markers. These latter results are consistent with cross sex bone marrow transfer human studies by Caplice et al.\textsuperscript{49} showing that >10% of ACTA2+ cells in human lesions are of myeloid origin. Taken together, although there remains some controversy about the extent to which myeloid cells express SMC markers within lesions, our interpretation of the studies is that at least some do but that the majority of SMC marker positive cells within lesions are not of myeloid origin.

A much more profound claim that challenge the conventional dogma that SMCs are the principal source of SMC lesions are not of myeloid origin. However, the main conclusions of this article were strongly refuted in an Editorial from a collective group of authors, who are leaders in the field including ourselves,\textsuperscript{55} on the basis of there being major technical and design flaws in those studies. More importantly, several subsequent rigorous lineage tracing studies by our group\textsuperscript{56,57} and several others\textsuperscript{58–60} directly refute the major conclusions of Tang et al and provide compelling evidence that SMCs are plastic and exhibit phenotypic switching in vivo (for a more complete review of this critical topic, please see recent reviews by Tabas et al.\textsuperscript{61} and Bennett et al.\textsuperscript{62}).

We recently used a novel SMC-specific conditional lineage tracing mouse model (ie, Myh11-CreER\textsuperscript{T2} ROSA floxed STOP eYFP mice, designated SMC YFP\textsuperscript{+}/+ mice) to show that >80% of SMC-derived cells within advanced ApoE\textsuperscript{−/−} brachiocephalic artery lesions lacked detectable expression of SMC markers, such as Acta2, and could only be detected using a rigorous SMC lineage tracing system.\textsuperscript{57} Moreover, we showed that approximately a third of these cells that were YFP+ but Acta2− expressed multiple markers of macrophages, and thus have likely been misidentified as being monocyte-derived macrophages in previous studies in the field. Most importantly, we showed that these phenotypic transitions of SMC were functionally important, in that we showed that SMC-specific conditional knockout of the pluripotency factor Klf4 did not prevent SMC phenotypic switching, but altered the nature of the phenotypic transitions, including a marked reduction in SMC-derived macrophage-like cells. Consistent with these results, Feil et al.\textsuperscript{58} used a Tagln CreERT\textsuperscript{T2} ROSA floxed STOP LacZ mouse model to show that a subset of SMC-derived cells expresses several macrophage markers, and SMC-derived cells within lesions underwent clonal expansion during atherosclerosis using R26R-Confetti multicolor Cre reporter mice. Additional evidence that SMCs are not terminally differentiated and undergo phenotypic transitions during vascular injury repair can be found in studies by Nemenoff et al.\textsuperscript{59} who used a conditional Myh11 creERT\textsuperscript{T2} ROSA26 LacZ reporter mouse to provide compelling evidence that the vast majority of neointimal cells after wire-induced injury of the femoral artery are derived from pre-existing differentiated (ie, Myh11+) SMCs.\textsuperscript{59} These latter observations have been confirmed and extended in elegant recent studies by Herring et al.\textsuperscript{60} who used conditional Myh11 and Acta2 creERT\textsuperscript{T2} mTmG mice to show that previously differentiated SMC dedifferentiate and constitute the main source of SMC within the neointima after carotid ligation injury.

Taken together, results indicate that typical methods for detecting SMC based on immunostaining for SMC marker genes not only greatly underestimate the number of SMC-derived cells within lesions but also misidentify many of these cells because they lack SMC markers and have activated markers of other cell types, including macrophages. Likewise, there is also clear evidence that cells other than SMC activate at least some SMC markers in the context of atherosclerotic lesions.\textsuperscript{50,51,53} As such, studies establish that reliable identification of SMC-derived cells within intimal lesions after injury or within atherosclerotic lesions must rely on the use of rigorous SMC lineage tracing methods which is absolutely dependent on (1) efficient and SMC-specific conditional activation of a sensitive lineage tracing gene; and (2) high resolution imaging to ensure the lineage tracing gene and markers of cell phenotype are present within the same cell (for a thorough review of requirements for definitive cell lineage tracing see this excellent review).\textsuperscript{63}

**Modifications of the SMC Epigenetic Programming Are Associated With SMC Phenotype Modulation**

There is evidence suggesting that epigenetic mechanisms play an important role in regulating phenotypic switching of SMC. For example, we showed that platelet-derived growth factor BB-induced phenotypic transition of cultured SMC was associated with (1) decreased histone acetylation mediated through Klf4-dependent recruitment of HDACs 2, 4, and 5 to multiple CArG-dependent SMC marker genes;\textsuperscript{10,42} and (2) enrichment of the silencing modification H3K9me3 on SMC promoters.\textsuperscript{10} Consistent with these findings, subsequent studies by our laboratory\textsuperscript{64} showed that silencing of the Tagln gene after ligation-induced carotid injury in vivo also seemed to be mediated through binding of HDAC2 to the CArG region, as well as pELK, and reduced H4 acetylation, mediated through Klf4-dependent binding to a G/C repressor element in the Tagln promoter that is also found in most other SMC CArG-dependent marker genes. That is, Klf4 is upregulated after injury, binds to G/C repressor elements, and recruits both HDACs and pElk that contribute to silencing of SMC marker genes. Of major interest, we have recently provided evidence, indicating that similar mechanisms contribute to silencing of SMC marker genes within phenotypically modulated SMC within atherosclerotic lesions, in that we showed marked enrichment of Klf4 on the promoters of Acta2, Myh11, and Tagln based on in vivo ChIP assays on chromatin extracted from advanced ApoE\textsuperscript{−/−} brachiocephalic artery lesions.\textsuperscript{57}
Although likely to be of critical importance, unfortunately little is currently known with respect to the contribution of DNA methylation in SMC phenotypic switching. Several lines of evidence suggest that changes in the DNA methylation pattern might play a role in major vascular diseases, such as atherosclerosis and SMC phenotypic switching. First, a process of global hypomethylation and decrease in DNA methyltransferases expression have been observed in human and mouse atherosclerotic lesions. Nevertheless, this study does not provide direct evidence that the hypomethylation phenomenon occurs in SMC and the full spectrum of genes affected by the hypomethylation is unknown. Second, SMC marker genes, including Tagln and Myh11 promoters, are hypomethylated in SMC compared with freshly isolated aortic SMC. These latter observations suggest that dynamic modifications of DNA methylation patterns of SMC marker genes may contribute to changes in expression of these genes during phenotypic transitions, at least during adaptation of cells to culture. Third, modulation of DNA demethylase TET2 expression and activity and the concomitant changes in hydroxymethylation of the SMC marker genes have been associated with SMC phenotypic switching within atherosclerotic lesions and after femoral artery injury. TET2 expression is reduced in injured and atherosclerotic vessels. Moreover, TET2 knockdown induced increased neointimal proliferation post injury, whereas overexpression of TET2 had the opposite effect. Taken together, the results suggest that changes in DNA methylation are likely to contribute to changes in gene expression during SMC phenotypic transitions but much further work is needed in this critical area.

Of major importance, we have observed that at least a part of the SMC epigenetic programming established during SMC differentiation is retained during phenotypic switching. Indeed, H3K4me2 is stably enriched on the Myh11 and Acta2 promoters in SMC treated with platelet-derived growth factor-BB or oxidized phospholipids compared with untreated SMC. Rigorous investigation of epigenetic modifications within SMC in vivo is particularly challenging considering the fact that normal and diseased tissues are made up of a large number of heterogeneous cell types. Indeed, this is a major general limitation in the epigenetic field that has greatly confounded efforts attempting to elucidate how any given epigenetic modification might alter the function of a particular cell type within a complex tissue. To circumvent this limitation, we developed a method that we refer to as ISH-PLA (in situ hybridization-proximity ligation assay), to visualize histone modifications at single genomic loci within individual cells in tissue sections. Importantly, because there was no precedent for doing these types of analyses within intact tissue sections, it was critical to rigorously validate the methodology including doing the following. First, we used our SMC-specific Myh11 YFP lineage tracing mice to demonstrate that the presence of H3K4me2 on the Myh11 promoter (H3K4me2 was completely restricted to SMC within all mouse and human tissues examined, including numerous arteries and arterioles. Second, an endothelial cell selective epigenetic modification, H3K4me2 of Cdh5 (vascular endothelial cadherin) is restricted to endothelial cells in all blood vessels examined in both mouse and human paraformaldehyde fixed tissue specimens. We subsequently used our ISH-PLA method in conjunction with our SMC lineage tracing mice to show that H3K4me2 is retained in phenotypically modulated SMC within atherosclerotic lesions that lack detectable expression of SMC marker genes (ie, YFP+ Acta2− lesion cells). Moreover, Myh11 H3K4me2 was maintained in SMC that had undergone transition to a macrophage-like state within mouse and human lesions. Remarkably, the combination of ISH-PLA and rigorous Myh11 YFP lineage tracing showed that >30% of Lgals3+ cells within advanced mouse lesions were of SMC and not myeloid origin as has been assumed in previous studies in the field. Moreover, using ISH-PLA, we provided evidence that nearly 20% of macrophage marker positive cells within advanced human coronary artery lesions were of SMC, not myeloid origin, and >35% of cells dual positive for Lgals3 and Acta2 were of SMC origin. The latter is of major significance in that a recent article from Allahverdian et al showed that 50% of foam cells within advanced human coronary artery lesions are dual positive for these markers. Taken together, these results resolve the decade long debate as to whether SMCs are a major source of foam cells within human lesions. Importantly, our findings were confirmed by performing a combination of ISH-PLA detection of the SMC-specific epigenetic mark, Myh11 H3K4me2, and Y-chromosome tracking in advanced coronary artery lesions of a male subject, who had received a female heart transplant. That is, we observed Y-chromosome negative CD68− cells in lesions from the female donor, which had a SMC Myh11 H3K4me2 epigenetic signature. Importantly, in no case Y-chromosome+ cells (ie, of myeloid origin from the male recipient) that had the SMC epigenetic signature were observed, indicating that myeloid cells do not acquire this mark in the context of atherosclerotic lesions thus further validating these method and findings. These observations lead us to the conclusion that H3K4me2 on the SMC marker genes is a restricted and stable epigenetic signature of the SMC lineage distinguishing SMC from other cell types independently of their state of differentiation. The next section will consider the possible functional significance of this observation (Figure).

Do Epigenetic Mechanisms Regulate Cell Lineage Memory?

Biologists have been challenged for centuries trying to elucidate the mechanisms that contribute to maintenance of cellular lineage identity and integration of environmental cues across cell generations, including during development of complex multicellular organisms, from a single fertilized egg. Whereas it has been assumed that this critical cell property must be regulated through epigenetic mechanisms, and this might be the case, there are several major limitations in the experimental evidence in support of this concept including the following.

First, the vast majority of the functional epigenetic studies have been based on genetic deletion of various histone-modifying enzymes and DNA methyltransferases that result in global and massive changes across the entire genome and which not surprisingly are often embryonic or postnatal lethal. Similarly,
pharmacological inhibition of histone-modifying enzymes (e.g., HDAC inhibitors) leads to global epigenetic reprogramming of the genome. Whereas these sorts of studies establish that epigenetic controls are critical, by no means can one ascertain that a particular change at a specific locus is functional.

Second, tremendous emphasis has been made on the identification of cell-specific epigenetic programming by high-throughput sequencing of the whole genome, and recent studies compiled and compared epigenetic profiles of somatic cells in culture or extracted from different tissues and ESC undergoing differentiation into primordial germ lines. These data showed that lineages acquire specific epigenetic programming signatures. However, these studies are correlative, and they fail to ascertain if acquisition of a cell-specific epigenome is the cause of cell differentiation or the consequence.

Third, by far, the majority of epigenomic studies have been performed on cultured cells systems that poorly replicate epigenetic controls in vivo. Indeed, cultured SMCs are poorly differentiated and exhibit a phenotype different than mature differentiated SMC in vivo because cell culture systems do not recapitulate complex environmental cues that regulate SMC differentiation in vivo under normal and pathological conditions. As a relevant illustration of this bias, studies by Zhu et al involving genome-wide mapping of the epigenome of various human tissues and cultured cells provide a striking confirmation of just how different cultured cells can be from their in vivo counterparts and how somatic cells from different lineages tend to undergo similar epigenetic reprogramming once they are cultured in vitro, becoming more and more like one another, while less and less like their in vivo brethren. Indeed, these latter
studies showed that variety of cultured cells from different origins acquired a similar epigenome during adaptation to growth in culture, and which was distinct from that exhibited in vivo in the rare cases where this could be ascertained.

Fourth, studies, including ChIP-seq, performed on chromatin derived from multicellular tissue samples have attempted to differentiate which epigenetic signals come from each respective cell type using bioinformatics approaches that use epigenomes identified from studies of cultured cells. Although these studies have the advantage that assays were performed on a homogenous cell population, as noted above, it is extremely unlikely the epigenome displayed accurately replicates that displayed by their in vivo counterparts because of many differences in environmental cues present in the 2 systems. Importantly, the latter includes the absence of heterotypic cell interactions. As a relevant example, ChIP and ChIP-seq cannot be used to rigorously investigate SMC-specific epigenetic regulation in vivo because chromatin obtained from SMC-rich tissues is not exclusively derived from SMCs but is rather contaminated by chromatin derived from multiple cell types. Consequently, ChIP analyses on an atherosclerotic lesion specimen represent a composite signal derived from many different cell types (eg, endothelial cells, monocytes, macrophages, adventitial fibroblasts) that are all susceptible to change their phenotype and their epigenetic programming during development of the disease. Importantly, this issue has also not been adequately addressed in nearly all studies involving analyses of genome-wide DNA methylation data and comparative studies using either ChIP-seq or DNA methylation data sets almost always rely on biased bioinformatics approaches. Importantly, we have identified a simple, albeit expensive and time-consuming means to at least partially resolve these limitations as exemplified in our recent studies examining the effect of SMC-specific conditional knockout of the stem cell pluripotency factor Klf4 within ApoE−/− mice fed a Western diet for 18 weeks to induce formation of advanced atherosclerotic lesions. In brief, we performed differential Klf4 ChIP-seq analyses of chromatin samples obtained from brachiocephalic lesions of our Klf4−/+ YFP+/+ ApoE−/−) versus SMC-specific Klf4 knockout atherosclerotic mice (ie, SMC Klf4Δ−/− YFP+/+ ApoE−/−), thus enabling us to identify putative Klf4 target genes selective for SMC.

The last and perhaps the most striking limitation of epigenetic studies in vivo is the failure to provide direct loss of function evidence that a given epigenetic modification has a specific functional effect in a given cell. Let us finish this review by addressing this considerable challenge.

**Development of New Methods for Locus-Specific Epigenetic Editing and Studying Functional Consequences**

As noted previously, functional epigenetic studies require the combination of locus-specific epigenomic editing with single cell epigenetic and functional analyses. While at one time this would have seemed impossible, it appears that all the pieces may finally be available, although as far as we are aware that no one has yet successfully combined them. Here are the pieces.

First, several methods are now available for doing single-cell epigenetics, including single-cell RNA-seq and single-cell genome-wide DNA methylation analysis. Our ISH-PLA method allows investigation of epigenetic modification in single cells in vivo in fixed histological sections and is powerful for validation purposes, including in both normal and diseased human tissue sections, but by no means is this method conducive for high throughput screens or initially defining the epigenome of a given cell type in vivo. That is, you must first define what you want to look for using other methods and approaches.

Second, several recent articles have described innovative new methods and tools that permit reliable cell-specific/locus-specific epigenetic modification and reproportioning in major human diseases, including cardiovascular pathologies. The use of technologies like Zinc fingers, TAL effector proteins (TALE), and CRISPR-Cas9 initially designed for genome editing have recently been used to modify the epigenome by altering DNA methylation or histone modification patterns at single genomic loci. Importantly, a recent *Nature Biotechnology* article from Bradley Bernstein’s laboratory described the first method to achieve locus-specific editing of the epigenome, which has become the Holy Grail for the epigenetic field and is essential for determining the functional role of specific epigenetic changes in disease states, including cardiovascular pathologies. The use of technologies like Zinc fingers, TAL effector proteins (TALE), and CRISPR-Cas9 initially designed for genome editing have recently been used to modify the epigenome by altering DNA methylation or histone modification patterns at single genomic loci. Importantly, a recent *Nature Biotechnology* article from Bradley Bernstein’s laboratory described the first method to achieve locus-specific editing of the epigenome. This study targets histone marks H3K4me2 and H3K27ac characteristic of active enhancers by the use of TALE fused with the histone demethylase responsible for removal of H3K4me2 LSD1 (referred as TALE-LSD1) to aim specific gene enhancer regions. With the 40 enhancers targeted, the authors observed a locus-specific decrease in H3K4me2 and H3K27ac in 65% of these enhancers. For 9 of the TALE-LSD1 constructs, a comparison between ChIP-seq and RNA-seq demonstrated a correlation between H3K4me2 removal on enhancers and downregulation of genes regulated by these enhancers. Taken together, these results provide a compelling proof of principle that locus-selective editing of the epigenome is possible although surprisingly this initial article did not go onto examine how these site-specific epigenomic modifications affected cell function, and importantly studies were done entirely in vitro. However, the field seems to be poised to finally be able to combine single-cell epigenetic assays with single-cell locus-specific epigenetic editing to directly and rigorously begin to assess the exact functional roles of cell-specific epigenetic controls in regulation of cell differentiation, cell lineage memory, and phenotypic transitions during tissue injury, repair, and adaptation, as well as how these critical processes are altered in disease states, including major cardiovascular diseases. Indeed, it is an exciting time for the field.

**Summary and New Perspective**

Observations of a SMC-specific epigenetic signature, including SMC-specific distribution of histone modification and DNA hydroxymethylation, are consistent with the long-standing dogma in the epigenetic field that differences in chromatin
organization and distribution of histone marks are crucial events modulating the reading of the unique genomic sequence and allowing generation of multiple somatic cell types from ESC. Importantly, there is evidence in vitro and in vivo that SMC retain their SMC-specific epigenetic signature (ie, enrichment of H3K4me2 on the SMC marker genes) during phenotypic switching and their transition to alternative phenotypes. Nevertheless, a major challenge for the field is to determine whether such epigenetic signatures play a critical functional role in controlling SMC lineage identity and functional phenotypes in vivo. Novel methodologies, including rigorous lineage tracing, single-cell epigenetic assay, and locus-specific epigenome editing tools, provide unique new opportunities to investigate the functional relevance of epigenetic programming in controlling SMC identity and plasticity in the context of major cardiovascular diseases, such as atherosclerosis.

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